INHIBITION OF NEUTROPHIL ACTIVATION: EFFECTS IN REPERFUSION INJURY AND CARDIOPULMONARY BYPASS

By

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Declaration

I hereby declare that the work described in this thesis was performed entirely by myself in the Department of Pharmacology (now Neuroscience), University of Edinburgh, Department of Dietetics and Nutrition, Queen Margaret University College and Department of Cardiac Surgery, University of Glasgow between 1995 and 1999.

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Abstract

It is known that neutrophils play an important role in host defence mechanisms especially in acute inflammation. Such neutrophil activation and accumulation in the myocardium are suggested to be major pathological events in myocardial ischaemia reperfusion (MI-R) injury. Endothelial dysfunction occurs very early after reperfusion followed by neutrophil infiltration into the myocardium and subsequent myocardial necrosis. Neutrophil products cause myocardial injury during MI-R. In experimental models, interventions that deplete neutrophils or inhibit their function cause a significant reduction in myocardial infarct size. Neutrophil activation is also associated with cardiopulmonary bypass (CPB) as well as a failure of platelets to form large, stable aggregates.

The aims of this thesis were: 1) to establish the role of nitric oxide (NO) and cyclic GMP in neutrophil chemotaxis and superoxide anion generation (SAG), 2) to investigate the effects of a novel NO donor GEA 3162, the A_{2A} receptor agonist 2-HE-NECA, and the PGI_2 analogue cicaprost on neutrophil accumulation and myocardial injury *in vivo*, in a rat model of MI-R, and 3) to identify the role of neutrophil activation in the formation of stable platelet aggregates and whether heparin, which is used systemically to anticoagulate for CPB, contributed to platelet dysfunction during CPB by interfering with neutrophil-platelet interactions. Effects of heparin *in vitro*, on neutrophil SAG and myeloperoxidase release were also determined.

The mechanisms responsible for chemotaxis and neutrophil activation are not fully understood. Selective inhibitors of the NO and cyclic GMP pathways have been used to elucidate their roles in the activation and inhibition of human neutrophils. In addition, the ability of NO donors to inhibit neutrophil chemotaxis was compared with their ability to increase neutrophil nitrate/nitrite and cyclic GMP levels. The results confirm that neutrophil activation results from the stimulation of several signal transduction systems. It appears that chemotaxis can occur via a NO-dependent as well as NO-independent pathway. Similar pathways appear to operate in SAG. The results also suggested that the small concentrations of NO and

cyclic GMP induced by fMLP activated neutrophils while large concentrations of NO and cyclic GMP are inhibitory.

The effects of GEA 3162, 2-HE-NECA, and cicaprost on neutrophil accumulation and myocardial injury in a rat model of MI-R were investigated. Myocardial ischaemia was induced by occlusion of the left main coronary artery (45 min) and then reperfused (120 min). Drugs or saline vehicle were infused intravenously for 130 min beginning 10 min before reperfusion. Neutrophil accumulation in the area at risk and normal area was assessed by myeloperoxidase assay. Infarct and perfusion area were determined by the triphenyltetrazolium chloride-Evans blue technique. The results demonstrated that reduction of neutrophil accumulation in the area at risk by all three drugs was associated with a reduction in myocardial necrosis.

To investigate the platelet defect associated with CBP, patients undergoing routine aortocoronary bypass grafting were studied before and after heparinisation, and at end-CPB. Macroaggregation in response to collagen or the neutrophil stimulant fMLP was determined by whole blood impedance aggregometry. Microaggregation was determined by counting unaggregated single platelets. Volunteers' blood was studied *in vitro*. The results demonstrated that the abolition of macroaggregation results from heparinisation *per se* rather than CBP, which had no additional effect. This major inhibition seen *ex vivo* was insensitive to heparinase and could not be fully reproduced *in vitro* suggesting that heparin released an inhibitory factor *in vivo*. While heparin inhibited neutrophil activation *in vitro* and inhibited fMLP induced neutrophil-dependent platelet aggregation *ex vivo*, this did not account for the inhibition of collagen macroaggregation.

Publications

Wanikiat, P., Woodward, D.F. & Armstrong, R.A. (1997). Investigation of the role of nitric oxide and cyclic GMP in both the activation and inhibition of human neutrophils. Br. J. Pharmacol., 122, 1135-1145.

Belcher, P.R., Muriithi, E.W., Milne, E.M., Wanikiat, P., Wheatley, D.J., Armstrong, R.A.(1999). Heparin, platelet aggregation, neutrophils and cardiopulmonary bypass. Thromb Res (in press).

Wanikiat, P., Armstrong, R.A. and Gray, G.A. (1999). Infarct size is reduced by inhibition of neutrophil accumulation in a rat model of myocardial ischaemia and reperfusion. Mediators of Inflammation, 8 supplement S 45. Presented as an oral communication at the 4th World Congress on Inflammation, Paris, June 1999.

Milne, E.M., Muriithi, E.W., Wanikiat, P., Armstrong, R.A., Wheatley, D.J., Belcher, P.R. Heparinisation, neutrophils and platelet activation in whole blood. Clin. Sci. 1999; 96 (Suppl 40): 3p. Presented at Medical Research Society, London, 1998.

Milne, E.M., Muriithi, E.W., Wanikiat, P., Armstrong, R.A.,Wheatley, D.J., Belcher, P.R. Heparin, neutrophil-platelet interaction, and cardiopulmonary bypass. Clin. Sci. 1999; 96 (Suppl 40): 3p. Presented at Medical Research Society, London, 1998.

Belcher, P.R., Milne, E.M., Muriithi, E.W., Wanikiat, P., Armstrong, R.A., Wheatley, D.J. Heparin, cardiopulmonary bypass and neutrophil-platlet interaction. Blood Coag Fibrinol 1999; 19 (8).

Presentations

Wanikiat, P., Armstrong, R. A. & Gray, G. A. Inhibition of neutrophil accumulation in a rat model of myocardial ischaemia and reperfusion. Presented as a poster at 2nd Annual Meeting of the Scottish Cardiovascular Forum, Edinburgh, October, 1998.

List of Abbreviations

5'-AMP	5'-adenosine monophosphate
5-HT	5-hydroxytryptamine
AAR	area at risk
AA	arachidonic acid
AC	adenylate cyclase
ADP	adenosine diphosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
BH_4	tetrahydrobiopterin
BPI protein	bactericidal permeability-increasing protein
BSA	bovine serum albumin
C5a	5^{th} component of complement
CD18	common β subunit of β_2 leukocyte integrin
CD62E	E-selectin
CD62L	L-selectin
CD62P	P-selectin
C-C chemokines	β chemokines
CFU-GM	colony-forming unit for granulocytes and monocytes
CPB	cardiopulmonary bypass
C-X-C chemokines	α chemokines
Cyclic AMP	cyclic adenosine monophosphate
Cyclic GMP	cyclic guanosine monophosphate
DAG	1,2 diacylglycerol
DARS	donkey anti-rabbit serum
DBP	diastolic blood pressure
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EC_{50}	concentration causing 50% of maximum response
EDRF	endothelium-derived relaxing factor
EEC	equi-effective concentration

EGF-like	epidermal growth factor-like
ELAM-1	endothelial-leukocyte adhesion molecule 1
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal related kinase
FAD	flavin adenine dinucleotide
fMLP	N-formyl methionyl leucyl phenylalanine
FMN	flavin mononucleotide
GC	guanylate cyclase
G-CSF	granulocyte colony-stimulating factor
GDP	guanosine diphosphate
GM-CSF	granulocyte macrophage colony-stimulating factor
GMP-140	granule membrane protein-140 (P-selectin)
G-protein	guanyl nucleotide binding protein
GP IIb-IIIa (αIIbβ3)	glycoprotein integrin
GRB-2	growth factor binding protein
GTP	guanosine triphosphate
gly-CAM-1	glycosylation-dependent cell adhesion molecule-1
H_2O_2	hydrogen peroxide
Hmac-1 (CD11b/CD18)	β_2 integrin
HTAB	hexadecyltrimethyl ammonium chloride
IBMX	isobutyl methylxanthine
ICAM-1	intercellular adhesion molecule-1
ICAM-2	intercellular adhesion molecule-2
ICAM-3	intercellular adhesion molecule-3
IL-1	interleukine-1
IL-3	interleukin-3
IL-8	interleukine-8
IP_3	inositol 1,4,5-trisphosphate
LAD-1	leukocyte adhesion molecule-1
LAM-1	lectin adhesion molecule-1
LECAM-1	lectin-like cell adhesion molecule-1
LFA-1 (CD11a/CD18)	lymphocyte function-associated antigen-1

L-NMMA	L-N^G-monomethyl arginine
LPS	bacterial lipopolysaccharide
LTB_4	leukotriene B_4
Mabs	monoclonal antibodies
MABP	mean arterial blood pressure
MadCAM-1	mucosal addressin cell adhesion molecule-1
MAPK	mitogen-activated protein kinase
MGSA	melanocyte growth-stimulating activity
MIP-1α	macrophage inflammatory protein-1α
MIP-1β	macrophage inflammatory protein-1β
MO-1 (CD11b/CD18)	β_2 integrin
MPO	myeloperoxidase
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NAP-1	neutrophil activating peptide-1
NAP-2	neutrophil activating peptide-2
nNOS	neuronal nitric oxide synthase
NBT	nitroblue tetrazolium chloride
NO	nitric oxide
NO_2^-	nitrite ion
NO_3^-	nitrate ion
NRS	normal rabbit serum
NSB	non-specific binding
O_2^-	superoxide anion
OCI^-	hypochlorite
$ONOO^-$	peroxynitrite
P150/95 (CD11c/CD18)	β_2 integrin
PA	phosphatidic acid
PADGEM (GM140)	platlet activation-dependent granule-external membrane
PAF	platelet activating factor
PBS	phosphate buffer saline
PC	phosphatidylcholine

PDE	phosphodiesterase
PDGF	platelet-derived growth factor
PECAM-1	platelet-endothelial cell adhesion molecule-1
PEG	polyethylene glycol
PG	prostaglandin
PGE_1	prostaglandin E_1
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGI_2	prostacyclin
PI3-kinase	phosphatidylinositol 3-(OH) kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIP_3	phosphatidylinositol 3,4,5-trisphosphate
PKA	protein kinase A
PKC	protein kinase C
PKCα	protein kinase Cα
PKCβ	protein kinase Cβ
PLA_2	phospholiapse A_2
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol myristate acetate
PSGL-1	Mucin-like P-selectin glycoprotein ligand 1
RIA	radioimmunoassay
SAG	superoxide anion generation
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SEM	standard error of the mean
SBP	systolic blood pressure
SCR	short consensus repeat
SMG	small molecular weight GTP-binding protein
SOD	superoxide dismutase
TMB	3,3',5,5'-tetramethylbenzidine
TNF-α	tumour necrosis factor-α

TNF-β	tumour necrosis factor-β
TTC	2,3,5-triphenyl tetrazolium chloride
VCAM-1	vascular cell adhesion molecule 1

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CHAPTER 1

GENERAL INTRODUCTION

1.1 NEUTROPHILS

Neutrophils comprise a fundamental component of the non-specific immune response to bacterial infection. The ability of neutrophils to combat infectious agents is due to a number of specific activities including adherence to blood vessel walls and transmigration into tissues, random (non-directed) migration and chemotaxis, phagocytosis, and microbial killing (Root & Cohen, 1981; Sawyer *et al.*, 1989). While these functions are essential for host defence against invading micro-organisms, it is now equally clear that inappropriate or excessive activation of neutrophils may contribute to inflammatory tissue injury in a variety of clinical scenarios e.g. the acute respiratory distress syndrome, rheumatoid arthritis and ischaemia-reperfusion injury. Thus modulation of the activation status of the neutrophil is of key importance in determining the balance between defence and injury.

1.1.1 Neutrophil production

Neutrophils are the most common type of leukocyte in blood and constitute 40-75% of circulating leukocytes. They are small cells with a diameter of 12-15 μm. The most prominent feature of the neutrophil is the highly lobulated nucleus. When mature, there are usually five lobes connected by fine strands of nuclear material. Under conditions of complete physical and mental relaxation, the usual basal level of neutrophils is 5 to 7 $\times$ 10^9 cells/l of blood. Their numbers can increase 10-fold during infection.

The development of mature neutrophils from stem cells involves the processes of differentiation, amplification of cell numbers and cellular maturation. Granulocyte progenetor cells (CFU-GM) in the bone marrow produce myeloblasts, which in turn differentiate via several recognisable morphological stages into mature non-dividing polymorphonuclear neutrophils. The myeloblasts, promyelocytes, myelocytes, band cells and polymorphonuclear neutrophils represent the stages in neutrophil development. Several factors that stimulate neutrophil release from the bone marrow have been identified including Granulocyte Colony-stimulating factor (G-CSF), Granulocyte-Macrophage Colony-stimulating factor (GM-CSF), the fifth component

of complement (C5a), tumour necrosis factor-α (TNF-α), tumour necrosis factor-β (TNF-β), and possibly a cleavage product of the third component of complement (Price *et al.*, 1994). Neutrophils are released from a storage pool in the bone marrow into the peripheral blood where they circulate for about 6-10 hours. In the blood there are two pools of about equal size: the circulating pool and the marginating pool. Finally, they enter the tissues where they perform their phagocytic function and probably live for 1-2 days before they are destroyed during defensive action or as a result of senescence.

1.1.2 Subcellular structure of neutrophils

Mature neutrophils contain several types of granules and other subcellular organelles. Four well-defined types of granules have been defined in neutrophils: primary granules (azurophilic granules), secondary granules (specific granules), tertiary granules (gelatinase granules), and secretory vesicles.

Many constituents of the neutrophil plasma membrane have been defined. These include membrane channels, adhesive proteins, and receptors for various ligands, ion pumps, and ectoenzymes. Many of membrane protein molecules probably play a role in regulating the neutrophil response.

Like many other cells, neutrophils contain a complex cytoskeleton. Alterations in the distribution of cytoskeletal elements may be important in chemotaxis, phagocytosis, and exocytosis. Many protein components of this cytoskeleton have been identified, including actin, actin-binding protein, α-actinin, gelsolin, profilin, myosin, tubulin, and tropomyosin. Actin accounts for approximately 10% of neutrophil protein (Southwick, 1983).

1.1.3 Neutrophil function

The main function of neutrophils is to destroy and remove invading micro-organisms or inflammatory debris. To carry out these functions, neutrophils exhibit a variety of rapid and co-ordinated responses designed to transport the cells rapidly to the area of inflammation and to deal with the inciting agent. Neutrophils are attracted to

inflammatory sites and/or sites of infection through the production of chemoattractant mediators at these sites (see section 1.1.3.1). As a result of chemoattractant receptor activation, neutrophils are stimulated to move, adhere and de-adhere, rearrange their cytoskeleton and ultimately to phagocytose infectious micro-organisms (see section 1.1.3.2). In addition they secrete granule contents containing proteolytic enzymes and antibacterial proteins (see section 1.1.3.3), and activate the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to generate toxic metabolites of oxygen (see section 1.1.3.4). These responses are collectively termed effector responses. Despite the continued presence of chemoattractant, neutrophils in suspension terminate these responses within 5-10 minutes after stimulation. Termination of the neutrophil response to chemoattractants has been attributed to the association of bound receptors with the cytoskeleton and the segregation of these receptors to domains of the plasma membrane, which is rich in actin and fodrin but depleted of the GTP-binding proteins required for further signal transduction (Jesaitis *et al.*, 1984, 1986, 1989).

1.1.3.1. Chemotaxis and chemotactic factor receptors

Chemotaxis is defined as the directed movement of a cell along a chemical gradient and is included in the more general phenomenon of cell motility. The ability of neutrophils to move along a chemotactic gradient is essential to their accumulation at sites of injury or infection. The initial step in the neutrophil response to infection is the detection of an appropriate signal. The interaction of bacteria with blood components, especially antibodies and the complement system, results in the formation of various chemotactic factors. In some instances the bacteria directly release factors that are chemotactic for neutrophils.

Several types of chemoattractant of different origin are known to activate neutrophils (Table 1.1). By 1986, the structural and functional properties of the "classical" chemoattractants N-formylmethionylleucylphenylalanine (fMLP), C5a, platelet activating factor (PAF) and leukotriene B_4 (LTB_4) had been extensively detailed (Hwang, 1990; Goldstein, 1992; see review in Snyderman *et al* 1992). N-formylmethionyl oligopeptides like fMLP are products of bacteria (Schiffmann *et*

al., 1975) or derive from mitochondria of damaged tissue (Carp, 1982). The complement fragment C5a is formed in blood plasma and inflammatory exudates upon complement activation (Fernandez *et al.*, 1978). PAF and LTB_4 are lipid mediators released by activated cells including the neutrophils themselves (Baggiolini *et al.*, 1988; Crooke *et al.*, 1991). Chemokines, a new class structurally and functionally related to cytokines, has been identified as chemotactic for leukocytes (Oppenheim *et al.*, 1991). These chemokines are produced by particular cell types. This family of chemokines has been divided in two groups according to the position of the first two cysteine residues, which are either separated by one amino acid residue (C-X-C or α chemokines) or are adjacent (C-C or β chemokines). The C-X-C subfamily of chemokines preferentially activates neutrophils and includes IL-8, neutrophil activating peptide-2 (NAP-2), melanocyte growth-stimulating activity (MGSA), granulocyte chemotactic protein-2 (GCP-2), and interferon-inducible protein-10 (IP-10). The C-C subfamily of chemokines activates a large number of cell types, including monocytes, lymphocytes, basophils and eosinophils (Baggiolini & Dahinden, 1994). This subfamily includes, macrophage inflammatory protein-1α (MIP-1α), and MIP-1β, monocyte chemotactic protein 1 (MCP-1) (Murphy, 1994).

Neutrophils express various receptors for a variety of chemotactic factors such as those for fMLP, C5a, PAF, LTB_4 (Baggiolini *et al.*, 1991) and IL-8 (see review Murphy, 1994) on their cell surface. All of these receptors are seven-transmembrane-domain-rhodopsin-like G protein-coupled receptors (Probst *et al.*, 1992; see review Murphy, 1994). The most extensively studied chemotactic receptors are those for the bacterial peptide fMLP which is a potent polymorphonuclear leukocyte secretagogue and chemotactic agent (Painter *et al.*, 1984). Both high and low affinity receptors for fMLP have been described (Snyderman *et al.*, 1985), and these receptors are subject to up and down regulation. The initial activation of the neutrophil occurs when soluble chemotactic factors bind to their receptors on the neutrophil surface. The association kinetics for these receptor-ligand interactions are very rapid. Typically, sufficient receptor-ligand interaction to initiate neutrophil activation occurs within seconds.

Table 1.1 *Neutrophil chemoattractants*

Chemoattractant	**Source**
Classic chemoattractants *(1)*	
N-formyl peptides	Bacterial (mitochondrial) protein synthesis
C5a	Complement activation
LTB_4	Arachidonic acid metabolism
PAF	Phosphatidylcholine metabolism
C-X-C chemokines *(2)*	
IL-8 (NAP-1)	Multiple cells including T-cells, monocytes, endothelial cells
β-Thromboglobulin (NAP-2/CTAP)	Degradation of platelet α-granule protein
gro-α (MGSA)	Multiple cells including endothelial cells, monocytes
ENA-78	Epithelial cells
C-C chemokines *(3)*	
MIP-1α, β	Monocytes, T cells

(1) References : Devreotes & Zigmond, (1988); Snyderman & Uhing, (1992).
(2) References : Oppenheim et al., (1991); Miller & Krangel, (1992); Kuna et al., (1993).
(3) References : Oppenheim et al., (1991); Miller & Krangel, (1992); Tanaka et al., (1993); Kuna et al., (1993).
Modified from Springer T. Traffic signal for lymphocyte recirculation and leukocyte emigration; the multistep paradigm. Cell, 1994; 76: 301-314

Occupancy of a critical number of receptors "activates" the neutrophil and leads to rapid shape changes (polarisation) and directional movement (Snyderman *et al.*, 1985). Binding of chemoattractants to receptors generates intracellular signals leading to the alteration in the cytoskeleton involved in the motile response. Neutrophils stimulated by chemoattractants line up, with the leading edge of the cells oriented in the direction of the highest concentration of the chemoattractant stimulus. Signals that emit from the oriented leading edge of the neutrophil to the cell "motor" result in appropriate cell movement. Occupancy of neutrophil chemotaxis receptors

appears to generate multifunctional signals. For example, exposure of neutrophils to a single stimulus (such as fMLP) can cause several different neutrophil activities, including chemotaxis, degranulation, and oxidative burst. The activity expressed appears to depend on the concentration of the stimulus and the modulation of the receptor. For example, occupancy of adenosine A_1 receptors enhances chemotaxis induced by fMLP (Rose, *et al.*, 1988).

Moving neutrophils assume a polarised morphology with an anterior lamellipodium or pseudopodium extended in the direction of movement, a cell body that is elongated parallel to the axis of lamellar protusion, and a knob-like tail or "uropod". The lamellipodium is a relatively broad flattened region that is the site of dynamic alteration in the actin cytoskeleton. Chemotaxis begins with the protusion of a pseudopodium at the front of the cell. This occurs where the submembraneous actin filament network (the cortex) becomes less filamentous. As the cell moves, the pseudopodium ruffles rapidly. Part of the pseudopodium adheres to the underlying surface and the contents of the cell move forward into the pseudopodium, making the pseudopodium less prominent. This cycle is then repeated with the protusion of another pseudopodium. Chemotaxis occurs by repetitions of this process, although often the process is so well co-ordinated as to appear as a continuous gliding motion. The mechanism of these cell movements appears to involve alterations in the polymerisation state of actin, regulated by several proteins, including actin binding protein, gelsolin, and others as well as ATP-dependent contraction of the actin network mediated by myosin. Local contraction of the cytoskeleton could move intracellular components forward into an area where the cortical gel has weakened because of shortening of actin filaments beneath the surface of the advancing pseudopodium. Gelsolin is important in neutrophil chemotaxis (Stossel, 1994), but other proteins can compensate to some extent in its absence. The increase in free calcium that alters the cytoskeleton by activating gelsolin, and thereby decreasing filamentous actin with a resultant decrease in viscosity, may play a role in locomotion. In addition, the transient dissolution of the submembraneous cytoskeletal network may allow closer contact of intracellular granules with the

plasma membrane, facilitating granule fusion and release. Some granule release occurs with chemotaxis.

The development of a two compartment chamber separated by a leukocyte-permeable membrane has enabled quantitation of chemotaxis *in vitro* and facilitated the investigation of chemotactic factors (Boyden, 1962; Baum, 1971). Such studies revealed that neutrophils show directional migration under the influence of chemotactic agents, but that a concentration gradient is needed for migration to occur.

1.1.3.2 Phagocytosis

Phagocytosis is the engulfment of microbial particles that takes place when phagocytes recognise serum opsonins (complement and immunoglobin) or specific sugars deposited on the surface of microbes. When a neutrophil meets a particle, it envelops the particle with pseudopodia, which fuse around it, forming a phagosome that rapidly fuses with azurophilic and specific granules. Phagocytosis facilitates effective killing by trapping a particle in a phagosome. The receptors that participate in the ingestion and killing of microbes can be divided into those that require the target particle to be coated by serum opsonins and those that do not (Ofek *et al.*, 1992). Nonopsonic phagocytosis allows ingestion and killing of microbes as a result of the presence of neutrophil surface receptors that recognise microbial sugar molecules (Ofek *et al.*, 1992). However, many microbes are most effectively killed in the presence of serum opsonins (Leijh *et al.*, 1979; Mannion *et al.*, 1990). The most critical serum opsonins are immunoglobulin and complement.

1.1.3.2.1 Immunoglobulin receptors

Binding and ingestion of some common bacterial pathogens depend on effective opsonisation with immunoglobulin G (IgG). A deficiency of IgG can increase the likelihood of severe infection with these organisms. Neutrophils express at least two types of receptors that recognise the Fc components of the IgG: FcRII and FcRIII. FcRII is a membrane glycoprotein, whereas FcRIII is a glycosylphosphatidylinositol-link protein (Huizinga *et al.*, 1990; Kimberly *et al.*,

1990). Occupancy of phagocytosis receptors allows particle engulfment and stimulates microbicidal mechanisms. Phagocytosis is also affected by occupancy of adenosine receptors; occupancy of adenosine A_2 receptors facilitates Fc-mediated phagocytosis, whereas occupancy of adenosine A_1 receptors has the opposite effect (Salmon & Cronstein, 1990).

1.1.3.2.2 Complement receptors

Neutrophils display several types of receptors for complement, each of which recognises a different component such as the first component of complement (CR1), and the third component of complement (CR3) (Fallman *et al.*, 1993). Binding of opsonically active complement component to microbial pathogens is essential, and CR3 is of greatest importance. CR3 mediates the binding of C3bi-coated particles. CR3 also known as Mac-1, is a member of the β_2 integrin family. CR1 is primarily responsible for recognition of C3b. Complement coating of a particle allows engulfment.

1.1.3.3 Granule release

Neutrophils contain four well-defined types of intracellular granules: azurophilic, specific, gelatinase granules and secretory vesicles. The azurophilic granules contain many antibacterial components, and the fusion of these granules with phagocytic vesicles is important in bacterial killing. Among the azurophilic granule contents is myeloperoxidase (MPO), a protein that catalyses the production of hypochlorite (OCl^-) from chloride and hydrogen peroxide (H_2O_2) produced by oxidative burst. The presence of MPO in azurophilic granules is of prime importance for full function of the oxygen-dependent bactericidal system. MPO constitutes approximately 5% of the dry weight of the neutrophil (Schultz & Kaminker, 1962). At the time of respiratory burst activation, MPO is excreted into the extracellular environment and has the capacity to generate an array of oxidising species with considerable cytotoxic potential. Defensins, a group of cationic proteins that kill a variety of bacteria, fungi, and viruses (Ganz & Lehrer, 1995; Levy *et al.*, 1995; Martin *et al.*, 1995), also constitute approximately 5% of total neutrophil protein (Lehrer *et al.*, 1988). Other components of azurophilic granules include lysozyme, which degrades bacterial

peptidoglycan (Arnheim *et al.*, 1973), bactericidal permeability-increasing-protein (BPI), azurocidine and the serine proteinases elastase that may alter locomotion by hydrolysing certain extracellular matrix components, cathepsin G, proteinase 3, and others (Baggiolini & Dewald, 1985; Tanaka *et al.*, 1985; Henson & Johnson, 1987; Kao *et al.*, 1988).

Although some specific (also called secondary) granules fuse with phagocytic vesicles, they are more readily released from the cell, suggesting an important function in the extracellular millieu. The known contents of these granules include apolactoferrin, the major specific granule protein, vitamin B_{12}-binding protein, plasminogen activator, and collagenase. Lysozyme and some gelatinase are also present in specific granules. About two-thirds of lysozyme is in the secondary granule (Quesenberry & Levitt, 1979). These secretory granules are involved in many inflammatory processes including complement activation (Wright & Gallin, 1977), leukocyte adhesion, collagen removal and bacterial cell wall lysis. Release of specific granule contents may modify the inflammatory process. For example, collagenase may degrade collagen, and thus augment movement through collagen and participate in tissue remodelling. Apolactoferrin, which binds iron, may exert an antibacterial effect by depriving bacteria of iron, alter hydroxyl radical formation, and alter cell adhesion (Oram & Reiter, 1968; Oseas *et al.*, 1981; Boxer *et al.*, 1982; Aruoma & Halliwell, 1987). Membrane components of secondary granules are upregulated during granule release and may play a role in regulating the expression of these membrane proteins on the cell surface.

Tertiary or gelatinase granules contain gelatinase in addition to other components and, like collagenase, this enzyme may play a role in extracellular matrix remodelling during locomotion. Secretory vesicles, which largely distribute in the plasma membrane fraction, have also been described. A defining feature of secretory vesicles is their rapid and complete translocation to the surface membrane with weak stimulation (Sengelov *et al.,* 1994). These secretory vesicles also contain alkaline phosphatase, cytochrome b_{558}, and fMLP receptors. Both tertiary granules and the secretory vesicles contain membrane proteins that can be rapidly up-regulated to the

cell surface (Kjeldsen *et al.*, 1994), and may play a role in alterations of the functional utility of these surface proteins following stimulation.

1.1.3.4 Respiratory burst

Neutrophil activation is accompanied by a prominent increase in molecular oxygen (O_2) use called the oxidative burst or respiratory burst. This phenomenon was first described by Baldrige and Aldo (cited in Karnovsky, 1962). The respiratory burst, a series of metabolic events, is a distinguishing property of phagocytes and takes place when they are appropriately stimulated. Most of the respiratory burst results from the assembly and activation of NADPH oxidase (Hurst & Barrette, 1989; Clark, 1990; Gallin, 1991; Heyworth *et al.*, 1991; Rotrosen *et al.*, 1992). This system is responsible for converting O_2 into O_2^- by transporting an electron from NADPH (Babior, 1984) and essential for the bactericidal function of neutrophils.

$$2O_2 + NADPH \xrightarrow[\text{Cyt } b_{558}]{\text{oxidase}} 2{\cdot}O_2^- + NADP^+ + H^+$$

A b-type cytochrome (cytochrome b_{558}), a heterodimer composed of 22 kDa and 91 kDa subunits, oxidises NADPH, leading to the formation of O_2^-. The O_2^- then rapidly dismutates to form H_2O_2, a reaction catalysed by superoxide dismutase. The hypothetical model of NADPH oxidase activation is shown in Figure 1.1.

In its dormant state, NADPH oxidase is composed of both membrane-bound and cytosolic components. The membrane bound components include $gp91^{phox}$ and $p22^{phox}$ subunits of cytochrome b_{558} (the term *phox* indicates that the protein is a component of phagocyte oxidase). The $gp91^{phox}$ contains binding sites for both a flavin adenine dinucleotide (FAD) and NADPH (Rotrosen *et al.*, 1992; Segal *et al.*, 1992).

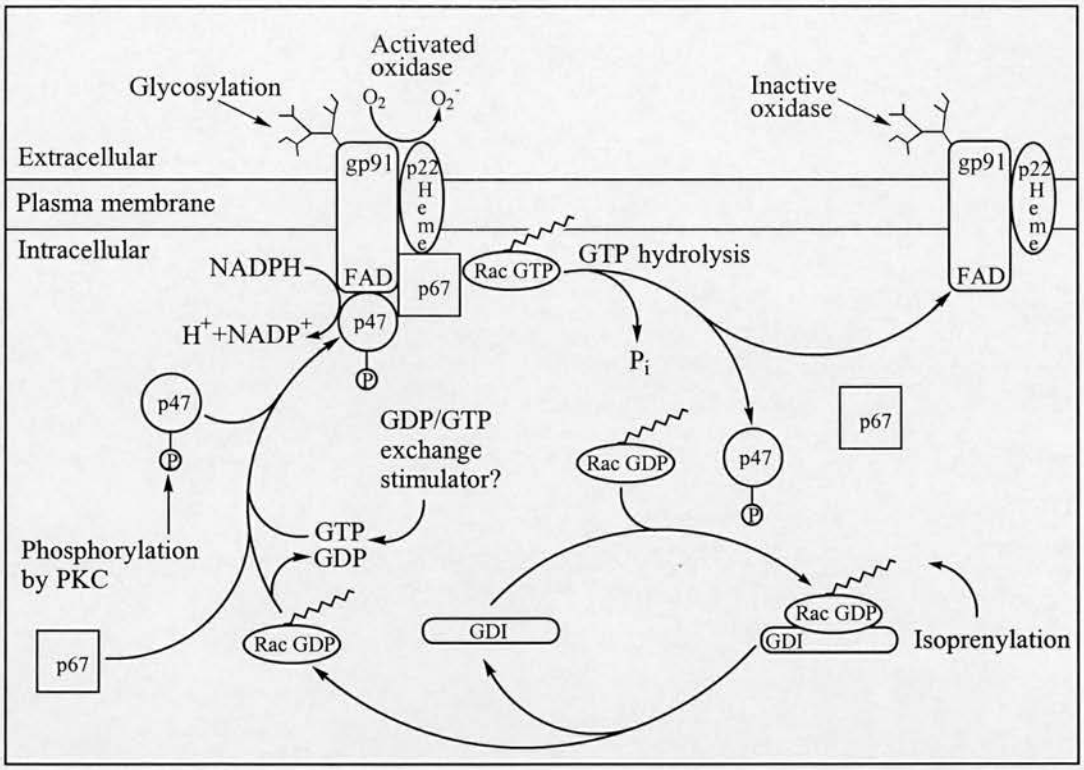


Figure 1.1 *Schematic representation of structural features in the activation of NADPH oxidase. The cytochrome $_{b558}$ is an integral membrane protein with two subunits, gp91^{phox} (gp91) and p22^{phox} (p22). The glycosylation site of the cytochrome is on the exterior, and the FAD and NADPH binding sites are on the interior, of the plasma membrane. On activation, cytosolic constituents p47^{phox} (p47), p67^{phox} (p67) and Rac 2 (Rac) are translocated to the plasma membrane where they bind to the cytochrome and initiate O_2^- synthesis. Activation requires dissociation of a GTP dissociation inhibitor (GDI) from Rac 2 followed by exchange of GDP for GTP. Oxidase activation also involves phosphorylation of at least one of the cytoplasmic subunits, p47^{phox}. Hydrolysis of GTP bound to Rac leads to inactivation and dissociation of the oxidase complex. Modified from Bastion, N.R.: Assembly and regulation of NADPH oxidase and nitric oxide synthase, Current Opinion in Immunology, 1994, 6:131-139.*

Several lines of evidence suggested that heme (iron protoporphyrin IX) in the cytochrome is located in the p22^{phox} (Segal, 1989). Three cytosolic components are also required for electron transfer to the cytochrome. They include 47 kDa (p47^{phox}), 67kDa (p67^{phox}) protein, and the low-molecular-mass GTP-binding protein, Rac 2. Rac 2 is also present in the cytosol in its inactive state (with GDP bound), presumably complexed with a GDP dissociation inhibitor that serves to keep Rac 2 in its inactive state. On activation, cytosolic components p47^{phox}, p67^{phox} and Rac 2

(Rac) are translocated to the plasma membrane (Dusi *et al.*, 1993) where they bind to the cytochrome and initiate O_2^- synthesis. Uhlinger *et al* (1993) proposed a model in which $p47^{phox}$ binds directly to cytochrome b_{558}, and $p67^{phox}$ interacts with both $p47^{phox}$ and the GTP-regulatory protein Rac 2. Activation requires dissociation of a GTP dissociation inhibitor (GDI) from Rac 2 followed by exchange of GDP for GTP. This exchange on Rac 2 is catalysed by a GDP/GTP dissociation stimulator in the intact cell (Knaus *et al.*, 1992). Oxidase activation also involves phosphorylation of at least one of the cytosolic subunits, $p47^{phox}$ (Heyworth *et al.*, 1991; El Benna *et al.*, 1994). Isoprenylation of the Rac protein (represented in the Figure 1.1 by a zigzag) is necessary for oxidase activity and may function as a membrane anchor that helps stabilise the assembled oxidase complex. Hydrolysis of GTP bound to Rac to GDP leads to inactivation and dissociation of the oxidase complex. Rac 2 has a very rapid intrinsic rate of GTP hydrolysis, which may confer unique regulatory properties to the Rac 2 regulated NADPH oxidase (Knaus *et al.*, 1992). Regulation of GTPase activity of Rac may be a key factor in the regulation of NADPH oxidase activity. In its active state, the FAD redox centre accepts electrons from NADPH and passes them on to O_2 via the heme groups in cytochrome b_{558}. The activated oxidase is readily detected by nitroblue tetrazolium (NBT) or cytochrome C reduction or the production of chemiluminescence.

Small concentrations of oxygen free radicals are present in normal oxidative metabolism but are controlled by the body's defence mechanisms, such as the enzymes superoxide dismutase, catalase, and glutathione peroxidase. Activated neutrophils can generate a number of oxygen-derived free radicals including : O_2^-, H_2O_2, and hydroxyl radical (OH). The O_2^- itself reacts with NO to form peroxynitrite ($ONOO^-$).

Though stable in ionic form, this compound rapidly decomposes when protonated to form OH, which has powerful oxidising activity (Beckman *et al.*, 1990). The proposed reactions are:

$$NO + O_2^- \longrightarrow ONOO^-$$

$$ONOO^- + H^+ \longleftrightarrow ONOOH \text{ (peroxynitrous acid)}$$

$$ONOOH \longleftrightarrow OH + NO_2 \text{ (nitrogen dioxide)}$$

$$ONOOH \longrightarrow NO_3^-$$

This reaction process ultimately produces nitrites and nitrates. Since neutrophils have been shown to produce NO in high concentration when activated, peroxynitrite, product of O_2^- and NO, may be of considerable importance in neutrophil cytotoxicity.

1.1.3.5 Bacterial killing and digestion

Phagocytic cells are ultimately expected to eliminate microbial pathogens. Their failure to do so permits the development of serious and possibly even fatal infection. Two types of system work in concert to eliminate pathogens: reactive oxygen intermediates and varieties of microbicidal proteins stored in granules in the cytoplasm of neutrophil. Although these two systems interact considerably, oxidative and nonoxidative mechanisms will be discussed separately.

1.1.3.5.1 Oxygen-dependent antimicrobial system

Optimal killing of many species of bacteria requires products from the oxidative burst. Bacterial killing decreases under anaerobic conditions, so the respiratory burst is important to bactericidal activity. As explained above, activated neutrophils produce O_2^- via a multicomponent NADPH-dependent oxidase. Subsequent reactions result in the formation of H_2O_2 and hypochlorous acid (HOCl), which increase bacterial killing.

Although O_2^- has some antibacterial activity, most O_2^- is rapidly converted to H_2O_2 by dismutation, either spontaneously or catalytically by superoxide dismutase (SOD):

$$2O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2$$

Of the microbial oxidants generated by the respiratory burst, O_2^- and H_2O_2 are not potent microbicides, but rather function as starting materials to generate more potent oxidising radicals, such as oxidised halogens (Babior, 1984).

1.1.3.5.1.1 Myeloperoxidase-mediated oxygen-dependent bacterial killing with oxidised halogens

Some of the granular proteins, especially MPO and lactoferrin, play an important role in the oxidative chemistry of the phagosome. MPO is released into the phagosome during granule-phagosome fusion. In the presence of a halide (chloride being of maximal importance for neutrophils), MPO converts H_2O_2 generated during phagocytosis to an oxidised halogen that is a potent antimicrobial, such as hypochlorite (OCl^-) (Harrison & Schultz, 1976):

$$Cl^- + H_2O_2 \xrightarrow{MPO} H_2O + OCl^-$$

HOCl appears as the most likely mediator of oxygen-dependent bacterial killing in the neutrophil phagosome but OCl^- can also react with amines to form long-lived chloramines. Some N-chloramines have greater microbicidal potency than O_2^- or H_2O_2 (Weiss & Lampert, 1983). OCl^- can interact with O_2^- to form OH, albeit in a low concentration (Ramos *et al.*, 1992). The oxidative chemistry of the phagosome is shown in Figure 1.2.

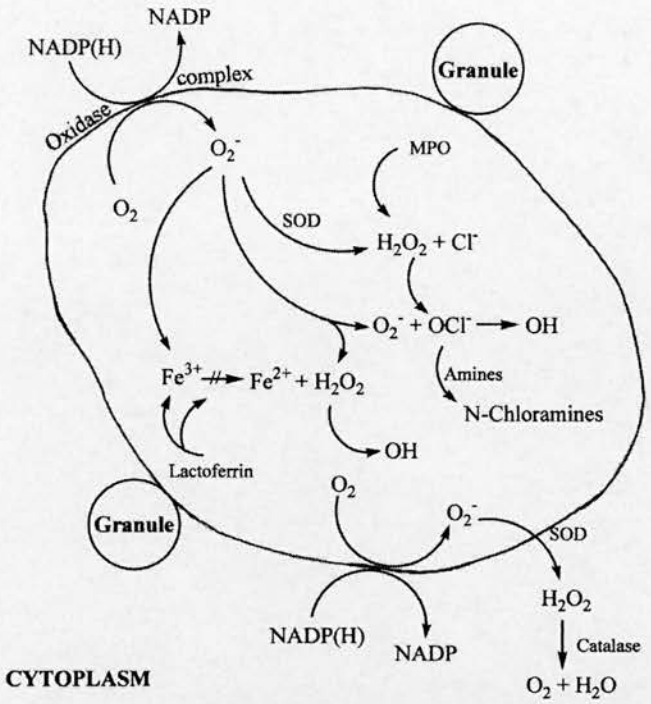


Figure 1.2 *Oxidative chemistry of the phagosome. A unique enzyme system catalyses the formation of O_2^- (see Figure 1.1). The acid pH within the phagosome favours the rapid dismutation of O_2^- to H_2O_2. This reaction can be quickly catalysed by superoxide dismutase (SOD). HOCl is formed from the oxidation of chloride by MPO-H_2O_2. OCl^- can react with O_2^- to form hydroxyl radical or with some amines to form N-chloramines, which are toxic and mutagenic to micro-organisms. O_2^- can reduce Fe^{3+} to Fe^{2+}, which then reduces H_2O_2 to hydroxyl radical. However, lactoferrin can bind Fe^{3+} to prevent the formation of hydroxyl radical. Modified from Cohen, M.S.: Molecular events in the activation of human neutrophils for microbial killing. Clinical Infectious Diseases, 1994, 18 (suppl. 2): S170-9.*

1.1.3.5.1.2. Myeloperoxidase-independent (but oxygen radical-dependent) bacterial killing

This antimicrobial system is important because cells with no detectable MPO activity retain antibacterial actions that require oxygen. Bacterial killing in MPO-deficient cells is associated with greater oxygen consumption than in normal cells.

1.1.3.5.2 Oxygen-independent antimicrobial system

A variety of microbes can be killed by phagocytes under strict anaerobic conditions. Both antimicrobial proteins and phagosomal acidity play critical roles in this process. The delivery of the wide array of antibacterial compounds to the

phagosome by fusion with azurophilic and specific granules generally results in bacterial killing caused by the direct actions of the granule contents. In addition, these effects are potentiated by the acidification of the phagosome, caused partly by the granule content themselves, as well as active translocation of H^+ ion into the phagosome by ion pumps. The antimicrobial proteins of neutrophils including defensins, lactoferrin, lysozyme, BPI, cationic proteins, azurocidin and the serine proteinases elastase, cathepsin G and proteinase 3 (Spitznagel, 1990), make an important contribution to the function of neutrophils. This contribution is reflected by the ability of neutrophils to kill a variety of microbes under anaerobic conditions. In addition, microbicidal proteins work in conjunction with free radicals. The acidification of the phagosome is an important part of the microbicidal process. Some microbes are killed by low pH, and some neutrophilic antimicrobial systems require a low pH for optimal activity (Hurst & Barrette, 1989).

1.1.3.6 Signalling

Chemoattractants (Table 1.1) and other inflammatory agents mediate their biological responses by binding to and activating their receptors on the surface of inflammatory cells (Gerard & Gerard, 1994). A variety of chemoattractant receptors have now been cloned, and these receptors belong to a large family of seven transmembrane domain receptors, which are coupled to heterotrimeric guanine nucleotide regulatory proteins (G proteins) (Thelen & Dewald, 1993; Gerard & Gerard, 1994; Murphy, 1994). These receptors mediate chemotaxis, degranulation and activation of the NADPH oxidase. The binding of chemoattractants to their receptor results in the activation of G proteins, which act as a molecular switch to relay information from the activated receptor to downstream effector molecules. These effector molecules may be enzymes that generate second messengers or ion channels. A large number of heterotrimeric G proteins, consisting of α ($G\alpha$) and $\beta\gamma$ ($G\beta\gamma$) subunits have been isolated so far, and many of these are present in leukocytes (Amatruda *et al.*, 1993).

In resting condition, G proteins exist as heterotrimeric complexes with GDP bound to the α subunit. Receptor activation leads to a conformational change in $G\alpha$,

resulting in an exchange of GTP for GDP. This interaction causes the dissociation of Gβγ from the heterotrimeric complex (Figure 1.3).

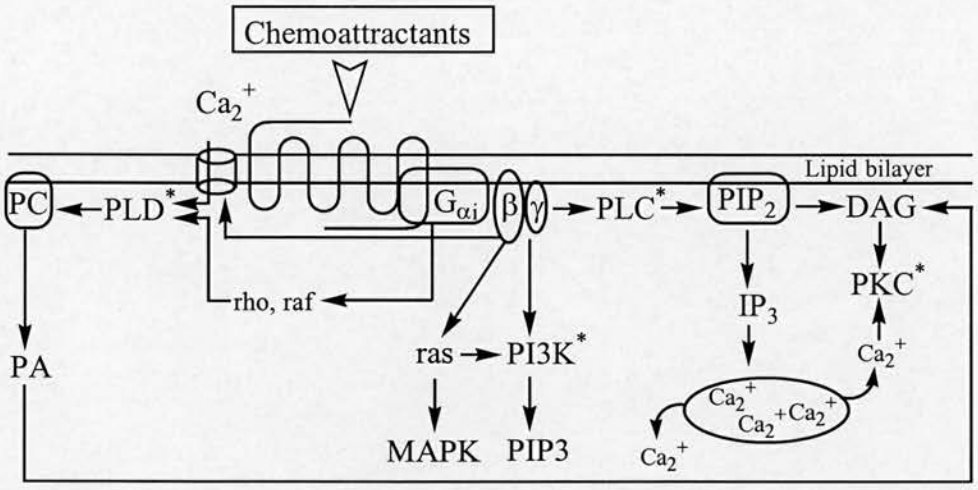


Figure 1.3 *Chemoattractant receptor signalling pathways. The receptor is indicated as the seven transmembrane-spanning structure at the cell surface within the lipid bilayer. The arrows indicate activation. $G_{\alpha i}$ and $\beta\gamma$ are the different subunits of the G protein Gi. PC, phosphatidylcholine; PIP_2 phosphatidylinositol 4,5-biphosphate; PLC, phospholipase C; PLD, phospholipase D; DAG, diacylglycerol; PKC, protein kinase C; IP_3, inositol 1,4,5-triphosphate; MAPK, mitogen activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol triphosphate. Enzymatically active forms are indicated by asterisks. Modified from Ali, H.: Mechanism of inflammation and leukocyte activation. Medical Clinics of North America, 1997, 81:1-28.*

Free α subunits were believed to transduce all signals of the activated receptors. It is now realised that $G_{\beta\gamma}$ also plays an essential role in mediating many of these events, including the activation of phospholipase C (PLC) (Lee *et al.*, 1995). Molecular cloning has revealed three classes of PLC: PLC_β, PLC_γ and PLC_δ, and each of these occurs in several isoforms (Lee *et al.*, 1995). $PLC_{\beta 2}$ is expressed predominantly in phagocytic leukocytes and is activated by the peptide chemoattractants, fMLP, C5a, and IL-8 (Camps *et al.*, 1992; Amatruda *et al.*, 1993). This enzyme catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate two second messengers, 1,2 diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG acts in conjunction with Ca^{2+} to activate various isoforms of protein kinase C (PKC),

whereas IP_3 mobilises Ca^{2+} from intracellular stores (Verghese *et al.*, 1987; Truet *et al.*, 1989; Gerard & Gerard, 1994).

The increase in the intracellular concentration of free calcium $[Ca^{2+}]i$ following stimulation by chemoattractants follows a biphasic course: a rapid and transient phase attributed to mobilisation of intracellular Ca^{2+} stores, followed by a more sustained phase that is dependent on the net influx of Ca^{2+} from the extracellular space due to an increase in plasma membrane permeability to Ca^{2+}. The role of intracellular calcium in the functional responses of neutrophils seems to be best established in the regulation of degranulation and secretion (Smolen, 1989) and phagolysosome fusion during phagocytosis (Jaconi *et al.*, 1991). In addition, transient increases in $[Ca^{2+}]i$ appear to play a role in modulation of adhesive events essential for effective cell motility. The role of calcium in other neutrophil functions such as activation of the NADPH oxidase has been a matter of debate. Certain neutrophil functions might be controlled by Ca^{2+} oscillations, rather than by sustained elevations of $[Ca^{2+}]i$ (Lew, 1989; Richter, 1990).

DAG accumulation in response to chemoattractants is biphasic in nature consisting of initial transient production of DAG that parallels PIP_2 hydrolysis via PLC. This is followed by phospholipase D (PLD) activation, which leads to DAG production from phosphatidylcholine (PC) via the sequential involvement of PLD and phosphatidate phosphohydrolase (Billah, 1993), which is more sustained and of greater magnitude (Truett, *et al.*, 1989; Snyderman & Uhing, 1992). Phosphatidic acid, generated from PC, may act as a second messenger itself or serve as a precursor for DAG (Billah, 1993). It may be involved in the activation of NADPH oxidase (Agwu *et al.*, 1991; Gelas *et al.*, 1992; Baggiolini *et al.*, 1993) and changes in the actin cytoskeleton (Ha & Exton, 1993). DAG may interact with putative DAG-binding proteins, leading to actin polymerisation (Shariff & Luna, 1992; Hitt & Luna, 1994).

In neutrophils, the most abundant isoform of PKC appears to be PKC_β, which translocates to the plasma membrane in a Ca^{2+}-dependent manner (Majumdar *et al.*,

1991). In intact cells, the activation of PKC_{α} and PKC_{β} results from either an increase in intracellular DAG alone or the synergistic action of increased $[Ca^{2+}]i$ and DAG. Activation of PKC, as well as various Ca^{2+}-sensitive protein kinases, catalyses protein phosphorylation, and this is believed to account for activation of the various neutrophil functions (Rossi, 1986; Lew, 1990; Billah, 1993; Thelen *et al.*, 1994). PKC has been postulated to be involved in a variety of effector pathways in neutrophils including: superoxide production, priming, chemotaxis, production of PAF and LTB_4, PLD activation, potentiation of arachidonic acid release, activation of the Na^+/H^+ antiporter, production of DAG, and regulation of secretion specific granules (reviewed by Huang, 1989).

A number of studies provided evidence for PKC- and Ca^{2+} independent mechanisms of leukocyte activation (McPhail *et al.*, 1984; Dewald *et al.*, 1988; Watson *et al.*, 1991). Therefore, chemoattractant signalling is more complex than originally envisioned, making use of alternate signalling pathways involving kinases and phosphatases, adapter molecules, and small GTP-binding proteins.

Other neutrophil plasma membrane receptors such as growth factors and cytokines and those receptors involved in phagocytosis: Fc and complement receptors, are linked to the cell interior by pathways primarily involving tyrosine phosphorylation. Tyrosine phosphorylation has been found to play an important role in signal transduction from various chemotactic factor receptors (Gomez-Cambronero *et al.*, 1989; Kusunoki *et al.*, 1992). Some chemotactic agents including fMLP, phorbol myristate acetate (PMA) (Berkow & Dodson, 1990), TNF-α (Akimaru *et al.*, 1992), GM-CSF (McColl *et al.*, 1991), and PAF (Rollet *et al.*, 1994) increase the tyrosine phosphorylation of a number of proteins in human neutrophils. This appears to be caused by both the activation of tyrosine kinases as well as by inhibition of tyrosine phosphatases (Berkow & Dodson, 1991). Diverse functional responses in neutrophils have been linked to pathways involving tyrosine phosphorylation including activation of the NADPH oxidase (Gaudry *et al.*, 1992; Laudanna *et al.*, 1993), migration (Gaudry *et al.*, 1992), and priming of neutrophils by G-CSF and TNF-α (Akimaru *et al.*, 1992). Neutrophils also express the *src* (*hck*, *fgr*, *fes* and

lyn) and *syk* families of nonreceptor protein tyrosine kinases (Bolen *et al.*, 1992). The involvement of protein tyrosine phosphatases in the regulation of tyrosine phosphorylation as well as their role in neutrophil responses is still unclear.

Serine and threonine kinases also appear to be involved in signalling, and some are activated by fMLP. These kinases may be involved in regulating early events in pathways leading to activation of the respiratory burst, cytoskeletal assembly and motility, and possibly secretion of granules.

Evidence suggests that chemoattractants stimulate the activity of a phosphatidylinositol 3-(OH) kinase (PI3-kinase) which catalyses the phosphorylation of (PIP_2) to generate phosphatidylinositol-3,4,5-trisphosphate (PIP_3) which has been implicated in signalling pathways leading to several functional responses including: the oxidative burst, secretion of granules, and cytoskeletal changes including membrane ruffling (cytoskeletal assembly) (Eberle *et al.*, 1990; Zhang *et al.*, 1993). This enzyme is activated by growth factors via its tyrosine phosphorylation. The G-protein-coupled chemoattractant receptors might directly regulate the activity of this PI3-kinase. In neutrophils, chemoattractant-mediated PI3-kinase activation requires G-protein activation, and the onset of PIP_3 generation coincides with actin polymerisation (Downey, 1994).

In addition to heterotrimeric G proteins, neutrophils express a number of small molecular-weight GTP-binding proteins (SMG), which are likely involved in cellular regulation. These proteins can be subdivided into three major families including the *Ras*, *Rho*, and *Rab* families of proteins (Polakis *et al.*, 1989; Bokoch *et al.*, 1994). Recent attention has focused on the pathway involving activation of SMG *Ras* and *Rho* in cytoskeletal regulation. Activation of *Ras* via receptor-coupled heterotrimeric GTP-binding proteins or via as yet unidentified tyrosine kinases binding to growth factor binding protein (GRB-2) and the guanine nucleotide exchange factor (SOS1), leads to alterations in the neutrophil cytoskeleton (Li *et al.*, 1993). *Ras* that has been activated either by tyrosine kinases receptors and/or G-protein-coupled receptors activates a number of enzymes including mitogen-activated protein kinase (MAPK).

Ras/MAPK pathway is likely to play an important role in the early signalling events leading to cell activation. However, the relationship of MAPK with cytoskeletal changes remains unproven. Other SMGs such as RhoA and Rac have also been implicated in activation of cell adhesion, formation of membrane ruffles and leukocyte chemotaxis (Ridley *et al.*, 1992; Downey, 1994; Laudanna *et al.*, 1996). A current view of chemoattractant receptor signalling is shown in Figure 1.4

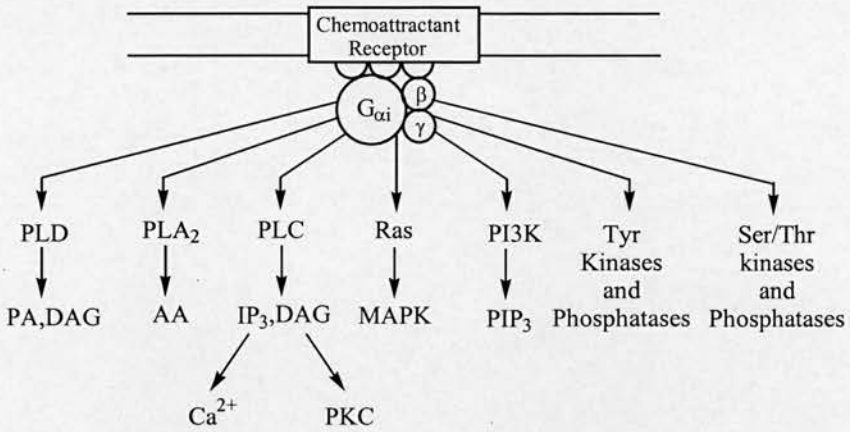


Figure 1.4 *A current view of chemoattractant receptor signalling: Multiple pathways. Schematic diagram summarising the known signalling pathways and effectors used by neutrophil chemoattractant receptor. Modified from Bokoch, G.M.: Chemoattractant signalling and leukocyte activation. Blood, 1995, 8: 1649-1660.*

1.1.4 Adhesion molecules: Neutrophil-endothelial adhesion

The migration of neutrophils from blood, through the endothelium, to the site of inflammation involves highly co-ordinated cell to cell adhesive interactions. This requires a series of activation steps mediated by inflammatory agents. Neutrophils have to interact with the vascular endothelium, traverse the endothelial cell layer, penetrate the basement membrane and move through the interstitial medium to reach the affected area. While this process is essential in destroying offending microbes as well as repairing injured tissue, abnormal or uncontrolled neutrophil infiltration contributes significantly to the pathology associated with inflammatory disorders. The interaction between neutrophils and endothelial cells is dependent on certain adhesion molecules that are expressed on the surface of both cell types (Springer,

1990). Some of the known adhesion molecules of neutrophils and endothelial cells are given in Table 1.2.

Three families of cell adhesion molecules are involved in leukocyte and endothelial cell interactions: selectins, integrins and the members of immunoglobulin (Ig-like) superfamily, which interact in a sequential manner to cause neutrophil transmigration.

1.1.4.1 Selectins

Selectins mediate the initial, low-affinity adherence of leukocytes to the endothelium, manifested by rolling along the endothelial cell surface under conditions of flow (Tedder *et al.*, 1995). There are three different selectins: L-selectin, E-selectin, and P-selectin. L-selectin (CD62L, LAM-1, LECAM-1) is constitutively expressed on the surface of most leukocytes (Butcher, 1991; McEver, 1991; Lasky, 1992; Bevilacqua & Nelson, 1993). The ligand for L-selectin is the glycoprotein known as glycosylation-dependent cell adhesion molecule-1 (Gly-CAM-1). E-selectin (CD62E, ELAM-1) is transiently synthesised and expressed on the surface of endothelial cells only following stimulation and its expression requires *de novo* protein synthesis. TNF-α, interleukin-1 (IL-1), substance P, bacterial endotoxin (lipopolysaccharide, LPS), and several other stimuli have been found to induce the expression of E-selectin (Bevilacqua *et al.*, 1987, 1989; Bevilacqua & Nelson, 1993; Carlos & Harlan, 1994; Springer, 1994; Imholf & Dunon, 1995; Tedder *et al.*, 1995). In contrast, P-selectin (CD62P, GMP-140, PADGEM) is constitutively synthesised and stored in the α granules of platelets and the Weibel-Palade bodies of endothelial cells (McEver, 1991; Lasky, 1992; Bevilacqua *et al.*, 1993). It can be rapidly mobilised to the cell surface by a number of cytokines and inflammatory mediators (Hattori *et al.*, 1989). Recent studies suggested that IL-1 and TNF also enhance biosynthesis of endothelial P-selectin (Gotsch *et al.*, 1994).

Table 1.2 *Neutrophil-Endothelial Cell Adhesion Proteins.*

Neutrophil Selectins		**Ligand**
L-selectin *(1)*	CD62L, LAM-1, Mel-14	Sialylated carbohydrates related to sLe^x (CD15s) and sLe^a *(12)*
Endothelial Selectins		**Ligand**
E-selectin *(2)*	CD62E, ELAM-1	Sialylated carbohydrates
P-selectin *(3)*	CD62P,GMP-140, PADGEM	Sialylated carbohydrates including PSGL-1 on neutrophils *(13)*
Neutrophil Integrins *(4)*		**Ligand**
αLβ2 *(5)*	LFA-1, CD11a/CD18	ICAM-1 (CD54) *(14)* ICAM-2 (CD102) *(15)* ICAM-3 (CD50)
αMβ2 *(6)*	Hmac-1, CD11b/CD18	ICAM-1, iC3b, fibrinogen, factor X
αXβ2	p150, 95, CD11c/CD18	iC3b, fibrinogen
Endothelial Ig Family *(7)*		**Ligand**
CD54 *(8)*	ICAM-1	CD11a/CD18, CD11b/CD18
CD102 *(9)*	ICAM-2	CD11a/CD18
CD31 *(10)*	PECAM-1	CD31/$\alpha_v\beta_3$?
Neutrophil Ig Family		**Ligand**
CD31	PECAM-1	CD31/$\alpha_v\beta_3$
CD50 *(11)*	ICAM-3	CD11a/CD18
CD66a	(Bilary glycoprotein)	CD66a, CD66c, CD66e
CD66b	(CGM6)	CD66c
CD66c	NCA50/90)	CD66a, CD66b, CD66c, CD66e

(1): Butcher, 1991; McEver, 1991; Lasky, 1992; Bevilacqua & Nelson, 1993.
(2): Bevilacqua et al., 1987, 1988; Bevilacqua & Nelson, 1993; Carlos & Harlan, 1994, Springer, 1994; Imholf & Dunon, 1995; Tedder et al., 1995.
(3): McEver, 1991; Lasky, 1992; Bevilacqua, 1993.
(4): Springer, 1990a; Arnaout, 1990; Kishimoto et al., 1992; Cronstein & Weissmann,1993.
(5): Larson & Springer, 1990. (6): Diamond et al., 1991; Diamond & Springer, 1993.
(7): Springer, 1990; Arnaout, 1990. (8): Pober & Cotran, 1990. (9): Springer, 1990.
(10): Muller et al., 1993.
(11): de Fougerolles et al., 1992,1994.
(12): Rosen, 1993; Baumhueter et al., 1993. (13): Moore et al., 1992; Sako et al., 1993.
(14): Diamond et al., 1991; Springer, 1990a. (15); de Fougerolles et al., 1994.

All three of these molecules contain an amino-terminal lectin domain, an epidermal growth factor (EGF)-like domain, and a variable number of short consensus repeat (SCR) sequences on the outside with a single transmembrane segment and a short cytoplasmic tail (Bevilacqua & Nelson, 1993). The selectins function by binding via their lectin domains to sialylated, fucosylated carbohydrate moieties contained on glycoproteins and glycolipids. It has been demonstrated that the tetrasaccharide sialyl Lewis^x (sLe^x, CD15s) and related terminal sugars expressed on neutrophils can act as ligands for E-selectin. sLe^x is also recognised by P-selectin and L-selectin, although the affinity of binding may differ substantially (Phillips *et al.*, 1990).

1.1.4.2 Integrins

Intregins are transmembrane heterodimers consisting of noncovalently linked α and β subunits (Kishimoto & Anderson, 1992; Shimizu *et al.*, 1992; Cronstein & Weissmann, 1993). There are at least 15 α and 8 β subunits, which combine to form many different integrin receptors. The major integrins of neutrophils are the β_2-integrins which are composed of a common β_2 subunit (CD18) that is noncovalently linked to one of three α subunits: CD11a (α_L), CD11b (α_m) or CD11c (α_x). This produces noncovalently linked β_2-integrins that are made up of $\alpha_L\beta_2$ (LFA-1, CD11a/CD18), $\alpha_m\beta_2$ (Hmac-1, MO-1, CD11b/CD18), and $\alpha_x\beta_2$ (p150/95, CD11c/CD18). In neutrophils there are intracellular stores of CD11b/CD18 and CD11c/CD18, and expression can rapidly be induced by a variety of stimuli, including IL-8. There are no intracellular stores of CD11a/CD18. Cell surface expression of β_2-integrins is not sufficient for adhesion of leukocytes to endothelial cells. Activation of the β_2-integrins due to a change in conformation or postreceptor events (e.g., cytoskeletal association) is required before adhesion can occur (Faull *et al.*, 1994). This activation is induced by soluble agents (cytokines, chemotactic factors and coagulation factors) and by ligation of other cell surface receptors. Ligands for the β_2-integrins include Ig-like surface proteins expressed on the endothelial cells such as intercellular adhesion molecule-1 (ICAM-1, CD54) for CD11b/CD18 and ICAM-1 and ICAM-2 (CD102) for CD11a / CD18. A definite role of β_2-integrins in neutrophil chemotaxis came from the identification of a

clinical syndrome called leukocyte adhesion deficiency, in which the β_2 subunit was either not expressed or was mutated (Arnaout *et al.*, 1982). Neutrophils from leukocyte adhesion deficiency patients are incapable of chemotaxis or H_2O_2 production *in vitro*, indicating a role for integrins in regulating neutrophil activation as well as mediating adherence.

1.1.4.3 Immunoglobulin superfamily

As shown in Table 1.2 several immunoglobulin (Ig) superfamily members are expressed on endothelial cells and are ligands for leukocyte integrins (Arnaout, 1990; Pober & Cotran, 1990; Shimizu *et al.*, 1992; Cronstein & Weissmann, 1993). Those most clearly associated with leukocyte adhesion and diapedesis are intercellular adhesion molecule 1 (ICAM-1, CD 54), ICAM-2, vascular cell adhesion molecule 1 (VCAM-1), mucosal addressin cell adhesion molecule 1 (MadCAM-1), and platelet-endothelial cell adhesion molecule-1 (PECAM-1, CD 31). ICAM-1 is a transmembrane glycoprotein containing five Ig domains found on hematopoietic and non-hematopoietic cells (Springer, 1990). It is constitutively expressed at low levels on endothelial cells under normal conditions, but its expression can be increased after stimulation of endothelial cells with cytokines (IL-1, TNF-α, and interferon-γ), LPS, thrombin, and phorbol esters (Pober *et al.*, 1986). Its upregulation requires *de novo* synthesis. ICAM-1 expression on endothelium and other cell types is a common characteristic of inflammatory and immune responses (Pober & Cotran, 1990; Springer, 1990). ICAM-2 is a ligand for CD11a/CD18. It contains two extracelluar Ig domains that are closely related to the two N-terminal domains of ICAM-1. Like ICAM-1, ICAM-2 is expressed in a variety of non-endothelial cell types (Springer, 1990). It is constitutively expressed on endothelial cells and its expression does not appear to be modified by inflammatory cytokines or LPS. PECAM-1 is constitutively expressed on leukocytes, platelets as well as at the junction of endothelial cells and its surface expression is not increased by stimulation with TNF-α or IL-1. Other immunoglobulin superfamily members are probably also involved in neutrophil-endothelial cell adhesion, including ICAM-3 (CD50) (expressed on the neutrophil but not the endothelial cell), and the CD66 family of neutrophil activation antigens.

1.1.4.4 Sequence of neutrophil-endothelial cell adhesion

The sequence of events involved in neutrophil adherence to vascular endothelium and transmigration into tissue is complex and has been termed the adhesion cascade (reviewed in Carlos & Harlan, 1994; Springer, 1994). A multistep model in which different adhesion molecules in conjunction with chemoattractants and cytokines mediate neutrophil adhesion, transmigration through vessel walls and accumulation at the site of inflammation has been proposed and is depicted in Figure 1.5.

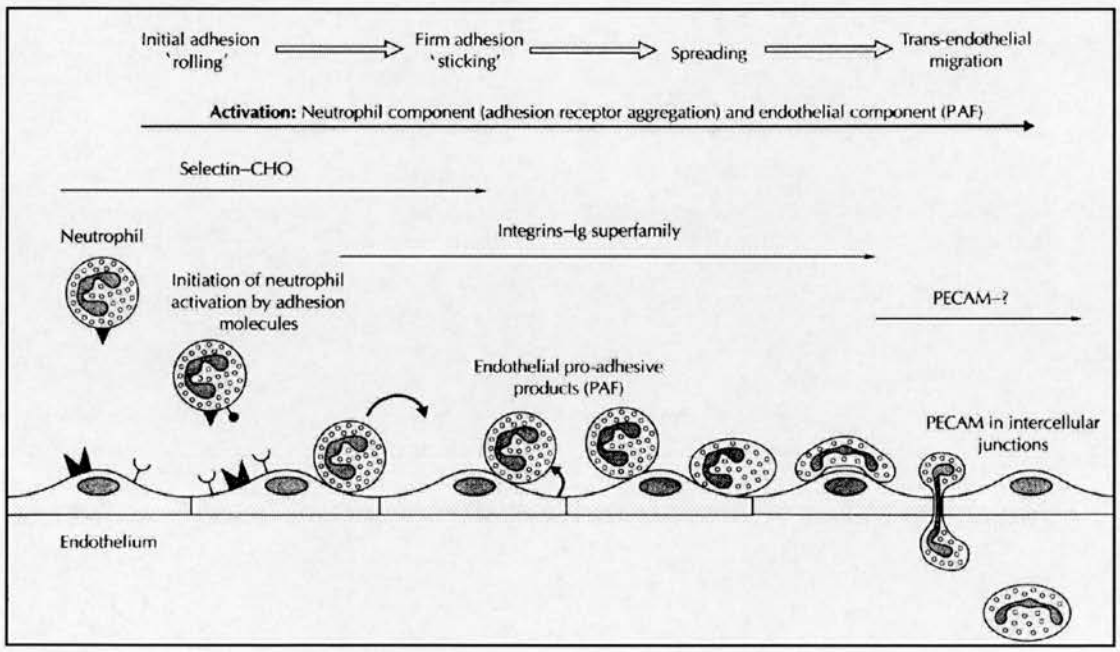


Figure 1.5 *The sequential steps model of neutrophil adherence to and transmigration across the endothelium. The initial interaction between neutrophils and the endothelium involves L-selectin on neutrophils and carbohydrate ligands (Gly-CAMS) on the endothelium. This is followed by an interaction between β_2-integrins on the neutrophil and Ig superfamily on the endothelium. During these adhesive interactions, the neutrophils become activated in part through intracellular signals, generated by adhesion molecules, as well as by chemoattractants (e.g. PAF) released by endothelial cells. The adhesive phase is followed by transmigration of neutrophils between the endothelial cells. The process of transmigration involves interaction of ligands on the neutrophil membrane with PECAM-1, which is located in the endothelial intercellular junctions. Modified from Downey, G.P. Mechanisms of leukocyte motility and chemotaxis. Current Opinion in Immunology 1994, 6:113-124.*

The cell adhesion cascade is initiated by the rapid interaction of selectins and their ligands by inflammatory mediators, which result in binding of selectins to cell surface carbohydrates allowing the flowing neutrophils to attach weakly to the vascular endothelium and roll over its surface (Springer *et al.*, 1994; Alon *et al.*, 1995). This loose adhesion brings the neutrophil in close proximity to the endothelial cell, where chemoattractants can be released or displayed on the cell surface. The next step involves further activation of the endothelial cell and neutrophils by cytokines, chemoattractants, and chemokines that are produced locally. This leads to upregulation and/or activation of the neutrophil β_2 integrins and the endothelial Ig-like molecules. These integrins can then bind their ligands such as ICAM-1 and/or ICAM-2 (which are expressed constitutively on the endothelial cell surface or that have been upregulated by cytokines, resulting in the firm adherence (i.e., sticking) of neutrophils to the endothelial cell and cessation of rolling (Lawrence & Springer, 1991; Von-Adrian *et al.*, 1991). At the site of inflammation, neutrophil rolling along the vessel wall is increased and cells may become more closely apposed to the vessel wall, allowing better interaction with chemoattractants. The firm adhesion through integrins allows chemoattractants to mediate migration of neutrophils through two adjacent endothelial cells along the gradient of chemoattractants being generated at the site of inflammation. This is the most complex and least understood process, requiring intense cytoplasmic and membrane reorganisation both in the extravasating neutrophil and in the endothelial cell. It is followed by passage through the basement membrane and migration into the subendothelial matrix to reach the site of inflammation. PECAM-1 has been shown to play a role in the process of neutrophil and monocyte diapedesis between endothelial cells, both *in vitro* (Muller *et al.*, 1993) and *in vivo* (Vaprociyan *et al.*, 1993). ICAM-1 expressed at endothelial cell junctions appears to be important for neutrophil migration, at least *in vitro* (Smith *et al.*, 1989; Furie *et al.*, 1991, 1992). There is also evidence that E selectin can mediate migration of neutrophils across endothelial cells (Luscinskas *et al.*, 1991), although other studies do not support these findings (Kishimoto *et al.*, 1991; Furie *et al.*, 1992).

1.1.4.5 Adhesion cascade and inflammation

While the adhesion cascade functions normally to recruit neutrophils to extravascular sites for host defence and repair, under some circumstances the adhesive interactions may lead to vascular and/or tissue damage. Once neutrophils are firmly adhered to the endothelium, a protected microenvironment is formed. In this microenvironment, inflammatory mediators produced by neutrophils may potentially reach high concentrations and overcome local and systemic anti-inflammatory protective mechanisms, thereby allowing endothelial cell injury to occur and resulting in an increase in microvascular permeability and haemorrhage. This series of events may initiate and sustain a cycle of inflammation, leading to further neutrophil recruitment and endothelial cell injury. The continued recruitment of neutrophils can lead to occlusion of the microvasculature by neutrophil aggregates, causing local ischaemia. Neutrophils may also diapedese between endothelial cells, gaining access to the extravascular space, where they can mediate further tissue damage, producing organ dysfunction.

1.2 ENDOGENOUS CYTOPROTECTIVE AGENTS

To maintain the patency of the blood vessels and the fluidity of blood, the endothelial cells synthesise many active substances. The major endothelial generated agents that are cytoprotective and thus preserve endothelial function are nitric oxide (NO), adenosine and prostacyclin (PGI_2).

1.2.1 Nitric oxide

In 1980, Furchgott & Zawadzki described the release of an endothelium-derived relaxing factor (EDRF) from the vascular endothelium. Based on the similarities in the pharmacological behaviour of EDRF and NO generated from acidified NO_2^-, Furchgott suggested in 1986 that EDRF might be NO. (See Furchgott, 1988). At the same time, Ignarro *et al.* also speculated that it may be NO or a closely related species (see Ignarro *et al.*, 1988). The final proof that nitric oxide was an EDRF was provided by Palmer *et al* and was quickly confirmed by Ignarro *et al* (Ignarro *et al.*, 1987; Palmer *et al.*, 1987). NO is a biologically active gas that is synthesised by a

variety of cells, including vascular endothelium, from one of the terminal guanidino nitrogen atoms of L-arginine in a stereospecific process catalysed by a family of enzymes, the nitric oxide synthases (NOS). The enzymes are large and complex haemoproteins that share similarities with cytochrome P-450 reductase, and require multiple cofactors for their activity (White & Marletta, 1992). Three families of isozymes have been identified and cloned, namely: constitutively expressed neuronal (nNOS) (Bredt & Synder, 1990) and endothelial (eNOS) isoforms (Pollock, *et al.*, 1990), as well as the inducible isoform (iNOS) (Stuehr *et al.*, 1991). According to a recent nomenclature, nNOS, iNOS and eNOS were designated as NOS-1, NOS-2 and NOS-3, respectively. Two constitutive isoforms (eNOS and nNOS) are present as normal constituents in some cell types whereas iNOS is not constitutively expressed but can be induced in a wide variety of cells after they are exposed to endotoxin or some cytokines (Stuehr & Griffith, 1992; Nussler & Billiar, 1993). The constitutive enzymes are Ca^{2+}-calmodulin-dependent, and present in the endothelium, platelets, myocardium, endocardium, neural tissue and skeletal muscle (Moncada, 1991; Kobzir *et al.*, 1994). These enzymes produce NO in relatively small amounts in response to receptor stimulation or physical activation of the cell. In contrast, the iNOS is functionally Ca^{2+}-independent and can be expressed in a variety of cells, including endothelial cells, myocytes, immune cells such as macrophages and neutrophils, and astrocytoma cells in the brain after these cells are exposed to inflammatory cytokines or endotoxin. The inducible iNOS produces large amounts of NO for longer periods of time that can result in tissue damage and cell death.

All three NOS isoforms synthesise NO from L-arginine and O_2 in the presence of NADPH and other cofactors: FAD, flavine mononucleotide (FMN), heme, and tetrahydrobiopterin (BH_4). All three NOS isoforms catalyse a five-electron oxidation of one of two equivalent guanidino nitrogens in L-arginine to yield nitric oxide at the cost of 1.5 mols of NADPH and 2 mols of dioxygen (for review, see Stuehr *et al.*, 1992). This process involves two successive mono-oxygenation steps (Kwon *et al.*, 1990; Leone *et al.*, 1991; Stuehr *et al.*, 1991; Mayer *et al.*, 1991; Stuehr & Griffith, 1992). The first step involves the oxidation of one NADPH and the reduction of one O_2 in the presence of BH_4 yielding N^{ω}-Hydroxyarginine as an isolatable intermediate.

Subsequent activation of another molecule of O_2 facilitates the further oxidation of N^{ω}-Hydroxyarginine to produce NO, citrulline, and H_2O. The flavins are used to deliver electrons singly to heme from the two-electron donor NADPH for the activation of dioxygen. A schematic diagram of nitric oxide synthesis is shown in Figure 1.6.

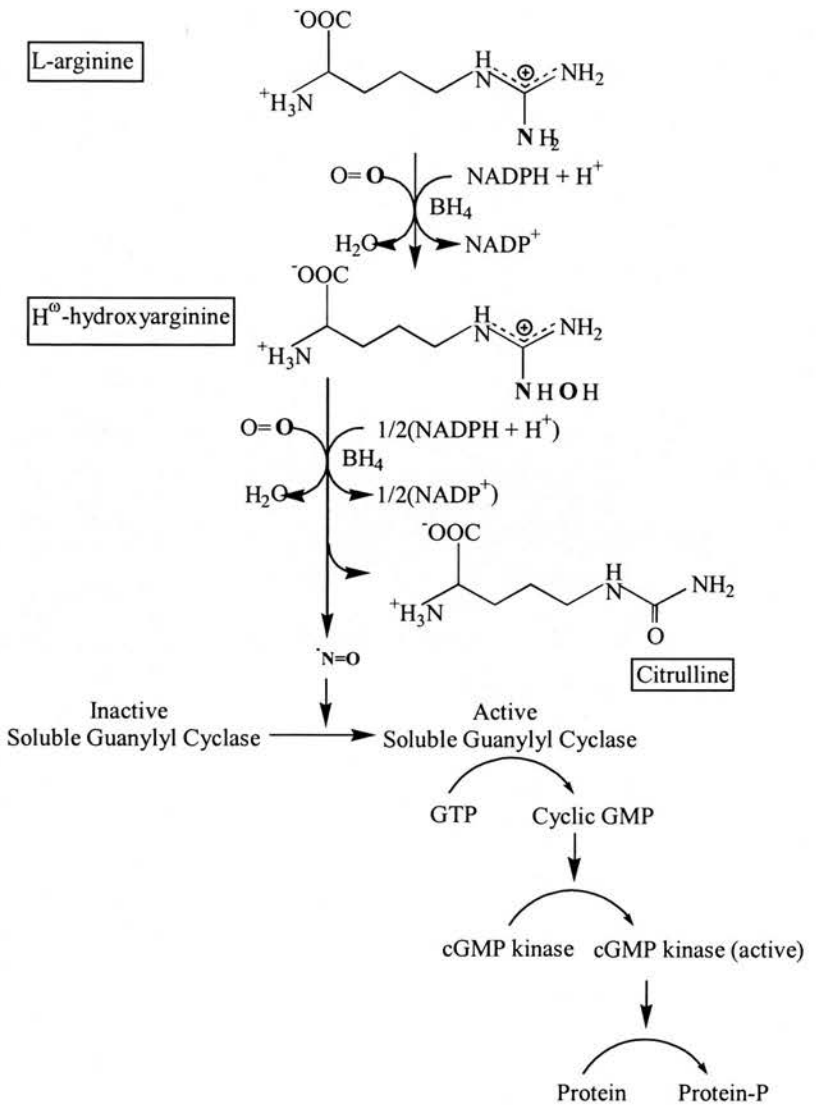


Figure 1.6 *Schematic diagram of NO synthesis and nitric-oxide cyclic GMP signal transduction system.*

NO has diverse important biological effects including: vasodilatation (Tesmafarium *et al.*, 1985; Furchgott, 1987), inhibition of platelet aggregation (Busse *et al.*, 1987; Radomski *et al.*, 1987b; Furlong *et al.*, 1987; Ignarro *et al.*, 1989), and platelet adhesion to endothelial cells (Radomski *et al.*, 1987c,d), inhibition of both neutrophil aggregation (McCall *et al.*, 1988) and adhesion to endothelial cells in postcapillary venules, (Schroder *et al.*, 1990; Kubes *et al.*, 1991), attenuation of cellular proliferation (Garg & Hassid, 1989); regulation of endogenous ADP-ribosylation (Brune & Lapetina, 1989; Clancy *et al.*,1993, 1995); neurotransmission and neuromodulation (Toda *et al.*, 1993; see review Garthwaite & Boulton, 1995), inflammatory response (Moilanen & Vapatalo, 1995) and immunological defence (Liew & Cox, 1991; Kolb & Bachofen, 1992). Normally, NO is released basally by the endothelial cells of the coronary vasculature and inhibits platelet and neutrophil adhesion to the endothelium, thereby preventing thrombus formation and microvascular embolisation. A study by Williams *et al* suggests that endogenous NO production exerts a tonic cardioprotective effect, reducing the extent of myocardial infarct following coronary reperfusion (Williams *et al*, 1995).

The physiological effects of NO are usually mediated via activation of soluble guanylate cyclase through direct binding of NO to its prosthetic haem group (Ignarro, 1991) and the resultant formation of cyclic GMP (Murad, 1986; McCall *et al.*, 1988; Schroder *et al.*, 1990; Moilanen *et al.*, 1993). In many cell types, the increases in cyclic GMP lead to cyclic GMP-dependent protein kinase activation and altered phosphorylation of many endogenous proteins (Rapaport & Murad, 1983b; Murad, 1986; Waldman & Murad, 1987). In addition, it is proposed that NO exerts some of its main physiological and pathological effects on cell functions by inhibiting cytochrome oxidase (Brown, 1995).

NO is a free radical and highly unstable, with an apparent half-life of 10-20 seconds, owing to reactions with O_2 and O_2^- (Ignarro *et al.*, 1987; Palmer *et al.*, 1987; Moncada *et al.*, 1989; Furchgott *et al.*, 1990). NO is oxidised to nitrite ion (NO_2^-) in tissue fluid and then converted to nitrate (NO_3^-) in whole blood. In biological systems, the dominant reaction of NO will be with another free radical (Ignarro *et al.*,

1981; Vanin, 1991; Stamler *et al.*, 1992). These NO adducts, their secondary reaction products, and products of NO oxidation and reduction are capable of reaction with metals, thiols, and additional targets to give further products, often with biological activity and relevance (Stamler *et al.*, 1992). Thus the biological chemistry of NO is complex. Reaction of NO with O_2^- leads to rapid destruction of NO with loss of biological activity or to the formation of other radicals, including peroxynitrite ($ONOO^-$) (Beckman *et al.*, 1990). $ONOO^-$ is a highly reactive species with a half-life of less than 1 sec under biological conditions, owing to its equilibrium with peroxynitrous acid (ONOOH) (Beckman *et al.*, 1990; Radi *et al.*, 1991a). ONOOH spontaneously decomposes to NO_2 and forms an extremely reactive species with hydroxyl-like properties (Beckman *et al.*, 1990). Cellular generation and reactivity of $ONOO^-$ and its derived chemical species suggest that this pathway is an important process in pathophysiological actions of NO. $ONOO^-$ has been demonstrated to cause lipid peroxidation, thiol oxidation, and nitrosation or nitration of several functional groups of amino acids (Radi *et al.*, 1991a; Radi *et al.*, 1991b; Ischiropoulos *et al.*, 1992). Reaction of NO with ions and macromolecules such as haemoglobin or glutathione can result either in its inactivation or, in some cases, in the formation of a relatively stable NO-carrier intermediate that stabilises NO and permits it to act at a greater distance from its site of synthesis than would otherwise be possible. NO reacts rapidly with oxygen-bound Fe^{2+}-haemoglobin to form the corresponding met-haemoprotein (Fe^{3+}-Hb) and nitrate (Doyle *et al.*, 1981). The reversible binding of NO to the iron of reduced (Fe^{2+}) haemoproteins is a well-established interaction that results in the formation of NO-heme. NO is known to inhibit key cellular enzymes with iron-sulphur complexes and cause the release of iron-NO-sulphur species (Hibbs *et al.*, 1990). Reaction of NO with macromolecules (nitrosylation) can result in cell damage and accounts for some of the effects of the large amounts of NO produced during host defence reactions and in some pathological states. Several of the oxidation products of NO have the potential to react with amines to yield products of pathophysiological significance

Cytotoxic and/or cytostatic effects of NO are important in non-specific host defence against numerous pathogens and tumour cells. High levels of NO may be cytotoxic

to some invading pathogens, cancer cells or normal host cells (Beckman *et al.*, 1990; Nguyen *et al.*, 1992; Vallance & Moncada, 1993). Neutrophils produce NO after induction of NOS, and some stimuli (e.g. fMLP) which stimulate NO biosynthesis also cause neutrophil O_2^- production. NO reacts with O_2^- to yield cytotoxic $ONOO^-$, a potent oxidant that can destroy invading organisms, but if produced in excess can also damage the host. Other mechanisms by which NO may mediate cytotoxicity include: the impairment of the function of mitochondrial and other iron-sulphur containing enzymes resulting in the depletion of cellular ATP stores (Stuehr & Nathan, 1989; Stadler *et al.*, 1991), impairment of DNA synthesis through the inhibition of ribonucleotide reductase (Kwon *et al.*, 1991) as well as by direct toxicity through deamination reactions (Nguyen *et al.*, 1992), inactivation of phosphoenolpyruvate carboxykinase and glyceraldehyde-3-phosphate dehydrogenase which are important enzymes in glucose metabolism (Nathan, 1992).

1.2.2 Adenosine

Adenosine is an endogenous nucleoside released by many different cell types and is present in physiologically relevant amounts in plasma; it regulates many physiological processes. It is produced primarily from the degradation of adenosine triphosphate (ATP) (Bern, 1980). A schematic diagram of adenosine metabolism is shown in Figure 1.7.

The major pathways of adenosine formation are the enzymatic dephosphorylation of 5'-adenosine monophosphate (5'-AMP) by 5'-nucleotidase and the hydrolysis of S-adenosylhomocysteine (SAH) by SAH-hydrolase (Achterberg *et al.*, 1985). During normoxia, a major source of adenosine is SAH formed from S-adenosylmethionine (SAM). During ischaemia and hypoxia, 5'-nucleotidase is activated and is thought to be primarily responsible for adenosine production (Frick & Lowenstein, 1976; Worku & Newby, 1983) aside from the accumulation of 5'-AMP. Adenosine that is produced from either SAH or 5'-AMP is rapidly phosphorylated by adenosine kinase or deaminated by adenosine deaminase (Figure 1.7).

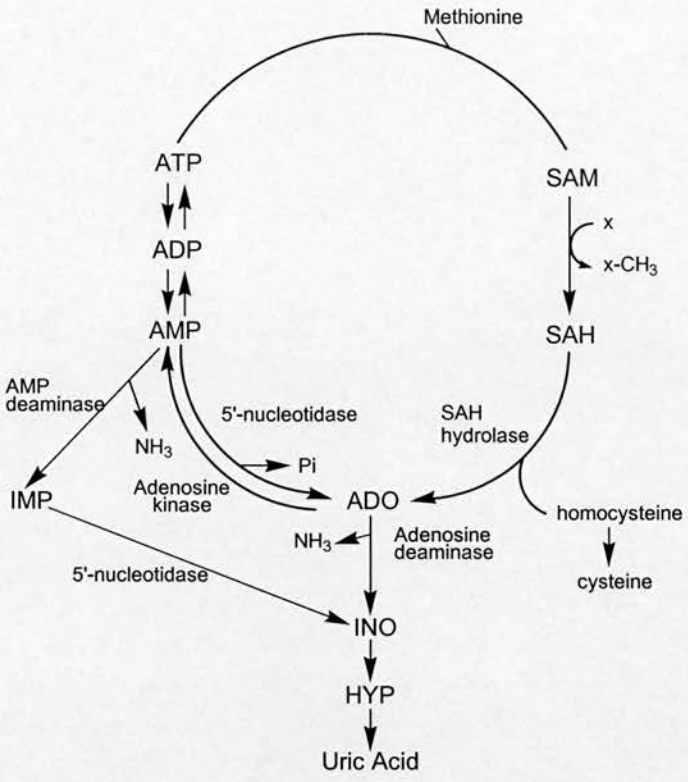


Figure 1.7 *Schematic diagram of adenosine metabolism. SAM, S-adenosylmethionine; SAH S-adenosylhomocysteine; ADO, adenosine; AMP, adenosine 5'-monophosphate; IMP, inosine 5'-monophosphate; INO inosine; HYP, hypoxantine. Modified from Schrader, J: Metabolism of adenosine and site of production in the heart, in Berne, R.M., Rall, T.W., Rubio, R. (eds): Regulatory Function of Adenosine. Boston, Marinus Nijhoff Publisher, 1983, pp 133-156.*

Adenosine acts as a neuromodulator in the central and peripheral nervous systems and as a homeostatic regulator in a variety of other tissues, including smooth muscle cells, heart muscle, platelets, coronary arteries and cells involved in immune and inflammatory reactions (Schrier & Imre, 1986; Liang, 1992; Hussain & Mustafa, 1993). Adenosine is a vasodilator in most vascular beds and regulates a variety of stimulated neutrophil functions. Adenosine inhibits O_2^- generation (Cronstein & Kramer, Cronstein *et al.*, 1983, 1985, 1990; Roberts, *et al.*, 1985; Gunther & Herring, 1991; Thiel & Bardenheuer, 1992; Burkey & Webster, 1993), β_2-integrin- and L-selectin-mediated adhesion to endothelial cells (Cronstein *et al.*, 1992; Firestein *et al.*, 1995), and phagocytosis (Salmon & Cronstein, 1990) but does not inhibit chemotaxis (Cronstein, 1994). Adenosine is reported to be a poor inhibitor of

granule release from stimulated neutrophils, that does not inhibit neutrophil aggregation (neutrophil-neutrophil adhesion) (Cronstein *et al.*, 1983, 1988; Cronstein, 1994; McGarrity *et al.*, 1989; Walker *et al.*, 1989). However, some investigators have reported that adenosine inhibits stimulated neutrophil degranulation and aggregation (Schmeichel & Thomas, 1987; Skubitz *et al.*, 1988; Richter, 1992). Adenosine inhibits platelet aggregation (Feoktistov *et al.*, 1991; Bullough *et al.*, 1994; Grenegard *et al.*, 1996; Jonzon *et al.*, 1997). Adenosine has been shown to play a role in protecting vascular endothelium from damage by neutrophils (Cronstein *et al.*, 1986) and exerts beneficial effects in ischaemia and reperfusion (Engler & Gruber, 1991; Pitarys *et al.*, 1991; Norton *et al.*, 1991, 1992; Toombs *et al.*, 1992).

Most effects of adenosine are exerted via cell surface receptors, which are present on most cells and organs. There are four pharmacologically distinct adenosine receptor subtypes, designated A_1, A_{2A}, A_{2B} and A_3, all of which have been cloned and their binding profiles characterised in various cell lines (Fredholm *et al.*, 1994; Olah & Stiles, 1995). In some tissues, however, only one subtype is present. For example, the A_1 receptor prevails in the heart, whereas the A_2 receptor is present mainly in vessels and platelets. In turn, the A_2 receptor has been further subdivided into A_{2A} (high affinity, in brain striatum) and A_{2B} (low affinity, in fibroblasts). It is known that stimulation of A_{2A} receptors leads to vasodilatation, inhibition of platelet aggregation and neutrophil adhesion to vascular endothelium, as well as reduction in generation of oxygen free radicals by activated neutrophils (Hori & Kitakaze, 1991; Cronstein, 1991; Sandoli *et al.*, 1994). In the nonischaemic heart, adenosine A_1 receptors, located primarily on cardiac myocytes, mediate the negative chronotropic/dromotropic and antiadrenergic effects. Adenosine A_2 receptors located predominantly on endothelial cells, mediate the effects on coronary blood flow (Lasley & Mentzer, 1995).

Earlier evidence from functional studies showed that neutrophils possess A_1 and A_{2A} receptor subtypes (Cronstein *et al.*, 1985). It has been demonstrated recently that neutrophils also posses A_{2B} and A_3 receptors subtypes (Fredholm *et al.*, 1996; Bouma

et al., 1997). Based on pharmacological data, it appears that the A_{2A} receptor on neutrophils mediates the inhibition of neutrophil O_2^- generation, adhesion to endothelial monolayers, and phagocytosis (Cronstein, 1994), whereas activation of A_1 receptors promotes chemotaxis (Cronstein *et al.*, 1990) and neutrophil adherence to endothelial cells (Cronstein *et al.*, 1992).

Regulatory G proteins are involved in the signal transduction for both the A_2 and A_1 receptors resulting in activation or inhibition of adenylate cyclase, respectively (Ramkumar & Stiles, 1988). Although adenosine A_2 receptors are classically linked to heterotrimeric G_s signalling proteins and stimulation of adenylate cyclase which leads to increase intracellular concentrations of cyclic AMP (see Fredholm *et al.*, 1994), it is clear that cyclic AMP does not act as the second messenger for the inhibitory actions of adenosine on the neutrophils (Cronstein, 1994). An inhibitory effect on the late increases in intracellular DAG and Ca^{2+} has also been proposed, but a more recent study has indicated that adenosine A_{2A} receptors on human neutrophils signal via a novel pathway, cyclic AMP-independent activation of a serine/threonine protein phosphatase in the plasma membrane (Revan *et al.*, 1996).

It has been only recently that potentially important functions have been discovered for the A_{2B} receptors. A_{2B} receptors have been implicated in mast cell activation, (Marquardt *et al.*, 1994; Feoktistov & Biaggioni, 1995; Feoktistov *et al.*, 1996), vasodilatation (Webb *et al.*, 1992), regulation of cell growth (Dubey *et al.*, 1996b; Boyle *et al.*, 1996; Feoktistov & Biaggioni, 1995), intestinal function (Stehle *et al.*, 1992; Nicholls *et al.*, 1996), and modulation of neurosecretion and neurotransmission (Walday & Aas, 1991; Phillis *et al.*, 1993; Okada *et al.*, 1996). A_{2A} and A_{2B} receptors are frequently found in the same tissue e.g. human neutrophils (Fredholm *et al.*, 1996). Functional A_{2B} receptors have been found in many cell types such as various vascular beds (Martin *et al.*, 1993; Chiang *et al.*, 1994; Prentice & Hourani, 1996; Dubey *et al.*, 1996), myocardial cells (Liang & Haltiwanger, 1995) endothelium (Iwamoto *et al.*, 1994), neurosecretory cells (Casado *et al.*, 1992; Mateo *et al.*, 1995). In addition to coupling to adenylate

cyclase through G_s proteins, A_{2B} receptors can also couple to other intracellular pathways, including calcium channels through G_s and phospholipase C.

The pathophysiological role of the A_3 receptor might be very different from the role of the A_1 and the A_2 subtypes, in that it may act as an endogenous regulator under conditions of more severe challenge (see review by Jacobson, 1998). The varied effects of A_3 receptor agonists, *in vitro* and *in vivo*, appear to be opposing, i.e. either cytoprotective or cytotoxic, depending on the level of receptor activation and the paradigm studied. A_3 receptors are potentially involved in apoptosis (programmed cell death). It appears that intense, acute activation of A_3 receptors acts as a lethal input to cells, while low concentrations of A_3 receptor agonists protect against apoptosis. Selective activation of A_3 receptors appears to inhibit human neutrophil degranulation, suggesting the anti-inflammatory potential of A_3 receptor agonists in neutrophil-mediated tissue injury (Bouma *et al.*, 1997). There might be an involvement of A_3 receptors in cancer (MacKenzie *et al.*, 1994). There are protective effects of A_3 receptor activation in heart cells administration both prior to (Strickler *et al.*, 1996; Tracey *et al.*, 1997) and during (Stambaugh *et al.*, 1997) an ischaemic episode (Tracey *et al.*, 1997). Activation by endogenous adenosine of both A_1 and A_3 receptors is thought to mediate preconditioning. As A_3 receptor activation protects both in a preconditioning model and during prolonged ischaemia, selective agonists might be of great clinical importance.

1.2.3 Prostacyclin

Prostacyclin (PGI_2) was discovered in 1976 by Moncada &Vane and their colleagues (Moncada *et al.*, 1976) and is a major active arachidonic acid metabolite produced mainly by the endothelial cells. In endothelial cells, pulsatile pressure, a number of endogenous mediators and some drugs stimulate PGI_2 generation. Some endogenous chemical stimulants include substances derived from plasma such as bradykinin and thrombin, and those liberated from stimulated platelets such as serotonin (5-HT), platelet-derived growth factor (PDGF), IL-1, and adenine nucleotides (Forsberg, *et al.*, 1987). PGI_2 production is initiated by the enzyme phospholipase A_2 (PLA_2),

which liberates arachidonic acid (AA) from membrane phospholipids (Walsh *et al.*, 1983) (Figure 1.8).

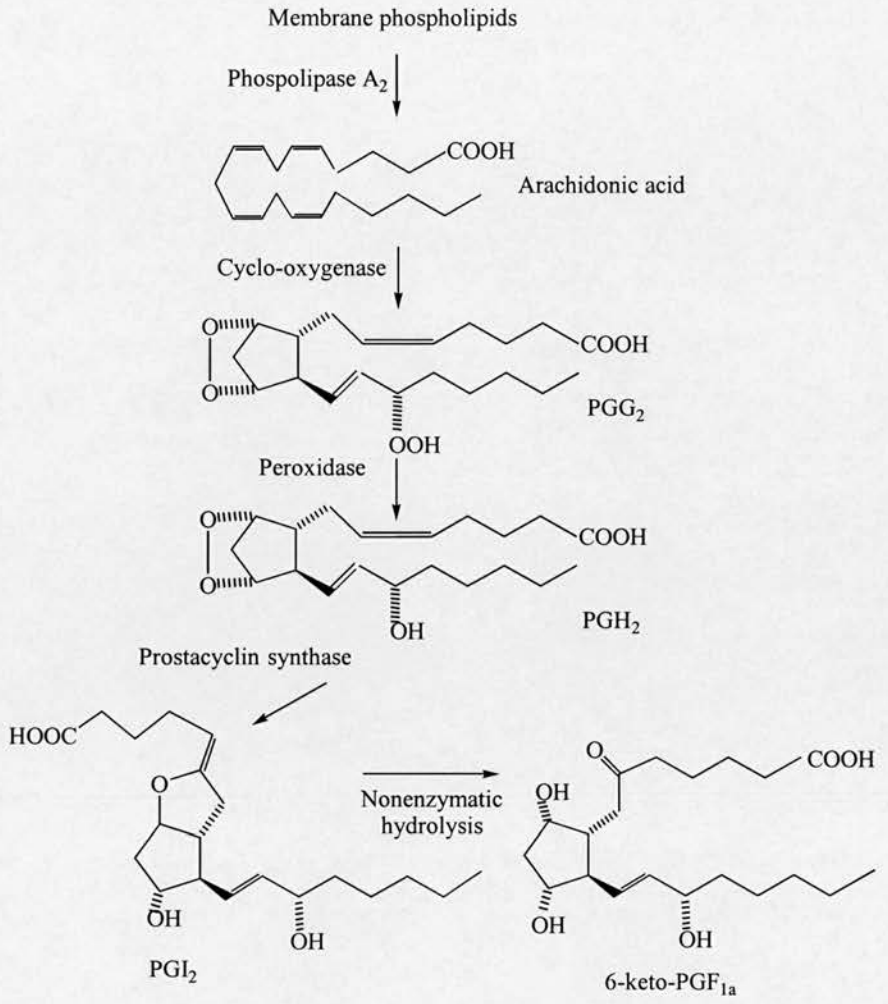


Figure 1.8 *Schematic diagram of Prostacyclin (PGI_2) biosynthesis. Prostacyclin is formed from arachidonic acid, with the endoperoxides and prostaglandins G_2 and H_2 as intermediates. PGI_2 is unstable and breaks down into the stable and less active 6-keto-$PGF_{1\alpha}$.*

Arachidonic acid is converted into prostaglandin G_2 (PGG_2) and then to prostaglandin H_2 (PGH_2) by the enzyme cyclooxygenase (prostaglandin G/H synthase) and peroxidase, respectively. Prostacyclin synthase subsequently forms prostacyclin (PGI_2) from the endoperoxide PGH_2. PGI_2 is hydrolysed rapidly in plasma to 6-keto-prostaglandin $F_{1\alpha}$; its metabolic half life is about 1-2 minutes at physiological pH in aqueous media.

PGI_2 has a number of physiological actions, including vasodilatation of most vasculatures (Fitzpatrick *et al.*, 1978; Lefer *et al.*, 1978; Moncada & Vane, 1979), inhibition of platelet aggregation (Tateson, *et al.*, 1977; Higgs *et al.*, 1978; Moncada & Vane, 1979), prevention of neutrophil adhesion, and stabilisation of membranes (Lefer *et al.*, 1978; Jones & Hurley, 1984). PGI_2 was found to inhibit fMLP-induced chemotaxis and superoxide anion generation in rat (Fantone & Kinnes, 1983; Kainoh, *et al.*, 1990) and human neutrophils (Claesson *et al.*, 1981). In addition, PGI_2 was also found to inhibit lysosomal enzyme release or LTB_4 release from fMLP-activated human neutrophils (Claesson *et al.*, 1981). Since it has been proposed that PGI_2 production by the endothelium is reduced following anoxia and reoxygenation (Hempel *et al.*, 1990) and endogenous PGI_2 seems to be an important protective prostanoid against myocardial injury inflicted by ischaemia and reperfusion, the use of PGI_2 or its analogues may offer cardioprotection to the ischaemic myocardium after reperfusion.

The effects of PGI_2 are mediated via specific cell surface IP receptors (Coleman *et al.*, 1994), activation of which generally leads to elevation of intracellular cyclic AMP (Gorman *et al.*, 1977) through G_s protein coupling to adenylate cyclase (Hashimoto *et al.*, 1990). The resultant increase in cyclic AMP level leads to activation of PKA and phosphorylation of several key proteins. For example, a PGI_2 analogue, beraprost, was shown to effectively inhibit fMLP-induced chemotaxis of neutrophils via the elevation of intracellular cAMP levels, which interferes with the signal transduction process, probably through the inhibition of influx of Ca^{2+} mobilization in neutrophils (Kainoh *et al.*, 1990). Recently, the inhibitory effect of this drug was shown to mediate through the inhibition of p47 phox phosphorylation and translocation by a Ca^{2+} dependent mechanism (Okuyama *et al.*, 1995).

Prostanoid receptors have highly conserved amino acid sequences and constitute a novel family of seven transmembrane domains receptors, which are coupled to heterotrimeric guanine nucleotide regulatory protein (G proteins) (Coleman *et al.*, 1994). Pharmacological studies have revealed that the responses to PGI_2 and its

analogues are somewhat different among tissues and cells of different species, indicating that the presence of several receptor subtypes (Corsini *et al.*, 1987) or coupling of the receptor to more than one species of G protein. However, current molecular biological knowledge of PGI_2 receptor (IP receptor) does not support the existence of subtypes for this receptor (see review Hirata *et al.*, 1995). In contrast, cloned IP receptors coupled to both adenylate cyclase stimulation and PLC activation (Katsuyama *et al.*, 1994) probably via Gs and Gq, respectively. Thus, the IP receptor belongs to a receptor that can couple to multiple G proteins. Recent evidence suggests that IP receptors can couple to Gs, Gq, as well as Gi (Schwaner *et al.*, 1995). Some evidence was presented for IP receptor agonists opening K_{ATP} channels by a process independent of cyclic AMP production, but possibly still involving a G protein (Jackson *et al.*, 1993).

PGI_2 is extremely labile under physiological conditions, with a half-life of 1-2 minutes, and so a variety of chemical stable PGI_2 analogues have been developed. Among them, iloprost, a member of carbacyclin, (Schror *et al.*, 1981) and cicaprost, another carbacyclin, (Sturzebecher *et al.*, 1985) are the more commonly used IP mimetics, but cicaprost is the analogue of choice, owing to its potency and high selectivity (Dong *et al.*, 1986). Iloprost is less selective, acting as a partial agonist at EP_1 receptors (Jones *et al.*, 1984; Dong *et al.*, 1986).

CHAPTER 2

INVESTIGATION OF THE ROLE OF NITRIC OXIDE AND CYCLIC GMP IN BOTH THE ACTIVATION AND INHIBITION OF HUMAN NEUTROPHILS

2.1 INTRODUCTION

Neutrophils comprise a fundamental component of the non-specific immune response. It is well established that neutrophils play an important role in host defences during tissue injury and inflammation, when neutrophils migrate from microvessels into the tissues to combat pathogens (Curie *et al.*, 1987; Weiss, 1989). Neutrophils are recruited from the bloodstream by chemotactic factors generated and released locally in injured tissue (Barten *et al.*, 1976; Weiss, 1989). Once at the site of inflammation, these cells respond to injurious agents by phagocytosis, the release of preformed granular enzymes and proteins, and by the *de novo* production of a range of potentially damaging, but ephemeral, reactive oxygen intermediates, such as $O_2^{\cdot-}$.

Neutrophil locomotion to a specific chemoattractant is a complex, multi-step process requiring ligation of a cell surface receptor, transduction of a signal from the receptor to intracellular effectors, reorganization of the cytoskeleton and finally a directed crawling movement towards the source of chemotaxin (Cassimeris & Sigmond, 1990). However, the effector signalling pathway activated in neutrophils to promote cell migration in response to these stimuli is still poorly understood. Several cellular pathways, as well as numerous specific macromolecules have been identified as being essential for the process of neutrophil movement (Cassimeris 1990; Sigmond, Gaudry *et al.*, 1992; Amatruda *et al.*, 1993). For example, the direct interaction of receptors with G protein activates PLC, and the subsequent release of calcium, as well as actin polymerisation, appears to be involved in the chemotactic response (Snyderman *et al.*, 1984, 1986; Becker *et al.*, 1985; Yasui *et al.*, 1988; Mark & Maxfied, 1990). Numerous studies have investigated the second messengers involved in $O_2^{\cdot-}$ generation in neutrophils, and these have revealed that protein tyrosine phosphorylation (tyrosine kinase) and the activation of PLD are the major factors involved in the activation of NADPH oxidase produced by stimulation with the chemoattractant fMLP (Pai *et al.*, 1988; Agwu *et al.*, 1989; Bonser *et al.*, 1989; Naccache *et al.*, 1990; English, 1992; Perry *et al.*, 1992; Kusunoki *et al.*, 1992). Further, it has been reported that tyrosine kinase precedes and induces the receptor-mediated activation of PLD, but not PLC activity, in human neutrophils

(Uings *et al.*, 1992). A study by Yasui *et al.* (1994) indicated that both the respiratory burst and the migration of neutrophils require tyrosine phosphorylation in the signalling pathway; however, the former needs PLD activation and the latter does not. Human neutrophils possess more than one pathway by which they exert their different functions.

Human neutrophils stimulated with Ca^{2+}-mobilizing agents such as fMLP or the ionophore A23187 undergo transient polarization followed by increased motility and degranulation. These actions appear to involve increases in cyclic GMP. The increase in $[Ca^{2+}]i$ stimulates NO formation. Unstimulated and primed human and rat neutrophils have been shown to generate and release factors with the pharmacological characteristics of nitric oxide (NO) (Stephen & Snyderman, 1982; Rimele *et al.*, 1988; Wright *et al.*, 1989; Schmidt *et al.*, 1989; Salvemini *et al.*, 1989; McCall *et al.*, 1989; Lee *et al.*, 1990; Mehta *et al.*, 1990; Myers *et al.*, 1990; Kadota *et al.*, 1991; Lopez *et al.*, 1991; Moncada *et al.*, 1991, 1991; Rimele *et al.*, 1991; Yui *et al.*, 1991). The release of NO is regulated by nitric oxide synthase (NOS), a cytosolic enzyme that catalyses the conversion of L-arginine to L-citrulline and NO (Moncada *et al.*, 1991b). A major molecular event in the signal transduction by NO seems to be activation of guanylyl cyclase (Garber, 1992; Schmidt *et al.*, 1993) by formation of an NO-haem iron complex, thus resulting in enhanced production of guanosine 3': 5'-cyclic monophosphate (cyclic GMP). Coincident with the elevations of cyclic GMP in activated neutrophils is the colocalisation of cyclic GMP-dependent protein kinase (G-kinase) and the intermediate filament cytoskeletal protein vimentin, this is followed by phosphorylation of vimentin by G-kinase (Wyatt *et al.*, 1991). The transient colocalisation of G-kinase and vimentin correlates well with phosphorylation, cell polarisation, and degranulation, suggesting a role for G-kinase in these events. However, the role of NO generated by neutrophils is still insufficiently understood.

Intracellular accumulation of cyclic GMP has been suggested to regulate neutrophil chemotaxis *in vitro* (Sandler *et al.*, 1975; Smith & Ignarro, 1975; Stephen & Snyderman, 1982; Anderson *et al.*, 1989; Kaplan *et al.*, 1989). Consistent with these

concepts, it has been shown that fMLP-mediated chemotaxis was decreased by an inhibitor of NOS, N^G-monomethyl-L-arginine (L-NMMA), involving most probably a cyclic GMP-dependent pathway as exogenous cyclic GMP reversed this inhibition (Kaplan *et al.*, 1989). Furthermore, it has been shown that NOS inhibitors significantly attenuate chemotaxis of unstimulated and primed human neutrophils *in vitro* and that these effects were specific and modulated by cyclic GMP (Belenky *et al.*, 1993). These two latter experiments suggested a role for NO as an intracellular messenger for neutrophil chemotaxis. In addition, cyclic GMP plays a major role in neutrophil chemotaxis, by increasing cell polarization (Caterina & Devreotes, 1991). Recently, it was demonstrated that exogenous NO could mediate the chemotaxis of neutrophils *in vitro* (Beauvais *et al.*, 1995).

However, there are some contradictory data indicating that NO or NO donors (at high concentrations, >10μM) can inhibit neutrophil functions e.g. chemotaxis, degranulation, leukotriene (LT) production and O_2^- generation. Some of these effects were suggested to be mediated, at least in part, by an increase of cyclic GMP due to soluble guanylyl cyclase activation (Ney *et al.*, 1990; Schroder *et al.*, 1990; Kubes *et al.*, 1991; Rubanyi & Vanhoutte, 1991; Wenzel-Seifert *et al.*, 1991; Clancy *et al.*, 1992; Moilanen *et al.*, 1993; Rengasamy & Johns, 1993).

2.2 AIMS

The aim of this study was to establish the role of NO and cyclic GMP in chemotaxis and superoxide anion (O_2^-) generation by human neutrophils. For this purpose, the inhibitory effects of the NOS inhibitors, L-NMMA and L-canavanine; the NO scavenger Carboxy-PTIO; the guanylyl cyclase inhibitor LY 83583; the G-kinase inhibitors, KT 5823 and Rp-8-cCPT-cGMPS and the phosphatase inhibitor, 2,3 Diphosphoglycerate (DPG) have been investigated. In addition, the NO donors, 3-morpholino-sydnonimine (SIN-1) and mesoionic 3-aryl-substituted oxatriazol derivatives (GEA 3162 and GEA 5024) have been tested for inhibition of neutrophil chemotaxis as well as for their ability to increase neutrophil nitrate/nitrite and cyclic

GMP levels. The ultimate aim of this work was to resolve the paradox that NO appears to be able to both activate and inhibit human neutrophils.

2.3 MATERIALS

The following compounds were gifts which are gratefully acknowledged: 3-aryl-substituted oxatriazol derivatives GEA 3162 and GEA 5024 from Dr S. B. Pedersoen, GEA Ltd, Copenhagen, Denmark; A primary antibody against acetylated cyclic GMP from Dr. I. Gow, Department of Physiology, University of Edinburgh and Dr.Brent Williams, Department of Medicine, University of Edinburgh, Western General Hospital; Donkey-Anti-Rabbit Serum (DARS) and Normal Rabbit Serum (NRS) from the Scottish Antibody Production Unit, Carluke.

fMLP, L-canavanine, PBS (containing Ca^{2+} and Mg^{2+}), 2,3-Diphosphoglyceric acid (DPG), trypan blue and guanosine 3': 5'-cyclic monophosphate were purchased from Sigma; polyethylene glycol, cytochrome C and cytochalasin B from Aldrich; Percoll from Pharmacia; RPMI 1640 from Gibco; N^G-monomethyl-L-arginine (L-NMMA), 6-anilinoquinoline-5-8-quinone (LY 83583), (8R,9S,11S)-(-)-9methoxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H, 8H, 11H-2, 7b, 11a-triazadibenzo (a,g) cycloocta (cde)-trinden-1-one (KT 5823) from Calbiochem; Rp-8-(4-Chlorophenylthio)-guanosine-3'-5'-cyclic mono phosphorothioate (Rp-8pCPT-cGMPS) from Biolog; Diff-Quik™ from Gamidor; 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (Carboxy-PTIO) and 3-morpholinosydnonimine (SIN-1) from Tocris Cookson; Cayman's Nitrate/Nitrite Assay Kit from Alexis corporation; Guanosine 3',5'-cyclic phosphoric acid 2'-O' succinyl-3-(^{125}I) iodo from Amersham; triethylamine and acetic anhydride from BDH.

The chemical structures of the drugs used in this study are shown in Figures 2.1 & 2.2.

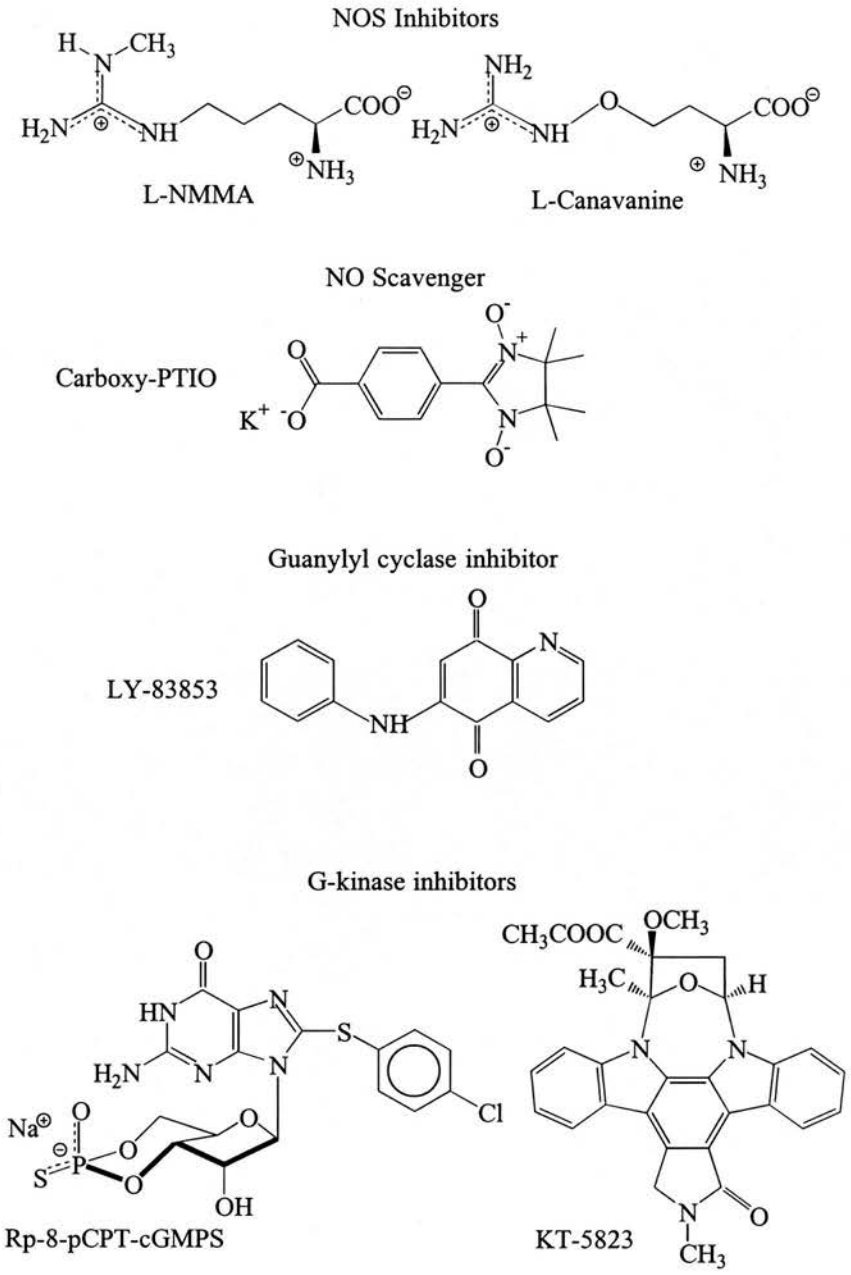
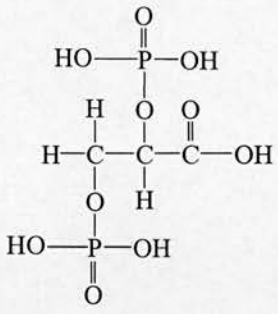


Figure 2.1 *Chemical structures of the NOS inhibitors, L-NMMA and L-Canavanine; the NO scavenger Carboxy-PTIO; the guanylyl cyclase inhibitor LY 83583 and the G-kinase inhibitors KT 5823 and Rp-8-cCPT-cGMPS.*

Phosphatase inhibitor



2,3-Diphospho-D-glyceric acid

NO donors

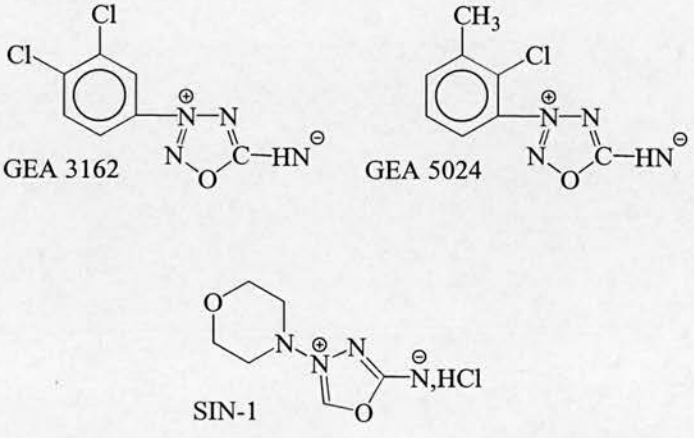


Figure 2.2 *Chemical structures of a phosphatase inhibitor 2,3-Diphospho-D-glyceric acid and the NO donors: GEA 3162, GEA 5024 and SIN-1.*

2.4 METHODS

2.4.1 Isolation of human neutrophils

Where possible and to avoid contamination, the following procedures were carried out within a laminar-flow cabinet using sterile materials and equipment. The isolation of human neutrophils is summarised in a simplified flow diagram (Figure 2.3).

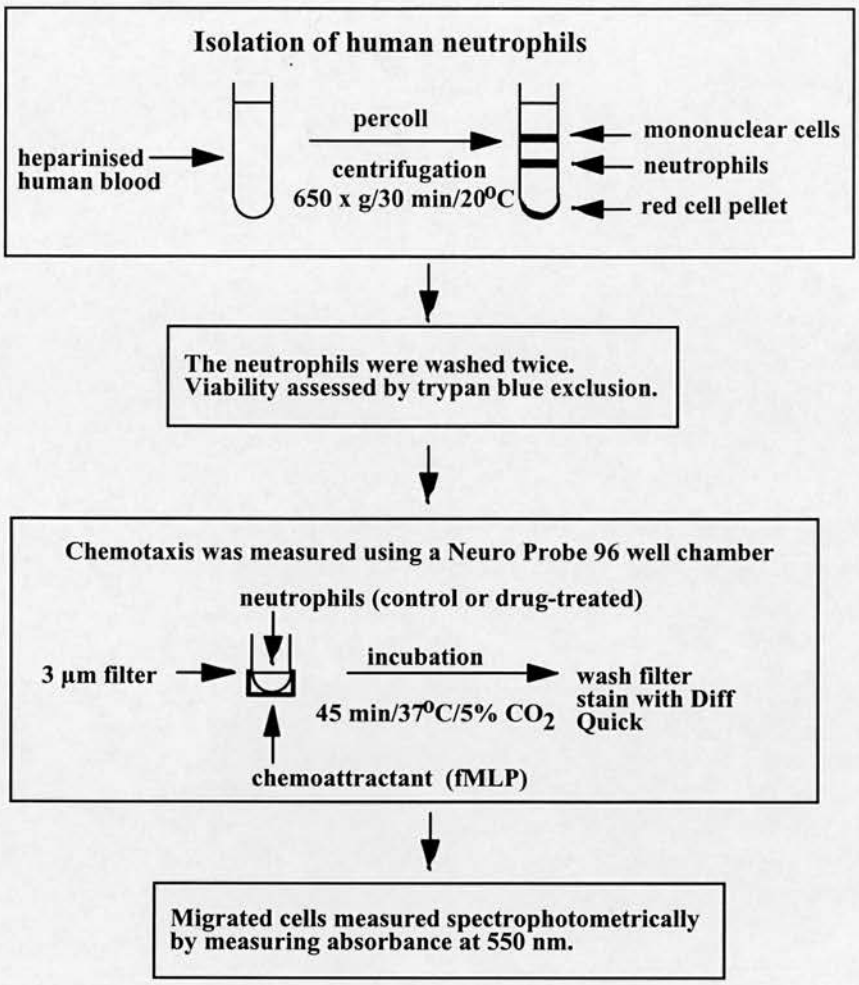


Figure 2.3 *Flow diagram of isolation of human neutrophils and measurement of neutrophil chemotaxis.*

Method

1. Venous blood (200 ml) was taken from the forearm of healthy volunteers, divided into 25 ml aliquots in eight 50 ml tubes and anti-coagulated with 50 units heparin per ml of blood, mixed with an equal volume of dextran (3% in 0.9% w/v saline) and left to stand at room temperature for 45 minutes to allow sedimentation of the red blood cells.

2. After 45 minutes, the leukocyte-rich plasma was then removed and dispensed into four tubes and then centrifuged at 280×g for 10 minutes. The resultant pellet was resuspended in 4×4 ml of 55% percoll. Using a syringe, the 55% percoll-leukocyte-rich solution was carefully layered onto each of the tubes

containing a discontinuous Percoll gradient (3 ml 70% Percoll layered on top of 5 ml 81% Percoll).

3. The tubes were then centrifuged for 30 minutes at 650×g to separate the polymorphonuclear leukocytes from the mononuclear cells. After centrifugation, the cells separated through the percoll concentration gradient into three visibly distinct layers; red blood cells at the bottom of the tubes, the cloudy neutrophil band settled in the middle layer and the cloudy monocyte band settled in the upper layer.

4. The neutrophil-rich layer was removed and washed with 0.9 % saline by centrifugation at 280×g, for 10 minutes, at room temperature, discarding the supernatant after spin.

5. Any contaminating red bood cells were lysed by resuspending the pellet in 10 ml ice cold 0.2% w/v NaCl solution for 20 seconds, after which 10 ml of ice cold 1.6% w/v NaCl solution was added to return the cells to isotonic conditions. To further wash the cells, 30 ml of 0.9% saline was added and the tubes were then centrifuged at 280×g, for 10 minutes, at room temperature. The pellet was then resuspended in 20 ml of PBS.

6. With this method of separation, neutrophils represented 84 ± 1.9% (n=9) of the polymorphonuclear leukocyte band, as determined by 1% of cells magnetically tagged with CD 16 microbeads (Eurogenetic).

7. Neutrophils were then counted and their viability assessed by trypan blue exclusion: 100 µl of the cell suspension was added to 400 µl of trypan blue and counted microscopically (×40) in an improved Neubauer chamber. The viability of the cells was more than 95%.

8. Finally the cells were resuspended, either at a concentration of 3×10^6 cells/ml in warm RPMI 1640 medium (without L-glutamine, Gibco) or at a concentration of

1.5×10^6 cells/ml in warm phosphate buffered saline (PBS containing Ca^{2+}/Mg^{2+}, Sigma) and kept at room temperature for determination of chemotaxis and O_2^- generation, respectively.

2.4.2 Chemotaxis procedure

The measurement of human neutrophil chemotaxis is summarised in a simplified flow diagram (Figure 2.3).

Method

1. The chemotaxis assay was performed using a 96 well chemotaxis chamber (Neuroprobe, Cabin John, Md). The bottom wells of the chamber were filled with chemoattractant (30 μl) which had been warmed to 37°C and vortexed to expel dissolved gases. To prevent excessive evaporative loss, the filling time was kept under 10 minutes. 30 μl of fMLP (1×10^{-7}M to 3×10^{-6}M) was used as the chemoattractant.

2. The top plate was fitted with a gasket and the framed filter (3 μm). The top plate, with the filter installed, was then inverted onto the filled bottom plate. The thumb nuts were installed and tightened gradually with equal force.

3. The upper wells were filled with 225 μl neutrophils (3×10^6 cells/ml) which had been treated with inhibitor or RPMI medium. In the case of carboxy-PTIO (100 μM), LY 83583 (10 and 100 μM), KT 5823(1 and 10 μM), Rp-8-pCPT-cGMPS (10 and 100 μM), and 2,3 diphosphoglyceric acid (DPG) (10 and 100 μM), neutrophils were treated with these inhibitors immediately prior to addition to the upper well. In the case of L-NMMA (500 μM), and NO donors (1-100μM), the cells were incubated with L-NMMA for 45 minutes and with NO donors for 10 minutes, prior to addition to the upper wells. The filled chamber was then incubated for 45 minutes at 37°C in a moist, 5% CO_2 atmosphere to allow neutrophil migration.

4. At the end of the incubation period, the filter was removed, washed, fixed and stained with Diff Quik™ (fixative-fast green in methanol for 5 minutes, eosin G in phosphate buffer for 5 minutes, thiazine dye in phosphate buffer for 5 minutes).

5. Chemotaxis was quantified spectrophotometrically by measuring absorbance at 550 nm (DYNATECH MR7000). The magnitude of the absorbance was taken as directly proportional to the amount of neutrophils, which had migrated and were trapped in the filter. An example of a filter with trapped neutrophils is shown in Figure 2.4. Basal absorbance was taken as cells without fMLP. Each incubation was carried out in triplicate and the values were averaged.

6. As LY 83583 and KT 5823 were dissolved in DMSO, the final percentages of DMSO in this assay of 10 μM and 100 μM LY 83583 treated cells were 0.05% and 0.5%, respectively and of 1 μM and 10 μM KT 83583 treated cells were 0.01% and 0.1%, respectively

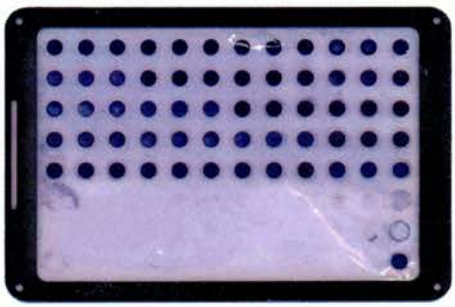


Figure 2.4 *An example of a filter with trapped neutrophils in each well.*

2.4.3 Superoxide anion generation

In this study, O_2^- generation by neutrophils was determined by spectrophotometric evaluation of the reduction of ferricytochrome C (Fe^{3+}) to ferrocytochrome C (Fe^{2+}) in the presence of cytochalasin B. Cytochalasin B has commonly been used in any experiments studying secretion and respiratory burst (Goldstein *et al.*, 1973) to

enhance the amount of O_2^- released during the reaction (Zurier *et al.*, 1974). In the neutrophils, association of bound chemoattractant receptors with the cytoskeleton dissociates bound receptors from the signal-transduction apparatus, thereby terminating the continued generation of intracellular signals required for neutrophil function. Cytochalasin B, an agent that depolymerises filamentous actin (F-actin), blocks the association of bound fMLP receptors with the cytoskeleton, permits increased generation of critical intracellular signals, and thereby amplifies the functional responses of the neutrophils to fMLP (Jesaitis *et al.*, 1986). The reduction of ferricytochrome C (Fe^{3+}) to ferrocytochrome C (Fe^{2+}) was previously shown to be inhibited by superoxide dismutase (SOD) (Babior *et al.*, 1973). The method that was used in this experiment is a modification of methods used by Gryglewski *et al.* (1987) and Babior *et al.* (1973). The measurement of O_2^- generation is summarised in a simplified flow diagram Figure 2.5 and the set up for this method is in Table 2.1

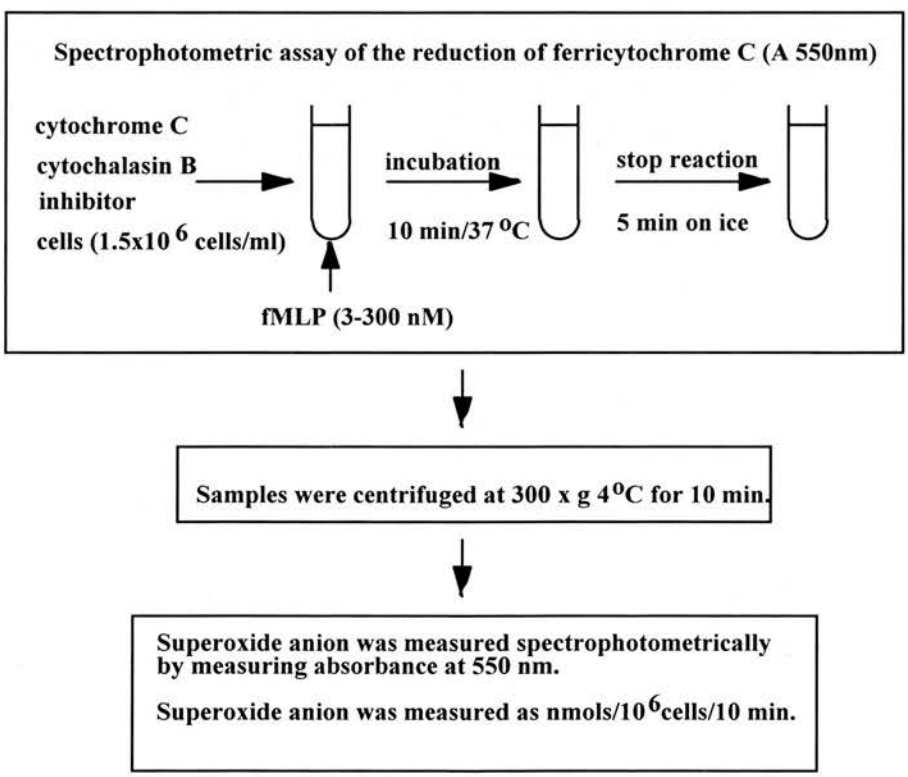


Figure 2.5 *Flow diagram of measurement of superoxide anion generation.*

Method

Neutrophils were isolated from human blood as discussed in section 2.4.1. For both control and sample tubes, cells (1.5×10^6cells/ml) were resuspended in PBS containing Ca^{2+} and Mg^{2+}, 5 mg/ml cytochrome C and 5 μg/ml cytochalasin B.

1. Cells (450 μl) were treated with PBS or inhibitor [Carboxy-PTIO (100 μM) or LY 83583 (10 and 100 μM) or KT 5823 (1 and 10 μM) or Rp-8-pCPT-cGMPs (100 μM)], immediately prior to the addition to tubes containing 50 μl of 3×10^{-8} to 3×10^{-6} M of fMLP (3-300 nM) and incubated for 10 minutes at 37°C. In the case of L-NMMA, L-canavanine, and DPG (all at contrations of 100 and 500 μM), the neutrophils were preincubated for 10-45 minutes, as appropriate, prior to addition to the tubes containing fMLP.

2. At the end of incubation period, the reaction was terminated by immersing the tubes in ice for 5 minutes and the samples were centrifuged at 320×g, at 4°C for 10 minutes, to sediment the cells.

3. Supernatant (200 μl) from each tube was dispensed into a 96 well plate and the absorbance at 550 nm was measured in a spectrophotometer (DYNATECH MR 7000). Basal absorbance was taken as cells without fMLP. Each sample was carried out in triplicate and the values were averaged. Results are expressed as nmol superoxide anions/10^6 cells/10 minute.

4. The final percentages of DMSO in this assay for 10 μM and 100 μM LY 83583 treated cells were 0.14% and 0.5%, respectively and for 1 μM and 10 μM KT 83583 treated cells were 0.01% and 0.18%, respectively

Table 2.1 : *Set up for superoxide anion generation*

Tube No.	Description	cells(µl)	Buffer(µl)	fMLP (50 µl)
1-3	Blank	450	50	_
4-6	Control cells	"	_	3×10^{-8} M
7-9	Control cells	"	_	10^{-7} M
10-12	Control cells	"	_	3×10^{-7} M
13-15	Control cells	"	_	10^{-6} M
16-18	Control cells	"	_	3×10^{-6} M
19-21	Blank	"	50	_
22-24	Treated cells	"	_	3×10^{-8} M
25-27	Treated cells	"	_	10^{-7} M
28-30	Treated cells	"	_	3×10^{-7} M
31-33	Treated cells	"	_	10^{-6} M
34-36	Treated cells	"	_	3×10^{-6} M

Cells (450 µl) which were resuspended in PBS containing Ca^{2+} and Mg^{2+}, 2.5 mg/ml cytochrome C and 5 µg/ml cytochalasin B were treated with PBS or inhibitor immediately prior to addition to tubes containing 50 µl of fMLP (3-300 nM) and incubated for 10 minutes at 37°C. In the case of L-NMMA, L- canavanine and DPG, the cells were preincubated for 10-45 minutes, as appropriate, prior to addition to the tubes containing 50 µl of fMLP. The reaction was terminated by immersing the tubes in ice for 5 minutes and the samples were centrifuged at 300×g, at 4°C. Samples (200 µl) from each tube were dispensed into 96 well plate and the absorbance at 550 nm was measured.

2.4.4 Measurement of guanosine 3' 5'-cyclic monophosphate (cyclic GMP)

In this study, radioimmunoassay (RIA) technique which was adapted from the method of Steiner and collaborators (Steiner *et al.*, 1972) and already set up for measurement of cyclic AMP in our laboratory (Armstrong & Talpain, 1994) was adapted for measurement of cyclic GMP production from neutrophils. The assay was based on competition of unlabelled cyclic GMP with a fixed quantity of labelled cyclic GMP for the same binding sites on the specific antibody (primary antibody). Incubation of unlabelled cyclic GMP, labelled cyclic GMP and primary antibody

allowed an equlibrium reaction to occur. The resulting solution then contained two more substances, the complex (bound) labelled cyclic GMP as well as the bound unlabelled. Separation of the bound from the unbound or "free" was achieved by the double antibody technique, followed by centrifugation. In our laboratory, Donkey Anti-Rabbit Serum (DARS) and Normal Rabbit Serum (NRS) were used as a second antibody. The addition of the second antibody to the reaction mixture resulted in the precipitation of the bound form, with the formation of cyclic GMP-primary antibody - second antibody complex. This complex was separated from the unbound form, which was in the supernatant, by centrifugation. After centrifugation, the pellet was counted in a gamma counter, giving an estimate of bound labelled cyclic GMP. This assay method measured the bound fraction and so the count obtained was inversely proportional to the amount of cyclic GMP presented in samples. By studying a series of standards of known amounts of unlabelled cyclic GMP, an unknown (cyclic GMP from neutrophils) was measured.

The dilution of primary antibody is often chosen so that the antibody binds approximately 50% of the labelled antigen. Therefore, before starting the RIA, dilution curves for the specific antibody were run to determine the optimum concentration for the RIA. A dilution curve was obtained by measuring the labelled cyclic GMP (count per minute, cpm) bound to increasing dilutions of the primary antibody. To get a dilution curve, 50 µl of 50 mM acetate buffer (pH 6.0) was incubated for 1 hour with 200 µl of 50 mM acetate buffer (pH 6.0) containing serially diluted primary antibody from 1:100 to 1:50000 and ^{125}I-cyclic GMP (10000 cpm/tube). DARS (50 µl, 1:10 dilution) and NRS (50 µl, 1:100 dilution) were added to all tubes and incubated overnight to achieve equilibrium binding. The assay tubes were washed with 6% polyethylene glycol in deionized water (1ml) and then centrifuged at 1900×g for 30 minutes at 4°C. The supernatant (unbound form) was aspirated and the residue (bound form) counted using a Gamma Counter. From these results a 1: 400 dilution of primary antibody which bound about 50% of the total counts was chosen for this study with the reason of it would produce the most significant changes in cpm (count per minute) values for a small change in displacment.

2.4.4.1 Extraction of cyclic GMP from neutrophils

The set up for extraction of cyclic GMP from neutrophils is summarised in Table 2.2.

Method

1. Neutrophils were isolated from human blood as discussed above in section 2.4.1. Neutrophils were resuspended to give 5×10^6 cells per ml in PBS containing 0.25 mM isobutylmethylxanthine (IBMX) (a non selective phosphodiesterase inhibitor) to block cyclic GMP breakdown by the enzyme phosphodiesterase.

2. 50 µl of 10^{-6} and 3×10^{-6} M fMLP (0.1 and 0.3 µM) or NO donors [GEA 3162 or GEA 5024 or SIN-1 (10-1000 µM)] or PBS were added to each tube.

3. 450 µl of cells (5×10^6 cells per ml) were added to each tube at 10 second intervals and each tube incubated for 10 minutes at 37°C. The reaction was then stopped by addition of 1.0 ml ethanol.

4. Five minutes later, the samples were centrifuged at 650×g, 20°C for 20 minutes, the ethanolic supernatants removed and evaporated to dryness at 55°C. The residue was dissolved in 0.5 ml 50 mM acetate buffer and centrifuged at 1900×g, 4°C, for 30 minutes to remove insoluble material.

Table 2.2 *Set up for cyclic GMP production from neutrophils*

Tubes No.	Description	cells (µl)	buffer (µl)	fMLP (50 µl)	No-donors (50 µl)
1-2	Control	450	50	_	_
3-4	0.1 µM fMLP	"	_	10^{-6} M	_
5-6	0.3 µM fMLP	"	_	3×10^{-6} M	_
7-8	10 µM GEA 3162	"	_	_	10^{-4} M
9-10	50 µM GEA 3162	"	_	_	5×10^{-4} M
11-12	100 µM GEA 3162	"	_	_	10^{-3} M
13-14	10 µM GEA 5024	"	_	_	10^{-4} M
15-16	50 µM GEA 5024	"	_	_	5×10^{-4} M
17-18	100 µM GEA 5024	"	_	_	10^{-3} M
19-20	10 µM SIN-1	"	_	_	10^{-4} M
21-22	50 µM SIN-1	"	_	_	5×10^{-4} M
23-24	100 µM SIN-1	"	_	_	10^{-3} M
25-26	1000 µM SIN-1	"	_	_	10^{-2} M

50 µl of fMLP (10^{-6} and 3×10^{-6}M) or NO donors: GEA 3162 or GEA 5024 (10^{-4}, 5×10^{-4} and 10^{-3} M) or PBS were added to each tube. 450 µl of cells (5×10^{6} cells/ml) were added to each tube at 10 second intervals and each tube incubated for 10 minutes at 37°C. The reaction was stopped by addition of 1 ml ethanol. The samples were then centrifuged at 650×g, 20°C for 20 minutes, the ethanolic supernatants removed and evaporated to dryness at 55°C. The residue was dissolved in 0.5 ml 50 mM acetate buffer and centrifuged at 1900×g, 4°C, for 30 minutes. The supernatant was assayed for cyclic GMP level by RIA.

2.4.4.2 Measurement of cyclic GMP production in neutrophils

The set up for measurement of cyclic GMP by RIA is shown in Table 2.3.

Method.

1. 500 µl aliquots of samples and standards were acetylated with 20 µl of a mixture of triethylamine/acetic anhydride (2:1), whirlimixed immediately and left for 2-3 minutes to acetylate, prior to the measurement of cyclic GMP by RIA.

2. A cyclic GMP standard curve (0.0625-32 nM) was performed in order to quantify cyclic GMP concentrations of the samples.

3. Both samples and standards were measured in duplicate.

4. ^{125}I-Cyclic GMP (about 10 µl) was counted and a volume that would give about 5000 cpm /tube was calculated.

5. Each assay tube contained the following substances: 50 µl of acetylated unlabelled cyclic GMP (Sigma) (0.0625-32 nM) or acetylated samples to be measured, 200 µl of 50 mM sodium acetate buffer (pH 6.0) (with 0.1% BSA) containing primary antibody (1:400 dilution) and guanosine 3',5'-cyclic phosphoric acid 2'-o' succinyl-3-(^{125}I) iodo tyrosine methyl ester (5000 cpm/tube, Amersham).

6. The assay tubes were equilibrated at 4°C for 1 hour, then 50 µl of DARS (1:10 dilution in 50 mM phosphate buffer) and 50 µl of NRS (1:100 dilution in 50 mM phosphate buffer) added into all tubes except T/T tube (total count) and the tubes whirlimixed, then incubated overnight at 4°C.

7. All assay tubes except T/T tube were washed with 1.0 ml 6 % polyethylene glycol (PEG) in deionized water, whirlimixed and then centrifuged at 1900×g for 30 minutes at 4°C. The supernatant was then aspirated.

8. The residue in all tubes, including T/T tube was counted on a Gamma Counter for 1 minute. This was pre-programmed to plot a standard curve from the standards [0.0625-32.0 nM in 50 mM sodium acetate buffer (pH6.0)] and to calculate a best-fit hyperbola from the points using a "Logit" fit of log × versus y. An example of a RIA standard curve of cyclic GMP is shown in Figure 2.24. From this curve the counter also calculated the amount of cyclic GMP (fmol) present in each sample. As experiments were carried out in duplicate and assayed for cyclic GMP in duplicate, the program calculated the mean for four points and also included the standard error for the four values.

Table 2.3 *Set up for cyclic GMP Radioimmunoassay.*

Tube No.	Description	acetate buffer (µl)	Standard/Unknown (50 µl)	^{125}I-cGMP/ 1°Ab mixture (µl)
1-3	Total bound (0 cGMP)	50		200
4-6	NSB (16000fM)	_	320 nM cGMP	"
7-8	3.125 fM cGMP	_	0.0625 nM cGMP	"
9-10	6.25 fM cGMP	_	0.125 nM cGMP	"
11-12	12.5 fM cGMP	_	0.25 nM cGMP	"
13-14	25 fM cGMP	_	0.50 nM cGMP	"
15-16	50 fM cGMP	_	1.0 nM cGMP	"
17-18	100 fM cGMP	_	2.0 nM cGMP	"
19-20	200 fM cGMP	_	4.0 nM cGMP	"
21-22	400 fM cGMP	_	8.0 nM cGMP	"
23-24	800 fM cGMP	_	16.0 nM cGMP	"
25-26	1600 fM cGMP	_	32.0 nM cGMP	"
27-28	unknown	_	unknown	"
29-30	unknown	_	"	"
-------	unknown	_	"	"
T/T	Total count	_		"

The assay tubes were equilibrated at 4°C for 1 hour, then 50 µl of DARS (1:10 dilution in 50 mM phosphate buffer) and 50 µl of NRS (1:100 dilution in 50 mM phosphate buffer) were added into all tubes except T/T tube (total count) and then incubated overnight at 4 ℃. All assay tubes except T/T tube were washed with 1.0 ml 6% polyethyene glycol and then centrifuged at 1900×g for 30 minutes at 4°C. The supernatant was aspirated. The residue in all tubes, including T/T tube, was counted on a Gamma Counter for 1 minute.

2.4.5 Nitrate and nitrite production

NO undergoes a series of reactions with several molecules present in biological fluids. The addition of NO to an aqueous saline environment under physiological condition of temperature, oxygen tension, and pH results in accumulation of nitrite (NO_2^-) and lesser amounts of nitrate (NO_3^-). To account for the formation of nitrite

as a primary metabolite of NO along with smaller amounts of NO_3^- (Furchgott *et al.*, 1990), the following reactions have been proposed:

$$NO + O_2^- \longrightarrow ONO_2^- + H^+ \longrightarrow NO_3^- + H^+$$

$$2NO + O_2 \longrightarrow 2\,NO_2 \longleftrightarrow N_2O_4$$

$$N_2O_4 + 2\,OH^- \longrightarrow NO_2^- + NO_3^- + H_2O$$

$$NO + NO_2 \longrightarrow N_2O_3$$

$$N_2O_3 + 2\,OH^- \longrightarrow 2NO_2^- + H_2O$$

NO_2, nitrogen dioxide; N_2O_3, dinitrogen trioxides; N_2O_4, dinitrogen tetraoxides

The final products of NO *in vivo* are nitrite (NO_2^-) and nitrate (NO_3^-). The relative proportion of NO_2^- and NO_3^- is variable and can not be predicted with certainty. Thus, the best index of total NO production is the sum of both NO_2^- and NO_3^-.

2.4.5.1. Total nitrate/nitrite producton in neutrophils: Effect of the NO donors and fMLP

There are two ways of measuring the generation of NO quantitatively: the oxidation of haemoglobin and the formation of NO_2^- and NO_3^-. We have used the latter method. The set up for total nitrate/nitrite production in neutrophils is summarised in Table 2.4.

Method

1. Neutrophils were isolated from human blood as discussed in section 2.4.1. For control and sample tubes, cells (1.5×10^6 cells/ml) were resuspensed in PBS.

2. In the study of the effect of NO donors (GEA 3162, GEA 5024 and SIN-1) on total nitrate/nitrite production, 450 µl of cells that were treated with these compounds (1-100 µM) for 10 minutes at 37°C were added to the tubes containing 50 µl PBS.

3. In the study of the effect of fMLP on total nitrate/nitrite production, 450 µl of cells that were treated with PBS for 10 minutes at 37°C were added to the tubes containing 50 µl of 10^{-7} to 3×10^{-6} M of fMLP (0.01-0.3 µM). This was done in this way to mimic the experimental protocol for O_2^- generation.

4. All tubes were incubated for 30 minutes at 37°C. At the end of incubation period, the reaction was terminated by immersing the tubes in ice for 5 minutes and the samples were centrifuged at 320×g, at 4°C for 10 minutes to sediment the cells. Supernatants containing nitrate and nitrite were used for the measurement of total nitrate/nitrite production.

Table 2.4 *Set up for total nitrate/nitrite production from neutrophils: Effect of the NO donors and fMLP.*

Tube No.	Description	Control/treated cells (µl)	PBS (µl)	fMLP (µl)
1-3	Control	450	50	_
4-6	1 µMGEA 3162	"	"	_
7-9	10 µM GEA 3162	"	"	_
10-12	50 µM GEA 3162	"	"	_
13-15	100 µM GEA 3162	"	"	_
16-18	1 µM GEA 5024	"	"	_
19-21	10 µM GEA 5024	"	"	_
22-24	50 µM GEA 5024	"	"	_
25-27	100 µM GEA 5024	"	"	_
28-30	1 µM SIN-1	"	"	_
31-33	10 µM SIN-1	"	"	_
34-36	50 µM SIN-1	"	"	_
37-39	100 µM SIN-1	"	"	_
40-42	Control	"	"	_
43-45	0.01 µM fMLP	"	_	50
46-48	0.03 µM fMLP	"	_	"
49-51	0.1 µM fMLP	"	_	"
52-54	0.3 µM fMLP	"	_	"

Cells (450 µl) that were treated with NO donors or PBS for 10 minutes at 37°C were added to the tubes containing 50 µl PBS or 50 µl fMLP (10-300 nM), respectively. All tubes were incubated for 30 minutes at 37°C and then immersed in ice for 5 minutes to stop the reaction. The samples were centrifuged at 300×g, at 4°C for 10 minutes. Supernatants were used for the measurement of total nitrate/nitrite production.

2.4.5.2 Measurement of nitrate/nitrite production

Formation of NO_2^- and NO_3^-, the oxygenation products of NO, can be measured by a variety of techniques. The simplest is to use reduction of NO_3^- to NO_2^- by nitrate reductase or metallic catalysts followed by the colorimetric Griess reaction (Davison *et al.*, 1978; Green *et al.*, 1982; Stuehr *et al.*, 1989) to measure NO_2^-. Other methods measure NO_2^- by chemiluminescence following reconversion to NO (Palmer *et al.*, 1987; Knowles *et al.*, 1989; Palacios *et al.*, 1989; Bush. *et al.*, 1992) or by h.p.l.c. techniques with u.v. absorption detection (Stein *et al.*, 1988; Wiklund *et al.*, 1993). In this study, total NO_3^- and NO_2^- production was measured spectrophotometrically by using Cayman's Nitrate/Nitrite Assay Kit (Alexis Corporation). The measurement was a simple two-step process (Figure 2.6).

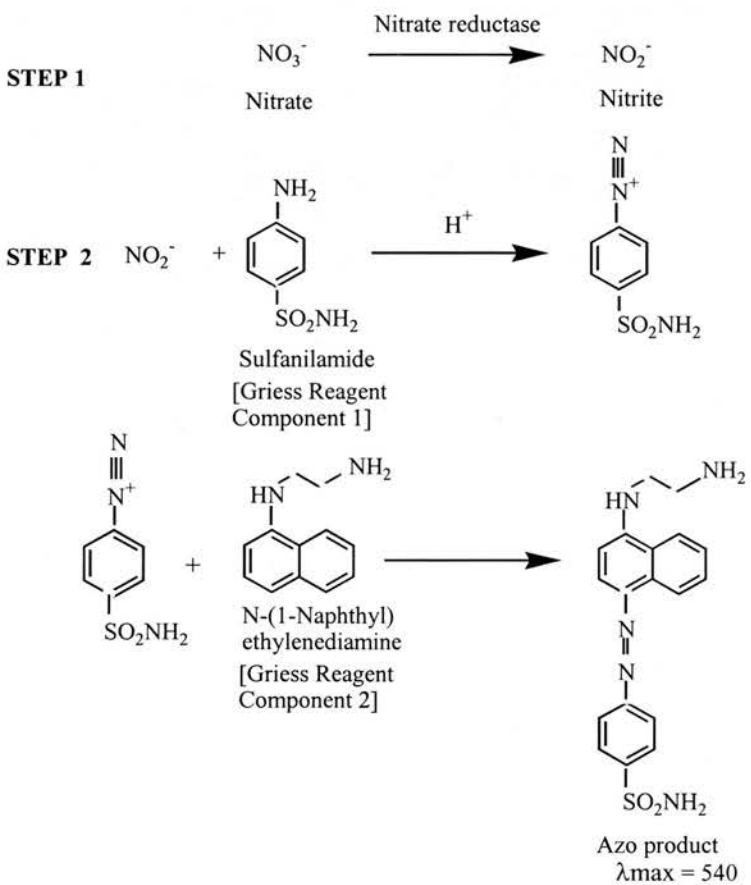


Figure 2.6 *Diagram of the simple two-step process of the conversion of nitrate to nitrite and the conversion of nitrite to an azo chromophore.*

The first step was the conversion of nitrate to nitrite utilizing nitrate reductase. The second step was the addition of the Griess Reagents which convert nitrite into a deep purple azo compound. Photometric measurement of the absorbance (540 nm) due to this azo chromophore accurately determined NO_2^- concentration. The measurement of total nitrate/nitrite is summarised in a simplified flow diagram (Figure 2.7).

Each time the samples were assayed for total nitrate and nitrite production, a nitrate standard curve was performed in order to quantify sample nitrate/nitrite concentrations. Both samples and standards were carried out in duplicate.

Method

1. Assay buffer (200 µl) was added to the blank wells. 80 µl of nitrate standards (5-35 µM) or samples were dispensed into a microtiter plate (96-well plate).

2. The enzyme co-factor mixture (10 µl) was added to each of the wells (standards and samples).

3. The nitrate reductase mixture (10 µl) was added to each of the wells (standards and samples). The microtiter plate was covered with the plate cover and incubated at room temperature for 1 hour.

4. After the required incubation time, 50 µl of Griess Reagent component 1 was added to each of the wells (standards and samples), followed by the addition of 50 µl of Griess Reagent Component 2 immediately after. The plate was left for 10 minutes at room temperature to allow the colour to develop.

5. The absorbance was read at 540 nM using the plate reader (DYNATECH MR 5000).

6. A nitrate standard curve was constructed and the absorbance of each sample was calculated back to the concentration of total nitrate/nitrite.

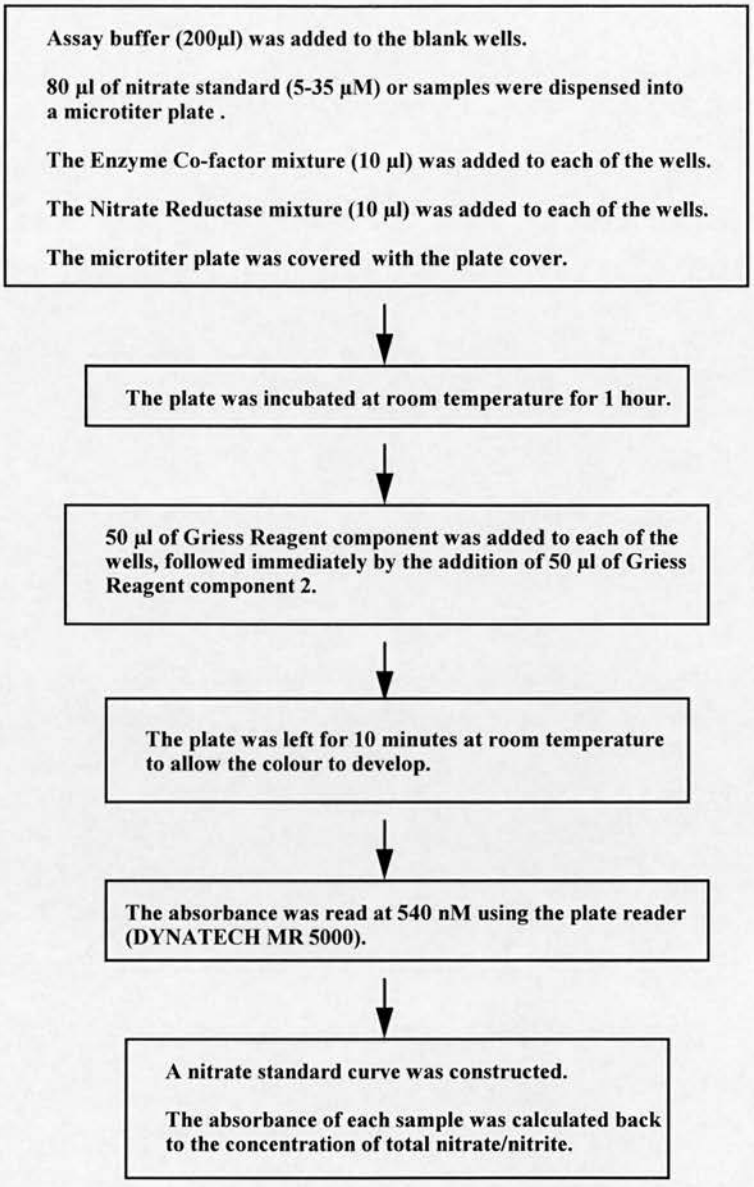


Figure 2.7 *Flow diagram for measurement of total nitrate/nitrite from neutrophils.*

2.4.6 Data analysis

2.4.6.1 Data analysis for chemotaxis

EC_{50} values (concentration of fMLP required to produce 50% of the maximal chemotactic effect produced by fMLP) were calculated for fMLP in the presence and absence of inhibitors using the Apple Macintosh programme "KaleidaGraph".

Kaleidagraph is not a graph-fitting but a graph-drawing programme and so uses the experimental maximum observed to determine the EC_{50} value.

Effects of the NO donors, GEA 3162, GEA 5024 and SIN-1, on fMLP-induced chemotaxis were expressed as the percentage inhibition of the response produced by a submaximally effective concentration of fMLP (100 nM). The EC_{50} for GEA 5024 and SIN-1 were expressed relative to the maximum effect achieved with GEA 3162. From EC_{50} values, equieffective concentration ratios (EEC) were calculated relative to the standard inhibitor, GEA 3162 (EEC=1).

$$\text{EEC for B} = \frac{[EC_{50}\,forB]}{[EC_{50}\,forA]} \quad \text{when [A] = a standard inhibtor}$$

2.4.6.2 Data analysis for superoxide anion generation

The amount of O_2^- generation (nmole per 10^6 neutrophils per 10 min) was calculated using the following equation:

$$\frac{dE}{Q \times d} \times \frac{1}{L} \times 10^6$$

L = 1.5×10^6 cells per ml (concentration of human neutrophils)

dE = absorbance of samples with fMLP - absorbance of sample without fMLP

Q = coefficient of molar extinction 21.1×10^3 per M per cm

d = thickness of well = 0.6 cm

Each incubation was carried out in triplicate and the values were averaged.

EC_{50} values were calculated as above.

2.4.6.3 Data analysis for total nitrate and nitrite production

Standard curve $y = mx + c$

m = slope

c = y-intercept

y = absorbance

x = amount of nitrate and nitrite

Determination of sample nitrate and nitrite concentrations

$$\text{nitrate} + \text{nitrite} = \frac{\lfloor A540 - y - \text{intercept} \rfloor}{\text{slope}} \times \frac{200\mu l}{\text{volume of sample}} \times \text{dilution}$$

2.4.7 Statistical analysis

All data are expressed as the mean ± standard error of the mean (s.e. mean), of the averaged result taken from a minimum of four separate experiments. Differences were assessed by using Student's two-tailed *t* test for paired observations. In addition, data involving multiple comparisons were analysed by ANOVA (two factors with replication) using Microsoft Excel. A value of $P < 0.05$ was regarded as denoting statistically significant differences.

2.5 RESULTS

2.5.1 Neutrophil chemotaxis

2.5.1.1 Effect of NOS inhibition

When neutrophils were preincubated with the NOS inhibitor, L-NMMA (500 μM) for 45 minutes at 37°C, significant attenuation ($P<0.001$, ANOVA) of fMLP-induced neutrophil chemotaxis occurred (Figure 2.8) (Table 2.5, page 67), giving EC_{50} for fMLP of 28.8 ± 5.6; 41.1 ± 4.8 pmol /10^6cells, n=5 ($P>0.05$) in the absence and presence of L-NMMA, respectively. The maximal effect of fMLP was reduced from 1.1 ± 0.1 to 0.7 ± 0.1 n=5 ($P<0.05$) (Table 2.5). Similarly, L-NMMA at a concentration of 100 μM induced a significant but less pronounced attenuation of neutrophil chemotaxis, (data not shown; $P<0.05$).

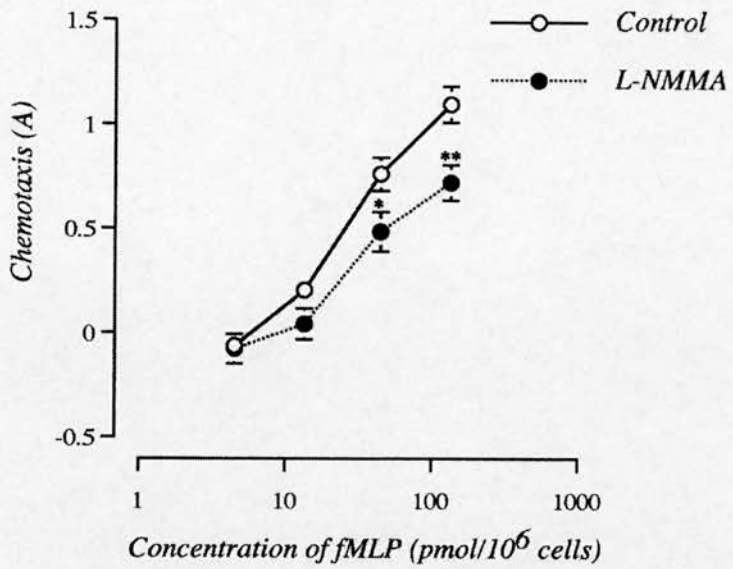


Figure 2.8 *Log concentration-effect curve for fMLP induced neutrophil chemotaxis in control cells and cells treated with 500 μM L-NMMA. Cells were preincubated with L-NMMA for 45 minutes at 37°C. The values are the mean ± s.e. mean of 5 different donors. Statistically significant differences* $^{*}P<0.05$ *and* $^{**}P<0.01$.

Table 2.5 *Effects of an NOS inhibitor, L-NMMA; a NO-scavenger, carboxy-PTIO; an inhibitor of guanylyl cyclase, LY 83583 on fMLP-induced human neutrophil chemotaxis.*

Drug	Number (n)	EC_{50} for fMLP (pmol/10^6 cells)	Maximal effect
Control	5	28.8 ± 5.6	1.1 ± 0.1
L-NMMA (500 μM)	5	41.1 ± 4.8	0.7 ± 0.1*
	5		
Control	5	19.7 ± 4.2	1.4 ± 0.1
Carboxy-PTIO (100 μM)	5	31.7 ± 8.5	1.0 ± 0.04*
	5		
Control	5	19.1 ± 4.3	1.1 ± 0.2
LY 83583 (10 μM)	5	47.0 ± 7.5*	0.9 ± 0.2
Control	5	32.5 ± 11.2	1.7 ± 0.01
LY 83583 (100 μM)	5	85.2 ± 10.4*	0.3 ± 0.1***

Data expressed as EC_{50} *for fMLP-induced neutrophil chemotaxis. The values are the mean ± s.e. mean of 5 different donors for each group. Statistically significant differences* $^{*}P<0.05$ *and* $^{***}P< 0.005$.

2.5.1.2 *Effect of a NO scavenger*

Carboxy-PTIO, a NO scavenger, at a concentration of 100 μM caused slight attenuation ($P<0.05$, ANOVA) of fMLP-induced neutrophil chemotaxis (Figure 2.9), giving EC_{50} for fMLP of 19.7 ± 4.2; 31.7 ± 8.5 pmol /10^6cells, n=5 ($n>0.05$) (Table 2.5, page 67) in the absence and presence of Carboxy-PTIO, respectively. However, this concentration of Carboxy-PTIO caused a significant reduction in the maximal effect of fMLP from 1.4 ± 0.1 to 1.0 ± 0.04, n = 5 ($P<0.05$) (Table 2.5).

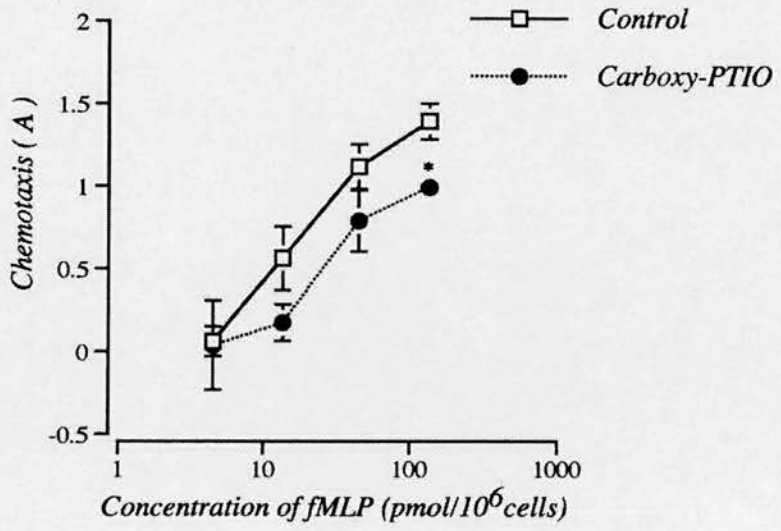


Figure 2.9 *Log concentration effect-curve for fMLP induced neutrophil chemotaxis in control cells and cells treated with 100 μM Carboxy-PTIO. The values are the mean ± s.e. mean of 5 different donors. Statistically significant difference *P<0.05.*

2.5.1.3 *Effect of guanylyl cyclase inhibition*

LY 83583, an inhibitor of guanylyl cyclase, at a concentration of 10 μM caused a significant attenuation ($P<0.01$, ANOVA) of fMLP-induced neutrophil chemotaxis (Figure 2.10a), giving EC_{50} for fMLP of 19.1 ± 4.3; 47.0 ± 7.5 pmol/10^6 cells, n=5 ($P<0.05$) (Table 2.5, page 67) in the absence and presence of LY-83583, respectively. This concentration of LY 83583 caused no significant reduction in the maximal effect of fMLP.

LY 83583 at a concentration of 100 μM caused a highly significant attenuation ($P<0.001$, ANOVA) of fMLP-induced neutrophil chemotaxis (Figure 2.10b), giving

EC_{50} for fMLP of 32.5 ± 11.2; 85.2 ± 10.4 pmol/10^6 cells, n=5 (P<0.05) (Table 2.5) in the absence and presence of LY-83583, respectively. LY 83583 at a concentration of 100 μM caused a significant reduction in the maximal effect of fMLP from 1.7 ± 0.01 to 0.3 ± 0.1, n = 5 (P<0.005) (Table 2.5).

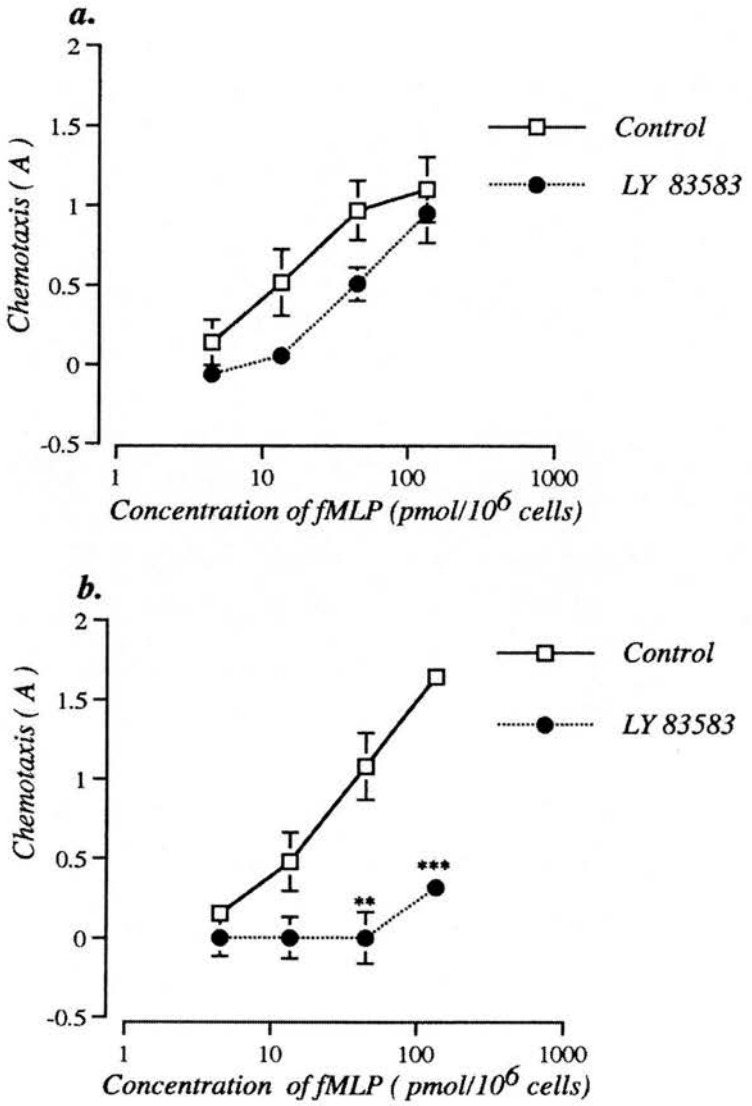


Figure 2.10 *Log concentration-effect curve for fMLP induced neutrophil chemotaxis in control cells and cells treated with LY 83583 (a) 10 μM and (b) 100 μM. The values are the mean ± s.e. mean of 5 different donors. Statistically significant differences* $^{**}P<0.01$ *and* $^{***}P<0.005$.

2.5.1.4 Effect of G-kinase inhibition

KT 5823, a specific inhibitor of G-kinase, at a concentration of 1 μM had no significant inhibitory effect ($P>0.05$, ANOVA) on fMLP-induced neutrophil chemotaxis (Figure 2.11a), giving EC_{50} for fMLP of 19.1 ± 4.3; 35.5 ± 13.5 pmol/10^6cells, n = 5 ($P>0.05$) (Table 2.6, page 74) in its absence and presence, respectively. However, at a concentration of 10 μM, KT 5823 completely inhibited ($P<0.001$, ANOVA) fMLP-induced neutrophil chemotaxis (Figure 2.11b), giving EC_{50} for fMLP of 32.2 ± 11.4; > 135 pmol/10^6cells, n = 5 ($P< 0.005$) (Table 2.6) in the absence and presence of KT 5823, respectively.

Rp-8-pCPT-cGMPS, another inhibitor of cyclic GMP-dependent protein kinase G1α both at concentrations of 10 μM and 100 μM had no significant inhibitory ($P>0.05$, ANOVA) effect on fMLP-induced neutrophil chemotaxis (Figures 2.12a & 2.12b), giving EC_{50} for fMLP of 19.1 ± 4.3; 32.7 ± 14.8, n = 5 ($P>0.05$) and 32.2 ± 11.4; 21.7 ± 4.2 pmol/10^6cells, n = 5 ($P>0.05$) (Table 2.6) in its absence and presence at the two concentrations, respectively.

2.5.1.5 Effect of phosphatase inhibition

DPG, an inhibitor of inositol polyphosphate-5-phosphatase, at a concentration of 10 μM caused significant attenuation ($P<0.001$, ANOVA) of fMLP-induced neutrophil chemotaxis (Figure 2.13a), giving EC_{50} for fMLP of 26.0 ± 4.3; 33.4 ± 4.0 pmol/10^6cells, n = 4 ($P>0.05$) (Table 2.6, page 74) in its absence and presence, respectively. The maximal effect of fMLP was reduced by 10 μM DPG from 1.4 ± 0.1 to 0.9 ± 0.1, n = 4 ($P<0.05$) (Table 2.6).

DPG at a concentration of 100 μM caused significant attenuation ($P<0.001$, ANOVA) of fMLP-induced neutrophil chemotaxis (Figure 2.13b), giving EC_{50} for fMLP of 19.2 ± 4.4; 61.5 ± 16.2 pmol/10^6cells, n = 4 ($P>0.05$) (Table 2.6) in its absence and presence, respectively. The maximal effect of fMLP was reduced by 100 μM DPG from 1.4 ± 0.1 to 0.7 ± 0.1, n = 4 ($P<0.05$) (Table 2.6), respectively.

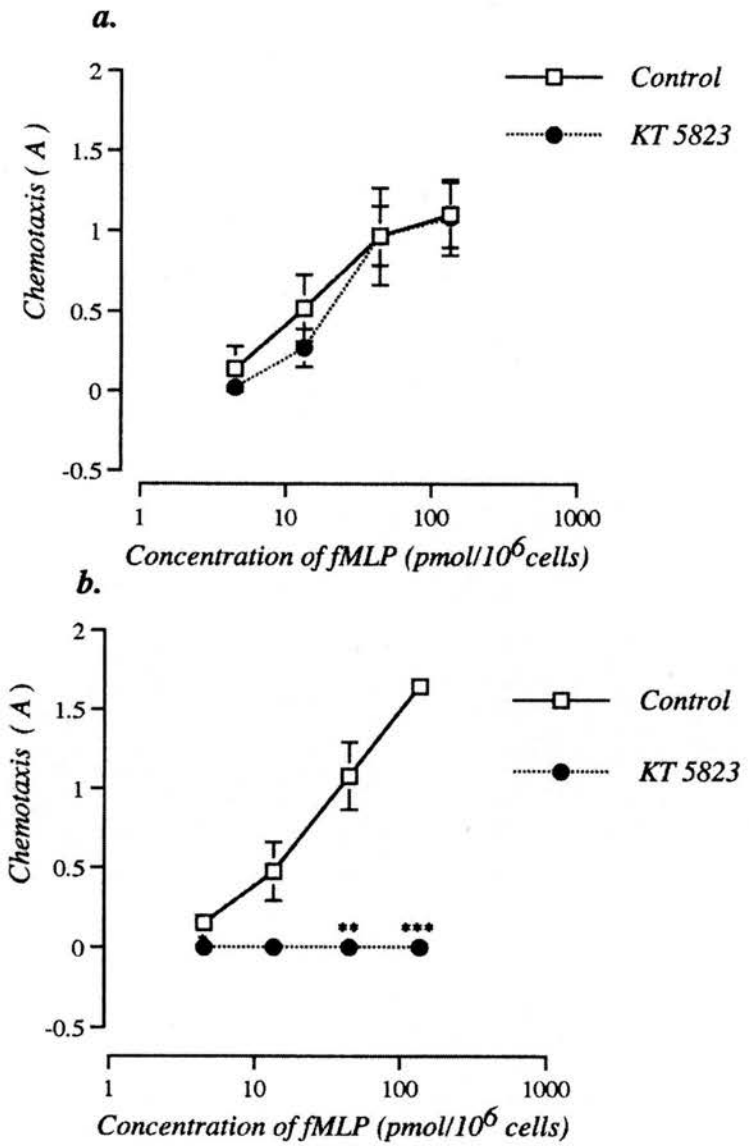


Figure 2.11 *Log concentration-effect curve for fMLP induced neutrophil chemotaxis in control cells and cells treated with KT 5823 (a) 1 μM and (b) 10 μM. The values are the mean ± s.e. mean of 4 different donors. Statistically significant differences* $^{**}P<0.01$ *and* $^{***}P<0.005$.

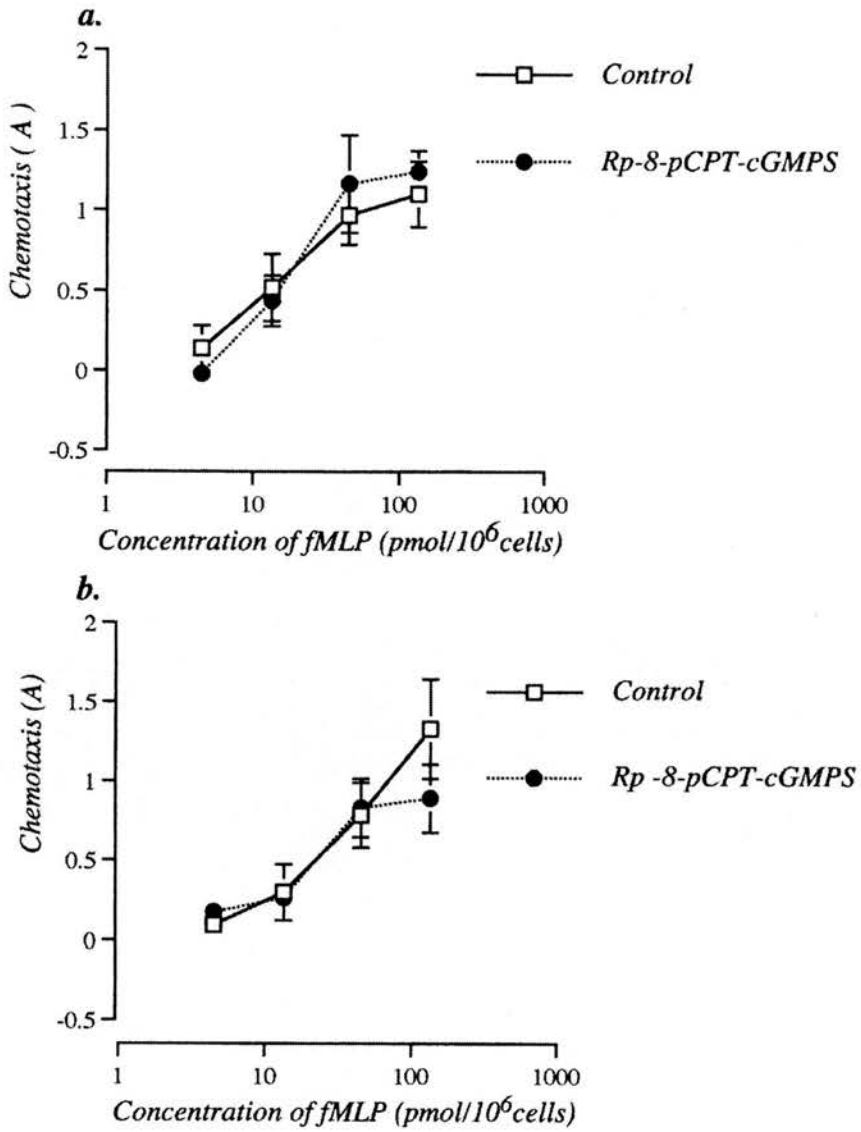


Figure 2.12 *Log concentration-effect curve for fMLP induced neutrophil chemotaxis in control cells and cells treated with Rp-8-pCPT-cGMPS (a) 10 μM and (b) 100 μM. The values are the mean ± s.e. mean of 5 different donors.*

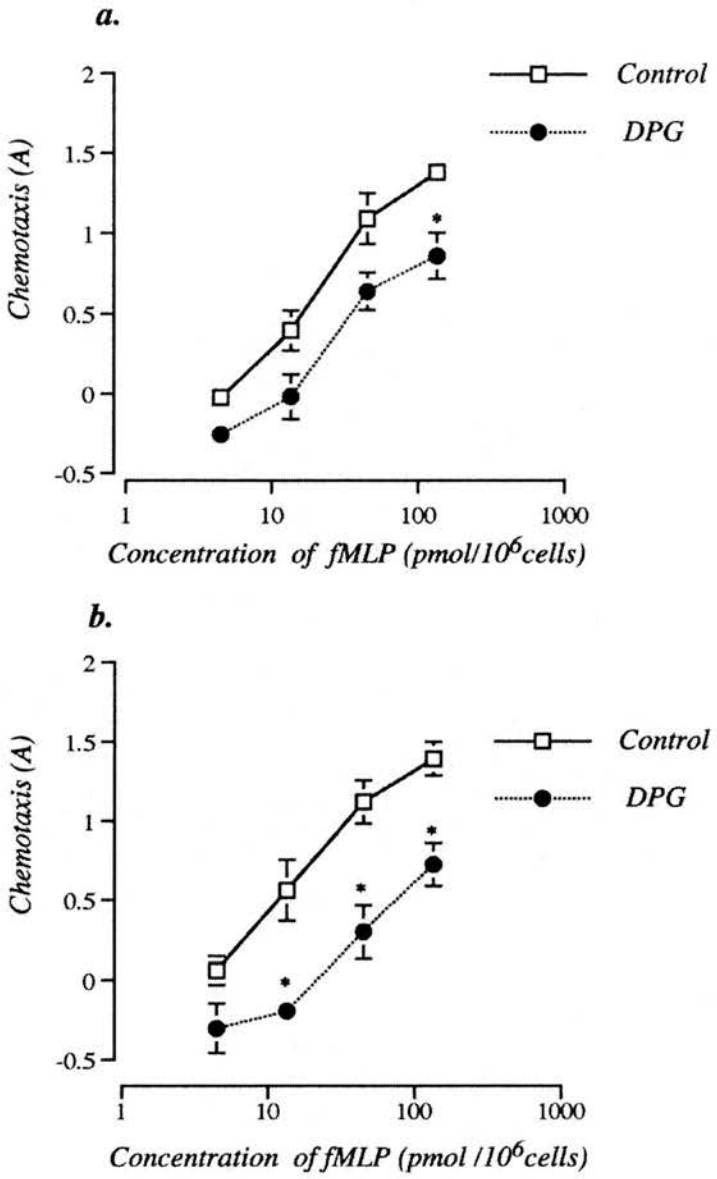


Figure 2.13 *Log concentration-effect curve for fMLP induced neutrophil chemotaxis in control cells and cells treated with DPG (a) 10 μM and (b) 100 μM. The values are the mean ± s.e. mean of 4 different donors. Statistically significant difference* $^{*}P<0.05$.

Table 2.6 *Effects of inhibitors of G-kinase, KT 5823 and Rp-8-pCPT-cGMPs and a phosphatase inhibitor, DPG on fMLP-induced human neutrophil chemotaxis.*

Drug	number (n)	EC_{50} for fMLP (pmol/10^6 cells)	Maximal effect
Control	5	19.1 ± 4.3	1.1 ± 0.2
KT 5823 (1 μM)	5	35.5 ± 13.5	1.1 ± 0.2
Control	5	32.2 ± 11.4	1.6 ± 0.0
KT 5823 (10 μM)	5	>135***	0.0
Control	5	19.1 ± 4.3	1.1 ± 0.2
Rp-8-pCPT-cGMPs (10 μM)	5	32.7 ± 14.8	1.2 ± 0.1
Control	5	32.2 ± 11.4	1.3 ± 0.3
Rp-8-pCPT-cGMPs (100 μM)	5	21.7 ± 4.2	0.9 ± 0.2
Control	4	26.0 ± 4.3	1.4 ± 0.1
DPG (10 μM)	4	33.4 ± 4.0	0.9 ± 0.1*
Control	4	19.2 ± 4.4	1.4 ± 0.1
DPG (100 μM)	4	61.5 ± 16.2	0.7 ± 0.1*

Data expressed as EC_{50} for fMLP-induced neutrophil chemotaxis. The values are the mean ± s.e. mean of 4-5 different donors for each group. Statistically significant differences $^{*}P<0.05$ *and* $^{***}P< 0.005$.

2.5.2 Neutrophil superoxide anion generation

2.5.2.1 Effect of NOS inhibition

When neutrophils were preincubated with L-NMMA at a concentration of 100 μM for 45 minutes at 37°C, L-NMMA caused no significant inhibition (P>0.05, ANOVA) of fMLP-induced O_2^- generation in human neutrophils (Figure 2.14), giving EC_{50} for fMLP of 54.2 ± 11.5; 57.1 ± 10.9 nM, n = 6 (P>0.05) (Table 2.7, page 75) in its absence and presence, respectively. Even when the concentration of L-NMMA was increased to 500 μM, no significant inhibition of fMLP-induced O_2^- generation was observed (Figure 2.14), giving EC_{50} for fMLP of 54.2 ± 11.5; 61.4 ± 12.9 nM, in its absence and presence, respectively n = 6 (P>0.05) (Table 2.7).

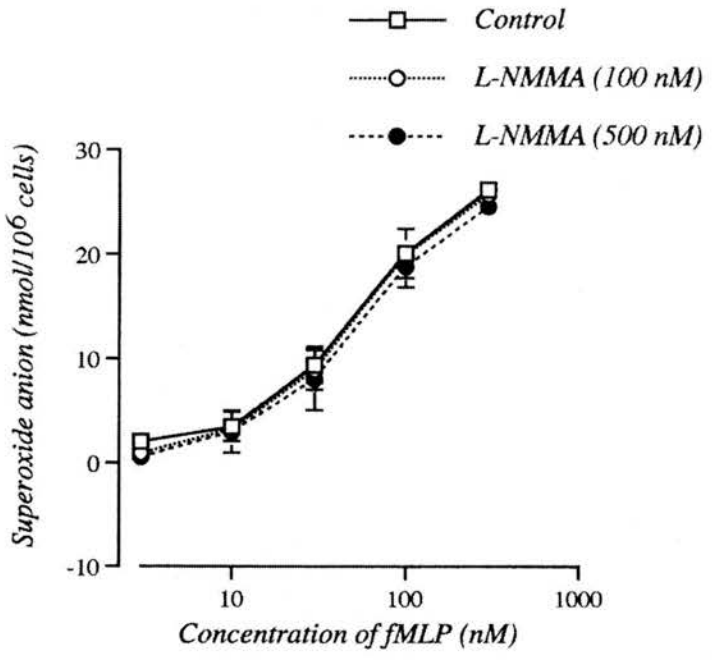


Figure 2.14 *Log concentration-effect curve for fMLP induced superoxide anion generation in control cells and cells treated with L-NMMA (100 μM and 500 μM). Cells were preincubated with L-NMMA for 45 minutes at 37°C. The values are the mean ± s.e. mean of 6 different donors.*

Table 2.7 *Effects of an NOS inhibitor, L-NMMA and L-canavanine; an NO-scavenger, carboxy-PTIO on fMLP-induced superoxide anion generation in human neutrophil.*

Drug	**number (n)**	**EC_{50} for fMLP nmol/10^6 cells/10 min**	**Maximal effect**
Control	6	54.2 ± 11.5	26.2 ± 1.0
L-NMMA (100 μM)	6	57.1 ± 10.9	25.1 ± 0.9
L-NMMA (500 μM)	6	61.4 ± 12.9	24.6 ± 1.3
Control	5	36.8 ± 7.9	22.9 ± 0.7
L-Canavanine (100 μM)	5	32.9 ± 5.4	25.5 ± 1.3
L-Canavanine (500 μM)	5	33.6 ± 8.3	24.1 ± 1.6
Control	6	36.2 ± 7.4	22.1 ± 1.5
Carboxy-PTIO (100 μM)	6	86.3 ± 14.1*	9.8 ± 1.6***

Data are expressed as EC_{50} for fMLP-induced superoxide anion generation (nmol/10^6 cells/10 min). The values are the mean ± s.e. mean of 4-6 different donors for each group. Statistically significant differences $^{*}P<0.05$ *and* $^{***}P< 0.005$.

Similarly, L-Canavanine at concentrations of 100 μM and 500 μM caused no significant inhibition (both P>0.05, ANOVA) of fMLP-induced O_2^- generation in human neutrophils (Figure 2.15), giving EC_{50} for fMLP of 36.8 ± 7.9; 32.9 ± 5.4, n =5 (P>0.05) and 36.8 ± 7.9; 33.60 ± 8.3 nM, n = 5 (P>0.05) (Table 2.7) in its absence and presence at the two concentrations, respectively.

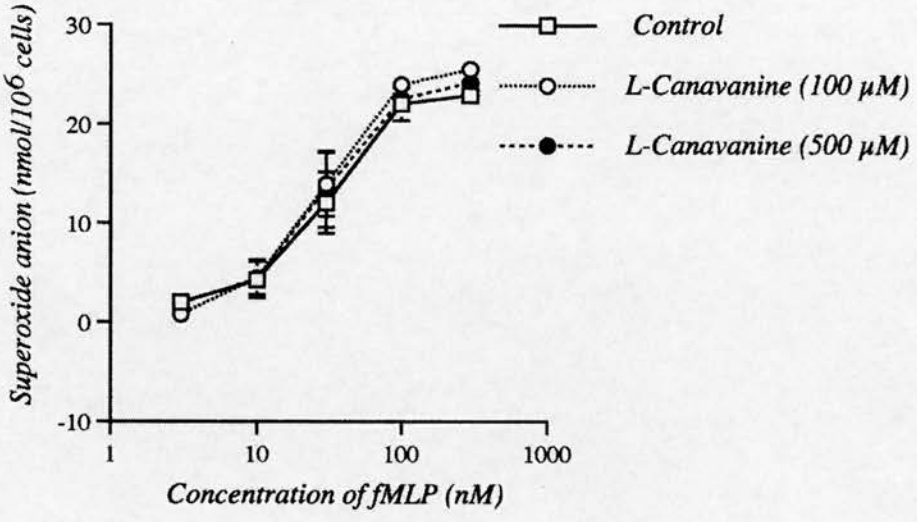


Figure 2.15 *Log concentration-effect curve for fMLP induced superoxide anion generation in control cells and cells treated with L-canavanine (100 μM and 500 μM). Cells were preincubated with L-canavanine for 45 minutes at 37°C. The values are the mean ± s.e. mean of 5 different donors.*

2.5.2.2 Effect of a NO scavenger

Carboxy-PTIO, a NO scavenger, at a concentration of 100 μM caused significant attenuation (P<0.05, ANOVA) of fMLP-induced O_2^- generation in human neutrophils (Figure 2.16), giving EC_{50} for fMLP of 36.2 ± 7.4; 86.3 ± 14.1 nM, n=6 (P<0.05) (Table 2.7) in its absence and presence, respectively. The maximal effect of fMLP was significantly reduced from 22.1 ± 1.5 to 9.8 ± 1.6 nmol $O_2^-/10^6$ cells/10 minutes at 300 nM (P<0.005) (Table 2.7).

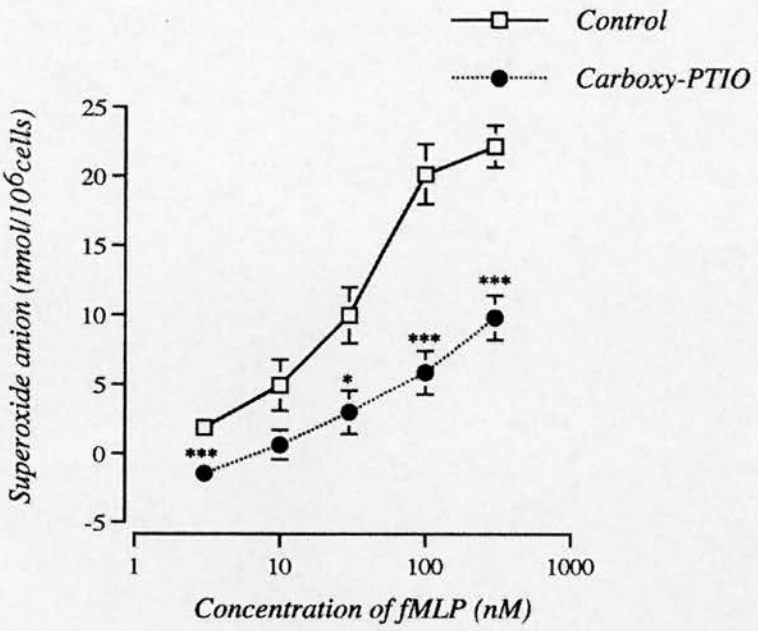


Figure 2.16 *Log concentration-effect curve for fMLP induced superoxide anion generation in control cells and cells treated with carboxy-PTIO (100 μM). The values are the mean ± s.e. mean of 6 different donors. Statistically significant differences* $^{*}P<0.05$ *and* $^{***}P<0.005$.

2.5.2.3 Effect of guanylyl cyclase inhibition

LY 83583, an inhibitor of guanylyl cyclase, at a concentration of 10 μM caused no significant inhibition of fMLP-induced O_2^- generation in human neutrophils (Figure 2.17a), giving EC_{50} for fMLP of 23.8 ± 1.8; 19.0 ± 4.5 nM, n = 4 (P>0.05) (Table 2.8, page 79) in its absence and presence, respectively. Even at the concentration of 100 μM, LY 83583 caused no significant inhibition of fMLP-induced O_2^- generation in human neutrophils (Figure 2.17b), giving EC_{50} for fMLP of 26.3 ± 1.4; 13.7 ± 3.3 nM, n=4 (P>0.05) (Table 2.8) in its absence and presence, respectively. However, a significant enhancement in fMLP-induced O_2^- generation at the low concentrations of fMLP tested (3-10 nM) was observed with 100 μM LY 83583 (P<0.05).

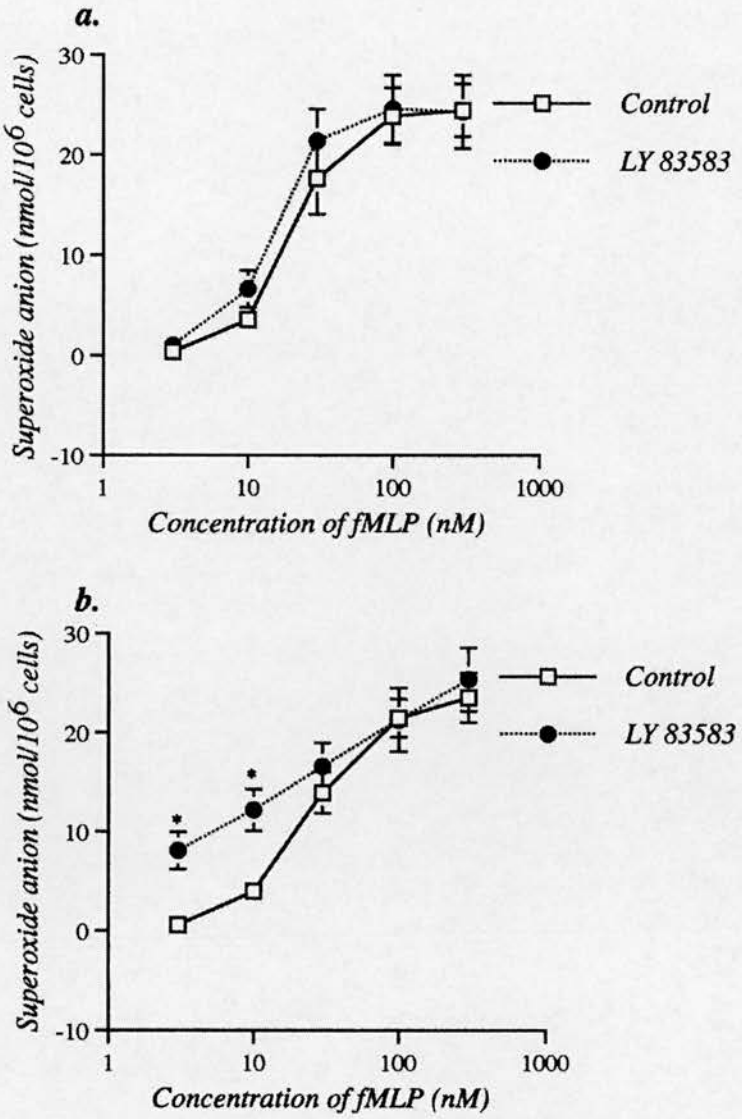


Figure 2.17 *Log concentration-effect curve for fMLP induced superoxide anion generation in control cells and cells treated with LY 83583 (a) 10 μM and (b) 100 μM. The values are the mean ± s.e. mean of 4 different donors. Statistically significant difference* $^{*}P<0.05$.

2.5.2.4 Effect of G-kinase inhibition

KT 5823, a specific inhibitor of G-Kinase, at a concentration of 1 μM caused significant inhibition ($P<0.0001$, ANOVA) of fMLP-induced O_2^- generation in human neutrophils (Figure 2.18a), giving EC_{50} for fMLP of 34.3 ± 8.9; 52.6 ± 4.9 nM, n = 5 ($P=0.05$) (Table 2.8) in its absence and presence, respectively. The maximal effect of fMLP 300 nM was significantly reduced by 1 μM KT 5823 from 22.2 ± 0.7 to 12.2 ± 1.4 nmol $O_2^-/10^6$ cells/10 minutes, n = 5, ($P<0.005$) (Table 2.8).

KT 5823 at a concentration of 10 μM caused significant inhibition ($P<0.0001$, ANOVA) of fMLP-induced O_2^- generation in human neutrophils (Figure 2.18b), giving EC_{50} for fMLP of 36.3 ± 8.8; and > 300 nM, n = 5 ($P<0.05$) (Table 2.8) in its absence and presence, respectively. The maximal effect of fMLP 300 nM was significantly reduced by 10 μM KT 5823 from 28.6 ± 4.2 to 6.6 ± 2.1 nmol $O_2^-/10^6$ cells/10 minutes, n = 5 ($P<0.001$) (Table 2.8).

Rp-8-pCPT-cGMPS (100 μM), a moderately potent inhibitor of cGMP-dependent protein kinase G1α caused no significant inhibition ($P=0.058$, ANOVA) of fMLP-induced O_2^- generation in human neutrophils (Figure 2.19), giving EC_{50} for fMLP of 28.4 ± 10.8; 49.3 ± 16.8 nM, n = 4 ($P>0.05$) (Table 2.8) in its absence and presence, respectively.

Table 2.8 *Effects of an inhibitor of guanylyl cyclase, LY 83583 and inhibitors of G-kinase, KT 5823 and Rp-8-pCPT-cGMPs on fMLP-induced superoxide anion generation in human neutrophil.*

Drug	**number (n)**	**EC_{50} for fMLP nmol/10^6 cells/10 min**	**Maximal effect**
Control	4	23.8 ± 1.8	24.3 ± 2.6
LY 83583 (10 μM)	4	19.0 ± 4.5	24.3 ± 3.7
Control	4	26.3 ± 1.4	23.5 ± 2.5
LY 83583 (100 μM)	4	13.7 ± 3.3	25.3 ± 3.2
Control	5	34.3 ± 8.9	22.2 ± 0.7
KT 5823 (1 μM)	5	52.6 ± 4.9*	12.2 ± 1.4***
Control	5	36.3 ± 8.8	28.6 ± 4.2
KT 5823(10 μM)	5	>300*	6.6 ± 2.1***
Control	4	28.4 ± 10.8	28.3 ± 0.9
Rp-8-pCPT-cGMPs (100 μM)	4	49.3 ± 16.8	27.3 ± 4.5

Data are expressed as EC_{50} for fMLP-induced superoxide anion generation (nmol/10^6 cells/10 min). The values are the mean ±s.e. mean of 4-5 different donors for each group. Statistically significant differences $^*P<0.05$ *and* $^{***}P< 0.005$.

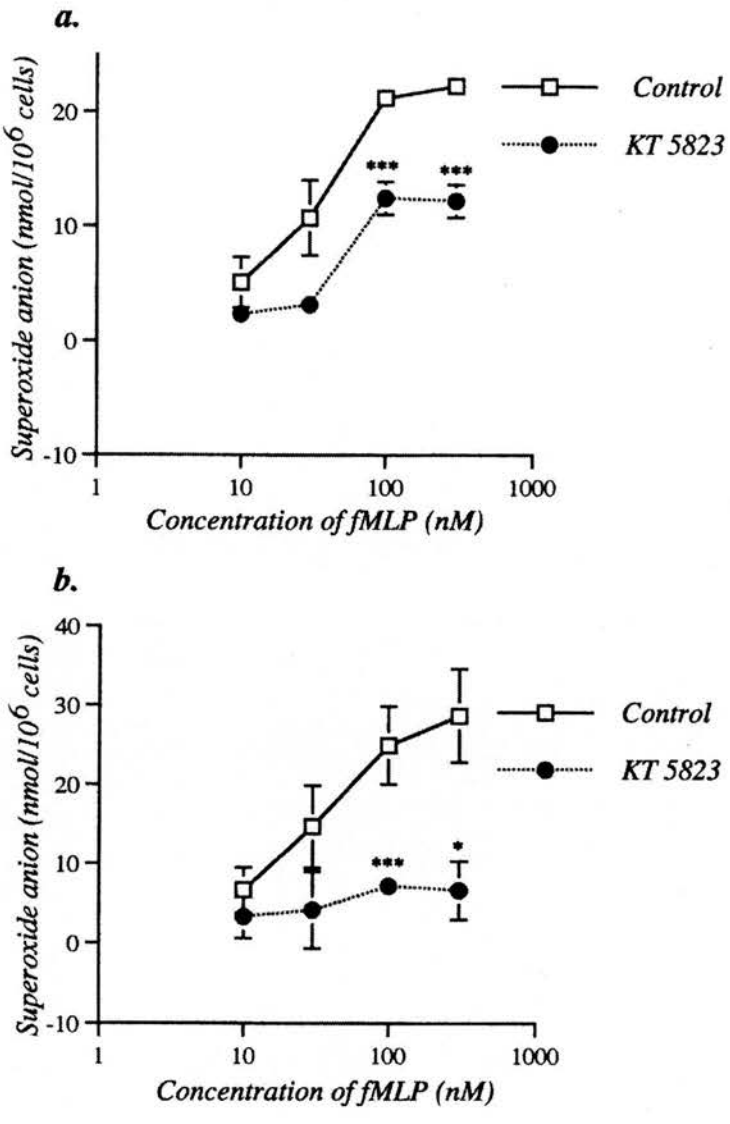


Figure 2.18 *Log concentration-effect curve for fMLP induced superoxide anion generation in control cells and cells treated with KT 5823 (a) 1μM and (b) 10 μM. The values are the mean ± s.e. mean of 5 different donors. Statistically significant differences* $^{*}P<0.05$ *and* $^{***}P<0.005$.

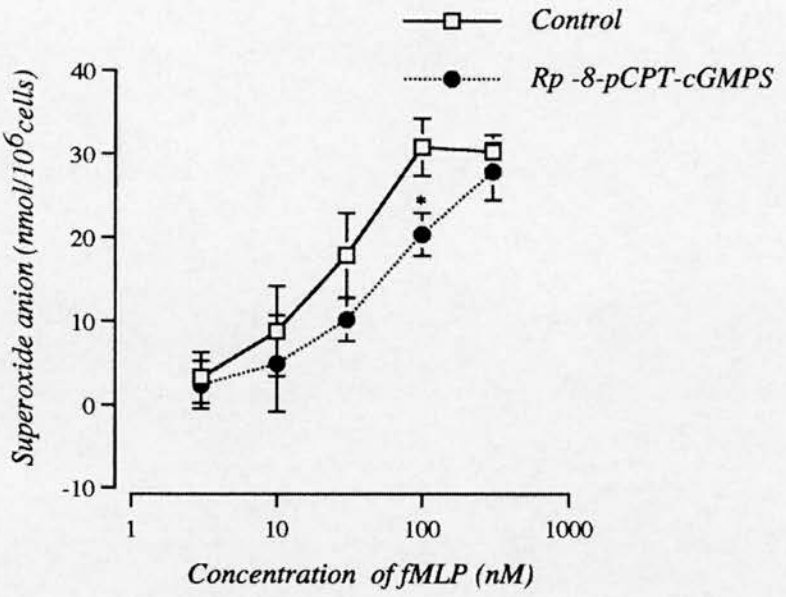


Figure 2.19 *Log concentration-effect curve for fMLP induced superoxide anion generation in control cells and cells treated with Rp-8-pCPT-cGMPS (100 μM). The values are the mean ± s.e. mean of 4 different donors. Statistically significant difference* $^{*}P<0.05$.

2.5.2.5 Effect of phosphatase inhibition

DPG, an inhibitor of inositol polyphosphate-5-phosphatase, at a concentration of 100 μM caused no significant inhibition of fMLP-induced O_2^- generation in human neutrophils (Figure 2.20a), giving EC_{50} for fMLP of 36.2 ± 9.1; 44.6 ± 8.9 nM, n = 4 (P> 0.05) (Table 2.9, page 84) was obtained in its absence and presence, respectively. DPG at concentration of 100 μM caused no significantly reduction in the maximal effects of fMLP, 25.6 ± 1.8 to 24.2 ± 2.2 nmol $O_2^-/10^6$ cells/10 minutes, n = 4 (P>0.05).

DPG at a concentration of 500 μM caused significant inhibition (P<0.005, ANOVA) of fMLP-induced O_2^- generation in human neutrophils (Figure 2.20b), giving EC_{50} for fMLP of 33.9 ± 4.2; 61.1 ± 14.4 nM, n =4 (P>0.05) (Table 2.9) in its absence and presence, respectively. This concentration of DPG significantly reduced the maximal effect of fMLP from 26.2 ± 2.7 to 20.6 ± 3.0 nmol $O_2^-/10^6$ cells/10 minutes, n = 4 (P<0.05).

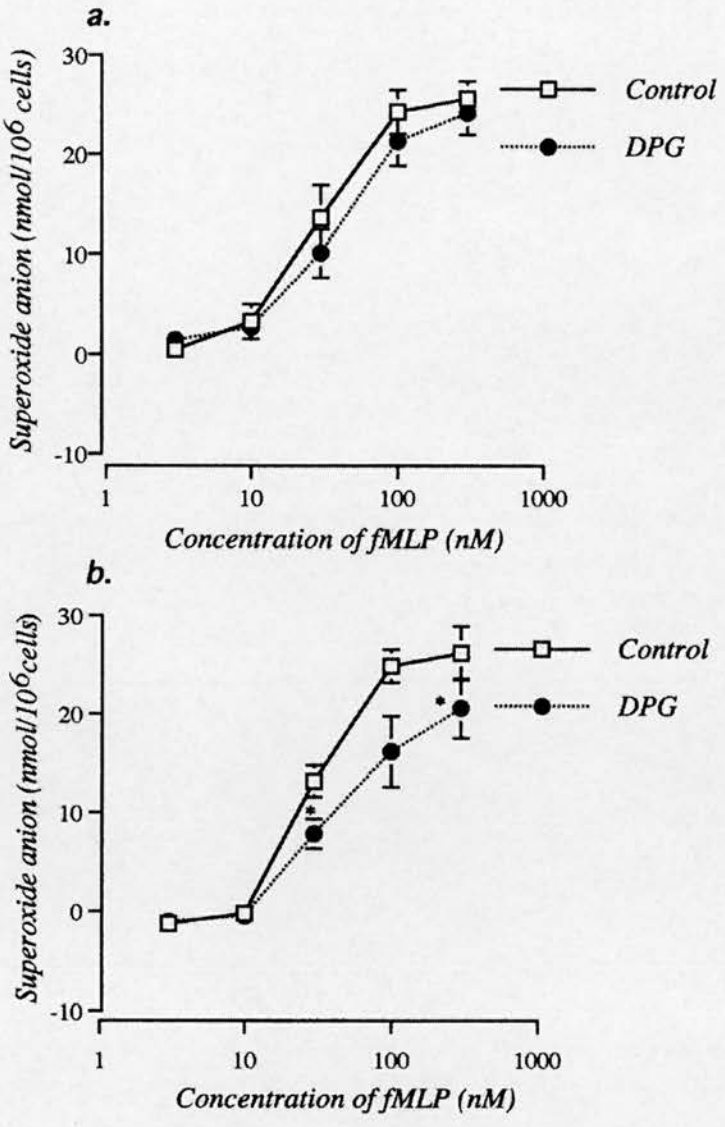


Figure 2.20 *Log concentration-effect curve for fMLP induced superoxide anion generation in control cells and cells treated with DPG (a) 100 μM and (b) 500 μM. The values are the mean ± s.e. mean of 4 different donors. Statistically significant difference* $^{*}P<0.05$.

In contrast, when neutrophils were preincubated with 100 μM and 500 μM DPG for 10 minutes at 37°C, neither concentration of DPG caused a significant effect on fMLP-induced O_2^- generation in human neutrophils (Figure 2.21a), giving EC_{50} for fMLP of 27.3 ± 6.1; 27.0 ± 6.3 nM, n = 4 (P>0.05) and 27.3 ± 6.1; 46.1 ± 23.1 nM, n = 4 (P>0.05) (Table 2.9, page 84) in its absence and presence at the two concentrations, respectively.

In addition, no significant effect on fMLP-induced O_2^- generation in human neutrophils was observed when neutrophils were preincubated with 100 μM and 500 μM for 20 minutes at 37°C (Figure.2.21b), giving EC_{50} for fMLP of 27.0 ± 2.3; 30.2 ± 10.0 nM, n = 4 (P>0.05) and 27.0 ± 2.3; 26.9 ± 9.5 nM, n = 4 (P>0.05) (Table 2.9) in its absence and presence at the two concentrations, respectively.

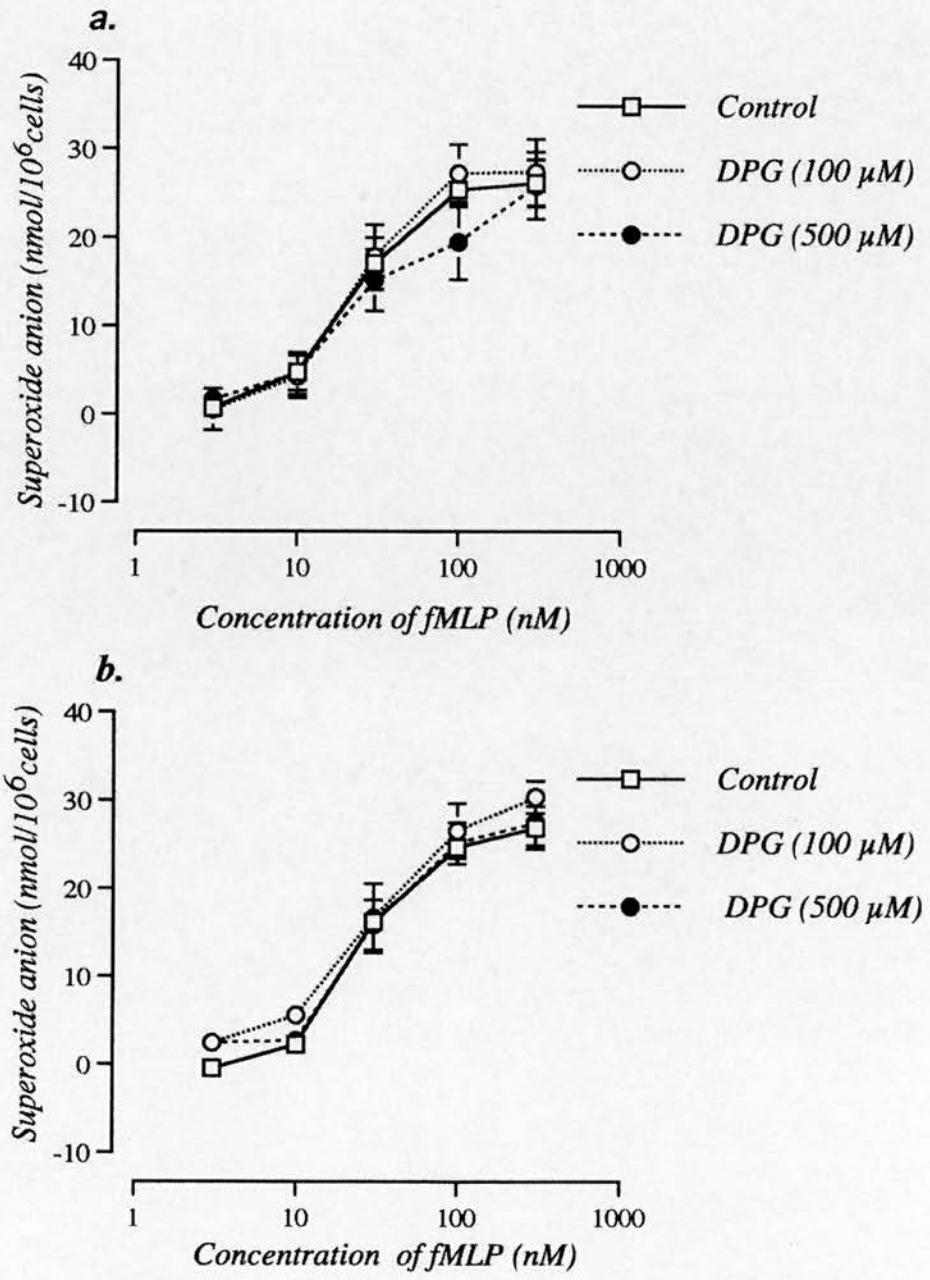


Figure 2.21 *Log concentration-effect curve for fMLP induced superoxide anion generation in control cells and cells treated with DPG (100 μM and 500 μM). Unlike Figure 2.20, these cells were preincubated with DPG at 37°C for (a)10 minutes and (b) 20 minutes. The values are the mean ± s.e. mean of 4 different donors.*

Table 2.9 *Effect of DPG on fMLP-induced superoxide anion generation in human neutrophils.*

Drug	incubation (min)	number (n)	EC_{50} for fMLP nmol/10^6 cells/10 min	Maximal effect
Control	-	4	36.2 ± 9.1	25.6 ± 1.8
DPG (100 μM)	-	4	44.6 ± 8.9	24.2 ± 2.2
Control	-	4	33.9 ± 4.2	26.2 ± 2.7
DPG (500 μM)	-	4	61.1 ± 14.4	20.6 ± 3.0*
Control	10	4	27.3 ± 6.1	26.1 ± 2.6
DPG (100 μM)	10	4	27.0 ± 6.3	27.4 ± 3.8
DPG (500 μM)	10	4	46.1 ± 23.1	25.9 ± 3.8
Control	20	4	27.0 ± 2.3	26.9 ± 2.5
DPG (100 μM)	20	4	30.2 ± 10.0	30.3 ± 1.8
DPG (500 μM)	20		26.9 ± 9.5	27.5 ± 2.3

*Cells were preincubated with DPG at 37 ℃ for 0, 10 and 20 minutes. Data are expressed as EC_{50} for fMLP-induced superoxide anion generation (nmol/10^6 cells/10 min) in neutrophils . The values are the mean ±s.e. mean of 4 different donors for each group. Statistically significant differences *P<0.05.*

2.5.3 Effects of the NO donors

2.5.3.1 Effect of the NO donors on neutrophil chemotaxis

fMLP at a concentration of 1 μM induced a submaximal migration of neutrophils. When neutrophils were preincubated with NO donors, GEA 3162 and GEA 5024, for 10 minutes at 37°C, these two compounds (1-100 μM) caused a concentration-related inhibition of fMLP-induced neutrophil chemotaxis (Figure 2.22), producing complete inhibition at a concentration of 100 μM. IC_{50} for GEA 3162 was 14.71 ± 1.6 μM, n = 5 and for GEA 5024 was 18.44 ± 0.43 μM, n = 5. When neutrophils were preincubated with GEA 3162 and SIN-1 for 10 minutes at 37°C, SIN-1 was found to be a significantly (P<0.05, ANOVA) less potent inhibitor of fMLP-induced neutrophil chemotaxis than GEA 3162. SIN-1 at concentration of 1000 μM induced a maximum inhibition of 24.99 ± 7.64%, n=8 (Figure 2.23). If the maximal effect of

GEA 3162 at 100 μM was taken to be 100% inhibition, the IC_{50} for SIN-1 was > 1000 μM (n=8), giving an EEC > 68.0.

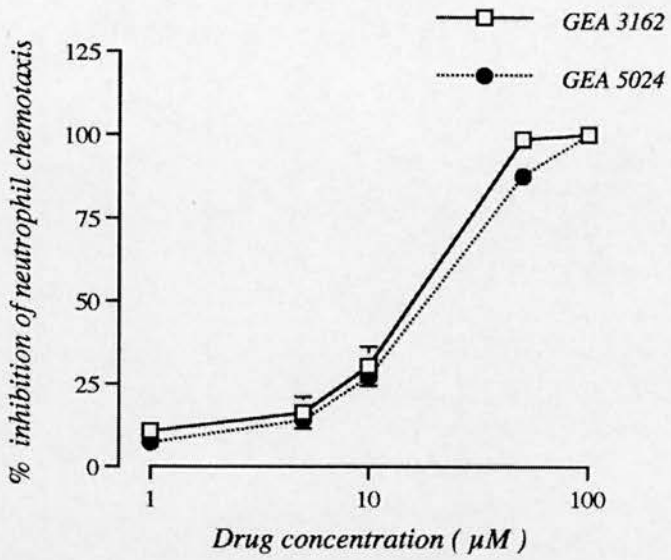


Figure 2.22 *Log concentration-effect curve for inhibition of fMLP (1 μM) induced neutrophil chemotaxis, observed with GEA 3162 and GEA 5024. Cells were preincubated with GEA 3162 and GEA 5024 for 10 minutes at 37°C, before being added to the chemotaxis chamber. The values are the mean ± s.e. mean of 5 different donors.*

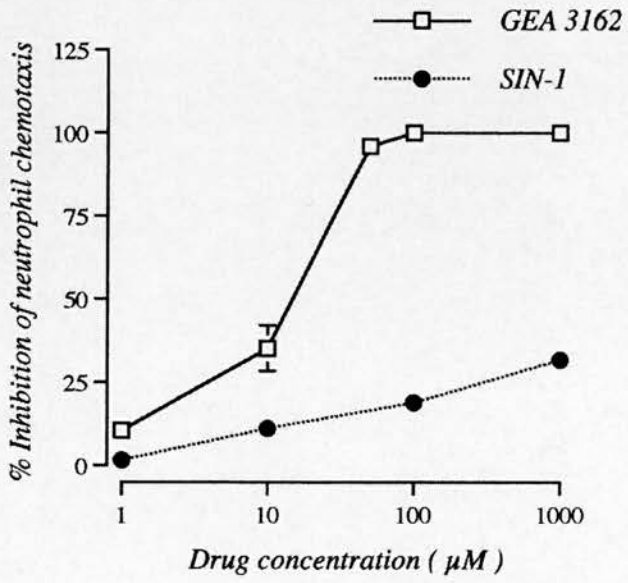


Figure 2.23 *Log concentration-effect curve for inhibition of fMLP (1 μM) induced neutrophil chemotaxis, observed with GEA 3162 and SIN-1. Cells were preincubated with GEA 3162 and SIN-1 for 10 minutes at 37°C, before being added to the chemotaxis chamber. The values are the mean ± s.e. mean of 8 different donors.*

2.5.3.2 Effect of the NO donors and fMLP on neutrophil cyclic GMP levels

An example of a RIA standard curve of cyclic GMP is shown in Figure 2.24. Incubation of neutrophils with the NO donors, GEA 3162 (1-100 μM), GEA 5024 (1-100 μM) and SIN-1 (1-1000 μM) for 10 minutes at 37°C induced concentration-dependent and significant increases in cyclic GMP production ($P< 0.05$, $P<0.01$ and $P<0.005$ as shown in Table 2.10). SIN-1 was considerably less potent than the GEA compounds at increasing cyclic GMP production. fMLP (0.1-0.3 μM) also induced concentration-dependent and significant increases in cyclic GMP production in human neutrophils when neutrophils were incubated with fMLP for 10 minutes at 37°C ($P<0.05$). However, all NO donors were found to be more potent than fMLP at increasing cyclic GMP production in human neutrophils (Table 2.10).

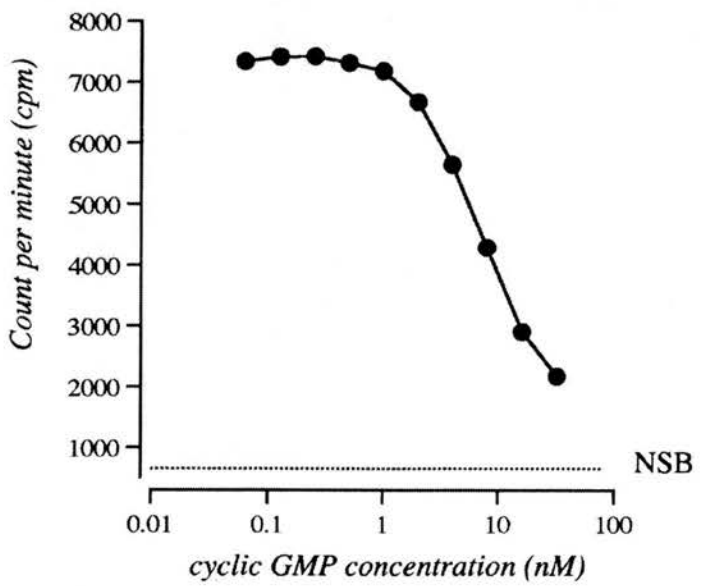


Figure 2.24 *An example of an RIA standard curve of cyclic GMP, n=1.*

Table 2.10 *The effects of NO-donors (GEA 3162, GEA 5024 and SIN-1) and fMLP on cyclic GMP levels in human neutrophils.*

Drug concentration (μM)	Cyclic GMP levels (pmol/10^6 cells) GEA 3162	GEA 5024	SIN-1	fMLP
0.0	0.10 ± 0.02	0.1 ± 0.01	0.09 ± 0.01	0.09 ± 0.01
0.1	-	-	-	0.18 ± 0.03*
0.3	-	-	-	0.22 ± 0.05*
10.0	0.87 ± 0.15**	0.59 ± 0.11*	0.25 ± 0.01*	-
50.0	1.13 ± 0.23**	0.73 ± 0.14*	0.29 ± 0.01*	-
100.0	1.32 ± 0.24**	0.85 ± 0.18*	0.40 ± 0.02*	-
1000.0	-	-	0.53 ± 0.01**	-

*Data are expressed as mean ± s.e. mean of 5 different donors, significant increase in cyclic GMP above basal values, *P<0.05, **P<0.01.*

2.5.3.3 Effect of the NO donors and fMLP on total nitrate and nitrite production

An example of a nitrate standard curve is shown in Figure 2.25. Incubation of neutrophils with the NO donors, GEA 3162, GEA 5024 and SIN-1 (1-100 μM) for 30 minutes at 37°C induced concentration-related increases in total nitrate/nitrite production (Table 2.11). GEA 3162 significantly increased total nitrate/nitrite concentration at all concentrations tested ($P<0.005$, except at 10 μM $P<0.05$). GEA 5024 and SIN-1 at 10-100 μM significantly increased total nitrate/nitrite concentration ($P<0.005$). Both GEA 3162 and GEA 5024 were less potent than SIN-1 at increasing total nitrate and nitrite production. With GEA 3162 as the standard agonist (EC_{50} = 39.70 ± 0.53 μM), apparent EC_{50} values calculated for SIN-1 and GEA 5024 were 37.62 ± 0.9 (n=4; EEC of 0.95) and 89.86 ± 1.62 μM (n=4; EEC of 2.26), respectively. fMLP at concentrations of 10-300 nM caused no significant increase in the total nitrate/nitrite levels.

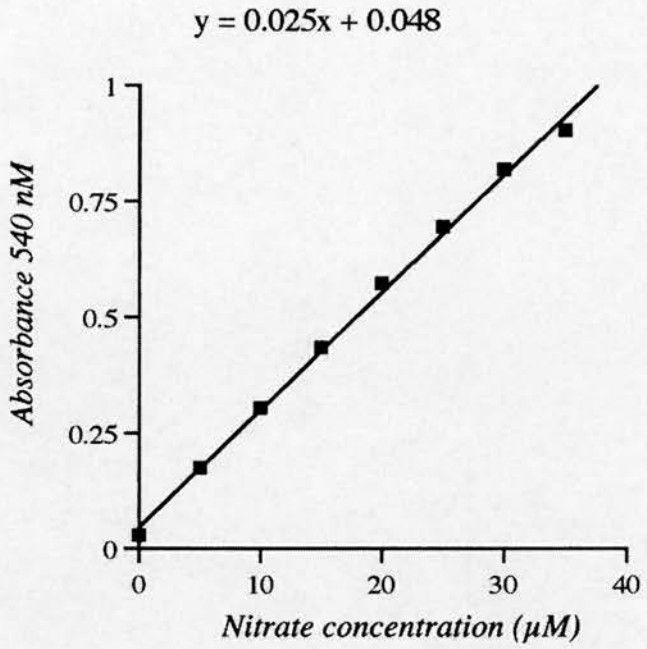


Figure 2.25 *An example of a nitrate standard curve, y is absorbance at 540 nM, x is nitrate concentration.*

Table 2.11 *The effects of NO-donors (GEA 3162, GEA 5024 and SIN-1) and fMLP on total nitrate/nitrite production in human neutrophils.*

Drug concentration (μM)	**Total nitrate and nitrite (μmol/10^6 cells)**			
	fMLP	**GEA 3162**	**GEA 5024**	**SIN-1**
0.0	4.1 ± 3.3	4.1 ± 3.3	4.1 ± 3.3	4.1 ± 3.3
0.1	4.1 ± 2.3	-	-	-
0.3	4.8 ± 2.3	-	-	-
1.0	-	11.3 ± 4.8	4.8 ± 0.0	9.4 ± 4.6
10.0	-	71.8 ± 3.6	30.5 ± 0.6***	55.4 ± 1.6*
50.0	-	282.2 ± 5.5	145.0 ± 1.4***	305.2 ± 5.1#
100.0	-	458.0 ± 3.6	251.4 ± 1.6***	552.0 ± 14.7##

Data are expressed as mean ± s.e. mean of 4 different donors. * *Significantly less nitrate/nitrite productuion than the equivalent concentration of GEA 3162,* *$P<0.05$, ***$P<0.005$. # *Significantly more nitrate/nitrite production than the equivalent concentration of GEA 3162,* #$P<0.05$, ##$P<0.01$. *GEA 3162 significantly increased nitrate/nitrite levels at all concentrations tested.*

2.6 DISCUSSION

In the neutrophil, several second messenger/signal transduction systems can become activated, and these may be involved in the regulation of a variety of neutrophil effector functions. It has been shown that fMLP induced chemotaxis in human neutrophils results from a rise in cyclic GMP levels subsequent to the production of NO (Kaplan *et al.*, 1989; Belenky *et al.*, 1993). Such a role for NO has been supported by the results of this study where an inhibitor of NO synthase, L-NMMA and the NO scavenger Carboxy-PTIO were used.

L-NMMA is a competitive inhibitor (competes with L-arginine) of all the NO synthases, K_i 1 μM (Schmidt *et al.*, 1991; Stuehr *et al.*, 1991; Pollock *et al.*, 1991; Stuehr & Griffith, 1992), this inhibition is enantiomer-specific. L-NMMA inhibits NOS by uncoupling NADPH from catalytic activity without influencing the reduction of the heme iron oxidation from catalytic activity. Inhibition of NO synthase with L-NMMA was found to inhibit fMLP-induced human neutrophil chemotaxis (Figure 2.8).

The NO scavenger Carboxy-PTIO (Akaike *et al.*, 1993), an imidazolineoxyl N-oxide derivative which is a stable radical compound, was found to antagonize NO produced in biological systems via a unique mechanism involving a radical-radical reaction in a completely stoichiometric manner in a neutral solution (sodium phosphate buffer, pH 7.4), followed by generation of NO_2^-/NO_3^- and imidazolineoxyls Carboxymethoxy-PTI (Akaike *et al.*, 1993). The result from the current study shows that chemical antagonism of NO with Carboxy-PTIO was found to inhibit fMLP-induced neutrophil chemotaxis (Figure 2.9).

However it must be noted that high concentrations of both of L-NMMA and Carboxy-PTIO have been used (500 and 100 μM, respectively). This suggests that NO represents only one of the pathways by which chemotaxis is induced, as complete blockage of NO only partially blocks the chemotactic response of fMLP.

It has been reported that G-kinase plays a physiological role in human neutrophils and the concept of compartmentalisation of cyclic nucleotides during neutrophil activation was suggested (Pryzwansky *et al.*, 1990). Cyclic GMP and G-kinase were demonstrated to regulate neutrophil activation in response to fMLP or A-23187 (Pryzwansky *et al.*, 1990; Wyatt *et al.*, 1990). A subsequent study by Wyatt *et al.* (1991) demonstrated that after activation by fMLP, G-kinase transiently co-localises with the intermediate filament subunit protein vimentin, resulting in the phosphorylation of vimentin by G-kinase. The transient redistribution of G-kinase may regulate neutrophil activation.

LY 83583, a quinolinedione compound, which is an inhibitor of guanylyl cyclase was used in this study to confirm the role of cyclic GMP and G-kinase in neutrophil chemotaxis. LY 83583 inhibits the activation of guanylyl cyclase in many cell types including macrophages (Naef *et al.*, 1984; Mulsch *et al.*, 1988) and effectively lowers intracellular cyclic GMP levels in a wide variety of cell types (Diamond & Chu, 1985; Schmidt *et al.*, 1985; O'Donnell & Owen, 1986). Although its mechanism of action in the reduction of cyclic GMP is not clear in all cases, LY-83583 has been reported to inhibit the release of NO in endothelial cells and inhibit the activation of soluble guanylyl cyclase in smooth muscle cells (Naef *et al.*, 1984). LY 83583 has been shown to inhibit the fMLP-stimulated increase in neutrophil cyclic GMP levels resulting in inhibition of vimentin and G-kinase co-localisation and subsequent phosphorylation of vimentin by G-kinase (Wyatt *et al.*, 1993). Whether LY-83583 acts on guanylyl cyclase itself or works through some other mechanism such as affecting the availability of ATP is not clear. In addition, it was demonstrated that LY 83583 can stimulate O_2^- production (Barbier & Lefebvre, 1992) and the stimulation of soluble guanylyl cyclase by NO mechanisms can be attenuated by extracellular and intracellular O_2^- (Cherry *et al.*, 1990; Furchgott *et al.*, 1990; Omar & Wolin, 1992), thus these effects of LY 83583 may be one of the mechanisms by which LY 83583 causes inhibition of fMLP-induced neutrophil chemotaxis. In this study, we found that LY 83583 significantly attenuated fMLP-induced neutrophil chemotaxis (Figures 2.9a & 2.9b), thus confirming that

vimentin and G-kinase co-localisation and phosphorylation of vimentin by G-kinase are involved in fMLP-induced neutrophil chemotaxis.

Two inhibitors of G-kinase were used to substantiate such a role for cyclic GMP, KT 5823 (Kase *et al.*, 1987; Jin *et al.*, 1993) and Rp-8-pCPT-cGMPS (Butt *et al.*, 1994). At a concentration of 10 μM KT 5823 completely inhibited fMLP-induced neutrophil chemotaxis (Figure 2.10b). The inhibition of neutrophil chemotaxis observed with both LY 83583 (100 μM) and KT 5823 (10 μM) was greater than would be expected if the rise in cyclic GMP resulted only from NO. This suggests either that fMLP can increase cyclic GMP levels independently of NO or that LY 83583 and KT 5823 at the concentrations used are not acting as selective inhibitors of guanylyl cyclase and G-kinase, respectively.

Rp-8-pCPT-cGMPS (10 and 100 μM) failed to block fMLP-induced neutrophil chemotaxis suggesting that the G-kinase activated in neutrophil chemotaxis is not type G1α. The inability of Rp-8-pCPT-cGMPS to block neutrophil chemotaxis is unlikely to result from too low a concentration being used as a significant effect was observed with Rp-8-pCPT-cGMPS (100 μM) on neutrophil superoxide anion generation (Figure 2.18).

It has been reported that more than 90% of the diglyceride formed in neutrophils in response to fMLP occurs through the activation of phospholipase D (PLD)/phosphatidic acid (PA) phosphohydrolase (Billah *et al.*, 1989). PLD catalyses the cleavage of the terminal phosphodiester bond of phosphatidylcholine to yield PA and an inactive choline molecule, and fMLP stimulation of a phosphatidylcholine-specific PLD activity is a well-documented response in neutrophils (Cockcroft *et al.*, 1984). PA is converted to DAG by phosphatidate phosphohydrolase (Billah *et al.*, 1989), and this results in a second, often more sustained phase of DAG generation than that resulting from the activation of PLC (Billah *et al.*, 1989; Truett *et al.*, 1988). The receptor-linked PLD can be regulated by a variety of mechanisms involving Ca^{2+} mobilisation (Olson *et al.*, 1991; Kessels *et al.*, 1991b), GTP-binding proteins (Van Der Meulen & Haslam, 1990), tyrosine

kinases (Uings *et al.*, 1992), PKC (Billah *et al.*, 1989b), and possibly cyclic AMP-dependent protein kinase (Agwu *et al.*, 1991a; Kessels *et al.*, 1991a).

In the present study, a phosphatase inhibitor, DPG was used to examine whether PLD plays a role in neutrophil chemotaxis. DPG is an inhibitor of inositol polyphosphate-5-phosphatase (Van Lookeren Campagne *et al.*, 1988; Rubiera *et al.*, 1988), as it possesses a pair of vicinal phosphate groups on a glycerol backbone. This structure shows some similarity to that of PA. DPG was found to significantly attenuate fMLP-induced neutrophil chemotaxis (Figures 2.12a & 2.12b) suggesting that activation of PLD is a major signal in neutrophil chemotaxis.

However the roles of NO and cyclic GMP are less clear in fMLP-induced O_2^- generation by human neutrophils and thus were investigated in this study. The effects of two NOS inhibitors, L-NMMA and L-canavanine on fMLP-induced O_2^- generation by human neutrophils were investigated. L-canavanine is a structural analogue of L arginine. It was demonstrated that L-canavanine inhibits the inducible macrophage NO synthase more potently than the constitutive brain and endothelial NO synthases (Knowles *et al.*, 1989; Stuehr & Griffith, 1992; Vallance *et al.*, 1992). The results from the current study showed that neither L-NMMA (Figure 2.14) nor L-canavanine (Figure 2.14) inhibited fMLP-induced O_2^- generation even when used at the concentration (500 μM) required to inhibit neutrophil chemotaxis. However the NO scavenger carboxy-PTIO (100 μM) significantly inhibited fMLP-induced O_2^- generation (Figure 2.15). The reason for this discrepancy is not clear, but these results suggest that NO may also play a role in O_2^- generation by fMLP.

Results with the guanylyl cyclase inhibitor LY 83583 did not show the inhibitory effect of LY 83583 on O_2^- generation but showed its ability to significantly enhance the amount of O_2^- generated by low concentrations of fMLP (3-10 nM) (Figure 2.16). These results are consistent with the report that LY 83583 can stimulate O_2^- production (Barbier & Lefebvre, 1992).

However, both inhibitors of G-kinase, KT 5823 (Figures 2.17a & 2.17b) and Rp-8-pCPT-cGMPS (Figure 2.18) significantly inhibited fMLP-induced O_2^- generation. As with chemotaxis, the effect of KT 5823 is quite dramatic. This suggests that cyclic GMP may play an additional role to NO. However, the selectivity of the inhibitors used is crucial to this interpretation and experiments looking at phosphorylation of G-kinase are required to substantiate these findings. Furthermore the different sensitivity to these G-kinase inhibitors observed with chemotaxis and O_2^- generation suggests that the G-kinase activated in the two processes may be different.

PLD catalyses the hydrolysis of phosphatidylcholine to generate PA, which is converted to DAG by phosphatidate phosphohydrolase (Billah, 1993). In particular PLD is thought to ensure that diacylglycerol levels are sustained, which is a requirement for O_2^- generation (Billah & Anthes. 1990). Although many studies have shown a correlation between DAG production and O_2^- formation in intact cells (Rider & Niedel, 1987), and cell-permeant DAG has been shown to stimulate NADPH oxidase, other studies have found a lack of correlation between DAG and O_2^- (Bauldry *et al.*, 1992). On the other hand, PA has been linked to oxidase activation (Bonser *et al.*, 1989; Rossi, 1990; Agwu *et al.*, 1991; Bauldry *et al.*, 1991, 1992; Baggiolini, 1993). For example, inhibition of PLD-mediated PA production prevents neutrophil fMLP-induced O_2^- release (Bonser *et al.*, 1989). Peptide chemoattractants were found to cause activation of PLD (Kanaho *et al.*, 1991). Studies by Kanaho *et al* (1993) in which the ability of the phosphatase inhibitor DPG to inhibit PLD activation in rabbit peritoneal neutrophils was examined and suggested that PLD plays an important role in fMLP stimulation of O_2^- generation in the primed neutrophils. However, a PLD-independent pathway plays a primary role in fMLP stimulation of O_2^- generation in nonprimed neutrophils. The inhibition of PLD by DPG appears to arise from direct interaction with the enzyme (Kanaho *et al,* 1993).

Such a role for PLD has been confirmed in these experiments using the phosphatase inhibitor DPG. It was found that DPG significantly inhibited fMLP-induced O_2^- generation in these cytochalasin B-treated neutrophils (Figures 2.19a & 2.19b). This

effect of DPG was lost if cells were pre-incubated with DPG for 10 or 20 min (Figures 2.20a & 2.20b). This result contrasts with those reported by Kanaho *et al* (1993) who observed greater inhibition of O_2^- generation with preincubation. A greater degree of inhibition was observed with the effect of DPG on neutrophil chemotaxis than with its effect on O_2^- generation of neutrophils.

While these results suggest that endogenous NO plays a role in mediating neutrophil chemotaxis, other evidence has been presented indicating that NO donors can inhibit neutrophil activation (Ney *et al.*, 1990; Schroder *et al.*, 1990; Kubes *et al.*, 1991; Wenzen-Seifert *et al.*, 1991). To resolve this apparent paradox the investigation of the effects of NO donors including GEA 3162, GEA 5024 and SIN-1 on neutrophil chemotaxis were performed. These NO donors have previously been shown to increase cellular cyclic GMP production concomitantly with the inhibition of neutrophil chemotaxis (Siminiak *et al.*, 1992; Moilanen *et al.*, 1993, 1994).

GEA 3162 and GEA 5024 at physiological pH were rapidly hydrolysed with a subsequent release of NO. At the standard conditions (GEA compounds at a concentration of 30 μM), the NO release reached its maximum in 10 minutes for GEA 3162, and 30 minutes for GEA 5024. GEA compounds consumed oxygen only when used at high concentrations (1000 μM), and thereby an oxygen-dependent mechanism of NO release may not be common with GEA compounds (Karup *et al.*, 1994). In contrast to GEA compounds, NO release from SIN-1 takes place spontaneously in aqueous solution and molecular oxygen plays a key role in the initiation of the decomposition of SIN-1 and thus for NO release (Feelisch *et al.*, 1989). The results from the current study showed that GEA 3162 and GEA 5024 (1-100 μM) caused significant concentration-dependent inhibition of fMLP-induced neutrophil chemotaxis (Figure 2.21). SIN-1 was less potent and caused significantly less inhibition of neutrophil chemotaxis than GEA 3162 (Figure 2.22). The rank order of potency was GEA 3162 (EC_{50}=14.7 ± 1.6 μM) > GEA 5024 (EC_{50}=18.4 ± 0.4 μM) > SIN-1 (EC_{50}=>1000 μM). One possible explanation for the difference in potency of these agents as inhibitors of chemotaxis may relate to the ability of SIN-1 to generate O_2^- during the liberation of NO (Feelisch *et al.*, 1989; Feelisch, 1991),

simultaneously generated O_2^- further reduced the half-life of released NO, presumably by a chemical reaction in which O_2^- reacts with NO to form peroxynitrite under alkaline conditions (Blough & Zafiriox, 1985). Gryglewski *et al* (1986) also showed that the concomitant release of NO and O_2^- by SIN-1 may well attenuate the inhibitory effects of NO on chemotaxis as NO is inactivated by O_2^-, to form peroxynitrite. Furthermore, peroxynitrite production by SIN-1 has been shown to enhance fMLP-induced neutrophil respiratory burst (measured as luminol-dependent chemiluminescence in whole blood) masking its otherwise inhibitory effects, such as a reduction in LTB_4 production (Bednar *et al.*, 1996). In addition, peroxynitrite formed from SIN-1 has been shown to stimulate phorbol ester-induced respiratory burst (Iha *et al.*, 1996). In contrast, GEA 3162 and GEA 5024 (in concentrations up to 100 mM) do not release significant amounts of O_2^-, to form peroxynitrite (Gryglewski *et al.*, 1986). Recently, a study by Holm *et al.*, also showed that GEA 3162 produced more NO than SIN-1 during 1-45 minutes incubation time. SIN-1 releases O_2^- in its decomposition process while GEA 3162 produces negligible amounts of O_2^- and $ONOO^-$ as compared to SIN-1 (Holm *et al.*, 1998). If peroxynitrite augments chemotaxis as well as O_2^- generation, this could explain why GEA 3162 and GEA 5024 are more potent inhibitors of neutrophil chemotaxis than SIN-1.

When the effect of these NO donors on the total nitrate and nitrite production were evaluated, it was found that each agent induced a concentration-related increase in total nitrate and nitrite (Table 2.6). The rank order of potency was SIN-1 (EC_{50}= 37.6 ± 0.9 μM) > GEA 3162 (EC_{50}=39.7 ± 0.5 μM) > GEA 5024 (EC_{50}=89.9 ± 1.7 μM). Taking GEA 3162 as the standard compound, this gives EEC values (equi-effective concentration value) for SIN-1 and GEA 5024 of 0.95 and 2.26 for nitrate and nitrite production compared to > 68.0 and 1.25 for inhibition of chemotaxis. This data showed that SIN-1 is more potent than GEA 3162 and GEA 5024 in production of nitrate and nitrite, but less potent than GEA 3162 and GEA 5024 in fMLP-induced inhibition of chemotaxis.

Clearly some reason is required to explain the lack of potency of SIN-1 at inhibiting chemotaxis compared with releasing NO (as measured by total nitrate/nitrite production). Interestingly, as reported by Moilanen *et al.* (1993), SIN-1 was much weaker than GEA 3162 and GEA 5024 at increasing cyclic GMP levels, these results are in agreement with the results from the present study which showed that SIN-1 (1000 μM) giving a maximal increase cyclic GMP levels of 5.3 fold over basal compared to 13.2 and 8.3 fold for GEA 3162 (100 μM) and GEA 5024 (100 μM), respectively (Table 2.5). Consequently, there is a better correlation between effects of these drugs on cyclic GMP and inhibition of neutrophil chemotaxis, than for effects on NO (total nitrate/nitrite production) and inhibition of neutrophil chemotaxis. Previous studies revealed that nitrate/nitrite which occurs, simultaneously with the release of NO, as metabolic products of SIN-1 breakdown did not account for the activation of guanylyl cyclase, because C 78-0698 (a sydnonimine compound) displayed a higher stimulating potency on guanylyl cyclase than SIN-1 while producing less nitrite/nitrate (Feelisch *et al.*, 1988).

These results suggest that neutrophil inhibition is likely to be related to increased cyclic GMP levels rather than ADP ribosylation by NO. Clancy *et al.* (1995) suggested that NO inhibited cytosketal assembly and adherence in human neutrophils in association with the ADP ribosylation of actin. These actions of NO regulate neutrophil responses such as margination, adhesion, and diapedesis (Clancy *et al.*, 1995). However, the role of peroxynitrite formed by SIN-1 requires further clarification, particularly with respect to chemotaxis. At present it is not clear whether peroxynitrite augments chemotaxis induced by fMLP as is the case for O_2^- generation (Iha *et al.*, 1996).

These results do not prove that GEA 3162, GEA 5024 and SIN-1 inhibit neutrophil chemotaxis by a NO-dependent mechanism. However, this is quite difficult to test. An NO scavenger such as Carboxy-PTIO will itself inhibit chemotaxis (Figure 2.9), so therefore the NO donor would be tested against a smaller fMLP stimulus. Because of the nature of physiological antagonism, it is easier to inhibit a smaller stimulus than a larger one (Kenakin, 1987) making comparision difficult. The

scavenger oxyhaemoglobin may prove useful in elucidating the role of NO. If this can be used at a low enough concentration not to affect endogenous NO and the control chemotactic response to fMLP.

2.7 CONCLUSIONS

These results confirm that neutrophil activation results from the stimulation of several signal transduction systems. We have shown that chemotaxis induced by fMLP can be attenuated by inhibitors of PLD, NO and cyclic GMP, suggesting a role for these agents in neutrophil chemotaxis. It appears that increases in cyclic GMP and activation of G-kinase resulting in chemotaxis can occur via a NO-dependent, as well as NO-independent pathway. As such, very small increases in cyclic GMP and NO were detectable after neutrophil activation by fMLP. Chemotaxis may occur via PLD activation. Similar pathways appear to operate in O_2^- generation. In contrast, the NO donors, GEA 3162, GEA 5024 and SIN-1, which produce large amounts of NO (measured as total nitrate/nitrite) and cyclic GMP compared with fMLP, inhibit neutrophil chemotaxis. The ability of SIN-1 to inhibit chemotaxis correlates better with effects on cyclic GMP than NO. This hypothesis, that low concentrations of NO activate whilst high concentrations inhibit neutrophils, is in agreement with that has been suggested by VanUfflen *et al.* (1996); they studied the effects of gaseous NO on rabbit peritoneal neutrophils and demonstrated that NO, not derived from NO donors but applied directly, may stimulate or inhibit neutrophil migration, depending on the concentration used. High concentrations of NO were found to inhibit chemotaxis induced by an optimal concentration of the chemotactic peptide fMLP.

CHAPTER 3

INHIBITION OF NEUTROPHIL ACCUMULATION AND MYOCARDIAL NECROSIS IN A RAT MODEL OF MYOCARDIAL ISCHAEMIA REPERFUSION

3.1 INTRODUCTION

Interruption of the blood supply to an area of tissue results in a reduction in the supply of oxygen and nutrients, and the accumulation of toxic waste products. Ischaemia leads to hypoxia, which initiates a series of events primarily related to activation of platelets and release of their vasoconstrictor mediators e.g. thromboxane A_2 (TxA_2), and 5-hydroxytryptamine (5-HT) that further restrict blood flow to the ischaemic area (Lefer, 1987). Initially, restoration of blood flow will result in complete recovery of normal function. If, however, the duration of ischaemia is extended beyond a critical period of time, the rate of metabolism is diminished and the generation of high energy compounds (e.g. ATP) subsequently declines. The reduced energy metabolism eventually leads to irreversible cellular injury and tissue necrosis. Tissue that has undergone a prolonged period of ischaemia shows characteristic features of an acute inflammatory response with increased microvascular permeability and leukocyte infiltration.

In the heart, irreversible injury in the subendocardium can be detected after 20 minutes of ischaemia (Jennings & Reimer, 1983). Restoration of blood flow is absolutely essential in order to arrest the process of necrosis. Ischaemia underlies many important diseases. The treatment of acute myocardial ischaemia is accomplished by coronary bypass grafting, coronary reperfusion with thrombolytic therapy, and percutaneous transluminal angioplasty procedures. These interventions are based upon animal studies in which early reperfusion of ischaemic myocardium decreases the infarct size and mortality after acute coronary artery occlusion (Flaherty *et al.*, 1982; Romson *et al.*, 1983; Guerci *et al.*, 1987) and patients after perfusion show better functional recovery and reduced loss of cardiac cell enzymes (Schwartz *et al.*, 1982). Recanalisation or reperfusion is an absolute requirement for the survival of the ischaemic myocardium. However, reperfusion is not always beneficial but may be detrimental (Braunwald & Kloner, 1985); abundant evidence suggests that reperfusion of the previously ischaemic myocardium, in particular the readmission of oxygen, is often followed by detrimental morphologic and functional changes in the affected coronary arteries and myocardial tissues which ultimately

results in tissue damage known as reperfusion injury (McCord, 1985). Although post ischaemic reperfusion is likely to increase necrosis, both animal (Reimer & Jennings, 1979) and human (Schwarz *et al.*, 1982) studies suggest that early reperfusion of ischaemic myocardium salvages the cardiac tissue. This has led to a debate as to whether reperfusion of ischaemic tissue can exacerbate tissue injury (Braunwald & Kloner, 1985; Lucchesi *et al.*, 1989; Forman *et al.*, 1990; Hearse & Bolli, 1992).

3.1.1 Reperfusion injury

Reperfusion injury refers to a causal event associated with reperfusion that had not occurred during the preceding ischaemic period. Injury of ischaemic-reperfused myocardium is complex, involving injury of vascular cells as well as cardiomyocytes. In a biochemical sense, reperfusion injury has been described as a complex interaction between substances that accumulate during ischaemia and those that are delivered as result of reperfusion. The degree of injury is dependent on the extent of the collateral blood flow and duration of ischaemia, as well as the influx of neutrophils and the generation of free radicals during reperfusion, which exacerbate the injury. There are four basic forms of reperfusion injury including: lethal reperfusion injury, vascular reperfusion injury, myocardial stunning and reperfusion arrhythmias.

Lethal (necrotic) reperfusion injury is defined as injury caused by restoration of blood flow after an ischaemic episode leading to death of cells that were only reversibly injured during the preceding ischaemic period (Kloner *et al.*, 1989). The existence of lethal reperfusion injury has been debated for years by scientists and is still controversial (Kloner, 1993; Ferrari & Hearse, 1997; Przyklenk, 1997).

Vascular reperfusion injury refers to progressive damage to the vasculature over time during the phase of reperfusion. Manifestations of vascular reperfusion injury include an expanding zone of no reflow and a deterioration of coronary flow reserve. The no reflow phenomenon is a reduction in perfusion despite restoration of blood flow in the conductance artery. No reflow is believed to be caused by neutrophil trapping and plugging of reperfused vessels and, possibly by compression or

obstruction of intramyocardial arteries by oedematous endothelial and perivascular cells. Microvascular damage can decrease formation of vasodilatory substances, such as NO, from the endothelium and promote formation of vasoconstrictors such as endothelin. Futhermore, endothelial damage could remove factors inhibiting platelet plugging and neutrophil adherence (Forman, 1989). This form of reperfusion injury has been documented in animal models and probably occurs in humans.

Myocardial stunning refers to postischaemic contractile dysfunction of the myocardium and probably represents a form of functional reperfusion injury. This form of dysfunction was first described by Heyndrickx *et al.* (1975) and was later studied by several investigators (Braunwald & Kloner, 1982; Bush *et al.*, 1983). Myocardial stunning is fully reversible and its full recovery can take a period of hours or days depending on the duration of ischaemia (Braunwald & Kloner, 1985). This phenomenon is well documented in both animals and humans.

One of the prominent features of reperfusion following reversible periods of ischaemia is the occurrence of reperfusion arrhythmias which include ventricular tachycardia and fibrillation occurring within seconds to minutes of restoration of coronary flow after brief episodes of myocardial ischaemia. In the human heart, reperfusion arrhythmias are not common during thrombolytic therapy for acute myocardial infarction (Maras *et al.*, 1986). Nevertheless, reperfusion arrhythmias may be important as a cause of sudden death in patients with coronary artery spasm.

Several mechanisms have been proposed for reperfusion injury, including the generation of oxygen-derived free radicals (Premaratne *et al.*, 1993), neutrophil-initiated damage, loss of antioxidant enzymes, calcium overload, loss of normal ATP concentration, vascular endothelial and myocyte oedema, and haemorrhage (Hudson, 1994). One that has attracted a great deal of attention is neutrophil infiltration.

3.1.2 Involvement of neutrophils in myocardial reperfusion injury

Neutrophils are important contributors to *in vivo* ischaemic reperfusion injury models of lethal injury produced by coronary occlusion and reperfusion (Romson *et al.*, 1983; Mehta *et al.*, 1988). Although occasional studies have failed to show the causative role of neutrophils in myocardial cell death (Reimer *et al.*, 1989; Tanaka *et al.*, 1993), more evidence lies in favour of this. Both by histological examination and enzyme assay, it can be shown that ischaemic myocardial injury is accompanied by a pronounced accumulation of neutrophils in the ischaemic zone (Engler *et al.*, 1986; Dreyer *et al.*, 1991). Studies in a canine model of coronary occlusion with and without reperfusion described neutrophil accumulation in the ischaemic endocardium and also in the epicardium. Accumulation in the endocardium was enhanced by reperfusion and the results suggested that collateral blood flow is an important mechanism of neutrophil arrival, early in ischaemic myocardium (Engler *et al.*, 1986). A subsequent study in a canine model supported the concepts that rapid neutrophil localisation during reperfusion occurs within the regions of previous myocardial ischaemia and neutrophils preferentially localise within the subendocardial region. The rate of neutrophil infiltration was found to be greatest in the first hour after the initiation of reperfusion (Dreyer *et al.*, 1991). Leukocyte accumulation during this period showed an inverse correlation with the blood flow during the ischaemic period. However, this relationship was no longer seen at later reperfusion times. A number of studies have shown a correlation between infarct size and the extent of neutrophil accumulation in ischaemic-reperfused myocardium (Mullane *et al.*, 1985; Chatelain *et al.*, 1987; Lucchesi *et al.*, 1989).

The possibility of infiltrating neutrophils having a deleterious effect in myocardial infarcts first attracted interest in the early 1980s. That neutrophil accumulation is a cause of myocardial injury rather than simply a response to it, has been demonstrated by studies of myocardial ischaemia reperfusion (MI-R) models in which the reperfusing blood was depleted of neutrophils. In 1983, Romson *et al* reported that systemic administration of a polyclonal antiserum to neutrophils reduced myocardial infarct size in a canine model of coronary artery occlusion and reperfusion. In

subsequent studies, neutrophil antiserum (Jolly *et al.*, 1986) and other interventions causing a reduction in circulating neutrophils, such as hydroxyurea (Mullane *et al.*, 1984) and leukocyte filters (Litt *et al.*, 1989), were found to be protective in experimental models of MI-R. However, in a few studies, antineutrophil interventions have not been found to confer protection against reperfusion injury (Reimer *et al.*, 1985; Chatelain *et al.*, 1987). In addition, a number of other interventions reported to be protective in experimental models of acute myocardial infarction were also found to have effects on neutrophil function (Dreyer *et al.*, 1991; Ma *et al.*, 1991).

In addition to having a possible role in causing myocardial necrosis following ischaemia reperfusion, neutrophils have also been implicated in other less severe changes in myocardial function, namely myocardial stunning and the no reflow phenomenon. Although an early study using leukocyte filters (Westlin & Mullane, 1989) suggested that neutrophils played a role in myocardial stunning, subsequent studies using either polyclonal antiserum, leukocyte filters or cyclophosphamide to deplete circulating neutrophils have not supported this (O'Neill *et al.*, 1989; Juneau *et al.*, 1993). Therefore, it seems unlikely that neutrophils are involved in myocardial stunning. Neutrophils have also been implicated in no reflow (Kloner *et al.*, 1974a, 1983; Willerson *et al.*, 1975; Hashimoto *et al.*, 1991). In a study carried out in a canine model of acute myocardial infarction, depletion of circulating neutrophils was reported to protect against the fall in coronary blood flow during reperfusion of ischaemic myocardium (Schmid-Schonbein & Engler, 1987; Litt *et al.*, 1989). However, in another study, depleting circulating neutrophils did not protect against no reflow in a canine model of acute myocardial infarction (de Lorgeril, 1989). The involvement of neutrophils in no reflow, therefore, still has to be resolved.

There is, therefore, evidence both direct and indirect, from a number of studies suggesting that infiltrating neutrophils exacerbate myocardial injury following ischaemia and reperfusion.

3.1.3 Endothelial dysfunction and myocardial ischaemia reperfusion

It is now established that the coronary vascular endothelium plays a vital role in maintenance of myocardial blood flow, prevention of intravascular thrombosis, and modulation of leukocyte function through elaboration of a variety of vasoactive substances such as adenosine, PGI_2 and NO. However, the coronary vascular endothelium also plays a role in the pathogenesis of ischaemic reperfusion injury and other inflammatory responses in which neutrophils participate.

Reperfusion leads to reoxygenation and the formation and activation of a variety of humoural mediators of injury and inflammation, including oxygen-derived free radicals (e.g. O_2^-, OH, H_2O_2), lipid mediators (e.g. PAF, and LTB_4), as well as polypeptide mediators (e.g. C5a). O_2^- and PAF originate to a large extent from endothelial cells.

Endothelial cell dysfunction is thought to be the "trigger" for reperfusion injury (Bulkley *et al.*, 1989). This endothelial dysfunction results in part from reduction of NO release by the endothelium which may be due to the inactivation by oxygen-derived free radicals from the reperfused coronary endothelium (Grygleski *et al.*, 1986; Rubanyi & Vanhoutte, 1987; Tsao & Lefer, 1990; Lefer *et al.*, 1991). Damage to the coronary vascular endothelium has been noted to occur within 2.5 to 5 mins after the initiation of reperfusion soon after the generation of O_2^- by the reperfused coronary endothelium. Several investigators have observed that myocardial ischaemia followed by reperfusion interferes with endothelium-dependent relaxation of coronary artery rings, (Ku, 1982; Van Benthuysen *et al.*, 1987; Mehta *et al.*, 1989).

NO may be an important endogenous modulator of neutrophil adherence in postcapillary venules and impairment of the release of NO results in a pattern of neutrophil adhesion and emigration that is characteristic of inflammation (Kubes *et al.*, 1991). Ma *et al.* showed that progressive reduction of the basal release of NO after MI-R was accompanied by enhanced neutrophil adherence to the coronary

endothelium, which may lead to neutrophil-induced myocardial injury (Ma *et al.*, 1993). In addition, endogenous PGI_2 seems to be an important protective prostanoid against myocardial injury inflicted by ischaemia and reperfusion. (Theimermann *et al.*, 1985). It was suggested that when the endothelium is injured, local production of PGI_2 may be inhibited sufficiently to permit adhesion of neutrophils to the injured endothelium and so induce the earliest stage in the emigration of neutrophils into the area of injury (Jones & Hurley, 1984). A number of investigations have reported that two manifestations of endothelial injury are loss of ability to produce NO and PGI_2 (Thiemermann *et al.*, 1985; Aoki *et al.*, 1988; Bitterman *et al.*, 1988; Nichols *et al.*, 1988). In addition, damage to the endothelium could jeopardise the release of adenosine as it does NO (Ma *et al.*, 1993; Nakanishi *et al.*, 1992).

3.1.4 Mechanisms of neutrophil accumulation and neutrophil-induced tissue damage

Decreased NO and PGI_2 along with chemotactic and cytotoxic agents (e.g PAF, LTB_4, IL_8, C5a, O_2^-, H_2O_2, and elastase) which are released or generated locally in the early reperfusion period of evolving myocardial infarction, promote neutrophil recruitment to the reperfusion site and adherence to the dysfunctional endothelium. These chemoattractants may act as inflammatory mediators causing neutrophil activation (Neumann *et al.*, 1994). The adherence of neutrophils to the dysfunctional endothelium is facilitated by cytokine upregulation of ICAM-1 and ELAM-1 receptors and PAF-induced upregulation of GMP-140 receptors on the endothelial cell surface (Bevilacqua *et al.*, 1985). These adhesive molecules accentuate neutrophil adherence and promote neutrophil diapedesis through the endothelium.

Since cardiac myocytes express ICAM-1 on their surface (Smith *et al.*, 1991), activated neutrophils that have diapedesed can adhere to cardiac cells and cause direct myocardial damage by release of cytotoxic metabolites including oxygen-derived free radicals, eicosanoids, cytokines and proteolytic enzymes (Weiss, 1989). Although the migration of neutrophils across the endothelium into the myocardium occurs much later, after reperfusion is initiated, the early

neutrophil-endothelial events produce microvascular injury and ultimately myocyte necrosis (Entman *et al.*, 1991; Lefer *et al.*, 1994; Lefer, 1995).

3.1.4.1 Oxygen-derived free radicals

The concept that oxygen free radicals are believed to play a major role in postischaemic injury has been debated extensively by both experimental and clinical investigators, as discussed by Werns (1994). $ONOO^-$ may be of considerable importance in neutrophil cytotoxicity. H_2O_2 was found to promote neutrophil myocyte adhesion (Gasic *et al.*, 1990). As discussed in Chapter 1, it can produce highly reactive toxic products such as OCl^- (Harrison & Schultz, 1976) which is thought to be the major product of oxidative metabolism by neutrophils (Weiss, 1989) and has ability to cause tissue damage. OCl^- can further produce monochloroamines which attack membrane unsaturated fatty acids causing lipid peroxidation resulting in loss of membrane associated functions (Kim & Akera, 1987). Lipid peroxidation of membranes induced by oxygen radicals generated during reperfusion causes functional alterations of various membrane enzymes (Konno *et al.*, 1987; Kim & Akera, 1987; Kako, 1987; Itoh *et al.*, 1991) and is accompanied by structural damage to the cardiac cells (Miki *et al.*, 1988; Oguro, 1992). Alterations in membrane proteins by free radicals are among the important factors in the evolution of myocardial ischaemia reperfusion damage. It has been proposed that a sudden burst of oxidant stress injured membrane proteins, leading to ionic imbalances and electrical instability (Pallandi *et al.*, 1987). However, the role of oxygen free radicals in the pathogenesis of reperfusion arrythmias has been questioned.

Experimental studies showed considerable evidence supporting an oxygen free radical-mediated mechanism for myocardial stunning and that O_2^-, H_2O_2 and OH are involved in the pathogenesis of stunning (Myers *et al.*, 1985, 1986; Przyklenk & Kloner, 1986; Farber *et al.*, 1988; Przyklenk *et al.*, 1990; Jeroudi *et al.*, 1990, 1994; Bolli, 1991; Triana *et al.*, 1991; Sekili *et al.*, 1991; Corretti *et al.*, 1991; Matheis *et al.*, 1992; Hess & Kukreja, 1995; Naseem *et al.*, 1995).

3.1.4.2 Neutrophil proteases

Three neutrophil enzymes have been of particular interest with respect to tissue damage: the serine proteinase elastase and the two metalloproteases collagenase and gelatinase. These enzymes are able to degrade key components of the extracellular matrix as discussed in Chapter 1. Elastase has been shown to alter barrier properties of endothelial monolayers *in vitro* and to cause detachment, or even lysis of cells (Harlan *et al.*, 1985; Smedly *et al.*, 1986). Under normal conditions, mechanisms are in place to prevent inappropriate action of these enzymes. Elastase can be expressed on the plasma membrane of adherent neutrophils, and its level of expression is inversely proportional to cell adhesion (Cai & Wright, 1996). Elastase inhibitors were shown to reduce neutrophil recruitment *in vivo*, and this study pointed to a pro-inflammatory action for this serine proteinase (Yoshimura *et al.*, 1994). Murohara *et al.,* investigated the effect of a novel serine protease inhibitor (serpin), LEX032 in a murine model of MI-R injury *in vivo*. LEX032 has the ability to inhibit neutrophil elastase and cathepsin G, two major neutral serine proteases in neutrophils as well as inhibit O_2^- generation. It was found that, the recombinant serine protease inhibitor, LEX032, appears to be an effective agent for attenuating MI-R injury by inhibiting neutrophil accumulation into the ischaemic-reperfused myocardium and by inactivating cytotoxic metabolites released from neutrophils (Murohara *et al.*, 1995).

3.1.5 Pharmacological approaches to MI-R injury

The ability of the heart to protect itself during ischaemia and reperfusion is impaired by a reduction in the endogenous oxygen radical scavengers and some endogenous agents such as NO and PGI_2. The cascade of events leading to vascular and tissue injury is dependent on neutrophil-endothelial adhesive interactions, and thus, interruping these interactions at the level of rolling, firm adherence, or diapedesis should decrease neutrophil-mediated injury. The goal of current research for management of the coronary heart disease patient is to go beyond the palliative treatments available by attacking the pathogenic determinants of reperfusion injury (Janero, 1995). Ongoing studies include the evaluation of antioxidants and free radical scavengers, membrane stabilizers, endogenous agents, protease inhibitors, calcium antagonists and nucleoside transport inhibition agents.

For example, it has been proposed that the use of monoclonal antibodies (Mabs) directed specifically towards leukocyte and endothelial adhesion molecules may alter the cascade of inflammatory events involved in neutrophil-mediated MI-R injury. They can be protective, as in animal models of MI-R injury, in which PMN accumulation and infarct size were reduced by anti-CD11b (Simpson *et al.*, 1988; Ma *et al.*, 1991), anti-P-selectin (Weyrich *et al.*, 1993), anti-L-selectin (Ma *et al.*, 1993), anti-ICAM-1 antibodies (Ma *et al.*, 1992; Yamazaki *et al.*, 1993) and sialyl Lewis^{x}-containing oligosaccharide (Buerke *et al.*, 1994; Lefer *et al* 1994, 1995; Silver *et al.*, 1995). Furthermore, anti-CD18 antibodies attenuated ischaemia-reperfusion injury in several other experimental models.

Studies with antioxidants have focused on the potential of these agents to lessen postischaemic myocardial injury (Janero, 1994, 1995). It has been proposed that vitamin E and its analogues may offer protection from reperfusion injury (Mickle & Weisel, 1993). Initial studies with oxygen-free radical scavengers, such as SOD and catalase, in MI-R showed enhanced salvage of the myocardium (Jolly *et al.*, 1984; Ambrosio *et al.*, 1987; Forman *et al.*, 1988), while subsequent studies in the conscious dog showed that free radical scavengers had no effect on infarct size after reperfusion (Gallagher *et al.*, 1986; Nejima *et al.*, 1989; Patel *et al.*, 1990; Downey, 1990). Thus, some researchers claim that no convincing evidence exists to show that antioxidant therapy can reduce infarct size after MI-R (Jeroudi *et al.*, 1994).

Early studies using antioxidant enzymes, iron chelating agent, deferoxamine (Bernier *et al.*, 1986), SOD and catalase (Woodward & Zakaria, 1985; Bernier *et al.*, 1989) and allopurinol (Bernier *et al.*, 1989) indicated that these antioxidants could be used successfully to reduce the incidence of reperfusion arrythmias. However, subsequent studies showed several free radical scavengers unable to reduce the incidence of reperfusion arrythmias in isolated rat (Coetzee *et al.*, 1990) and rabbit hearts (Maxwell *et al.*, 1989). Experiments with open-chest dogs showed protection against myocardial stunning with SOD and catalase (Myers *et al.*, 1985; Przyklenk & Kloner, 1986; Jeroudi *et al.*, 1990, 1994), dimethylthiourea (Bolli, 1991),

N-2-mercaptopropionyl glycine (Triana *et al.*, 1991; Sekili *et al.*, 1991), and the iron chelator deferoxamine (Farber *et al.*, 1988).

Impairment of NO release after reperfusion of ischaemic myocardium has led several investigators to treat MI-R injury with authentic NO (Johnson *et al.*, 1991), the NO synthase substrate L-arginine (Nakanishi *et al.*, 1992; Weyrich *et al.*, 1992; Pernow *et al.*, 1994) and various NO donor compounds including: nitroglycerine (Flaherty, 1983), sydnonimines such as SIN-1 and C87-3754 (Siegfried *et al.,* 1992a; Fung *et al.*, 1994), N-nitratopivaloly-S-(N'-acetylalanyl)-cysteine ester (SPM 5185) (Siegried *et al* ., 1992b; Lefer *et al* ., 1993; Williams *et al.*, 1995). Results from all of these studies demonstrated the cardioprotective role of these drugs in various animal models of MI-R injury except the study by Williams *et al* (1995) with L-arginine in a rabbit model of MI-R, which showed equivocal effects on infarct size.

Intravenous adenosine in large and moderate doses during the early reperfusion period significantly enhanced myocardial salvage in both canine and rabbit models of regional ischaemia (Pitarys *et al.*, 1991; Norton *et al.*, 1991). The effects of a selective A_1 receptor agonist, cyclopentyladenosine (Norton *et al.*, 1992) and a selective A_2 receptor agonist, CGS 21680 (Norton *et al.*, 1992; Schlack *et al.*, 1993) were investigated in rabbit and canine models of MI-R. The results from these studies demonstrated cardioprotective roles of these drugs in these animal models of MI-R. However, other investigators demonstrated no effect of adenosine on infarct size (Goto *et al.*, 1991) or even, an increased infarct size (Eliseev *et al.*, 1988) in rabbit models of MI-R. Selective adenosine A_1 receptor agonists such as BN-063 (1-cyclopropylisoguanosine) were also investigated in a rat models of MI-R and found to exert, through activation of adenosine A_1 receptors, antiarrhythmic and anti-infarct effects (Lee *et al.*, 1995). In addition, the selective A_1 adenosine receptor antagonists bamiphylline or xanthine amine congener were found to reduce infarct size in an *in vivo* feline model of MI-R (DiPierro *et al.*, 1995). Adenosine has also been shown to reduce postischaemic myocardial stunning (Lasley & Mentzer, 1995). Other approaches with the aim of potentiating the adenosine pathways have also been successful. Enhancement of adenosine levels through blocking of adenosine

deaminase, inhibited O_2^- generation from adherent neutrophils (Cronstein *et al.*, 1986, Cronstein, 1994). More recently, an inhibitor of adenosine kinase enhanced adenosine concentrations, inhibited neutrophil adhesion to endothelial monolayers *in vitro* (Firestein *et al.*, 1995) and reduced neutrophil elicitation in experimental inflammation (Cronstein *et al.*, 1995).

PGE_1 (Simpson *et al.*, 1988; Schror *et al.*, 1988) PGI_2 (Simpson *et al.*, 1987; Ogletree et al., 1979) and iloprost (ZK 36374), a chemically stable PGI_2 analogue which has a profile of actions similar to that of PGI_2, (Simpson *et al.*, 1987a; Chiariello *et al.*, 1988) were tested as therapeutic agents in experimental myocardial ischaemia, the results showed beneficial effects of these drugs in various models of acute myocardial ischaemia or postischaemic reperfusion. The cytoprotective effect of PGI_2 during MI-R in a canine model was suggested to be related to an inhibition of neutrophil migration and the production of cytotoxic activated oxygen species (Simpson *et al.*, 1987). Defibrotide, a compound that enhances PGI_2 release from the vascular endothelium, was also investigated and found to have a beneficial effect in experimental ischaemic myocardial injury in open-chest minipigs subjected to MI-R (Hohlfeld, 1993).

Preservation or replenishment of NO, adenosine, PGI_2 concentrations in the coronary vasculature should be one of the effective treatments of reperfusion injury. Therefore, it was postulated that a novel NO donor, GEA 3162, a selective adenosine A_{2A} receptor agonist, 2-HE-NECA, and a selective IP analogue, PGI_2, could inhibit neutrophil infiltration into the ischaemic area and the inhibitory effects of these drugs on neutrophil infiltration could reduce infarct size in ischaemic myocardial tissue in a rat model of MI-R.

3.2 AIMS

The aims of this study are:

1) to investigate the *in vivo* effect of a novel NO donor, GEA 3162 on neutrophil accumulation in a rat model of MI-R.

2) to compare the *in vivo* effects of GEA 3162 with the effects of the selective adenosine A_{2A} receptor agonist, 2-HE-NECA and the selective IP analogue cicaprost on neutrophil accumulation in a rat model of MI-R.

3) to investigate the effects of inhibition of neutrophil accumulation by these drugs on myocardial injury in a rat model of MI-R.

3.3 MATERIALS

The following compounds were generous gifts: 3-aryl-substituted oxatriazol derivatives GEA 3162 [3-(3',4'-dichlorophenyl)-1,2,3,4-oxatriazol-5-imine] from Dr S. B. Pederson (GEA Ltd, Copenhagen, Denmark). 2-HE –NECA (2-Hexynyl-5'-N-ethylcarboxamidoadenosine) from Dr G. Cristalli, Department of Chemical Sciences, University of Camerino, Italy. Cicaprost from Dr E. Schillinger, Schering AG, Berlin, Germany.

Hexadecyltrimethyl ammonium bromide (HTAB), 2,3,5-triphenyltetrazolium chloride (TTC), 3,3,',5,5'-tetramethyl benzidine (TMB), trypan blue, Evan's blue, PBS (containing Ca^{2+} and Mg^{2+}), 30% hydrogen peroxide, 10% formalin in saline and casein (sodium salt) were purchased from Sigma Chemical Co. Ltd. (UK). Polymorphprep™ was purchased from NYCOMED PHARMA AS. Diff Quick was purchased from Baxter Diagnostic AG. Heparin sodium was purchased from CP Pharmaceuticals Ltd. Wrexham, (UK). Lignocaine hydrochloride was purchased from Martindale Pharmaceuticals. Pentobarbitone sodium was purchased from Rhone Merieux (Ireland).

The chemical structures of GEA 3162, 2-HE-NECA and cicaprost are shown in Figure 3.1

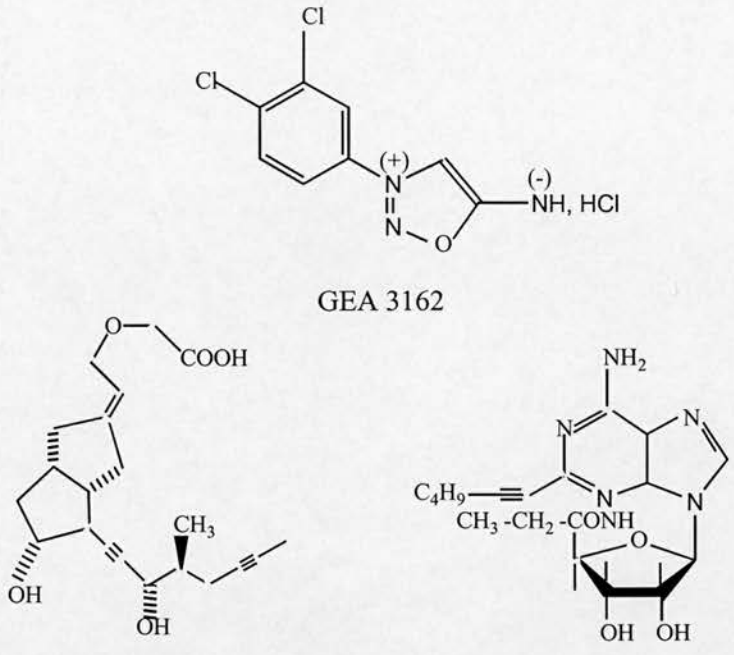


Figure 3.1 *The chemical structures of GEA 3162, 2-HE-NECA and cicaprost*

A mesoionic 3-aryl-1,2,3,4-oxatriazole-5-imine derivative (GEA 3162), is a potent NO donor which releases NO spontaneously in aqueous solutions in a dose-dependent manner as measured by ozone-chemiluminescence (Kankaanranta *et al.*, 1996). GEA 3162 has been shown to inhibit neutrophil functions such as chemotaxis, superoxide anion generation, and degranulation of neutrophils (Moilanen *et al.*, 1993, 1994; Wanikiat *et al.*, 1997). GEA 3162 has vasodilator, antiplatelet and fibrinolytic activity (Corell *et al.*, 1994). It also inhibits endothelial cell-mediated oxidation of low density lipoprotein (Malo-Ranta *et al.*, 1994). GEA 3162 has also been shown to suppress the release of histamine and LTB_4 from neutrophils (Corell *et al.*, 1994).

2-hexynyl-5'-N-ethylcarboxamidoadenosine (2-HE-NECA), a new potent selective A_{2A} receptor agonist, is more potent on A_{2A} than the reference A_{2A} agonist, 2-[4-(2-carboxyethyl)-pheneethylaminoadeno]-5'-N-ehtylcarboxamidoadenosine (CPEC) or

the non-selective adenosine agonist 5'-N-ethylcarboxamidoadenosine (NECA). 2-HE-NECA is a potent vasodilating agent manifested in both *in vitro* and *in vivo* models (Dionisotti *et al.*, 1992; Conti *et al.*, 1993; Monopoli *et al.*, 1994) and inhibits platelet aggregation *in vitro*, *in vivo* and *ex vivo* studies (Dionisotti *et al.*, 1992; Conti *et al.*, 1993; Sandoli *et al.*, 1994). In the anaesthetised rabbit, 2-HE-NECA induced dose-related blood pressure reduction and moderate increase in heart rate, as well as exhibiting potential anti-ischaemic action (Monopoli *et al.*, 1994). In the rat Langendorff model, in which global ischaemia was induced, 2-HE-NECA showed significant prevention in the rise of diastolic pressure occuring during postischaemic reperfusion (Monololi *et al.*, 1994). This anti-ischaemic action of 2-HE-NECA may support the beneficial effects of 2-HE-NECA in MI-R injury.

Cicaprost, a metabolically and chemically stable PGI_2 analogue, has a pharmacological profile comparable to PGI_2 and iloprost. As compared with iloprost, cicaprost is 5-12 fold more potent with respect to *in vivo* hypotensive and anti-aggregatory effects (Sturzebecher, *et al.*, 1986). It is an orally active PGI_2 analogue with high oral availability and long lasting biological activity. Due to its high metabolic stability, cicaprost is more potent and exhibits a longer duration of action than PGI_2 and iloprost with respect to vasodilating and platelet-inhibiting effects (Sturzebecher, *et al.*, 1986). Following intravenous application cicaprost lowers diastolic blood pressure in a dose dependent manner. Cicaprost inhibited fMLP-stimulated rat neutrophil aggregation and produced concentration-related increases in cyclic AMP accumulation (Wise, 1996).

3.4 METHODS

As no model of *in vivo* myocardial ischaemia reperfusion injury was available in our laboratory, it was necessary for me to set up the rat MI-R model with advice from Dr. C. Wainwright, Department of Physiology and Pharmacology, University of Strathclyde (surgical procedure), Dr F. Williams, Imperial College School of Medicine at the National Heart & Lung Institute (extraction and assay of myeloperoxidase) and Dr T. Stevens Department of Pharmacology, Astra Charnwood (isolation of rat peritoneal neutrophils and myeloperoxidase assay). All experiments were performed in accordance with the United Kingdom Home Office Guide on the Operation of Animals (Scientific Procedures) Act 1986.

3.4.1 Left main coronary artery occlusion and reperfusion

Male Wistar rats weighing 250-350 g were used in this study. Animals were anaesthetised with sodium pentobarbitone (Sagatal, 60mg/kg intraperitoneally, i.p.) and the level of anaesthesia was maintained throughout the experiment by intravenous administration of anaesthetic as required. The trachea was cannulated (using a polythene cannula, Portex) for artificial respiration. The right carotid artery was cannulated with a heparin solution (100 iu/ml in 0.9% NaCl)-filled polyethylene cannula. This was connected to a pressure transducer (Sensor Nor a.s.) for continuous recording of systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial blood pressure (MABP) and heart rate via a Maclab system (MacLab/4e, AD Instruments). Heart rate was obtained from the blood pressure traces. The external jugular and femoral veins were also cannulated for administration of anaesthetic and drugs or vehicle, respectively. Body temperature was kept at 37 ± 0.5°C by a heating pad placed under the animal which was thermostatically controlled by a probe inserted into the rectum (Homeothermic Blanket Control Unit, Harvard). The rats were artificially ventilated with room air using a respirator (C.F.Palmer) with the rate of 60 strokes/minute and a tidal volume of 1.5 ml/100 g. This was sufficient to maintain PCO_2, 18-24 mmHg, PO_2, 100-130 mmHg and pH within normal limits, 7.4 units (Clark *et al*, 1980).

The left main coronary artery was occluded according to a modification of the technique previously described by Selye *et al.*, 1960. Briefly, a skin incision was made over the thorax and the pectoral muscles were retracted to expose the thoracic wall. A left thoracotomy incision was performed by cutting through the fourth and fifth ribs at approximately 5 mm to the left of the sternum. The pericardium was cut to expose the heart and the heart was exteriorised by application of gentle pressure to the ribs on either side of the incision in the thorax (using two forceps). A 5/0 silk ligature was passed around the left main coronary artery just below the atrial appendage then the heart was immediately replaced into the thoracic cavity. Both ends of the thread were passed through a short length of polythene tube to form a snare. Lignocaine was intravenously injected at a dose of 2.0 mg/kg prior to thoracotomy incision in order to prevent the occurrence of ischaemic arrhythmias.

After completion of all surgical procedures, rats were allowed to equilibrate for 15 minutes to ensure that all haemodynamic variables had stabilized and baseline haemodynamic data were recorded. The left main coronary artery was then reversibly occluded by tightening the snare and clamping the ends against the polythene tubing with a vascular clamp. This induced myocardial ischaemia and was designated as time zero of occlusion. Ischaemia was confirmed visually by cyanosis in the area at risk. Previous blood flow analyses with radiolabelled microspheres showed that this procedure produced a 98% reduction in blood flow to the area at risk (Toombs *et al.*, 1992). Blood pressure and heart rate were determined pre-occlusion and at 30 minutes and 45 minutes post-occlusion. After 45 minutes of ischaemia, the ligature around the left main coronary artery was completely loosened by taking the vascular clamp off to allow reperfusion of the myocardium for a further 2 hours. Blood pressure and heart rate were determined at 30 minutes, 1 hour, 1.5 hours and 2 hours of the reperfusion period.

Ten minutes before reperfusion, the infusion of each drug or vehicle (0.9% saline, 0.5 ml/h) was begun and continued until the end of the reperfusion period. A variety of infusion rates of these drugs were initially used to obtain an infusion rate that produced minimal hemodynamic (i.e. vasodilatation) effects in intact rats. The

optimal rate of GEA 3162 infusion was determined to be 1 μg/kg/min whereas both cicaprost and 2-HE-NECA were infused at 0.1 and 1 μg/kg/min.

At the end of the reperfusion period, the left main coronary artery was retightened at the same site to completely occlude the vessel. At that time 1 ml of 40 mg/ml of Evans blue dye was injected into the jugular vein to stain the area of the myocardium perfused by the patent coronary arteries. A schematic diagram of the experimental protocol is shown in Figure 3.2. The area at risk, due to its anatomical dependence on the left main coronary artery for blood flow, was identified by negative staining (the lack of Evans blue in this region) as shown in Figure 3.3. The rat was then sacrificed and the heart was rapidly removed. The atria and great vessels were trimmed and the remaining tissue weighed, frozen and stored at -70°C for measurement of infarct size. In this study, infarct size was not measured in the same hearts as neutrophil infiltration. The hearts required for determination of neutrophil accumulation were sectioned in transverse rings 0.5 cm thick, from the apex to the base. The right ventricle was separated and discarded. The unstained portion of the myocardium (i.e. the total area at risk) was separated from the stained portion (i.e. the area not at risk or the normal area). They were then collected, weighed and frozen rapidly in dry ice. Samples were then stored at -20°C until assayed for myeloperoxidase content, within 2 weeks of each experiment.

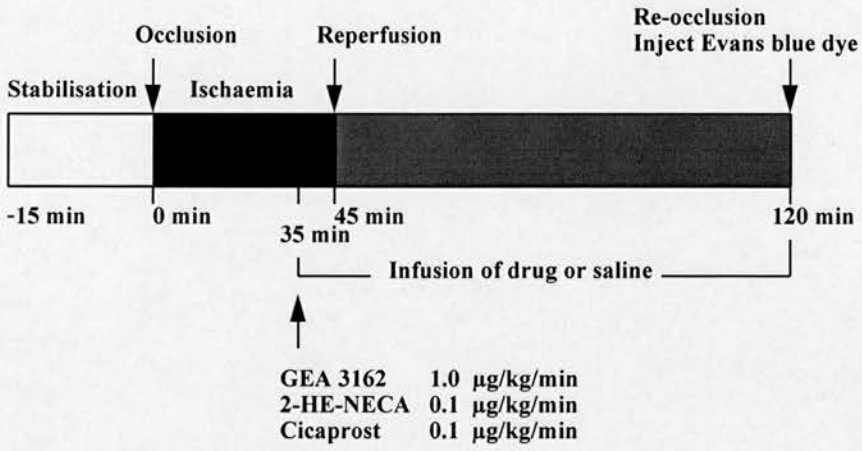


Figure 3.2 *Schematic diagram of the experimental protocol. After15 minutes of stabilisation, the left main coronary artery was occluded for 45 minutes. Ten minutes before reperfusion, the infusion of each drug or vehicle (0.9% saline) was begun and continued until the end of the reperfusion period. After 45 minutes of occlusion, the ligature around the left main coronary artery was loosened to allow reperfusion of the myocardium for 2 hours. On completion of the 2-hour reperfusion period, the coronary artery was reoccluded and Evan's blue dye was injected via the jugular vein to differentiate the area at risk from the normal area.*

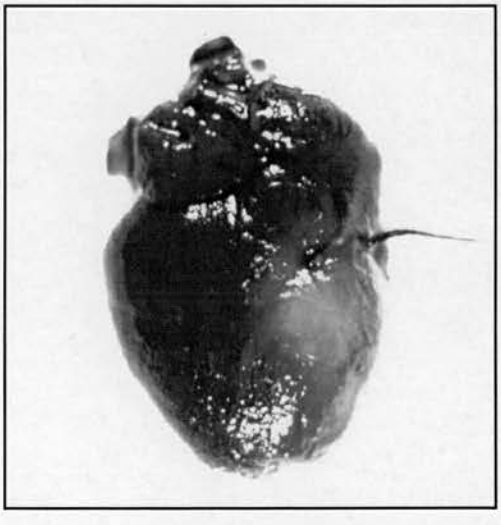


Figure 3.3 *Photograph of the rat heart subjected to 45 minutes occlusion of the left main coronary artery, followed by 2 hours reperfusion. Shows the area at risk (pink) and normal area (blue) on the surface of the heart after staining Evan's blue dye.*

3.4.2 Determination of neutrophil accumulation

Myeloperoxidase (MPO), an enzyme that is associated with the azurophilic granule of neutrophils, with less being found in eosinophils and monocytes, has been utilized as a convenient marker for infiltration of neutrophils into inflamed tissue (Bradley *et al.*, 1982; Allan *et al.*, 1985; Mullane *et al.*, 1985). Therefore, neutrophil accumulation in the heart tissue was assessed by measurement of its MPO content. The non-ischaemic area (area not at risk) and the ischaemic area (area at risk) of the left ventricle were measured for their MPO content.

In order to express results as the number of neutrophils per g tissue, a standard curve for MPO content [Absorbance at 460 nm versus neutrophil concentration (10^3-10^4 cells)] was constructed using MPO extracted from rat neutrophils. Originally, rat neutrophils were isolated from peripheral blood using Polymorphprep™.

3.4.2.1 Isolation of rat neutrophils from whole blood using Polymorphprep ™

The isolation of rat neutrophils from whole blood using Polymorphprep™ is summarised in a simplified flow diagram (Figure 3.4). Polymorphprep™ is a ready made sterile solution for the isolation of polymorphonuclear granulocytes from whole blood. It contains sodium metrizoate and Dextran 500. After centrifugation of anticoagulated whole blood, although two leukocyte bands (the top band of mononuclear cells and the lower band of polymorphonuclear cells) should be clearly distinquishable in the supernatant, only the one cloudy band (band A) was evident (Figure 3.4). In order to check their cellular content, the cells from the upper cloudy band A and the cells from the lower part of the supernatant which was closest to the cloudy band A (band B) were harvested separately and were spread onto slides using a Cytospin centrifuge (Centurion). After the slides were dried, fixed and stained with Diff Quik™, the cells were identified microscopically. It was found that most of the cells from band A were monocytes and lymphocytes and most of neutrophils were found in the lower part of supernatant which was closest to the upper cloudy band (band B) (Figure 3.5).

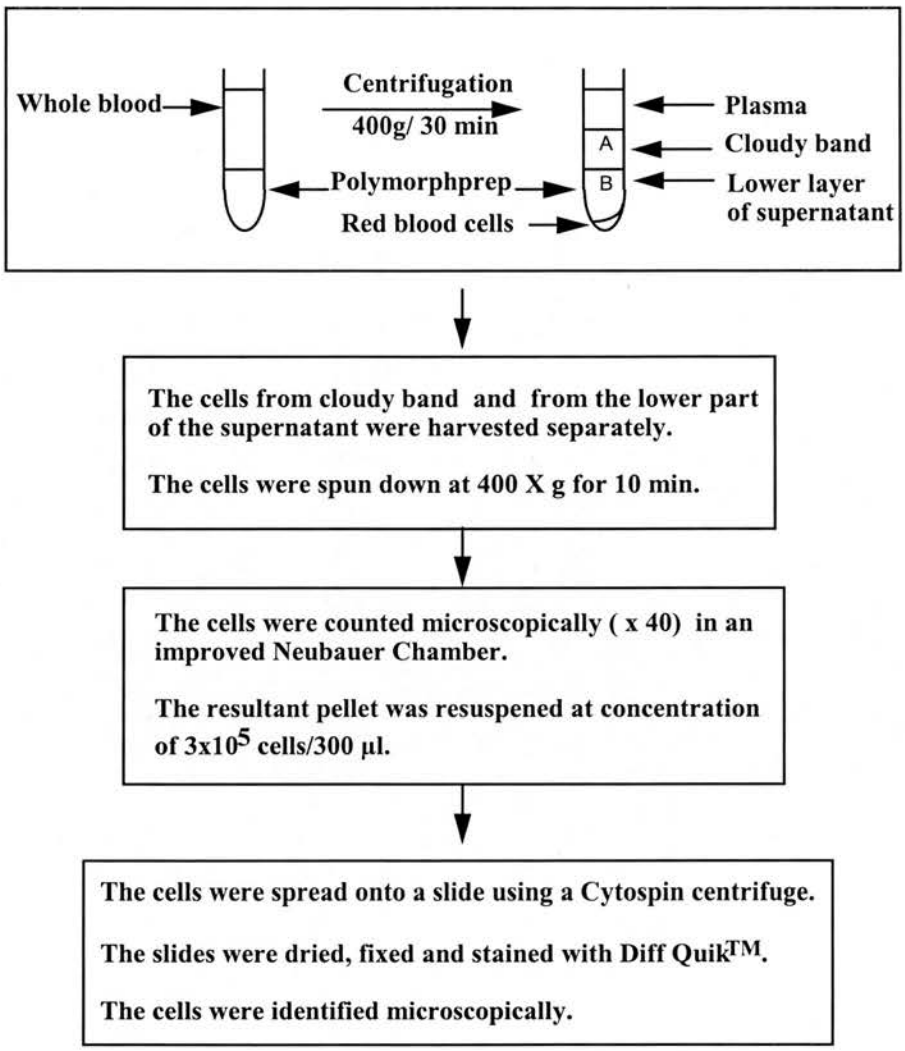


Figure 3.4 *Flow diagram showing isolation of rat neutrophils from whole blood using Polymorphprep ™. Most of the cells from the cloudy band (band A) were found to be monocytes and lymphocytes. Neutrophils were found in the lower part of the supernatant (band B).*

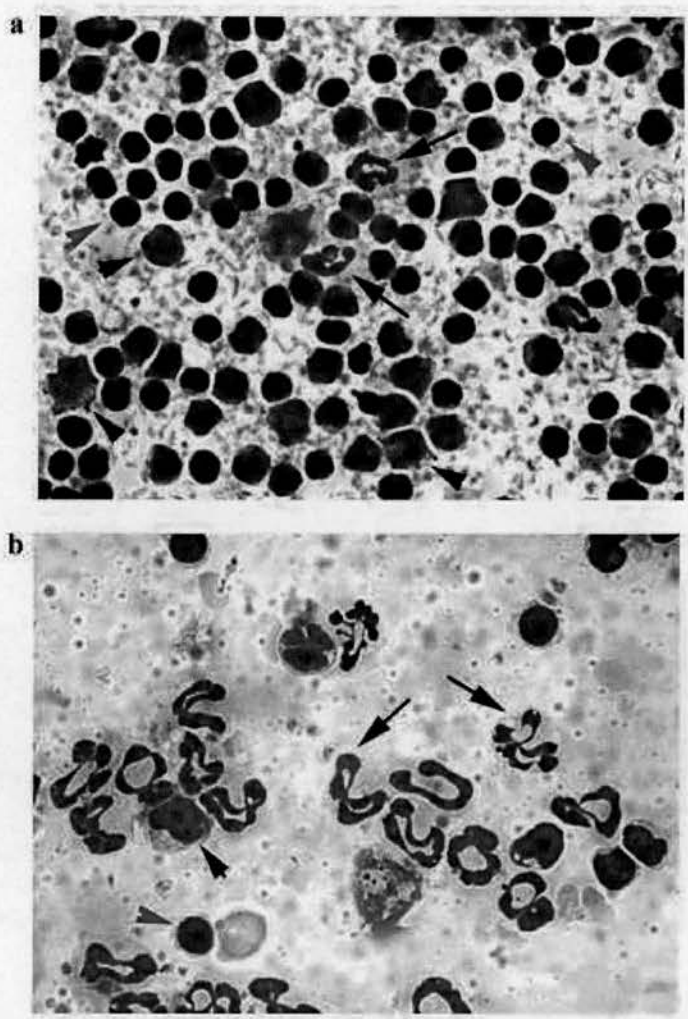


Figure 3.5 *Photograph showing rat neutrophils monocytes and lymphocytes isolated from whole blood using polymorphprep™. (a) cells from upper cloudy band (band A), (b) cells from the lower supernatant layer (band B). The black arrows indicate neutrophils, the black arrow heads indicate monocytes and the red arrow heads indicate lymphocytes (original magnification × 2500).*

Although this method works well with human blood, it is not suitable for isolation of neutrophils from rats for the following reasons: (a) the small volume of blood obtained from the rats, (b) the low yield of neutrophils and (c) the subtle difference in density among monocytes, neutrophils and lymphocytes that causes poor separation of the mononuclear and polymorphonuclear cell bands. Subsequently, rat neutrophils were isolated from the peritoneal cavity instead as described below.

3.4.2.2 Isolation of rat peritoneal neutrophils

The isolation of rat peritoneal neutrophils was carried out according to the method previously described by Cunningham, with some modification (Cunningham *et al.*, 1979). The extraction of rat peritoneal neutrophils is summarised in a simplified flow diagram (Figure 3.6).

Method

1. Male Wistar rats weighing 400-500 g were injected intraperitoneally with 15 ml of 5% casein (sodium salt) in 0.9% NaCl.

2. After 16 hours, the rats were terminated by anaesthetic overdose and 25 ml of sterile PBS containing 0.2% glucose and 20 U/ml heparin injected intraperitoneally to wash the peritoneal cavity. The peritoneum was then opened and the lavage fluid aspirated and collected in centrifuge tubes. The lavage fluid from each rat was kept separately and the volume of each tube increased to 50 ml with PBS-gluccose.

3. The cell suspension was centrifuged at 400 × g for 5 minutes and the supernatant was discarded. Red blood cells remaining in the neutrophil pellet were lysed by resuspending the pellet in 10 ml of ice cold 0.2% w/v NaCl solution for 20 seconds, after which 10 ml of ice cold 1.6% w/v NaCl was added to return the cells to isotonic conditions.

4. The cells were counted and their viability checked by trypan blue exclusion: 100 µl of the cell suspension were added to 400 µl of trypan blue and were counted under a microscope (× 40) in an improved Neubauer Chamber. The number of neutrophils that could be isolated from one rat was usually in the range of 5×10^6 to 15×10^6 cells. The viability of the neutrophils thus obtained were over 95% as estimated by the trypan blue dye exclusion test.

5. The cells were washed twice with PBS containing 0.1% bovine serum albumin (to maintain viability and decrease aggregation). The cell pellet was stored at

-20°C for determination of MPO content. The cells from at least two rats were pooled together for each standard curve repetition. The standard curve for MPO content represents the results of six repetitions. The method for extraction of rat neutrophil MPO was the same as that for heart tissue (discussed below).

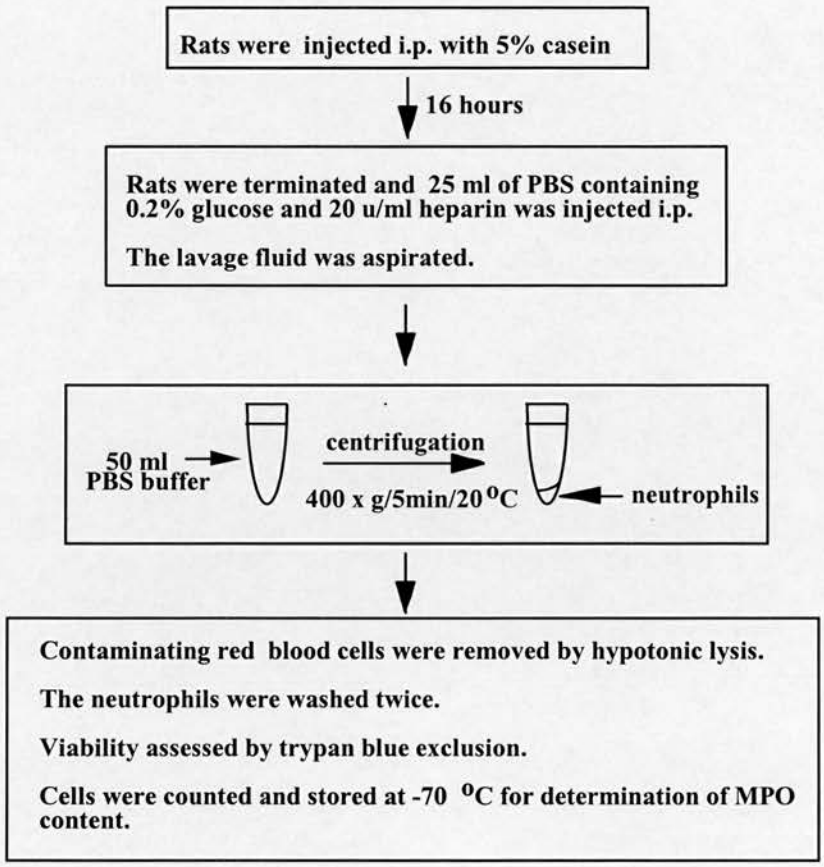


Figure 3.6 *Flow diagram showing isolation of rat peritoneal neutrophils.*

3.4.2.3 Extraction of myeloperoxidase from heart tissue and neutrophils

The extraction of myeloperoxidase from heart tissue and neutrophils was carried out according to a previously described method (Williams *et al.*, 1994) with some modifications. The extraction of MPO from heart tissue and neutrophils is summarised in a simplified flow diagram (Figure 3.7).

Method

1. The frozen myocardial and rat peritoneal neutrophil samples were thawed and kept on ice. Cold homogenisation buffer 1 containing 20 mM of sodium phosphate buffer (pH 4.7), 0.015 M EDTA, 0.1M NaCl, was added to the tissue samples in the proportion of 50 mg/ml for tissue samples from the area at risk and for rat peritoneal neutrophils and 100 mg/ml for tissue samples from the area not at risk (normal area).

2. The tissue samples were first homogenised using an Ultra-Turrax homogeniser until evenly ground.

3. The homogenate was centrifuged for 15 minutes at 10,000 × g at 4°C (IEC micromax RF) and the supernatant from each sample, containing mainly the haemoglobin, was discarded.

4. Cold homogenisation buffer 2 consisting of 0.5% hexadecyltrimethyl ammonium bromide (HTAB) dissolved in 50 mM sodium phosphate buffer (pH 5.4) was immediately added to the remaining pellet in the proportion of 100 mg/ml for pellet from myocardial area at risk and from rat peritoneal neutrophils and 200 mg/ml for pellet from myocardial normal area. Each sample was homogenised a second time for 20 seconds and the homogenate was then taken through three cycles of freeze/thaw followed by brief sonication (× 10 seconds) to ensure that cell lysis and MPO release from storage granules had occured.

5. The homogenates were then centrifuged for 15 minutes at 10,000 × g at 4°C and the supernatant assayed for MPO.

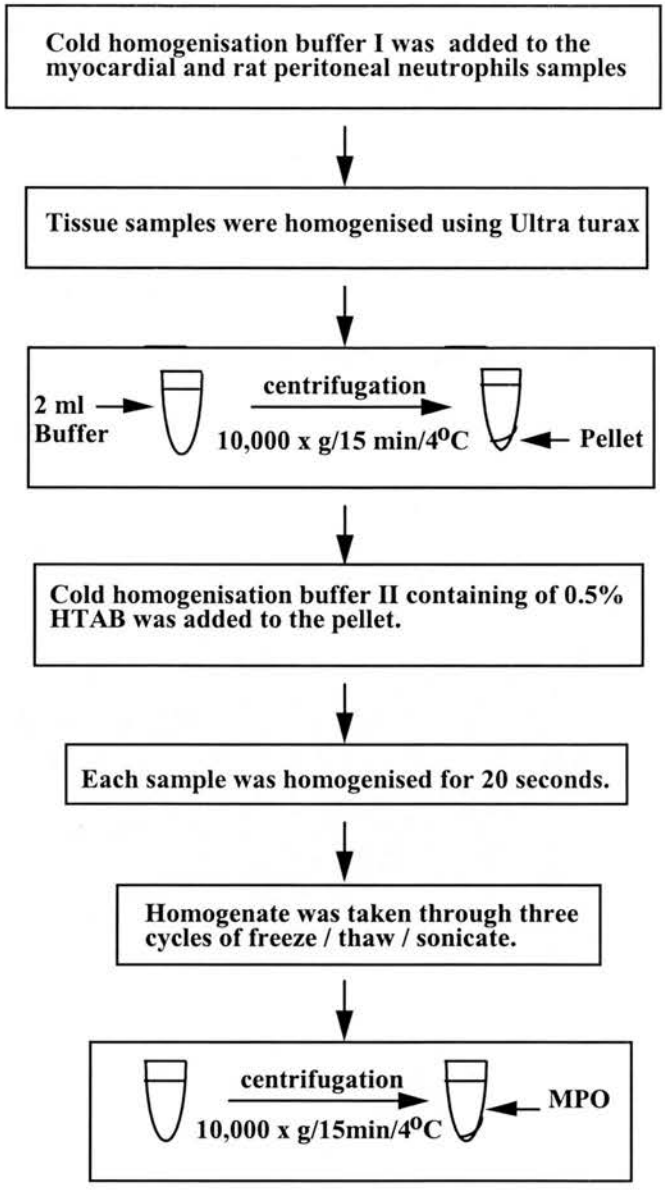


Figure 3.7 *Flow diagram showing extraction of myeloperoxidase from heart tissue and rat peritoneal neutrophil .*

3.4.2.4 Myeloperoxidase Assay

In this study, MPO was assayed spectrophotometrically by a modification of the method of Bos, *et al.* (1981). MPO uses H_2O_2 to oxidise a variety of aromatic compounds by a 1-electron mechanism to give substrate radical. 3,3',5,5'-tetramethylbenzidine (TMB) is a suitable substrate for the assessment of MPO activity or content. It can be oxidised both enzymatically by MPO as well as

chemically by HOCl produced by the MPO-catalysed oxidation of Cl^-. The proposed reactions are:

$$TMB \xrightarrow{MPO/H_2O_2} TMB_{OXI}$$

$$H_2O_2 + Cl^- \xrightarrow{MPO} HOCl + OH^-$$

$$TMB \xrightarrow{HOCL} TMB_{OXI} \text{ (oxidised product of TMB)}$$

Method

1. The supernatant from each sample was diluted (one in two, one in five and one in ten) so that the content of MPO was within the range that could be measured.

2. Aliquots of supernatant (30 µl) were pipetted in duplicate on to a 96 well plate.

3. 200 µl of reaction mixture [0.1 mg/ml 3,3',5,5'- tetramethylbenzidine (TMB) in 0.05 M citrate phosphate buffer (pH 5.0) supplemented with 0.012% (v/v) hydrogen peroxide] was immediately added. The reaction was allowed to develop for 5 minutes where the product from the MPO-dependent reaction of TMB and H_2O_2 appeared as a bright blue color.

4. The reaction was then stopped by adding 50 µl of 4M H_2SO_4, and the colour of the product turned from blue colour to a yellow colour. The absorbance was read in a spectrophotometer (DYNATECH MR7000) at 460 nm.

5. A standard curve for MPO content of rat peritoneal neutrophils was generated by plotting absorbance (460 nm) against number of neutrophils (10^3 - 5×10^4 cells) (Figure 3.8).

6. The average value of absorbance (460 nm) of each sample was calculated back to number of neutrophils (10^6 cells/g tissue) using the MPO standard curve of rat peritoneal neutrophils.

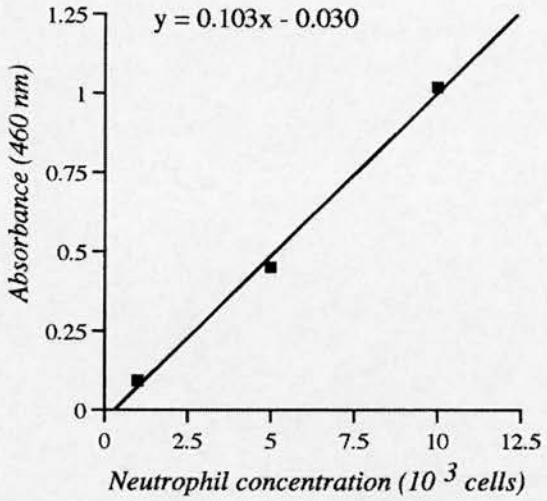


Figure 3.8 *Standard curve of MPO content of rat peritoneal neutrophils. Equation shows absorbance (460 nm), y and number of neutrophils (×10³ cells), x.*

3.4.3 Measurement of myocardial injury

To ascertain the effect of drugs on the degree of actual myocardial salvage of ischaemic tissue after reperfusion, an anatomical measurement of necrotic tissue using histochemical staining was used as an index of ischaemia reperfusion injury. Myocardial injury was evaluated with the triphenyltetrazolium chloride-Evans blue technique (Ytrehus *et al.*, 1994), with some modification.

Method

1. The frozen heart was rinsed in cold isotonic saline solution and cut into 2-3 mm transverse slices from the apex to the base, and the slices were then incubated at 37°C for 15 minutes in 2,3,5-triphenyltetrazolium chloride (TTC, Sigma; 1 % w/v in normal saline). This produced a bright red colouration in the presence of the dehydrogenase enzyme in nonnecrotic myocardium, whereas necrotic regions remained unstained (pale colour) due to the lack of their enzymes and the nonischaemic region stayed blue.

2. The slices were fixed in 10% formalin in saline for 10 minutes to distinguish stained and non-stained regions more clearly (Figure 3.9).

3. The area of myocardium at risk (ischaemic area) was separated from the non-ischaemic area within each slide then the necrotic area of the myocardium at risk that did not stain was carefully separated from the stained area (the ischaemic but non-necrotic area).

4. All three portions of left ventricular myocardium (i.e. non-ischaemic, ischaemic non-necrotic, and ischaemic necrotic) were weighed and the results expressed as the area at risk (AAR) determined as a percentage of the total left ventricular wet weight and the necrotic area expressed as a percentage of the area at risk (AAR) and as a percentage of the total left ventricular wet weight. The gravimetric method has been found to correlate closely with the planimetric method (Vinten-Johansen *et al.*,1992; Toombs *et al.*,1992)

Figure 3.9 *Photograph showing four transverse sections from the apex to the base of left ventricle of a heart (from control group) subjected to 45 minutes occlusion of the left main coronary artery followed by 2 hours reperfusion. The blue area represents the non-ischaemic area, whilst the pink and pale areas represent the ischaemic non-necrotic and ischaemic necrotic areas respectively.*

3.4.4 Assignment of animals to study groups

In this study, we used three drugs, an NO donor, GEA 3162 (Kankaanranta *et al.*, 1996), a selective adenosine A_{2A} receptor agonist, 2-HE-NECA (Monopoli *et al.*, 1994) and a selective IP analogue, cicaprost (Sturzebecher *et al.*, 1985, 1987;

Skuballa *et al.*, 1986). The study of the effect of these drugs on neutrophil accumulation in myocardial tissue in a rat model of myocardial ischaemia reperfusion was divided into seven experimental groups: Control for GEA treated group (0.9% saline, n=7); GEA 3162 treated group (1μg/kg/min, n=7); another control group for 2-HE-NECA and cicaprost (0.9% saline, n=7); 2-HE-NECA treated group (0.1μg/kg/min, n=6); 2-HE-NECA treated group (1 μg/kg/min, n=6); cicaprost treated group (0.1μg/kg/min, n=5) and cicaprost treated group (1μg/kg/min, n=9);

The study of the effect of these drugs on myocardial injury in a rat model of myocardial ischaemia reperfusion was divided into five experimental groups: Control for GEA 3162 treated group (n=7); GEA 3162 treated group (1μg/kg/min, n=7); control for 2-HE-NECA and cicaprost treated group (0.9% saline n=9); 2-HE-NECA treated group (0.1μg/kg/min, n=7) and cicaprost treated group (0.1μg/kg/min, n=7).

3.4.5 Statistical analysis

All results are presented as the mean ± s.e. mean of n experiments. Statistical analysis was performed by two-tailed unpaired Student's *t* test for comparing the following variables between control and treatment groups: myocardial necrotic area (expressed as a percentage of AAR, or a percentage of total left ventricular wet weight), neutrophil accumulation in the area at risk or in the normal area, mean arterial blood pressure and heart rate. A two-tailed paired Student's *t* test was used when comparing neutrophil accumulation in the area at risk and normal area within the control and treatment groups. One-way ANOVA followed by a Dunnett's test was used to compare the change in mean arterial blood pressure and heart rate within individual groups with their respective baseline values (30 minutes occlusion value). A value of $P<0.05$ was considered to be statistically significant.

3.5 RESULTS

3.5.1 Neutrophil accumulation

3.5.1.1 Effect of GEA 3162 on neutrophil accumulation

Occlusion of the left main coronary artery for 45 minutes followed by 2 hours reperfusion of control animals receiving saline (n=7) resulted in a significant increase in the number of neutrophils (as assessed by the MPO content) in the area at risk of the myocardium compared with the normal area of left ventricle, $8.4 \pm 0.8 \times 10^6$ cells/g tissue and $2.9 \pm 0.3 \times 10^6$ cells/g tissue, respectively ($P<0.005$, Figure 3.10) (Table 3.1, page 138).

After administration of GEA 3162 (1 μg/kg/min i.v., n=7) there was no longer a significant difference between the number of neutrophils within the area at risk compared with the normal area of the left ventricle, $5.9 \pm 1.1 \times 10^6$ cells/g tissue and $3.8 \pm 0.4 \times 10^6$ cells/g tissue, respectively ($P>0.05$, Figure 3.10) (Table 3.1). GEA 3162 (1 μg/kg/min) infusion produced a significant decrease in the number of neutrophils in the area at risk of the myocardium, when compared with the control group, $5.9 \pm 1.1 \times 10^6$ cells/g tissue and $8.4 \pm 0.8 \times 10^6$ cells/g tissue, respectively ($P<0.05$, Table 3.1).

There was no significant difference between the number of neutrophils in the normal area of the left ventricle of the GEA 3162 treated groups compared with the control group, $3.8 \pm 0.4 \times 10^6$ cells/g tissue and $2.9 \pm 0.3 \times 10^6$ cells/g tissue, respectively ($P>0.05$).

In the control group, neither blood pressure nor heart rate changed significantly during saline infusion ($P>0.05$ ANOVA, Figure 3.11 & Figure 3.12). Infusion of GEA 3162 (1μg/kg/min) significantly reduced blood pressure ($P<0.005$, ANOVA) but had no significant effect on heart rate ($P>0.005$, ANOVA) (Figures 3.11 & 3.12). However, there was no significant difference in blood pressure between the control and GEA 3162 treated group, except at 1.5 hours of reperfusion.

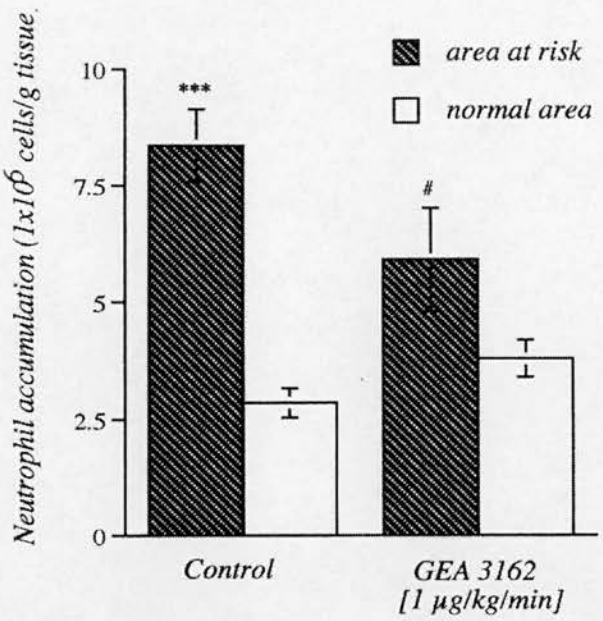


Figure 3.10 *Neutrophil accumulation in AAR and NA of left ventricle in control and GEA 3162 (1µg/kg/min) treated group. Values shown are the mean ± s.e. mean of 7 animals in each group.* $^{***}P<0.005$ *and* $^{\#}P<0.05$ *indicates significantly different from NA and AAR of control group, respectively.*

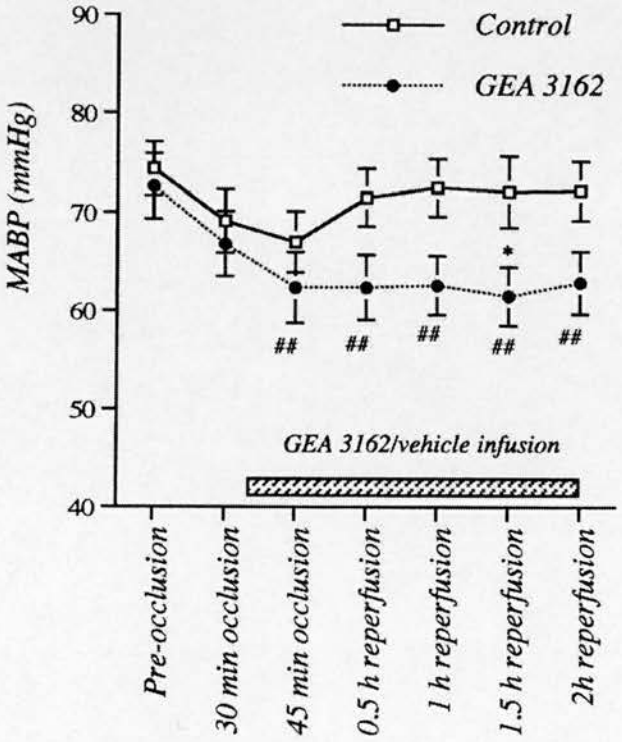


Figure 3.11 *MABP recorded before, 30 and 45 minutes of left main coronary artery occlusion and during 2 hours reperfusion in control and GEA 3162 (1µg/kg/min) treated group. Values shown are mean ± s.e. mean of 7 animals in each group.* $^{*}P<0.05$ *indicates significantly different from control group.* $^{\#\#}P<0.01$ *indicates significantly different from 30 minutes of occlusion of the left main coronary artery.*

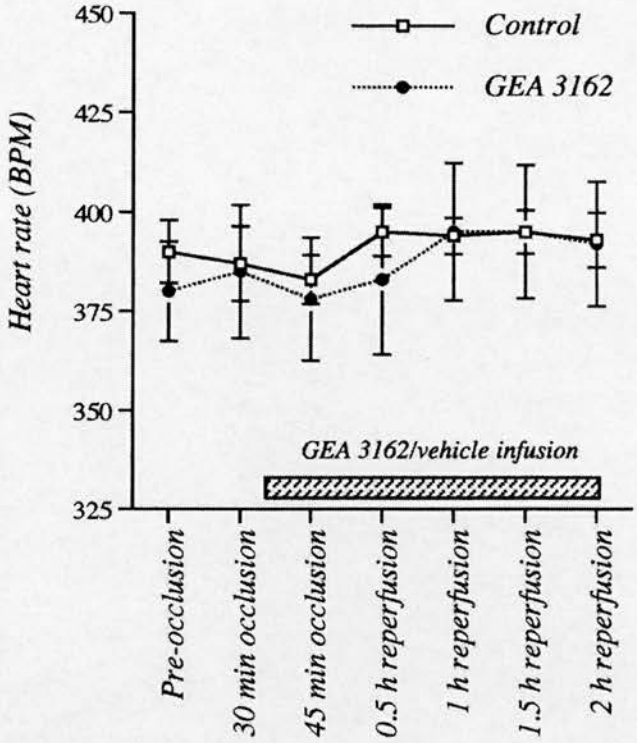


Figure 3.12 *Heart rate recorded before, 30 and 45 minutes of left main coronary artery occlusion and during 2 hours reperfusion in control and GEA 3162 (1 μg/kg/min) treated group. Values shown are mean ± s.e. mean of 7 animals in each group.*

3.5.1.2 Effect of 2-HE-NECA on neutrophil accumulation

In control animals receiving saline (n=7) occlusion of the left main coronary artery for 45 minutes followed by 2 hours reperfusion, resulted in a significant increase in the number of neutrophils in the area at risk of myocardium compared with the normal area of left ventricle, $8.9 \pm 0.3 \times 10^6$ cells/g tissue and $3.3 \pm 0.3 \times 10^6$ cells/g tissue, respectively ($P<0.005$, Figure 3.13, Table 3.1, page 138).

After infusion of 2-HE-NECA (1 μg/kg/min i.v., n=6) there was no longer a significant difference between the number of neutrophils in the area at risk compared with the normal area of the left ventricle, $3.1 \pm 0.5 \times 10^6$ cells/g tissue and $3.5 \pm 0.4 \times 10^6$ cells/g tissue, respectively ($P>0.05$, Figure 3.13, Table 3.1).

Infusion of a lower concentration of 2-HE-NECA (0.1 μg/kg/min i.v., n=6) also inhibited neutrophil accumulation within the area at risk, 4.5 ± 0.5 × 10^6 cells/g tissue and 3.7 ± 0.2 × 10^6 cells/g tissue, in the area at risk and normal area, respectivly (P>0.05, Figure 3.13, Table 3.1).

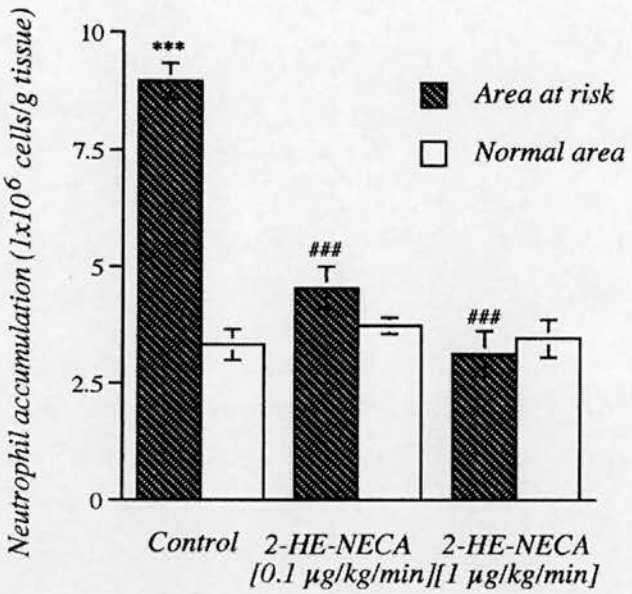


Figure 3.13 *Neutrophil accumulation in AAR and NA of left ventricle in control group (n=7), 2-HE-NECA (0.1μg/kg/min, n=6) and 2-HE-NECA (1μg/kg/min, n=6) treated groups. Values shown are the mean ±s.e. mean for each group.* $^{***}P<0.005$ *and* $^{\#\#\#}P<0.05$ *indicates significantly different from NA and AAR of control group respectively.*

There was no significant difference between the number of neutrophils in the normal area of the left ventricle of both concentrations of 2-HE-NECA (1 μg/kg/min and 0.1 μg/kg/min) treated groups compared with the control groups, 3.5 ± 0.4 × 10^6 cells/g tissue, 3.7 ± 0.2 × 10^6 cells/g tissue and 3.3 ± 0.3 × 10^6 cells/g tissue (P<0.05, ANOVA).

In the control group, both blood pressure and heart rate were significantly changed during infusion of saline (0.5 ml/h) (P<0.05, ANOVA, Figures 3.14 & 3.15). Infusion of 2-HE-NECA (1μg/kg/min) had significant effects on blood pressure and heart rate (P<0.005, ANOVA). This concentration of 2-HE-NECA caused

significant reduction in blood pressure during 45 minutes occlusion to 2 hours of reperfusion and significant increase in heart rate during 0.5 hour to 2 hours of reperfusion when compared with the control groups ($P<0.05$ and $P<0.005$ as indicated in Figures 3.14 & 3.15).

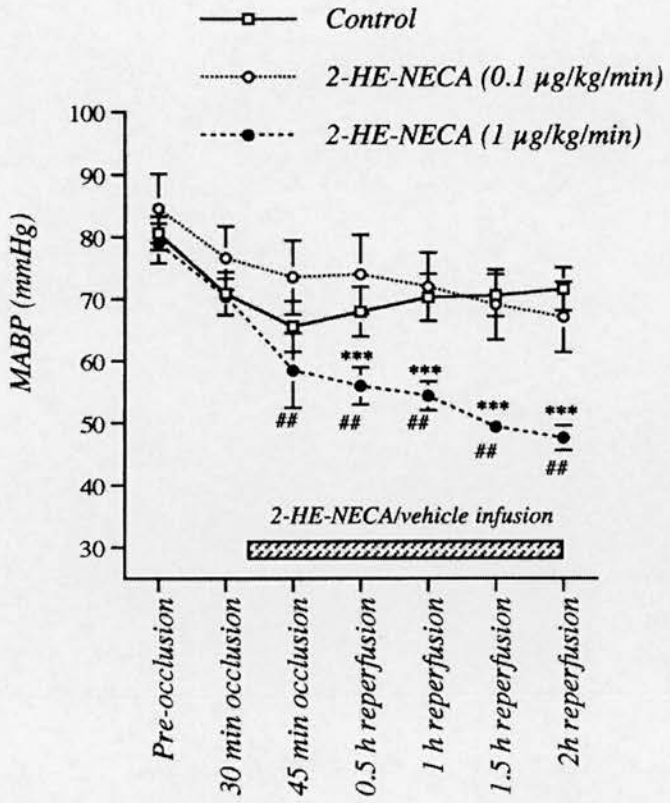


Figure 3.14 *MABP recorded before, 30 and 45 minutes of left main coronary artery occlusion and during 2 hours reperfusion in control group(n=7), 2-HE-NECA (0.1µg/kg/min, n=6) and 2-HE-NECA (1µg/kg/min, n=6) treated groups. Values shown are the mean ± s.e. mean for each group.* $^{***}P<0.005$ *indicates significantly different from control group.* $^{\#\#}P<0.01$ *indicates significantly different from 30 minutes of occlusion of the left main coronary artery.*

Although, infusion of 0.1 µg/kg/min 2-HE-NECA caused a small but significant decrease in blood pressure and increase in heart rate ($P<0.05$, ANOVA), neither blood pressure nor heart rate were significantly different from the control groups except for the heart rate recorded at the 1.5 and 2 hours of reperfusion ($P<0.05$).

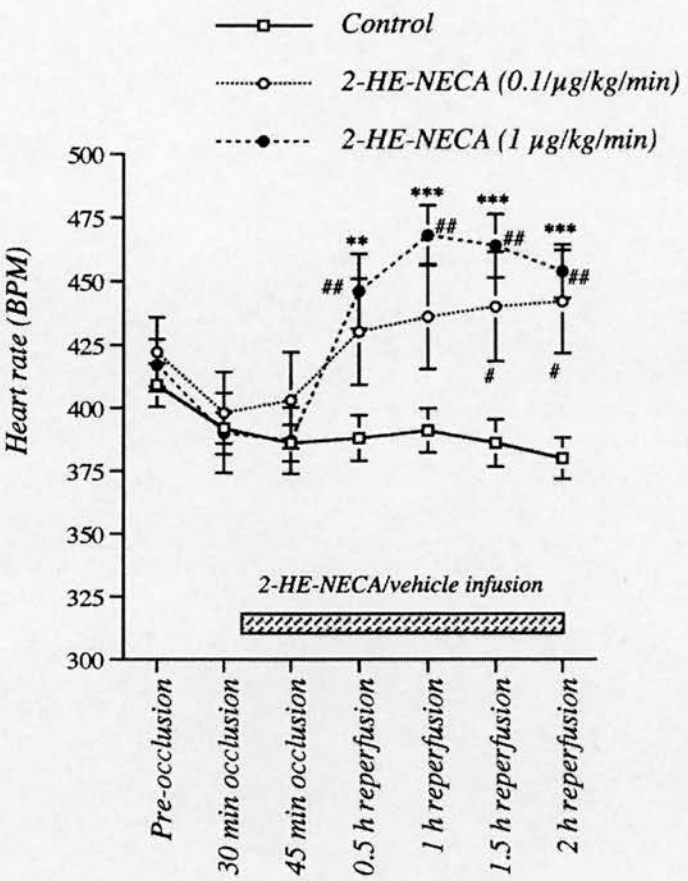


Figure 3.15 *Heart rate recorded before, 30 and 45 minutes of left main coronary artery occlusion and during 2 hours reperfusion in control group (n=7), 2-HE-NECA (0.1μg/kg/min, n=6) and 2-HE-NECA (1μg/kg/min, n=6) treated groups. Values shown are the mean ± s.e. mean for each group.* $^{*}P<0.05$, $^{**}P<0.01$ *and* $^{***}P<0.005$ *indicates significantly different from control group.* $^{\#}P<0.05$ *and* $^{\#\#}P<0.01$ *indicates significantly different from 30 minutes of occlusion of the left main coronary artery.*

3.5.1.3 Effect of cicaprost on neutrophil accumulation

In control animals receiving saline (n=7), occlusion of the left main coronary artery for 45 minutes followed by 2 hours reperfusion, resulted in a significant increase in the number of neutrophils in the area at risk of myocardium compared with the normal area of left ventricle, $8.9 \pm 0.3 \times 10^6$ cells/g tissue and $3.3 \pm 0.3 \times 10^6$ cells/g tissue, respectively ($P< 0.005$, Figure 3.16, Table 3.1, page 138).

In animals receiving an infusion of cicaprost (1 μg/kg/min i.v., n=9) there was no significant difference in the number of neutrophils in the area at risk compared with the normal area of the left ventricle, 4.7 ± 0.7 × 10^6 cells/g tissue and 3.9 ± 0.4 × 10^6 cells/g tissue, respectively (P>0.05, Table 3.1).

In animals receiving an infusion of a lower concentration of cicaprost (0.1 μg/kg/min i.v., n=5) the number of neutrophils within the area at risk was significantly lower than the number within the normal area of the left ventricle, 1.9 ± 0.3 × 10^6 cells/g tissue and 3.6 ± 0.2 × 10^6 cells/g tissue, respectively (P<0.05, Figure 3.16, Table 3.1).

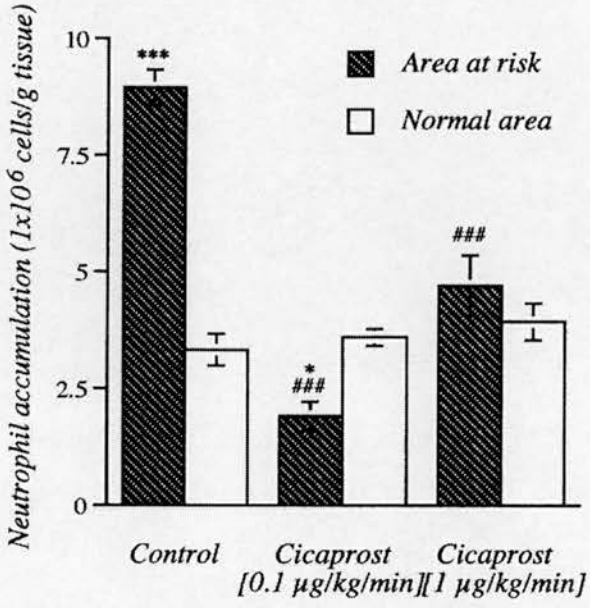


Figure 3.16 *Neutrophil accumulation in AAR and NA of left ventricle in control group (n=7), cicaprost (0.1μg/kg/min, n=5) and cicaprost (1μg/kg/min, n=9) treated groups. Values shown are the mean ± s.e. mean for each group.* $^{***}P<0.005$ *and* $^{*}P<0.05$ *indicates significantly different from NA of control and cicaprost (0.1μg/kg/min) treated group, respectively.* $^{###}P<0.005$ *indicates significantly different from AAR of control group.*

There was no significant difference in the number of neutrophils in the normal area of the left ventricle of both concentrations of cicaprost (1 μg/kg/min and 0.1 μg/kg/min) treated groups when compared with the control groups, 3.9 ± 0.4 × 10^6

cells/g tissue, 3.6 ± 0.2 × 10^6 cells/g tissue and 3.3 ± 0.3 × 10^6 cells/g tissue ($P<0.05$, ANOVA).

In the control group, blood pressure decreased significantly during the occlusion period. Both blood pressure and heart rate were changed significantly during saline infusion (0.5 ml/h) ($P<0.05$, ANOVA, Figures 3.17 & 3.18).

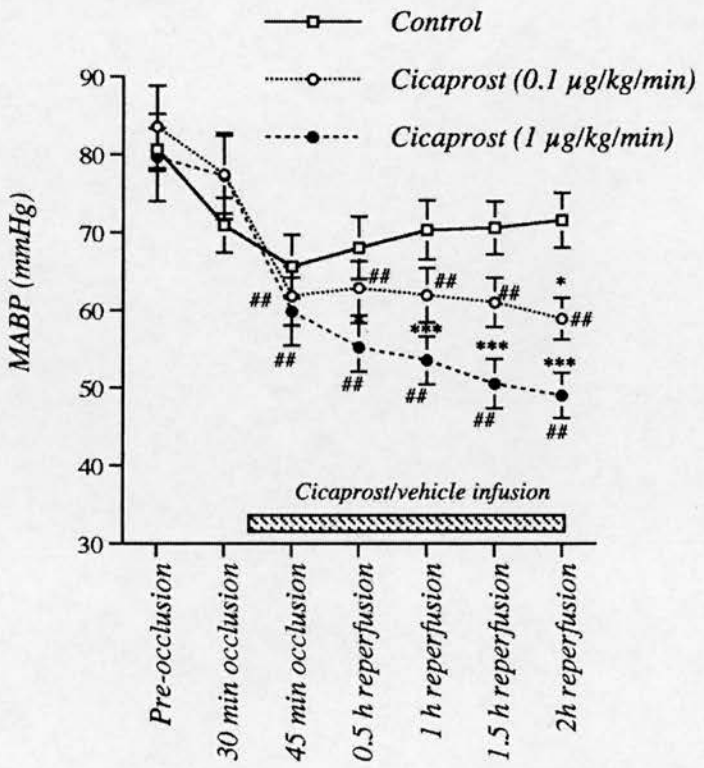


Figure 3.17 *MABP recorded before, 30 and 45 minutes of left main coronary artery occlusion and during 2 hours reperfusion in control group(n=7), cicaprost (0.1µg/kg/min, n=5) and cicaprost (1µg/kg/min, n=9) treated groups. Values shown are the mean ± s.e. mean for each group.* $^{*}P<0.05$ *and* $^{***}P<0.005$ *indicates significantly different from control group.* $^{\#\#}P<0.01$ *indicates significantly different from 30 minutes of occlusion of the left main coronary artery.*

Infusion of cicaprost (1µg/kg/min) caused a significant drop in blood pressure ($P<0.005$, ANOVA) but had no significant effects on heart rate ($P>0.05$, ANOVA). This concentration of cicaprost caused a significant reduction in blood pressure

during 0.5 to 2 hours of reperfusion when compared with the control group ($P<0.05$ and $P<0.005$ as indicated in Figure 3.17) and caused a significant increase in heart rate from 30 minutes occlusion throughout the reperfusion period ($P<0.05$, $P<0.01$ and $P<0.005$ as indicated in Figure 3.18).

Infusion of cicaprost (0.1 μg/kg/min) also caused a significant reduction in blood pressure ($P<0.005$, ANOVA) but had no significant effects on heart rate ($P>0.05$, ANOVA). However, this concentration of cicaprost did not cause significant difference in both blood pressure and heart rate when compared with the control group except the blood pressure recorded at the 2 hours of reperfusion ($P<0.05$, ANOVA).

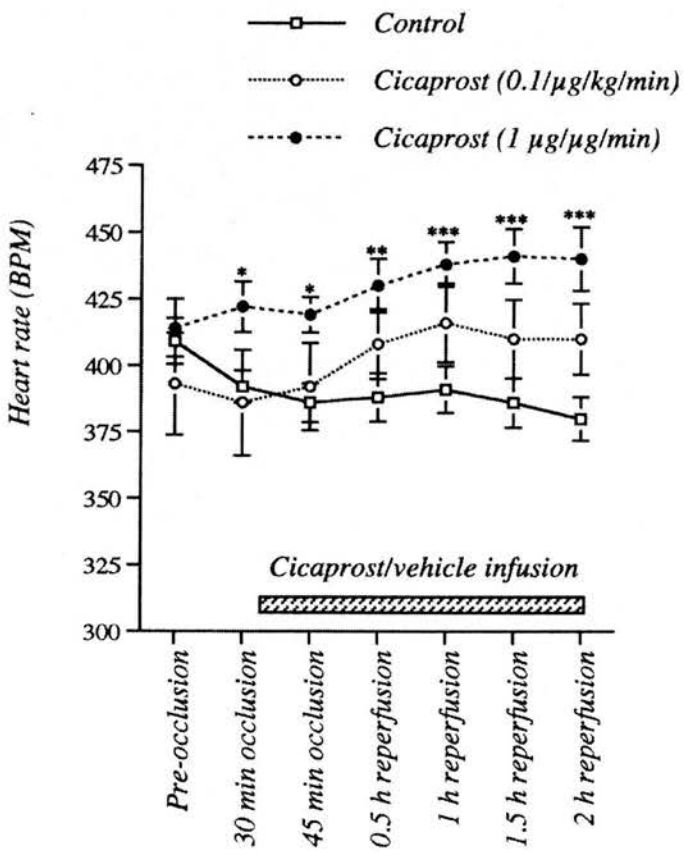


Figure 3.18 *Heart rate recorded before, 30 and 45 minutes of left main coronary artery occlusion and during 2 hours reperfusion in control group (n=7), cicaprost (0.1μg/kg/min, n=5) and cicaprost (1μg/kg/min, n=9) treated groups. Values shown are the mean ± s.e. mean for each group. *P<0.05, **P<0.01 and ***P<0.005 indicate significantly different from control group.*

Table 3.1 *The effect of GEA3162, 2-HE-NECA and cicaprost on neutrophil accumulation in area at risk and normal area of the left ventricle of the heart subjected to 45 minutes occlusion of the left main coronary artery followed by 2 hours reperfusion.*

Drug/vehicle	Concentration (μg/kg/min)	number (n)	Neutrophil accumulation (10^6 cells/g tissue) AAR	NA
Saline (Control 1)	0.5 ml/h	7	$8.4 \pm 0.8^{***}$	2.9 ± 0.3
GEA 3162	1	7	$5.9 \pm 0.1^{\#}$	3.8 ± 0.4
Saline (Control 2)	0.5 ml/h	7	$8.9 \pm 0.3^{***}$	3.3 ± 0.3
2-HE-NECA	1	6	$3.1 \pm 0.5^{\#\#\#}$	3.5 ± 0.4
2-HE-NECA	0.1	6	$4.5 \pm 0.5^{\#\#\#}$	3.7 ± 1.2
Cicaprost	1	9	$4.7 \pm 0.7^{\#\#\#}$	3.9 ± 0.4
Cicaprost	0.1	5	$1.9 \pm 0.3^{\#\#\#,*}$	3.6 ± 0.2

Control 1 is for GEA 3162 treated group; control 2 is for 2-HE-NECA and cicaprost treated group. $^{*}P<0.05$ *and* $^{***}P<0.005$ *indicates significantly different from NA of cicaprost (0.1μg/kg/min) treated group and control groups, respectively.* $^{\#}P<0.05$ *and* $^{\#\#\#}P<0.005$ *indicate significantly different from AAR of control 1 and control 2, respectively.*

3.5.2. Myocardial infarct size

Occlusion of the left main coronary artery was confirmed by changes in the colour of the distal portion of the artery and of the epicardial surface. Occlusion of the left main coronary artery for a period of 45 minutes followed by 2 hours reperfusion resulted in extensive injury to the myocardium. Figure 3.3 shows the rat heart subjected to 45 minutes ischaemia followed by 2 hours reperfusion with a clearly defined area at risk (pink) and normal area (blue) on the surface of the heart after Evan's blue dye injection. Occlusion of the left main coronary artery in the control group produced the area at risk (AAR) of 43.0% ± 1.9% of the total left ventricle wet

weight and necrotic myocardium of 35.1% ± 2.6% of the AAR or 15.0% ± 1.1% of the total left ventricle (Figure 3.19). The ischaemic necrotic, ischaemic non-necrotic and non-ischaemic area of the left ventricle subjected to ischaemia and reperfusion of a control group are clearly seen from the transverse sections from the apex to the base of the left ventricle (Figure 3.9).

3.5.2.1 Effect of GEA 3162 on myocardial infarct size

The area at risk expressed as a percentage of the total left ventricular wet weight was not significantly different between the control and GEA 3162 treated groups, 42.9% ± 1.9% and 43.2% ± 3.2% respectively (n=7, Figure 3.19) (Table 3.2, page 149), indicating that the region of myocardium subjected to ischaemia was comparable in both groups.

Infusion of GEA 3162 (1 μg/kg/min) significantly reduced the necrotic myocardial tissue expressed as a percentage of the myocardial AAR, 19.7% ± 1.1% compared with the control group, 35.1% ± 2.6%, (n=7, $P<0.005$) (Table 3.2) and reduced necrotic area expressed as a percentage of the total left ventricular wet weight 8.5% ± 0.8% compared with the control group, 15.0% ± 1.1% (n=7, $P<0.005$) (Table 3.2). Thus, GEA 3162 infusion resulted in significant attenuation of necrotic tissue in the AAR myocardium after ischaemia reperfusion.

In the control group, there was a significant change in heart rate ($P<0.01$, ANOVA, Figure 3.21) but there was no significant effect on blood pressure ($P>0.05$, ANOVA, Figure 3.20) during infusion of saline (0.5 ml/h).

Infusion of GEA 3162 (1μg/kg/min) caused a significant reduction in blood pressure ($P<0.005$ ANOVA) and had significant effects on heart rate ($P<0.005$). However, there was no significant difference in blood pressure and heart rate at any time between the control and GEA 3162 treated group (Figures 3.20 & 3.21).

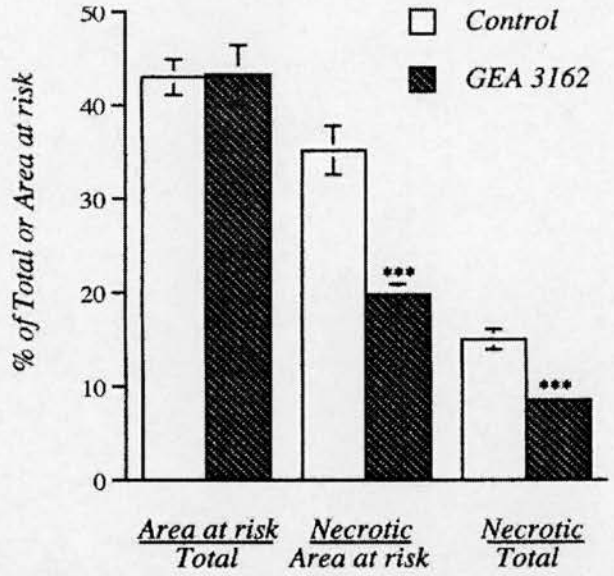


Figure 3.19 *Tissue wet weight of area at risk as a percentage of the total left ventricular wet weight, and of necrotic area as a percentage of area at risk and of the total left ventricular wet weight for control and GEA 3162 (1 μg/kg/min) treated group. Values shown are the mean ± s.e. mean of seven animals in each group.* $^{***}P<0.005$ *indicate significantly different from control group.*

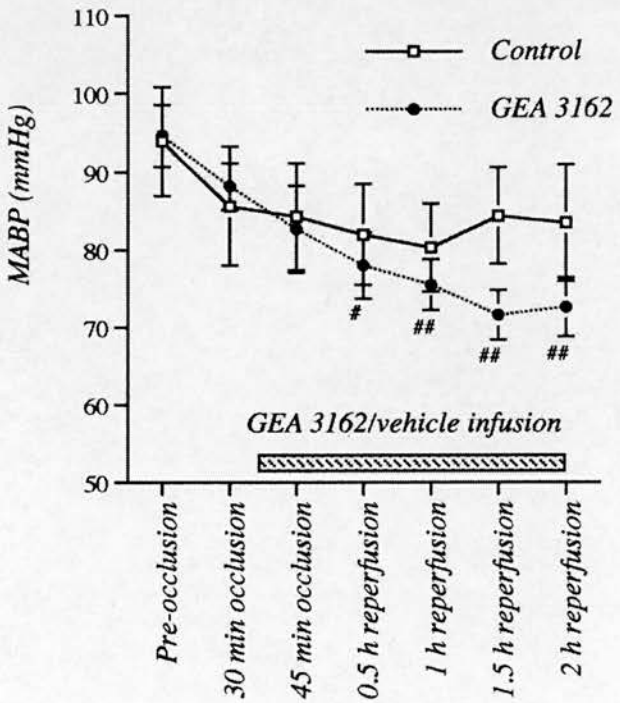


Figure 3.20 *MABP recorded before, 30 and 45 minutes of left main coronary artery occlusion and during 2 hours reperfusion in control group and GEA 3162 (1 μg/kg/min) treated group. Values shown are mean ± s.e. mean of 7 animals in each group.* $^{\#}P<0.05$ *and* $^{\#\#}P<0.01$ *indicates significantly different from 30 minutes of occlusion of the left main coronary artery.*

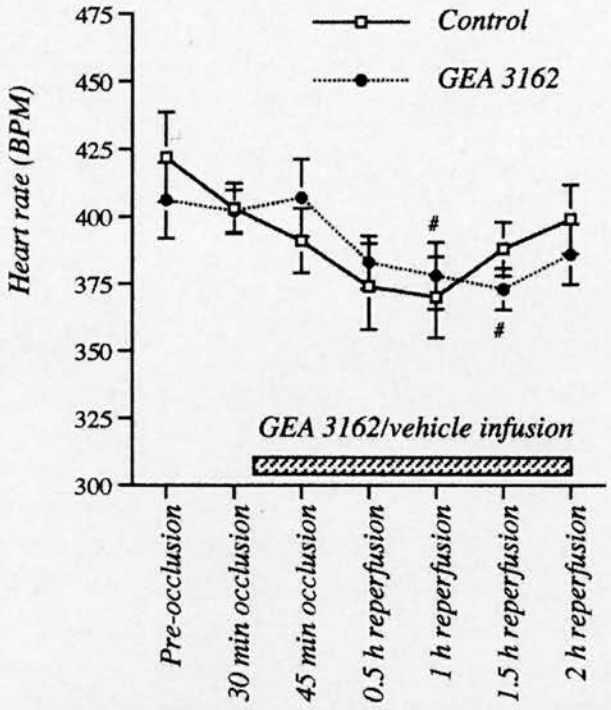


Figure 3.21 *Heart rate recorded before, 30 and 45 minutes of left main coronary artery occlusion and during 2 hours reperfusion in control group and GEA 3162 (1 μg/kg/min) treated group. Values shown are mean ± s.e. mean of 7 animals in each group.* $^{\#}P<0.05$ *indicates significantly different from 30 minutes occlusion of the left main coronary artery.*

3.5.2.2 Effect of 2-HE-NECA on myocardial infarct size

The area at risk expressed as a percentage of the total left ventricular wet weight was not significantly different between the control and 2-HE-NECA treated groups, 42.5% ± 1.6% (n=9) and 38.1% ± 2.6% (n=7), respectively (Figure 3.22, Table 3.2). Infusion of 2-HE-NECA (0.1μg/kg/min) significantly reduced the necrotic myocardial tissue expressed as a percentage of the myocardial AAR, 17.6% ± 1.2% compared with the control group, 35.2% ± 1.8% ($P<0.005$) and of 6.8% ± 0.8% as expressed as a percentage of the total left ventricular wet weight compared with the control group, 15.0% ± 0.9% ($P<0.005$) (Table 3.2). Thus, 2-HE-NECA infusion resulted in significant attenuation of necrotic tissue in the rat after ischaemia reperfusion. The effect of 2-HE-NECA (0.1 μg/kg/min) on reduction of necrotic myocardial tissue is shown in Figure 3.23.

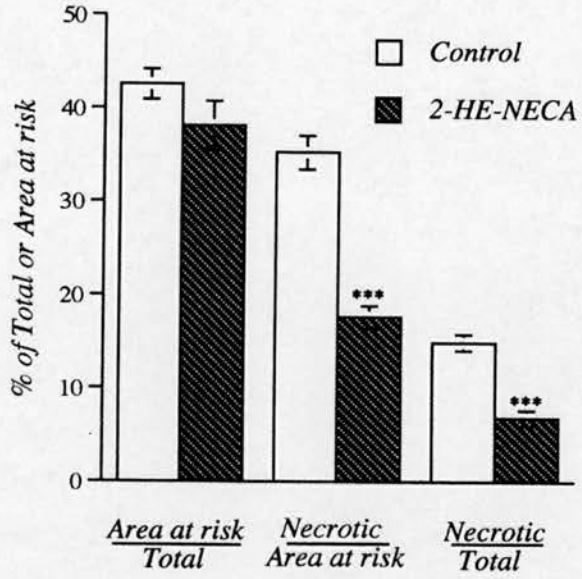


Figure 3.22 *Tissue wet weight of area at risk as a percentage of the total left ventricular wet weight, and of necrotic area as a percentage of area at risk and of the total left ventricular wet weight for control (n=9) and 2-HE-NECA (0.1 1μg/kg/min, n=7) treated group. Values shown are the mean ± s.e. mean for each group.* $^{***}P<0.005$ *indicate significantly different from control group.*

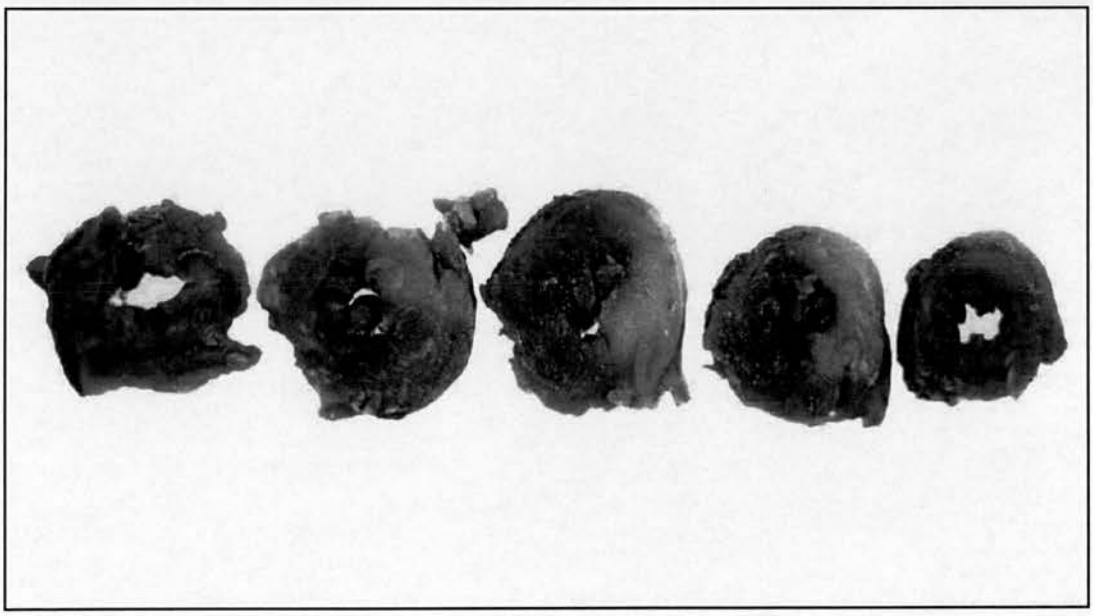


Figure 3.23 *Photograph showing five transverse sections (from apex to base) of left ventricle of a heart subjected to 45 minutes occlusion of the left main coronary artery followed by 2 hours reperfusion of 2-HE-NECA (0.1 μg/kg/min). The blue area represents the non-ischaemic area, whilst the pink and pale areas represent the ischaemic non-necrotic and ischaemic necrotic areas, respectively.*

However, infusion of 2-HE-NECA (0.1 μg/kg/min) had significant effects on blood pressure and heart rate ($P<0.005$, ANOVA) (Figures 3.24 & 3.25). 2-HE-NECA caused a significant reduction in blood pressure from 45 minutes occlusion until the end of the reperfusion period when compared with the control group ($P<0.005$ and $P<0.01$ as indicated in Figure 3.24). Infusion of this drug also caused a significant increase in heart rate compared with the control group ($P<0.01$, measured at 0.5 hour reperfusion and $P<0.005$, measured at 1, 1.5 and 2 hours of reperfusion) (Figure 3.25)

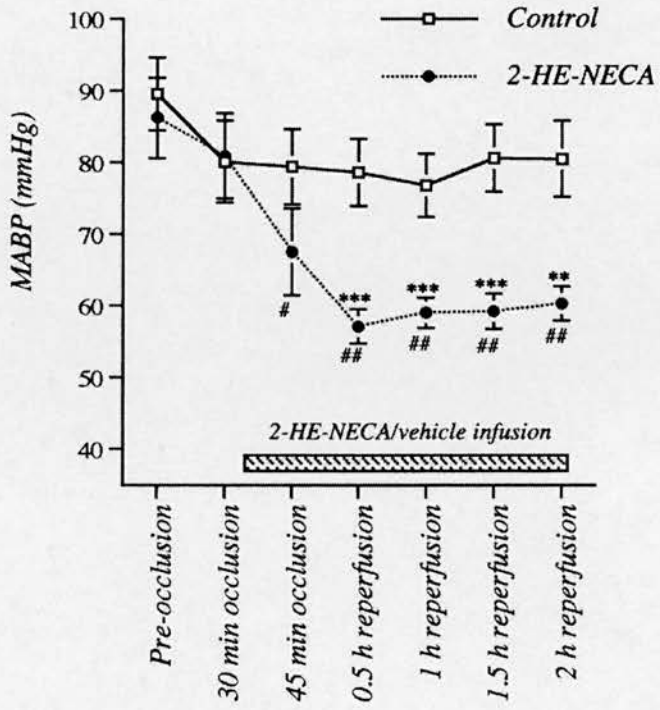


Figure 3.24 *MABP recorded before, 30 and 45 minutes of left main coronary artery occlusion and during 2 hours reperfusion in control group (n=9) and 2-HE-NECA (0.1μg/kg/min, n=7) treated group. Values shown are mean ± s.e. mean for each group.* $^{**}P<0.01$ *and* $^{***}P<0.005$ *indicate significantly different from control group.* $^{\#}P<0.05$ *and* $^{\#\#}P < 0.01$ *indicates significantly different from 30 minutes of occlusion of the left main coronary artery.*

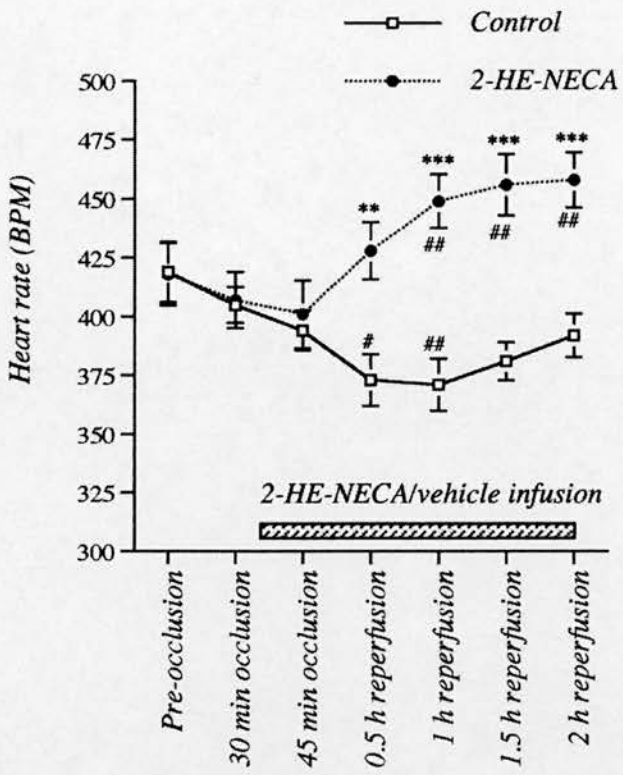


Figure 3.25 *Heart rate recorded before, 30 and 45 minutes of left main coronary artery occlusion and during 2 hours reperfusion in control group (n=9) and and 2-HE-NECA (0.1μg/kg/min, n=7) treated group. Values shown are mean ± s.e. mean for each group.* $^{**}P<0.01$ *and* $^{***}P<0.005$ *indicate significantly different from control group.* $^{\#}P<0.05$ *and* $^{\#\#}P<0.01$ *indicates significantly different from 30 minutes of occlusion of the left main coronary artery.*

3.5.2.3 Effect of cicaprost on myocardial infarct size

The area at risk expressed as a percentage of the total left ventricular wet weight was not significantly different between the control and cicaprost treated group, 42.5% ± 1.6% (n=9) and 38.2% ± 3.1% (n=7), respectively (Figure 3.26, Table 3.2).

Infusion of cicaprost at the concentration of 0.1μg/kg/min significantly reduced the necrotic myocardial tissue expressed as a percentage of the myocardial AAR, 15.6% ± 0.7% compared with the control group, 35.2% ± 1.8% (P<0.005) and as expressed as a percentage of the total left ventricle, 6.0% ± 0.6% compared with the control group, 15.0% ± 0.9% (P<0.005) (Table 3.2). Thus, cicaprost infusion resulted in

significant attenuation of necrotic myocardial tissue in the rat after myocardal ischaemia reperfusion.

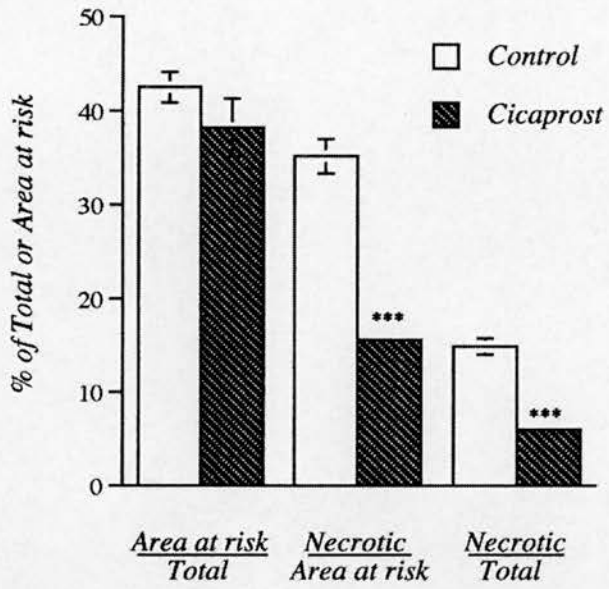


Figure 3.26 *Tissue wet weight of area at risk as a percentage of the total left ventricular wet weight, and of necrotic area as a percentage of area at risk and of the total left ventricular wet weight for control (n=9) and cicaprost (0.1 μg/kg/min, n=7) treated group. Values shown are the mean ± s.e. mean for each group.* $^{***}P<0.005$ *indicate significantly different from control group.*

However, infusion of cicaprost (0.1 μg/kg/min) caused significant changes in blood pressure and heart rate ($P<0.005$, ANOVA) (Figures 3.27 & 3.28). Cicaprost caused a significant reduction in blood pressure when compared with the control group ($P<0.005$, measured at 0.5 and 1 hour reperfusion and $P<0.01$, measured at 1.5 and 2 hours reperfusion). Infusion of cicaprost also caused a significant increase in heart rate compared with the control group ($P<0.01$, measured at 0.5 hour reperfusion and $P<0.005$, measured at pre-occlusion, 1, 1.5 and 2 hours of reperfusion).

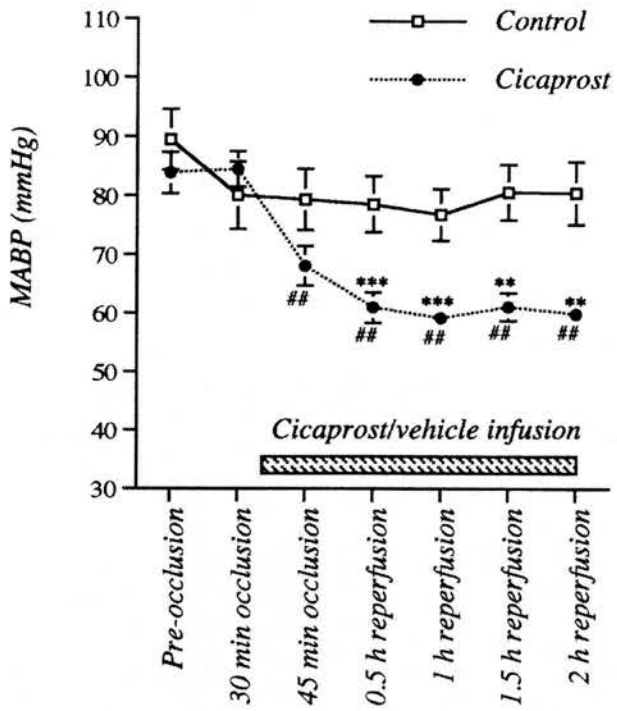


Figure 3.27 *MABP recorded before, 30 and 45 minutes of left main coronary artery occlusion and during 2 hours reperfusion in control group(n=9) and and cicaprost (0.1μg/kg/min, n=7) treated group. Values shown are mean ± s.e. mean for each group.* $^{**}P<0.01$ *and* $^{***}P<0.005$ *indicate significantly different from control group.* $^{\#\#}P<0.01$ *indicates significantly different from 30 minutes of occlusion of the left main coronary artery.*

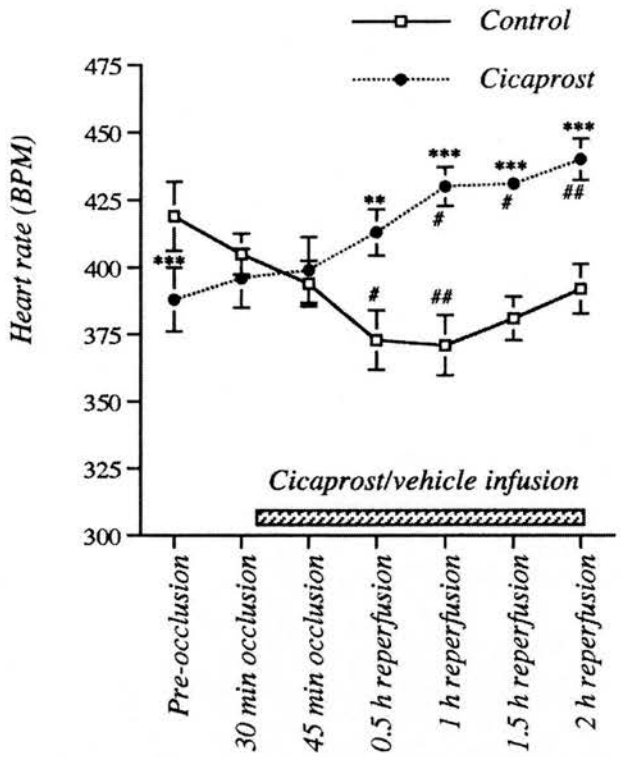


Figure 3.28 *Heart rate recorded before, 30 and 45 minutes of left main coronary artery occlusion and during 2 hours reperfusion in control group (n=9) and cicaprost (0.1 μg/kg/min, n=7) treated group. Values shown are mean ± s.e. mean for each group.* $^{**}P<0.01$ *and* $^{***}P<0.005$ *indicate significantly different from control group.* $^{\#}P<0.05$ *and* $^{\#\#}P<0.01$ *indicates significantly different from 30 minutes of occlusion of the left main coronary artery.*

Table 3.2 *The effects of GEA 3162, 2-HE-NECA and cicaprost on myocardial necrosis.*

Drug/vehicle	Concentration (μg/kg/min)	number (n)	%AAR Total	%Necrotic AAR	%Necrotic Total
Saline (Control 1)	0.5 ml/h	7	42.9 ± 1.9	35.1 ± 2.6	15.0 ± 1.1
GEA 3162	1	7	43.2 ± 3.2	19.7 ± 1.1***	8.5 ± 0.8***
Saline (Control 2)	0.5 ml/h	9	42.5 ± 1.6	35.2 ± 1.8	15.0 ±0.9
2-HE-NECA	0.1	7	38.1 ± 2.6	17.6 ± 1.2***	6.8 ± 0.8***
Cicaprost	0.1	7	38.3 ± 3.1	15.6 ± 0.7***	6.0 ± 0.6***

% AAR/Total: Tissue wet weight of area at risk as a percentage of the total left ventricular wet weight. %Necrotic/AAR: Tissue wet weight of necrotic area as a percentage of the area at risk. %Necrotic/Total: Tissue wet weight of necrotic area as a percentage of the total left ventricular wet weight. Control 1 is for GEA 3162 treated group and control 2 is for 2-HE-NECA and cicaprost treated groups. ***$P<0.005$ *indicates significantly different from each control group.*

3.6 DISCUSSION

Neutrophil activation and accumulation in the myocardium are suggested to be major pathological events in reperfusion injury (Engler *et al.*, 1983). Emigration of neutrophils into the ischaemic-reperfused myocardium is associated with microvascular injury and myocyte damage (Lucchesi, 1990; Mullane, 1988). A critical event in the early phase of reperfusion injury is endothelial dysfunction; manifested as a loss of ability to produce NO and PGI_2 (Thiemermann *et al.*, 1985; Aoki *et al.*, 1988; Bitterman *et al.*, 1988; Nichols *et al.*, 1988), both of which are inhibitors of neutrophil adhesion to vascular endothelium (Lefer *et al.*, 1978; Jones & Hurley, 1984; McCall *et al.*, 1988; Kubes *et al.*, 1991). Adherence of neutrophils to the endothelium can lead to further endothelial damage and represents a critical step in the pathogenesis of ischaemia reperfusion injury (Harlan, 1987). As endothelial

dysfunction occurs early in reperfusion, efforts have been made to supplement NO, PGI_2 and adenosine during MI-R.

As shown in the results of Chapter 2, the novel NO donor, GEA 3162, caused significant concentration-dependent inhibition of fMLP-induced human neutrophil chemotaxis *in vitro* and it was therefore decided to determine whether treatment with GEA 3162 during reperfusion would reduce neutrophil infiltration in a rat model of MI-R. The *in vivo* effects of GEA 3162 on neutrophil infiltration were also compared with the effects of drugs that are known to inhibit neutrophil function: a potent selective A_{2A} receptor agonist 2-HE-NECA, and a metabolically and chemically stable PGI_2 analogue cicaprost (ZK 96 480). In addition, the effects of inhibition of neutrophil accumulation by these drugs on myocardial injury in a rat model of MI-R were also investigated.

To study the effects of these drugs on neutrophil infiltration to the ischaemic area of the heart, a rat model of MI-R was set up. Myocardial ischaemia of the left ventricle of the heart was induced by occlusion of the left main coronary artery to induce an infarcted area of the left ventricle. During the surgical procedures, which included a left thoracotomy incision and exteriorisation of the heart, a reduction in blood pressure always occurred. However, blood presure and heart rate returned to baseline after rats were allowed to equilibrate for 15 minutes after completion of all surgical procedures. Reduction in blood pressure always occurred during the occlusion period within the control and treated groups. After the ischaemic myocardium was reperfused, an increase in blood pressure was observed within the control group. However, blood pressure was seen to decrease in the treated group following the infusion of test drugs and remained low until the end of the reperfusion period. Although lignocaine was injected intravenously prior to thoracotomy incision to prevent the occurrence of arrhythmias, during occlusion of the left main coronary artery, some animals developed arrhythmias and were given further lignocaine 0.5-1.0 mg/kg iv. After 45 minutes of occlusion of the left main coronary artery, the ischaemic myocardium was reperfused for 2 hours, reperfusion for a shorter period may show less infarct size than a longer period. Within a few seconds

after reperfusion of the ischaemic myocardium, some animals were found to develop reversible arrhythmias, but some animals underwent irreversible ventricular fibrillation. Oxygen radical production appears to peak within the first few minutes of reperfusion, but continues at a lower level for hours (Bolli *et al.*, 1988). The rapid production of oxygen-derived free radicals during reperfusion of ischaemic myocardium contributes to irregularities of rhythm (Manning & Hearse, 1984; Kusuma *et al.*, 1990). Pallandi *et al.* (1987) proposed that a sudden burst of oxidant stress, injured membrane proteins, leading to ionic imbalances and electrical instability. In this study, electrocardiography was not used and therefore, only the area at risk demarcated with Evan's blue dye was used to confirm the left main coronary artery occlusion. Animals that did not achieve occlusion of the left main coronary artery or underwent irreversible ventricular fibrillation were excluded from this study.

Several factors should be taken into account when examining the ability of these drugs to limit neutrophil accumulation and myocardial injury. It was suggested that myocardial injury depended directly on the severity and duration of ischaemia; a longer duration of ischaemia is likely to cause more cell death, making it impossible for tissue salvage by any intervention. After prolongation of ischaemic duration, cell necrosis starts from the subendocardial region, spreads slowly towards the subepicardium (Reimer & Jennings, 1979), and becomes transmural within 2 hours (Fujiwara *et al.*, 1982). The duration of the preceding ischaemia may also be important in determining the effectiveness of antineutrophil interventions (Jolly *et al.*, 1986; Williams *et al.*, 1994). In this study, myocardial ischaemia was induced for 45 minutes and analysis of the data showed that both control and drug-treated groups were exposed to comparable degrees of ischaemia. The area at risk (ischaemic area) of the control and the drug-treated groups was 38-43% of the total area of left ventricle wet weight.

The degree of injury is dependent on the extent of the collateral blood flow and duration of ischaemia. In some species, such as the guinea pig, collateral flow is so extensive that even complete coronary artery occlusion by ligation results in no

detectable ischaemia. In other species, such as the rat, the coronary collateral flow is so low that severe ischaemia occurs (Schaper, 1984) and therefore, coronary occlusion and reperfusion results in a uniform degree of myocardial injury.

Tissue wet weight was used as a measurement of the size of area at risk and infarct area in this study. The techniques of assessing injury vary considerably. The method used for measuring the infarct size is a potential source of error. Schaper, (1979) demonstrated that infarct size measured histochemically, correlated very well with the size determined by histological examination. 2,3,5-triphenyltetrazolium chloride was used in this study to demarcate the necrotic and non-necrotic areas. The use of tetrazolium is a standard choice for delineating the transmural infarct area, which is not easily measurable by using normal histological techniques (Miura *et al.*, 1988). It has been suggested that there is a close correlation between the size of area at risk and infarct area measured by either area (planimetric method) or tissue wet weight (gravimetric method) (Mullane & Moncada, 1982; Vinten-Johnansen *et al.*, 1992; Toombs *et al.*, 1992).

Dreyer *et al.* (1991) demonstrated that neutrophils are localised into the previously ischaemic myocardium, predominantly during the first hour of reperfusion. In the present study, each drug or saline vehicle was infused 10 minutes before reperfusion of the ischaemic myocardium. Infusion was continued throughout the period of reperfusion (2 hours) because the drugs have to be present in the ischaemic myocardium prior to reperfusion in order to be effective.

The open-chest rat model of MI-R that was set up in this study enabled the inducement of myocardial ischaemia of the left ventricle after occlusion of the left main coronary artery, which led to necrosis of ischaemic myocardial tissue after reperfusion of the ischaemic myocardium. It is therefore, reasonable to assume that this rat model of MI-R is useful for the study of the *in vivo* effects of GEA 3162, 2-HE-NECA and cicaprost on neutrophil infiltration and myocardial injury.

At physiological concentrations, NO possesses a number of properties which may make it ideal for the treatment of MI-R injury. A study by Williams *et al.,* suggested that endogenous NO production exerted a tonic cardiprotective effect on myocardial infarction following coronary reperfusion (Williams *et al.*, 1995). Exogenous administration of NO or organic NO donors provides another means of replacing a naturally occuring endothelially produced humural agent and attenuating the degree of ischaemia reperfusion injury. Several investigators have attempted to treat MI-R injury with authentic NO (Johnson *et al*, 1991), the NO synthase substrate L-arginine (Nakanishi *et al.*, 1992; Weyrich *et al.*, 1992; Pernow *et al.*, 1994) and various nitrovasodilators. A number of experimental studies have provided evidence for the cardioprotective role of these drugs in MI-R.

In the present study, the ability of GEA 3162 to inhibit neutrophil infiltration *in vivo* was investigated in a rat model of MI-R injury and the results demonstrated that the new potent NO donor, GEA 3162 was able to inhibit neutrophil accumulation in the ischaemic area after MI-R. Furthermore, this was associated with a reduction in myocardial necrosis. Thus, the reduction of necrotic area within the ischaemic area may be attributed in part to the inhibitory action of GEA 3162 on neutrophil accumulation.

These findings are in agreement with those of other investigators using different classes of NO donors. For example, a novel cysteine-containing NO donor N-nitratopivaloly-S- (N'-acetylalanyl)-cysteine ester (SPM 5185) (Noak, *et al.*, 1992) was shown to reduce myocardial necrosis in a feline model of MI-R injury and attenuation of neutrophil-induced endothelial dysfunction was suggested to be a likely mechanism for the cardioprotection of this drug (Siegfried *et al.*, 1992b). The cardioprotective effect of this drug was also demonstrated in a canine model of MI-R by Lefer *et al.* (1993) who showed that intracoronary administration of SPM 5185 during reperfusion reduced neutrophil accumulation and myocardial necrosis. The reduction in myocardial injury may be partially related to the inhibitory action of this drug on neutrophil adherence to the coronary endothelium which leads to attenuation of neutrophil-dependent endothelial cell damage (Lefer *et al.*, 1993). A study by

Fung *et al.* (1994) showed that SIN-1 (Feelisch & Noack, 1987) was an effective *in vivo* cardioprotector in a rabbit model of MI-R. In contrast, Siegfried *et al.* (1992a) demonstrated that SIN-1 and C87-3754, the sydnonimine class of NO donors which release NO spontaneously and nonenzymatically at physiological pH in aqueous systems (Feelisch, *et al.*, 1989), decreased myocardial necrosis and reperfusion-induced endothelial dysfunction in a feline model of MI-R. This was not however, associated with the accumulation of neutrophils in the necrotic area. From this, it seems likely that other mechanisms may contribute to the cardioprotective effect of the SIN-1 and C87-3754. It is thought that MI-R injury is caused largely by neutrophil-derived mediators. However, there is evidence indicating that another important source of the oxygen-derived free radicals in reperfusion injury may be the endothelium itself (Tsao & Lefer, 1990) which suggests that neutrophils might not be the only source of toxic mediators in reperfusion injury.

Both attenuation of neutrophil accumulation in ischaemic myocardium and reduction of myocardial injury produced by GEA 3162 were associated with a reduction in MABP and change in heart rate. It was previously shown that intravenously administered GEA 3162 induced a dose-dependent and short lasting hypotensive effect accompanied by a transient tachycardia (Nurminen & Vapaata, 1996). Unfortunately, the concentration chosen had no significant effect on blood pressure and heart rate during pilot studies because these used intact rats. The decrease in MABP in the rat model of MI-R after GEA 3162 was infused may be due to its vasodilating effect, but other factors such as the depth of anaesthesia and the duration of infusion of the drug may also have contributed to the decrease in MABP.

As GEA 3162 is an effective vasodilator (Corell *et al.*, 1994), it might be expected that the cardioprotection of this drug occured through either systemic or coronary vasodilation. It is possible that NO donors could dilate large epicardial conductance vessels or collateral vessels to increase the flow to the area at risk (Bassenge & Mulsch, 1989). Unfortunately, both coronary and collateral flow were not measured during MI-R in this study, therefore, we cannot conclude whether or not coronary and collateral flow to the ischaemic-reperfused myocardium contributed to the

attenuation of neutrophil accumulation and myocardial necrosis. With regard to the possibility of collateral flow, Schaper (1984) noted that the collateral circulation of the rat is poorly developed and unlikely to be a factor. It seems unlikely that colleral flow contributes a major role in this model of MI-R injury and_GEA 3162 seems unlikely to achieve its protective effects by dilating collateral arteries in the myocardium. As the coronary blood flow was not measured during MI-R, it is not possible to state the extent to which the coronary flow was affected by the vasodilating activity of GEA 3162. Furthermore, it is also not possible to conclude whether or not the increase in coronary blood flow affected neutrophil infiltration and myocardial necrosis. However, if the cardioprotective effect of this drug occured through either systemic or coronary vasodilation, the admistration of GEA 3162 should result in the attenuation of neutrophil accumulation in both the area at risk and the normal area, but it was found that only the number of neutrophils in the area at risk was reduced after GEA 3162 was administered. In addition, according to Bassenge & Mulsch (1989), the epicardial dilatation produced by SIN-1 was not accompanied by any long-term increase in coronary flow and resistance. Thus, systemic or coronary vasodilation seems unlikely to be the mechanism by which GEA 3162 exerted cardioprotection. Further investigations using GEA 3162 at doses lower than 1 μg/kg/min are required to find the dose of GEA 3162 that exerts a cardioprotective effect without causing a significant haemodynamic change.

GEA 3162 has previously been shown to cause significant concentration-dependent inhibition of fMLP-stimulated human neutrophil chemotaxis (Moilanen *et al.*, 1993; Wanikiat *et al.*, 1997). It is probable that the inhibitory effect of GEA 3162 on neutrophil chemotaxis may be related to the attenuation of neutrophil accumulation in the ischaemic area in the rat model of MI-R injury.

As GEA 3162 is a potent NO donor which releases NO spontaneously in aqueous solutions, the probable mechanism of the cardioprotective effect of GEA 3162 is the replacement of decreased endogenous NO to protect endothelial cell function. NO is known to inhibit neutrophil aggregation and quench O_2^- (McCall *et al.*, 1988; Moncada *et al.*, 1989). It is possible that these actions protect endothelial cells from

injury. It was previously demonstrated that the anti-adhesion properties of NO are related to its ability to inactivate O_2^- (Gaboury *et al.*, 1993). GEA 3162 has previously been shown to suppress dose-dependent zymosan-induced O_2^- production in human blood cells as measured by luminol-enhanced chemiluminescence (Moilanen *et al.*, 1993). In addition, it was demonstrated that NO donors can block NADPH oxidase and the resulting superoxide burst in neutrophils (Clancy *et al.*, 1992). Thus, the possible mechanisms for the cardioprotective effects of GEA 3162 in MI-R may be the supression of O_2^- generation and/or the direct quenching of O_2^- by the NO released from GEA 3162. This may lead to the prevention of adherence of neutrophils to the endothelium and finally the attenuation of neutrophil accumulation.

Although GEA 3162 was shown to protect the ischaemic myocardium from reperfusion injury, there are reports that NO can also combine with O_2^- *in vitro* to produce a toxic free radical, $ONOO^-$ (Beckman *et al.*, 1990). This radical has been shown to oxidise sulfhydryl groups and peroxidise membrane lipids (Radi *et al.*, 1991), both of which are harmful to cells. However, it is unlikely that $ONOO^-$ plays a significant role in MI-R injury due to the high concentration of $ONOO^-$ required to produce lipid peroxidation and its short half-life (1.9 sec) (Beckman *et al.*, 1990). Furthermore, if binding of NO to O_2^- produced significant quantities of $ONOO^-$, an increase in reperfusion injury would be expected, with the additional NO that GEA 3162 provides, rather than attenuation of myocardial injury as was observed with the administration of GEA 3162. Moreover, NO donors can block NADPH oxidase and the resulting superoxide burst in neutrophils, suggesting that NO can simultaneously disrupt signal pathways, that are initiated by reactive oxygen species and reduce the potential for toxic levels of $ONOO^-$ synthesis (Clancy *et al.*, 1992). In addition, recent studies have shown that physiologically relevant concentrations of $ONOO^-$ exert significant cardioprotective effects in animal models of MI-R, in part by inhibition of endothelial cell surface expression of P-selectin and attenuation of neutrophil-endothelial cell interactions (Lefer *et al.*, 1997; Nossuli *et al.*, 1997, 1998).

Like other endothelially released vasodilators, adenosine has been shown to protect the myocardium during both ischaemia and reperfusion (Engler *et al.*, 1991). Although several mechanisms may contribute to the cardioprotective role of adenosine, inhibition of neutrophil activation is of some importance (Rudolphi *et al.*, 1992). Gunther & Herring, (1991) found that endogenous adenosine is released by the vascular endothelium in sufficient quantities to inhibit O_2^- generation by neutrophils, via an A_2-mediated mechanism. Adenosine was shown to directly inhibit the production of O_2^- from stimulated neutrophils (an endothelium-independent action) by interacting with specific adenosine A_2 surface receptors (Cronstein *et al.*, 1983, 1985a, 1985b, 1986, 1990, 1992; Nakanishi *et al.*, 1994; Zhao *et al.*, 1996), as well as inhibit neutrophil adherence and subsequent damage to the endothelium (Nolte *et al.*, 1991, 1992; Nakanishi *et al.*, 1994; Zhao *et al.*, 1996). In addition to inhibition of adherence and injury to vascular endothelium, Bullough *et al.* (1995) have shown that adenosine inhibits neutrophil-myocyte adhesion and adherence-dependent injury to myocytes. Adenosine was found to act via A_2 receptors to inhibit the upregulation of Mac-1 (integrin) expression of fMLP-stimulated neutrophils (Wollner *et al.*, 1993). This effect of adenosine may help to limit Mac-1-dependent neutrophil exudation at sites of inflammation or ischaemic-reperfusion (Wollner *et al.*, 1993). Recently, Zhao *et al.* demonstrated that adenosine reduced neutrophil-mediated injury to the coronary endothelium by A_2-receptor-mediated inhibition of O_2^- generation and adherence (Zhao *et al.*, 1996). Based on pharmacological data, it appears that the A_{2A} receptor on neutrophils mediates the inhibition of neutrophil O_2^- generation, adhesion to endothelial monolayers, and phagocytosis (Cronstein, 1994). Stimulation of adenosine A_{2A} receptors, therefore, may account for most of the beneficial effects of adenosine in reperfusion injury (Cronstein, 1994).

In the present study, the *in vivo* effect of 2-HE-NECA on neutrophil infiltration and the inhibitory effect of neutrophil infiltration by 2-HE-NECA on myocardial necrosis, were examined in a rat model of MI-R injury. The results demonstrated that 2-HE-NECA inhibited neutrophil accumulation in ischaemic myocardium after MI-R, and that this was associated with a reduction in myocardial necrosis. Thus,

the reduction of necrotic area in the ischaemic area by 2-HE-NECA may be attributed in part to its inhibitory action on neutrophil accumulation.

These results concur with other studies that have shown the cardioprotective effects of other selective A_{2A} receptor agonists on MI-R. For example, CGS-21680, a selective adenosine A_{2A} receptor agonist, was shown to reduce infarct size in the postischaemic area as much as adenosine, in a rabbit model of MI-R (Norton *et al.*, 1992) and in a canine model of MI-R (Schlack *et al.*, 1993). The cardioprotective effects of this drug were recently confirmed by Jordan *et al.* (1997) who demonstrated that CGS-21680 reduced infarct size, O_2^- generation *in vitro*, neutrophil adherence to the coronary vascular endothelium and neutrophil accumulation in the area at risk in a canine model of MI-R. Jordan *et al.* suggested that selective activation of adenosine A_2 receptors during reperfusion, with the A_2-selective agonist CGS-21680, reduced reperfusion injury by inhibiting neutrophil-mediated damage.

The inhibitory effect of 2-HE-NECA on human neutrophil O_2^- generation was examined and the results demonstrated that 2-HE-NECA (0.001-10 μM) produced a concentration-related inhibition of fMLP-induced O_2^- generation in human neutrophils (unpublished data from our group). The inhibitory effect of 2-HE-NECA on neutrophil O_2^- generation and the suggestion that stimulation of adenosine A_{2A} receptors may account for most of the beneficial effects of adenosine in reperfusion injury, leads one to presume that 2-HE-NECA administration during reperfusion may reduce myocardial injury by inhibiting neutrophil-related processes. For example: neutrophil O_2^- generation, neutrophil adherence to the coronary endothelium and neutrophil accumulation in the area at risk.

While administration of 2-HE-NECA caused significant attenuation of infarct size and neutrophil accumulation, it also caused a significant decrease in MABP and an increase in heart rate during the reperfusion period, particularly at the higher dose. The decrease in MABP seen with 2-HE-NECA may not only be due to its vasodilating effect, but other factors (e.g., depth of anaesthesia), previously discussed for GEA 3162, may have contributed to these haemodynamic changes. Since

2-HE-NECA is a potent vasodilating agent in both *in vitro* and *in vivo* models, it might be expected that the increase in coronary blood flow to the ischaemic-reperfused myocardium plays a role in the cardioprotection elicited by 2-HE-NECA as in the case of GEA 3162. If this is the case, 2-HE-NECA infusion should have resulted in the attenuation of neutrophil accumulation in the area at risk, as well as in the normal area. However, the results showed only a significant reduction of neutrophil accumulation in the area at risk. Therefore, it seems unlikely that the inhibitory effect of 2-HE-NECA on neutrophil infiltration and myocardial necrosis was related to its vasodilating effect. This assumption is further supported by other studies. Vinten-Johansen *et al.* and Hori & Kitakaze. demonstrated a marked reduction in infarct size (Vinten-Johansen *et al.*, 1992, 1995b) and increase in functional recovery (Hori & Kitakaze, 1991; Vinten-Johansen *et al.*, 1992) when reperfusion was gradually, rather than abruptly, restored. With this gradual reperfusion protocol, coronary blood flow is intentionally limited to approximtely 20% of base line during the first 10 minutes of reflow and is gradually increased to unimpaired levels over 30 minutes. Therefore, enhanced blood flow during early reperfusion is not clearly associated with a reduction in morphological injury to the myocardium, although a transient improvement in contractile function may be observed. In addition, Toombs *et al.* (1992) showed that the pretreatment with adenosine before ischaemia conferred cardioprotection independent of haemodynamic effects, in a rabbit model of MI-R. From these findings, it is reasonable to assume that coronary vasodilation seems unlikely to be a mechanism by which 2-HE-NECA exerts cardioprotection. However, 2-HE-NECA at doses lower than 0.1 μg/kg/ml will be needed for further investigation to discover the appropriate dose of 2-HE-NECA that exerts cardioprotection without change in haemodynamic parameters.

In the present study, the ability of cicaprost (ZK 96 480) to inhibit neutrophil infiltration into ischaemic myocardium *in vivo* in a rat model of MI-R injury was demonstrated and this was associated with a reduction in myocardial necrosis. Thus, the reduction of myocardial necrosis in the ischaemic area by cicaprost may be attributed in part to the inhibitory effect of cicaprost on neutrophil accumulation.

These results are in agreement with the results from other investigators' work where PGI_2 and its stable anlogues were used. For example, a stable PGI_2 analogue, taprostene, which is more potent than PGI_2 in its cytoprotective and antiplatelet effects (Lefer & Darius, 1989), exerted a significant cardioprotection in a feline model of MI-R. This cardioprotection was related to protection of coronary endothelium integrity and prevention of neutrophil accumulation in the ischaemic myocardium (Johnson, *et al.*, 1990). The beneficial effect of taprostene in ischaemia-reperfusion was later confirmed by Lefer *et al.* (1994) who showed that taprostene exerted a profound inhibitory effect on neutrophil-endothelium interaction and subsequent neutrophil-mediated coronary endothelial dysfunction. Another synthetic PGI_2 analogue, beraprost was shown to exert cytoprotective effect in a canine model of MIR. This effect may be the consequence of the inhibition of neutrophil migration (Ueno *et al.*, 1994). This drug was previously shown to effectively inhibit fMLP-induced chemotaxis of neutrophils. Cicaprost has previously been shown to cause concentration-dependent inhibition of fMLP-induced human neutrophil chemotaxis (Armstrong, 1995) and fMLP-induced O_2^- generation in human neutrophils (unpublished data). It is probable that the inhibitory effect of cicaprost on neutrophil chemotaxis and O_2^- generation may be related to the attenuation of neutrophil accumulation in the ischaemic myocardium, and myocardial necrosis in the rat model of MI-R injury.

As with GEA3162 and 2-HE-NECA, the inhibitory effects of cicaprost on neutrophil infiltration and myocardial necrosis were associated with a decrease in MABP and increase in heart rate. Cicaprost caused attenuation of neutrophil accumulation only in the area at risk but not in the normal area. Interestingly, it reduced the number of neutrophils in area at risk to a level lower than that of the normal area. This suggests that the cardioprotective effect of cicaprost seems to be related to inhibition of neutrophil function and not related to its haemodynamic effects. This assumption is supported by other investigators' work, as dicussed below.

Several prostaglandins and their metabolites or synthetic analogues have been tested as therapeutic agents in experimental myocardial ischaemia. PGE_1 (Simpson *et al.*,

1988; Schror *et al.*, 1988), PGI_2 (Ogletree *et al.*, 1979) and iloprost (Schror *et al.*, 1981; Simpson *et al.*, 1987a) but not PGE_2, showed beneficial effects in various models of acute myocardial ischaemia or postischaemic reperfusion. These drugs are all vasodilators. In theory these effects might be attributed in part to the vasodilator property of these drugs. If the vasodilator action of prostanoids alone (dilation of the coronary vascular bed or reduction in blood pressure, which reduces myocardial oxygen demand) is responsible for their cardioprotective effect then one would expect that all of these agents would salvage the ischaemic myocardium. This was not the case as PGE_2 has been reported to be ineffective.

Beneficial effects of PGI_2 and its analogue iloprost on experimental infarct size followed by reperfusion were shown to be related to modification of neutrophil function (Simpson *et al.*, 1987a,b; Farber *et al.*, 1988) and were independent from their haemodynamic effects (Simpson *et al.*, 1987a,b) as vasodilators (Berti *et al.*, 1988; Ruocco *et al.*, 1988). Werns & Lucchesi, (1988) found that another stable analogue of PGI_2, SC39902, which exerted haemodynamic effects similar to PGI_2, did not inhibit neutrophil accumulation and reduce infarct size. This evidence suggests that PGI_2 and iloprost decreased myocardial injury by inhibition of neutrophil-mediated damage (Simpson *et al.*, 1987a,b). In addition, Werns *et al.* demonstrated that PGI_2 which inhibited O_2^- generation from neutrophils, also limited canine myocardial injury despite having no effect on collateral blood flow (Werns & Lucchesi, 1988). Recently, a study by Aitchison *et al.* demonstrated that in a rabbit model of MI-R, the reduction in myocardial infarct size by iloprost and SIN-1 was not dependent on the effects of the drug on haemodynamics or platelet aggregation (Aitchison & Coker, 1999). An additional mechanism of limitation by PGI_2 of the injurious effect of neutrophils on the ischaemic myocardium is stabilisation of lysosomal membranes (Hieda *et al.*, 1988), leading to limited release of proteolytic enzymes that injure myocytes.

3.7 CONCLUSIONS

In the present study, the NO donor GEA 3162, a potent selective A_{2A} receptor agonist 2-HE-NECA, and stable PGI_2 analogue cicaprost (ZK 96 480) have been shown to inhibit neutrophil accumulation in ischaemic myocardium and myocardial necrosis in a rat model of MI-R injury. The attenuation of myocardial necrosis by these drugs may be attributed in part to their inhibitory action on neutrophil accumulation in ischaemic myocardium. Although, all three drugs at the concentrations used, in this study caused a significant attenuation in neutrophil accumulation and myocardial necrosis, they also caused a significant change in blood pressure and heart rate. Further study using lower doses of these drugs will be required to find doses that would exert cardioprotection without causing any haemodynamic change.

CHAPTER 4

CARDIOPULMONARY BYPASS, HEPARIN, NEUTROPHIL AND PLATELET AGGREGATION

4.1 INTRODUCTION

4.1.1 Platelets

4.1.1.1 Platelet structure, functional anatomy and physiology

Platelets are small, anucleate blood elements with a diameter of 3-4 μM, and under normal conditions constitute a small fraction of the circulating cells, the platelet count in healthy human blood ranging from $1.3\text{-}4.0\times10^{11}$ platelets/l. Classically they were thought to be derived from megakaryocytes in the bone marrow by the process of fragmentation (Wright, 1990). However, it has more recently been suggested that megakaryocytes travel to the lung vasculature from the bone marrow where they physically become fragmented following impact with the extensive capillary network (Martin & Levine, 1991). Platelet production is under the control of a humoural agent known as thrombopoietin. The platelet lifespan has been estimated at 8-12 days.

The plasma membrane represents the site of platelet interactions with the external environment and is ultimately involved in the control or generation of the many specialised functional properties of the cell. The platelet plasma membrane is a typical trilaminar membrane with glycoproteins, glycolipids and cholesterol embedded in a phospholipid bilayer. 57% of total human platelet phospholipids are present in the plasma membrane (Perret *et al.*, 1979). Platelet membrane glycoproteins (GPs) mediate a wide number of adhesive cellular interactions. These GPs function as receptors that can receive signals from outside the platelet, facilitating cell-cell interactions. Binding of specific ligands to these receptors results in distinct platelet responses to the external environment. GP IIb-IIIa is the principal receptor on the platelet plasma membrane (Philips *et al.*, 1988). It is a member of the integrin family of proteins, which are heterodimeric, transmembrane complexes made up of an α subunit containing 3 or 4 divalent cation binding domains and a disulfide bond-rich β subunit which has a recognition sequence for RGD (Arg-Gly-Asp) amino acid sequences (Uzan *et al.*, 1988). All ligands known to bind GP IIb-IIIa, including fibrinogen, fibronectin, von Willebrand factor (vWF), and thrombospondin, contain this cell recognition sequence (Plow *et al.*, 1985). GP Ib is

present on platelet surfaces in a 1:1 ratio with GP IX (Hickey *et al.*, 1989). Stable platelet adhesion requires the interaction of the GP Ib-IX complex with vWF (Bennett, 1990). GP V forms a noncovalent complex with GP Ib-IX in the platelet membrane. Membrane GPs such as GP Ia-IIb, GP Ic-IIa, and αvβ3 mediate platelet adhesion to collagen, fibronectin, laminin, and vitronectin.

Platelet cytoplasm contains a number of different organelles essential to the maintenance of normal haemostasis. Two membrane systems weave throughout the cell interior, effectively increasing the platelet surface area. The functions of the surface-connected canalicular system (open canalicular system) are to provide a route of entry and egress for molecules, an internal reservoir of membrane to facilitate platelet spreading and filopodia formation after adhesion and a storage reservoir for membrane glycoproteins that increase on the platelet surface after activation (Suzuki *et al.*, 1992). The dense tubular system is associated with the circumferential microtubule band. This system is involved in the regulation of intracellular calcium transport (Cutler, *et al.*, 1978).

The platelet cytoskeleton is made up of three major structural components: an actin microfilament network present throughout the cytoplasm, a microtubule coil localised at the platelet periphery, and a membrane skeleton comprising a network of short actin filament that underlies the inner surface of the plasma membrane (Fox *et al.*, 1991). Platelet stimulation results in profound changes in cytosketal organisation.

Platelets contain four distinct populations of granules: α-granules, dense bodies, lysosomes, and microperoxisomes. After platelet stimulation by agonists, granules fuse with channels of the surface-connected canalicular system and extrude their contents (White, 1972, 1973). Proteins present in α-granules include β-TG, PF_4 (Stenberg *et al.*, 1984; Hegyi & Nakeff, 1989; Harrision *et al.*, 1990), vWF (Wencel-Drake *et al.*, 1985; Cramer *et al.*, 1985), thrombospondin and fibrinogen (Cramer *et al.*, 1985; Suzuki *et al.*, 1988, 1990), albumin, IgG, fibronectin, platelet-derived growth factor (PDGF) (Kaplan *et al.*, 1979), GP IIb-IIIa (Cramer *et*

al., 1990,1991; Suzuki *et al.*, 1992). Proteins present on the α-granule membrane include P-selectin, GP IIb-IIIa, granule membrane protein-33, CD9, PECAM-1. Dense granules contain ADP and ATP, PPi, 5-HT and calcium. Platelet lysosomes contain a large variety of enzymes (Holmsen, 1994) such as β-hexosaminidase, β-glycerophosphatase, cathepsins, collagenase. Microperoxisomes contain enzyme catalase, the function of which is to break down H_2O_2.

4.1.1.2 Platelets in haemostasis and thrombosis

Blood platelets play an essential role in haemostasis, thrombosis, and coagulation of blood. Intact blood vessels are lined by haemostatically inert endothelial cells and as a consequence, subendothelial structures do not normally come into contact with flowing blood. Vascular injury (either spontaneous or traumatic interruption of vascular continuity) is the stimulus required to initiate a series of complex and interdependent reactions. Platelets adhere to the injured blood vessel wall to prevent blood loss. Platelets perform this task through a discrete series of steps involving platelet adhesion to the wounded area and platelet activation, i.e. the generation of intracellular chemical signals that are initiated by platelet adhesion and by soluble factors that stimulate the platelet through specific receptors. These signals cause rapid morphological changes e.g. the extension of pseudopodia, platelet-platelet aggregation, and granule secretion. ADP discharged from the dense granules and TXA_2 generated by the activation of platelet membrane PLA_2 influence the recruitment of additional circulating platelets to clump on those already adhered to the injured site. If the flow conditions are sufficiently disturbed, platelet aggregates form on the vessel wall and serve as a focus for the acceleration of the coagulation reaction via platelet factor 3 (PF_3). Contact of blood with the subendothelium and release of the tissue factor (thromboplastin) from the damaged vessels initiates the cascade of proteolytic reactions in the intrinsic coagulation pathway, culminating in the formation of thrombin. The newly formed thrombin acts synergistically with ADP and TXA_2 to promote further aggregation of platelets to form an enlarging platelet mass as the haemostatic plug (Zucker, 1980). Thrombin converts fibrinogen, present in plasma and released from platelets, into fibrin monomers, which polymerise to stabilise and reinforce the platelet plug.

4.1.1.2.1 Platelet adhesion and adhesion receptors on platelets

Vascular injury disrupts the single layer of endothelial cells that line blood vessel walls and normally resists platelet aggregate formation, exposing a rich matrix of subendothelial proteins. A few of the many proteins that make up the subendothelium include collagen, vWF, and fibronectin. Upon blood vessel injury, platelet surfaces will adhere to the exposed proteins such as collagen fibres and vWF, through several membrane GPs adhesion receptors.

Adhesion receptors on platelets include, the GPIb-V-IX which interacts with vWF and mediates adherence of the platelet to the vessel wall under conditions of normal wound healing following arterial injury (Hickey *et al.*, 1993; Berndt *et al.*, 1995; Siedlecki *et al.*, 1996; Savage *et al.*, 1996); integrin αIIbβ3 (GPIIb-IIIa) which can bind fibrinogen, fibronectin, vWF, and thrombospondin (Smyth *et al.*, 1993); other platelet integrins such as α2β1 integrin (GPIa-IIb) (VLA-2) which is a receptor for collagen (Santoro, 1986; Kirchhofer *et al.*, 1990; Saelman *et al.*, 1994). Platelets also have on their surface, three other integrins, all of which are present in low numbers per platelet. These integrins are the α5β1 integrin (GPIc-IIa) (VLA-5), which is a fibronectin receptor, the α6β1 integrin (GPIc'-IIa) (VLA-6) which is a laminin receptor, and the αvβ3 integrin, which binds vitronectin, fibronectin, fibrinogen, thrombospondin, vWF etc. (Felding-Habermann & Cheresh, 1993). Other platelet GPs include, GP VI which is thought to be a collagen receptor and GP IV which has been proposed to be a receptor for collagen (Tandon *et al.*, 1989) and thrombospondin (Asch *et al.*, 1987).

4.1.1.2.2 Platelet aggregation

Platelets circulate as disc-shaped cells but when they come into contact with the exposed subendothelium, agonists that activate platelets are exposed, generated, or released. These agonists include collagen, which is present in the subendothelium; thrombin, which is generated on the surface of activated platelets and elsewhere; ADP, which is released from damaged red blood cells and secreted from activated platelet-dense granules; circulating epinephrine and; arachidonic acid, which is released from lipid stores in platelets and metabolised to the potent agonist TXA_2.

These agonists generally cause platelets to change shape preceding platelet aggregation. Platelets change shape from discoid to a more spherical form. This process is mediated by the contractile microtubular system and characterised morphologically by the extension of short and long dendritic pseudopodia (White, 1987). Platelet aggregation requires activation of the platelet integrin adhesion receptor GPIIb-IIIa so that it can bind soluble fibrinogen or vWF in plasma and link adjacent platelets together in an aggregate. Platelets circulate freely in the blood in a resting state with GPIIb-IIIa existing in a low affinity conformation that is unable to bind soluble fibrinogen, thus preventing spontaneous aggregation. In order to bind fibrinogen, platelets must be activated by locally generated or exposed agonists such as thrombin, ADP, or collagen. Binding of these agonists to their receptors on the platelet surface generates a cascade of signalling events, which leads to activation of the fibrinogen receptor GPIIb-IIIa. This cascade of inside-out signalling events induces a conformational change in the GPIIb-IIIa, resulting in activation of the receptor and rendering it capable of binding soluble fibrinogen (Sims *et al.*, 1991). Calcium is important in maintaining a functional conformation (Loftus *et al.*, 1990).

Under high shear stress flow conditions, the initial platelet attachment to the subendothelium is mediated by the interaction of vWF with the platelet GPIb-IX complex (Weiss *et al.*, 1978), subsequent platelet aggregatiom requires binding of vWF to the platelet GPIIb-IIIa complex (Weiss, 1989; Ikeda *et al.*, 1991). Under conditions of low shear stress, platelet aggregation is mediated by the interaction of GPIIb-IIIa with fibrinogen (Ikeda *et al.*, 1991).

Platelet shape change can be monitored with an optical aggregometer, which records the change as a decrease in light transmission through a suspension of platelets. Platelet aggregation in platelet rich plasma can be measured both spectrophotometrically as an increase in light transmission and by electrical impedance. PRP is slightly turbid due to the presence of platelets in suspension. When an aggregating agent is added, the turbidity decreases because of the clumping action of the platelets. In plasma, this reduces the optical density of the PRP allowing more light (infra red) to pass through. The instrument develops a voltage

proportional to the transmittance of light through the plasma. This voltage is recorded on a strip chart recorder as a function of time. Platelet aggregation in whole blood cannot be measured spectrophotometrically because it is opaque. However, it can be measured by electrical impedance. The impedance method detects aggregation by passing a very small electric current between two electrodes immersed in a sample of blood and measuring the electrical impedance between the electrodes. The electrodes become coated with a monolayer of platelets during initial contact with the blood or PRP. When an aggregating agent is added, platelets aggregate to the monolayer coated on the electrodes. This coats the electrodes, increasing the impedance (resistance)

4.1.1.2.2.1 General signalling mechanisms of platelet activation

Some signalling pathways involved in platelet activation are reasonably well understood, whereas others are not. Many, but not all, platelet agonists activate platelets by occupying seven transmembrane-spanning G protein-coupled receptors. Activation of these receptors generally results in phosphoinositide hydrolysis and the activation of phospholipase Cβ. PLC hydrolyses PIP_2, which generates IP_3 and DAG. Both IP_3 and DAG appear to play important roles in pathways leading to various aspects of platelet activation. IP_3 is believed to interact with specific receptors to induce intracellular Ca^{2+} release from the dense tubular system. However, the exact mechanism by which this response contributes to platelet aggregation is not entirely clear because IP_3-induced platelet aggregation is also dependent on TXA_2 production and ADP release (Knezevic *et al.*, 1992). DAG interacts directly with PKC, leading to PKC activation. Activated PKC appears to play a crucial role in pathways of some agonists, for example, the activation of GPIIb-IIIa and fibrinogen binding.

Collagen stimulation of platelets results in several intracellular signalling events that lead to platelet activation and aggregation. Collagen treatment of platelets leads to the rapid activation (less than a minute) of two nonreceptor protein tyrosine kinase, *Syk* (Fujii *et al.*, 1994) and *Src* (Huang *et al.*, 1992; Shattil *et al.*, 1994). Following *Syk* and *Src* activation, collagen stimulation of platelets results in the tyrosine

phospholyration and activation of PLC γ2 (Daniel *et al.*, 1994; Keely & parise, 1996). PLC γ2 activation, in turn, catalyses the cleavage of PIP_2 into IP_3, which causes Ca^{2+} release in the platelet (Smith *et al.*, 1992; Daniel *et al.*, 1994), and DAG, which activates PKC. The co-ordinated action of the, GPIa-IIb, GP VI, and perhaps other receptors for collagen, leads to collagen-induced platelet aggregation.

4.1.1.2.3 Platelet release reaction

After platelets are activated by agonists, they undergo a release reaction, secreting granular contents. The release reaction is associated with the production of TXA_2 (Smith *et al.*, 1973; Hamberg *et al.*, 1975). The secretion of α-granule and dense granule contents occurs by centralisation of secretory granules followed by exocytosis. The extent of secretion depends on the strength of the agonist. Strong agonists (such as thrombin and collagen) at high concentrations induce platelet aggregation secretion that is independent of cyclooxygenase activity (Krishnamurthi *et al.*, 1984), whereas at low concentrations induce aggregation and secretion that is entirely dependent on cyclooxygenase activity and released ADP.

4.1.1.3 Neutrophil-platelet interaction

Interactions between platelets and neutrophils occur at sites of vascular damage, as in haemostasis. Platelets bind to prothrombotic endothelium and to the underlying basement membrane in vascular injury. Binding of neutrophils to platelets in a thrombus may facilitate emigration into thrombosed areas, wound healing, tissue repair, or protection from infection, and may contribute to the maintenance of vascular integrity, as well as to its impairment in pathological states. Platelets and neutrophils do indeed co-localise at sites of haemorrhage, vascular grafts, atherosclerotic lesions, and myocardial infarction. Activation of neutrophils with platelet adhesion occurs after coronary angioplasty and has been associated with late clinical events (Mickelson *et al.*, 1996).

Physical interaction of neutrophils with platelets was previously described by Jungi *et al* (1986). Platelet-neutrophil interaction can also be mediated by P-selectin (Belvilacqua *et al.*, 1991) and Lewis X (CD15) could be a component of the ligand

for P-selectin (Larsen *et al.*, 1990). Rolling and arrest of neutrophils on activated platelets in flow has been shown to require the sequential action of P-selectin and β_2 integrins, respectively (Buttrum *et al.*, 1993; Yeo *et al.*, 1994; Lalor & Nash, 1995; Diacovo *et al.*, 1996). The dynamic interaction of activated platelets and neutrophils in stirred suspension also involves a P-selectin-dependent step and a functional signal that proceeds through tyrosine kinase activation to stimulate adhesiveness of Mac-1 (Evangelista *et al.*, 1996). It has been shown that, in stasis, adhesion strengthening of neutrophil on thrombin-stimulated, surface-adherent platelets was mediated by binding of Mac-1 ($\alpha M\beta_2$) on neutrophils to unidentified ligands on platelets (Diacovo *et al.*, 1996). Fibrinogen, a Mac-1 ligand (Wright *et al.*, 1988; Altieri *et al.*, 1990), that can bind to activated platelets via GPIIb-IIIa (Pytela *et al.*, 1986), has been implicated in platelet-neutrophil interactions in cell suspension or whole blood (Ruf *et al.*, 1992). Recently it was reported that neutrophil accumulation on thrombin-stimulated platelets in flow is mediated by interactions of Mac-1 with fibrinogen presented by GPIIb-IIIa on platelets, activation by PAF, and possibly tethering on platelet ICAM-2 (Weber & Springer, 1997).

There is considerable evidence to suggest that platelet aggregation *in vivo* is a multicellular process (Marcus *et al.*, 1995). In particular, considerable cross talk between platelets and neutrophils has been observed (Ott *et al.*, 1996), suggesting that neutrophil activation contributes to platelet activation *in vivo* since a number of neutrophil-derived agents trigger platelet aggregation (Cerletti *et al.*, 1995). These agents include PAF (Koltai & Braquet, 1992), cathepsin G (LaRosa *et al.*, 1994a; Selak, 1994), elastase (Renesto & Chignard, 1993) and superoxide anions (Iuliano *et al.*, 1997). Evidence suggests that neutrophil activation may promote the formation of giant mixed aggregates by increasing platelet P-selectin and platelet-leukocyte interaction (Maugeri *et al.*, 1994; Gawaz *et al.*, 1996; Brown *et al.*, 1998).

Human neutrophils activated *in vitro* by several agonists are able to stimulate coincubated, autologous platelets (Chignard *et al.*, 1986; Del Maschio *et al.*, 1990). This effect is largely mediated by cathepsin G, a neutral serine protease stored in the azurophilic granules and released after neutrophil stimulation (Selak *et al.*, 1988;

Ferrer-Lopez *et al.*, 1990; Renesto *et al.*, 1990; Evangelista *et al.*, 1991). Cathepsin G induces platelet calcium mobilisation and serotonin release (Chignard *et al.*, 1986). Cathepsin G is a potent platelet activator (Selak *et al.*, 1988; Renesto & Chignard, 1995) and degranulator which enhances binding to neutrophils (LaRosa *et al.*, 1994a) as well as platelet macroaggregation (Rabhisabile *et al.*, 1996) by increasing surface expression of P-selectin and GPIIb-IIIa (LaRosa *et al.*, 1994b). Human neutrophil elastase, a neutral serine proteinase, at physiologically relevant concentrations may inhibit thrombin-stimulated platelet aggregation and serotonin release as well as ristocetin-mediated platelet agglutination (Brower *et al.*, 1985).

Proteolytic enzymes released from activated neutrophils affect platelet-dependent haemostasis by several mechanisms, including inactivation of plasma inhibitors of coagulation and complement components as well as direct effects on platelets (Weksler, 1988). Cathepsin G has been shown to degrade the GPIb receptor of washed platelets (Aziz *et al.*, 1995) by cleavage of the GPIb-IX receptor at the GPIα sub-unit which decreases the ability of the platelet to interact with vWF (Pidard *et al.*, 1994). Neutrophil elastase as well as cathepsin G also cleave the GPIIb moiety and upregulate fibrinogen receptor activity. This modulation of the surface expression of the GPIb-IX complex transforms the platelet from a state favouring adhesion to one which favors binding to GPIIb-IIIa (LaRosa *et al.*, 1994b). Cathepsin G has also been shown to promote platelet activation by opening platelet membrane divalent cation channels (Cerletti *et al.*, 1995).

Free oxygen radicals released by activated neutrophils alter platelet function and platelets exposed to phagocytosing neutrophils exhibit decreased aggregatory responses. In contrast, there is evidence that O_2^-, a major free oxygen radical formed by activated neutrophils, induces platelet serotonin release and acts synergistically with thrombin to activate platelets (Handin *et al.*, 1977). Evidence that catalase reverses the inhibitory effect of neutrophils on platelet aggregation suggested that H_2O_2 released from neutrophils may mediate this effect on platelets (Levine *et al.*, 1976).

There is increasing evidence to support the mutual activation of neutrophils and platelets (Aziz *et al.*, 1995b; Ruf & Patscheke, 1995). For example, the precursor of neutrophil-activating peptide-2 (NAP-2), a cytokine that causes neutrophil degranulation and chemotaxis, is released by activated platelets (Cohen *et al.*, 1992). Platelet α-granule membranes contain P-selectin (also refered to as guanosine monophosphate-140, PADGEM protein, and CD62, (Hsu-Lin *et al.*, 1984; Sternberg *et al.*, 1985; Belvillacqua *et al.*, 1991), which is translocated to the platelet surface during stimulated secretion and enables adhesion of stimulated platelets to neutrophils (Hamburger & McEver, 1990; Palabrica *et al.*, 1992). Platelets expressing P-selectin can induce the production of O_2^- in neutrophils upon binding (Tsuji *et al.*, 1994; Ruf & Patscheke, 1995).

4.1.2 Cardiopulmonary bypass (CPB)

4.1.2.1 Cardiopulmonary bypass (CPB) and platelet dysfunction

CPB alters haemostasis and results in excessive postoperative bleeding (Khuri *et al.*, 1994) which led to increased tranfusion requirements in 29% of patients undergoing operation using CPB (Belisle & Hardy, 1996). The most important factor contributing to the haemostatic defect associated with CPB is considered to be platelet dysfunction (Woodman & Harker, 1990; Michelson, 1990; Kestin *et al.*, 1993; Khuri *et al.*, 1994). CPB adversely affects both platelet count and function. Haemodilution causes platelet counts to decrease rapidly soon after starting CPB. Within minutes after starting CPB, the bleeding time (BT) is prolonged significantly and platelet aggregation response to ADP or collagen is impaired (Harker *et al.*, 1980; Zilla *et al.*, 1989). Some intrinsic platelet defects have been reported in association with CPB. For example, some studies have reported that CPB results in partial platelet degranulation (Rinder *et al.*, 1991). Although contact with the extracorporeal circuit results in platelet loss secondary to platelet activation, secretion, and degranulation, the resultant thrombocytopenia encountered in the majority of patients undergoing CPB is not severe enough to account for the platelet dysfunction observed in these patients, which is manifested by a marked prolongation of the postoperative bleeding time (Khuri *et al.*, 1992). It was

postulated that CPB induced platelet dysfunction by altering the platelet membrane receptors (GP Ib-IX complex and GPIIb-IIIa complex) responsible for platelet adhesion and aggregation. Loss of platelet membrane receptors for both the vWF and fibrinogen were reported during and after CPB (George *et al.*, 1986; Wenger *et al.*, 1989; Rinder *et al.*, 1991). In contrast to these studies, Kestin *et al.* (1993) demonstrated that membrane receptors were intact in platelets circulating during CPB. It was suggested that the platelet dysfunction of CPB is not a defect intrinsic to the platelet, such as a loss of platelet surface GPIb and GPIIb-IIIa complex, but factors extrinsic to the platelet, such as an *in vivo* lack of availability of platelet agonists, might be important determinants of the platelet dysfunction observed during and after CPB (Kestin *et al.*, 1993). Two such factors are hypothermia and heparin (Valeri *et al.*, 1992).

4.1.2.2 Cardiopulmonary bypass, heparin and heparinase

Heparin, a highly sulfated glycosaminoglycan (Hook *et al.*, 1984) synthesised by different cells and organs (Jacques, 1979), is used systematically as an anticoagulant in patients undergoing CPB to prevent clotting in the extracorporeal oxygenator. The anticoagulant action of heparin resides in its ability to potentiate the activity of an endogenous coagulation cofactor antithrombin-III (AT-III) (reviewed in Bourin & Lindahl, 1993). Antithrombin-III inhibits many of the serine proteases involved in the coagulation cascade, particularly factor IIa (thrombin) and factor Xa. Heparin interacts with AT-III to form a complex that inhibits thrombin and Xa much more effectively than AT-III alone.

Of the three distinct phases of platelet aggregation namely: shape change, microaggregation and macroaggregation (Pedvis *et al.*, 1988), it is macroaggregation, the consolidation of small aggregates into large stable aggregates (Pedvis *et al.*, 1988) that is impaired after cardiopulmonary bypass, while microaggregation, the formation of aggregates containging up to 100 platelets per aggregate is not (Menys *et al.*, 1994, 1995; Kawahito *et al.*, 1999).

Persistent levels of circulating heparin, secondary to inadequate neutralisation (Shanberge *et al.*, 1987; Gundry *et al.*, 1989) or heparin rebound (Gravlee *et al.*, 1990), can contribute to excessive bleeding in the period following CPB. Protamine is currently the most widely used drug for the reversal of heparin anticoagulation, but it causes multiple adverse reactions. Heparinase I, a specific enzyme that inactivates heparin, is a possible alternative to protamine and is currently under clinical development. Heparinase I neutralises heparin by enzymatic cleavage of α-glycosidic linkages at the antithrombin III binding site on heparin (Choay, 1989; Baugh *et al.*, 1992; Desai *et al.*, 1993), resulting in di-, tetra-, and hexa-saccharide fragments. Heparinase I was shown *in vitro*, to effectively reverse heparin-induced anticoagulation in residual blood obtained from the extracorporeal circuit immediately after surgery, using CPB in humans as determined by activated clotting time (ACT) assay (Michelsen *et al.*, 1996). Heparinase was also highly effective in eliminating the anticoagulant effects of even large amounts of heparin in plasma from cardiac surgical patients (Despotis *et al.*, 1994). Other studies by Dehmer *et al.* (1995) and Levy *et al.* (1995) also demonstrated the effectiveness of Heparinase I in reversing heparin anticoagulation *in vitro*.

4.1.2.3 The involvement of neutrophils in cardiopulmonary bypass

Cardiopulmonary bypass is also associated with the activation of neutrophils and formation of circulating platelet-neutrophil complexes (Kestin *et al.*, 1993; Rinder *et al.*, 1992, 1994; Larson *et al.*, 1996; Morse *et al.*, 1998). Neutrophil activation, a feature of extracorporeal circulation, is associated with generation of O_2^- and release of proteinases such as cathepsin G, elastase, and tumour necrosis factor (Del Maschio *et al.*, 1990; Butler *et al.*, 1993).

Using washed platelets and neutrophils, neutrophil-derived proteases can cause platelet aggregation but this has not been demonstrated in whole blood (Selak, 1994; Molino *et al.*, 1995). Human platelet aggregation induced by purified neutrophil-derived cathepsin G is blocked in a dose-dependent manner by heparin (Evangelista *et al.*, 1992; Ferrer-Lopez *et al.*, 1992). The inhibitory effect of heparin was not related to its anticoagulant property, since a heparin preparation with an

inactivated active site for antithrombin III was also effective, but may be explained by a blockade of protease activity of cathepsin G. A study by Hind *et al.* (1988), showed that the heparin, rather than the CPB procedure was implicated in the activation of neutrophils (manifested as an increase in plasma elastase levels, a marker of neutrophil activation which at physiologically relevant concentrations may inhibit thrombin-stimulated platelet aggregation, following heparinisation).

In addition, recent *in vivo* research suggests that the complications associated with CPB are a result of multicellular activity, and are particularly associated with the respiratory burst, which initiates the production of oxygen-dependent free radicals from stimulated neutrophils. In contrast, *in vitro* studies have shown that heparin has an inhibitory effect on the respiratory burst.

Therefore, it was postulated that the neutrophils played a role in platelet aggregation and that heparin contributed to platelet dysfunction during CPB by interfering with a possible neutrophil-platelet interaction that contributes to platelet macroaggregation.

4.2 AIMS

The aims of this study are to determine if heparin, which is used systemically as an anticoagulant for CPB, contributes to the dysfunction of platelet macroaggregation observable with patients undergoing CPB, by interfering with neutrophil-platelet interactions and if heparinase can reverse the *ex vivo* and *in vitro* effects of heparin on platelet dysfunction. For this purpose the following investigations were performed.

1. The *ex vivo* effects of heparin on a direct platelet stimulant, collagen- and a direct neutrophil stimulant, fMLP-induced platelet aggregation (macroaggregation and microaggregation) in whole blood from patients undergoing CPB were investigated.

2. The *in vitro* effects of different concentrations of heparin on collagen and fMLP-induced platelet aggregation (macroaggregation and microaggregation) in whole blood from normal volunteers were also investigated.
3. The ability of heparinase I to neutralise the effects of heparin on platelet aggregation (macroaggregation and microaggregation) induced by collagen and fMLP in whole blood from both patients and normal volunteers were investigated.

4. The *in vitro* effects of heparin upon macroaggregation in PRP induced by collagen and fMLP from volunteers were investigated.

5. The *in vitro* effects of heparin on neutrophil activation using neutrophil production of MPO and superoxide anion generation as markers of neutrophil activation were investigated.

4.3 MATERIALS

The following compounds were kindly donated and are gratefully acknowledged: recombinant desulphatohirudin (r-hirudin, CGP 39393), specific activity 11,700 ATU/mg from Dr A. Suter, Novartis Pharma AG, CH 4002 Basel, Switzerland, Heparinase I (lot # G61-64) from Dr R. Vickers and Dr C. Poulin of IBEX Technologies Inc., Montreal, Quebec, Canada. R-hirudin was dissolved in PBS (pH 7.4) containing 0.1% polyethylene glycol 600 and stored at -20°C at a concentration of 20,000U/ml. Heparinase I was aliquoted and stored at -20°C. Just before use, it was dissolved in distilled water (specific activity 123U/ml).

Equine microfibrillar collagen and diluent were purchased from Hormon-Chemie, Munich, Germany. Collagen was stored at 4°C and diluted with the manufacturer's glucose buffer before use. fMLP was purchased from Sigma and dissolved in DMSO at a concentration of 10^{-2} M, aliquoted and stored at -20°C. Heparin was purchased from Leo Laboratories, Risborough, UK. Isoton II was purchased from Coulter Electronics Ltd, Luton, UK. Glutaraldehyde was purchased from Agar Scientific,

Stanstead, UK. Percoll from Pharmacia. Cytochrome C and cytochalasin B were purchased from Aldrich. 3,3,',5,5'-Tetramethyl benzidine (TMB), trypan blue, PBS (containing Ca^{2+} and Mg^{2+}), 30% hydrogen peroxide, were purchased from Sigma chemical Co. Ltd. (UK). Diff Quick was purchased from Baxter Diagnostic AG.

4.4 METHODS

All studies were approved by the Royal Infirmary of Glasgow Research Ethics Committee. Informed consent was obtained from all patients. In this study, macroaggregation and microaggregation in whole blood were studied using blood samples from patients and volunteers. In addition, volunteers' blood was used in the study of macroaggregation in PRP and superoxide anion generation from neutrophils.

4.4.1 Studies in whole blood

4.4.1.1 r-hirudin anticoagulation concentration

Recombinant hirudin (r-hirudin), a direct thrombin inhibitor, was used to anticoagulate blood samples in this study, to maintain normocalcaemia and to avoid artefacts associated with the use of citrate (Packham *et al.*, 1989; Wallen *et al.*, 1993) or heparin (Lages *et al.*, 1981). In order to find the appropriate concentration of r-hirudin to use for anticoagulation of the blood from both patients and volunteers, the thrombin clotting times for blood samples were determined using different concentrations of r-hirudin. Venous blood taken from a normal volunteer (n=1) was added to tubes preloaded with r-hirudin at concentrations of 0, 10, 50, 100, 200 U/ml. The thrombin clotting times for these samples were <9 sec, 175 sec, 285 sec, >25 mins, and >25 minutes respectively. It is therefore confirmed that r-hirudin concentration of 200 U/ml would give adequate anticoagulation.

4.4.1.2 Collagen and fMLP concentration

Collagen, a direct platelet stimulant, and fMLP, a direct neutrophil stimulant, were used as agonists in this study. Collagen was chosen on the basis that platelets adhere to collagen in the damaged vessel wall *in vivo*. Following this, there is formation of

TXA_2 and release of ADP and serotonin. At normocalcemia it has been shown that TXA_2 formation and release of ADP and serotonin largely account for platelet aggregation with collagen *in vitro* as determined by platelet counting (Menys *et al.*, 1993). Thus collagen-stimulation provides a means of assessing platelet response *in vitro*.

In order to find the appropriate concentration of collagen and fMLP used in this study, four concentrations of collagen and two concentrations of fMLP were tested upon platelet macroaggregation. The procedure of platelet macroaggregation in whole blood by impedance aggregometry is detailed in section 4.4.1.4. Briefly, 500 µl of whole blood from volunteers was diluted with the same volume of 0.9% saline in plastic cuvettes and samples were then equilibrated for 5 minutes at 37°C before measurement. Each sample was then stirred at 1000 rpm for 3 minutes at 37°C in the aggregometer to allow for spontaneous platelet aggregation and then 10 µl of different concentrations of collagen (40-80 µg/ml) or fMLP (10^{-5}-10^{-4} M) were added to each sample. The platelet macroaggregatory response to different concentrations of collagen or fMLP was then determined as the scale deflection in centimetres at 5 minutes for collagen and at 15 minutes for fMLP. It was found that collagen induced a dose-related platelet macroaggregation in whole blood (n=7) at concentrations 0.4, 0.6, 0.7 and 0.8 µg/ml. The median aggregatory responses were 6.8, 11.9, 12.8 and 11.5 ohms respectively. This indicated that a sub-maximal response would be obtained in whole blood with a collagen concentration of 0.6 µg/ml. fMLP, at both concentrations caused a platelet macroaggregatory response that was less marked and slower than that seen following collagen stimulation. The median macroaggregatory response achieved with 10^{-7} M and 10^{-6} M of fMLP were 3.8 and 3.6 ohms, respectively. The macroaggregatory response was not increased when a higher concentration of fMLP (10^{-6} M) was used. Thus, 10^{-7} M of fMLP was chosen for this study.

4.4.1.3 Blood samples

4.4.1.3.1 Patients

Patients undergoing elective operations using CPB were recruited into these studies. They had not received aspirin or other non-steroidal anti-inflammatory drugs for at least seven days. Diabetics or patients on anticoagulants, intravenous nitrates or heparin were excluded. Blood samples from these patients were studied. A total of 33 patients were studied, fourteen of whom took part in the full protocol. Blood from the others was used in validation studies and other experiments. Of the 14, there were 12 men and 2 women, median [interquartile range] age 60 [54-64] years, height 170 [165-177] cm and weight 79 [73-84] kg.

Preparation for and conduct of CPB was undertaken according to the individual surgeon's normal practice. These patients underwent a standard CPB procedure using the CPB circuit which consisted of an avecor tubing set, an affinity 40 μM arterial line filter (Avecor Ltd, Bellshill, Strathclyde, UK) and a membrane oxygenator (Duo-Cobe) driven by a Stockert roller pump. The pump was primed with 2.0 l of lactated Ringer's solution, 50 mM $NaHCO_3$ and 5,000 u sodium heparin. Ringer lactate solution was used to maintain pump reservoir volume. Flows were between 2.2 and 2.4 L/min/m^2. Perfusion pressure was maintained between 40 and 80 mmHg by use of methoxamine. Cardiopulmonary bypass duration ranged from 67-114 minutes (median 84 [76-98] minutes). Heparin sodium at a concentration of 300 u/kg was used as an anticoagulant for cardiopulmonary bypass and was administered through a central venous cannula, just before cannulation of the aorta, about 5 minutes before commencement of bypass. Anticoagulation was monitored regularly during CPB by the activated clotting time (ACT) in whole blood using a Hemacron system (Hemacron™, International Technodyne Corporation, Metuchen NJ). Additional doses of heparin were given if the ACT was less than 400 secs. After returning blood from the extracorporeal oxygenator to the patient at the end of CPB, heparin was neutralised with protamine sulfate.

The heparin concentration present in the blood was estimated using the Hepcon system (Hepcon™, Medtronic Ltd, Watford, UK), an automated protamine titration

assay, according to the manufacturers instructions. The median concentration of heparin in the blood after heparinisation was 4.1 [3.4-4.8] U/ml. The end-CPB value was significantly lower at 2.7 [2.7-3.4] U/ml. This allowed estimation of the amount of heparinase I required for neutralisation of heparin in the blood sample. By the addition of heparinase it was possible to remove heparin from the samples while maintaining thrombin inhibition with r-hirudin. It was indicated that 0.05 IU/ml of heparinase I completely neutralised therapeutic levels of heparin (unfractionated or LMWH) (3.5 U/ml) *in vitro*. Heparinase was added at the appropriate concentration to determine the effect of heparin neutralisation.

Venous blood samples were taken through an indwelling 14g catheter in the internal jugular vein.

1. before heparinisation with the chest open (pre-heparin)
2. after heparinisation and before the onset of extracorporeal circulation
3. at the end of cardiopulmonary bypass (still heparinised but fully rewarmed and before protamine administration) (end-CPB)

10 ml of blood was taken at each time point for the platelet aggregation studies. The blood was anticoagulated with r-hirudin (200 U/ml) to maintain normocalcemia. An extra 4 ml of blood was taken in EDTA (5mM) for determination of the total platelet count.

4.4.1.3.2 Volunteers

Venous blood (25 ml) was withdrawn, using a 19G needle without a tourniquet, from the antecubital fossae of 14 healthy male volunteers who had not taken non-steroidal anti-inflammatory drugs or any other antiplatelet therapy for at least 7 days. The blood was placed in an r-hirudin (200 U/ml)-containing siliconised glass tube, for platelet aggregation studies. 4 ml of blood was anticoagulated with EDTA (5mM) for determination of the total platelet count. In an additional experiment, 4 ml of blood was anticoagulated with sodium citrate (3.8%) for studies of platelet aggregation.

4.4.1.4 Macroaggregation in whole blood by impedance aggregometry

Platelet macroaggregation, in whole blood from both patients and volunteers was performed on an impedance aggregometer (Chrono-log 500-VS, Chronolog Corporation, UK) (Mackie *et al.*, 1984). This aggregometer measured platelet aggregation in whole blood by changes in impedance (ohms) across two electrodes immersed in the samples. The aggregometer was calibrated so that 20 ohms change in electrical impedance would give a deflection of the recorder 16 cm, giving a conversion factor of 1.25 ohms per centimetre.

4.4.1.4.1 Macroaggregation in whole blood from patients

Method

1. 10 ml of venous blood was sampled, at each time point (at pre-heparin, 5 minutes after heparinisation and at the end-CPB), from patients. Blood was then anticoagulated with r-hirudin (200 U/ml).

2. Hirudinised whole blood from patients was then divided into two 5 ml aliquots. The first was used for the measurment of platelet macroaggregation in the presence of heparinase I and the second aliquot was used for measurement of platelet macroaggregation in the absence of heparinase I. According to the manufacturer, 0.05U/ml of heparinase completely neutralised 3.5 U/ml of heparin.

3. 500 µl of whole blood from both aliquots was placed in plastic cuvettes and diluted with the same volume of 0.9% saline. The samples from the end-CPB, due to high haemodilution, were not diluted with 0.9% saline. The samples were then equilibrated for 5 minutes at 37°C before measurement of platelet macroaggregation.

4. Each sample was then stirred at 1000 rpm for 3 minutes at 37°C in the aggregometer to allow for spontaneous platelet aggregation.

5. 10 µl of collagen (60 µg/ml) or 10 µl of fMLP (10^{-5} M) was added to each sample. The platelet macroaggregatory response to collagen (0.6 µg/ml), and fMLP (10^{-7} M) were then determined for each sample. Stirring rate was kept constant at 1000 rpm and results are recorded as ohms of scale deflection. The macroaggregatory response to collagen was read as the scale deflection in centimetres at 5 minutes from the start of aggregation. *Ex vivo* neutrophil stimulation by fMLP caused platelet macroaggregation, which was less pronounced than with collagen and took a longer time, therefore these readings were taken at 15 minutes.

4.4.1.4.2 Macroaggregation in whole blood from volunteers

Method

1. 25 ml of venous blood was sampled from healthy volunteers. Blood was then anticoagulated with r-hirudin (200 U/ml). In an additional experiment, 4 ml of blood was anticoagulated with sodium citrate (3.8%) for studies of platelet aggregation.

2. 2.5 ml of hirudinised whole blood from volunteers was then pipetted into nine plastic tubes, which were divided into two groups. The first group contained 6 samples in plastic tubes. The different concentrations of heparin were then added to the 2^{nd}-6^{th} plastic tubes to produce final concentrations of heparin at 0.1-10 U/ml. The same volume of 0.9% saline was added into the 1^{st} plastic tube and this tube served as a control. These samples were used for measurement of the effects of different concentrations of heparin on platelet macroaggregation in the absence of heparinase I.

3. Heparin at concentrations of 0.4 U/ml and 4 U/ml were added into the 8^{th} and 9^{th} plastic tubes, respectively. The same volume of 0.9% of saline was added into the 7^{th} plastic tube which served as a control sample. The appropriate amounts of heparinase I (as discussed in section 4.4.1.5.1) sufficient to neutralise 0.4 U/ml and 4 U/ml of heparin in the blood samples were then added to the 8^{th}, 9^{th} plastic tubes, respectively. The 7^{th} plastic tube was added with the appropriate amounts

of heparinase I sufficient to neutralise 4 U/ml of heparin in the blood sample. These samples were used for the measurement of the effects of heparin on platelet macroaggregation in the presence of heparinase I.

4. 2.5 ml of citrated blood from volunteers was then pipetted into the 10th plastic tube. The citrated blood was used to investigate the macroaggregatory response induced by a direct platelet stimulant, collagen, and, a direct neutrophil stimulant, fMLP.

5. 500 µl of hirudinised whole blood from each tube and 500 µl of citrated blood from the 10th plastic tube were diluted with the same volume of 0.9% saline in plastic cuvettes. The samples were then equilibrated for 5 minutes at 37°C before measurement of platelet macroaggregation.

6. Each sample was then stirred at 1000 rpm for 3 minutes at 37°C in the aggregometer to allow for spontaneous platelet aggregation.

7. 10 µl of collagen (60 µg/ml) or 10 µl of fMLP (10^{-5} M) was added to each sample. The platelet macroaggregatory response to collagen (0.6 µg/ml), and fMLP (10^{-7} M) were then determined for each sample. Stirring rate was kept constant at 1000 rpm and results are recorded as ohms of scale deflection. The macroaggregatory response to collagen and fMLP were read as the scale deflection in centimetres at 5 and 15 minutes, respectively from the start of aggregation.

4.4.1.5 Microaggregation in whole blood (Single platelet counting)

Spontaneous aggregation and the microaggregatory response induced by collagen and fMLP in whole blood from both patients and volunteers were determined by counting unaggregated single platelets with a Coulter Counter ZM (Falcon *et al.*, 1989; Pedvis *et al.*, 1988; Menys *et al.*, 1994). Platelet counts were performed on samples of EDTA-anticoagulated blood to determine the total platelet count (A). In hirudinised blood, platelet counts were performed before stirring, after 3 minutes

stirring in the cuvette of aggregometer and after agonists had been added. This determined platelet count (B), spontaneous aggregation (C), and platelet count with agonists (D), respectively.

Method

1. 100 µl aliquots of blood were taken from the aggregometer cuvette during impedance aggregometry: before stirring (B), 3 minutes after stirring (C), and after exposure to agonists (D) (5 minutes for collagen and 15 minutes for fMLP).

2. The aliquots were added to 400 µl of 1% glutaraldehyde in isoton II in a 1 ml eppendorf tube. Blood samples in glutaraldehyde were then left at room temperature for an hour to settle.

3. After an hour, 10 µl of platelet suspension was taken from the supernatant (platelet rich layer) and added to 10 ml of isoton II.

4. Counts were performed in duplicate and corrected for dilution. The results are expressed as number of platelets $\times 10^9$/l. The extent of spontaneous aggregation in stirred blood was calculated with reference to the platelet count found for unstirred blood. The extent of agonists-induced aggregation was calculated with reference to the platelet count measured following stirring with vehicle alone.

The results are expressed as:

Percentage of aggregation = (A-B) × 100 / A

Percentage of spontaneous aggregation = (B-C) × 100 /B

Percentage of agonist induced-aggregation = (C-D) × 100 /C

4.4.2 *In vitro* studies in platelet-rich plasma (PRP)

4.4.2.1 PRP preparation

Method

1. Venous blood (45 ml) was taken, using a 19G needle without a tourniquet, from the antecubital fossae of 11 healthy male volunteers who had not taken non-steroidal anti-inflammatory drugs for at least 7 days.

2. The blood was anticoagulated with r-hirudin (200 U/ml). After collection, hirudinised whole blood (40 ml) was split into 2 aliquots (2×20 ml) which were treated with 4 U/ml heparin and 0.9% saline, respectively. 2×1 ml of hirudinised whole blood was collected into eppendorf tubes for isolation of platelet poor plasma (PPP).
3. Both treated and untreated hirudinised blood was left at room temperature for 5 minutes before centrifugation at 120×g for 18 minutes at room temperature to obtain the PRP. Aliquots of PRP were dispensed into siliconised glass cuvettes and kept at room temperature before assessment of macroaggregation.

4. PPP was obtained by centrifugation of the r-hirudin-anticoagulated blood in a microcentrifuge at 9000×g for 1 minute.

4.4.2.2 Macroaggregation in PRP

Before measurement of PRP macroaggregation, calibration of the aggregometer was carried out according to the manufacturer's instructions using PPP (16 cm scale deflection) and PRP (0 cm scale deflection). Aliqouts of PRP (0.4 ml) were equlibrated at 37°C for 3 minutes, 100 µl of collagen (2-10 µg/ml) or fMLP (10^{-6}-10^{-5}M) were added and stirred at 1000 rpm, then platelet macroaggregation was recorded using a Payton Dual Channel Aggregometer with a potentiometric recorder (Payton Associates Ltd, Ontario, Canada). The aggregation response was quantified in terms of amplitude (cm) of the increase in light transmission through the sample after addition of the agonist (at 3 minutes after the initial 'shape change' response).

4.4.3 *In vitro* studies of neutrophils

4.4.3.1 Isolation of human neutrophils

Venous blood (160 ml) was taken from six healthy volunteers who were free from non-steroidal anti-inflammatory drugs for at least 7 days. The blood was anticoagulated with r-hirudin (200 U/ml). Neutrophils were isolated from human blood as discussed in section 2.4.3 of Chapter 2. After neutrophils were counted microscopically in a counting chamber, cells were resuspended at a concentration of 1.5×10^6 cells/ml in PBS containing Ca^{2+} and Mg^{2+}, 5 mg/ml cytochrome C and 5 μg per/ml cytochalasin B and then divided into 4×8 ml aliquots.

4.4.3.2 Measurement of superoxide anion generation

Method

1. Neutrophils (450 μl) in hirudinised whole blood were treated with PBS or different concentrations of heparin to produce final concentrations of 0, 1, 4, 10 U/ml prior to the addition to tubes containing 50 μl of 3×10^{-8} to 10^{-5} M fMLP (3-1000 nM) and incubated for 10 minutes at 37°C.

2. At the end of incubation period, the reaction was terminated by immersing the tubes in ice for 5 minutes and the samples were centrifuged at 320×g, at 4°C for 10 minutes, to sediment the cells.

3. Supernatant (200 μl) from each tube was dispensed into a 96 well plate and the absorbance at 550 nm was measured in a spectrophotometer (DYNATECH MR 7000). Basal absorbance was taken as cells without fMLP. Each sample was done in triplicate and the values were averaged. The amount of O_2^- generation was calculated as discussed in section 2.3.6.2. Results are expressed as nmol superoxide anions/10^6 cells/10 minutes. In this assay the final percentage of DMSO was 0.09%.

4.4.3.3 Measurement of Myeloperoxidase (MPO) production

Venous blood (160ml) was taken from five healthy volunteers, who were free of non-steroidal anti-inflammatory drugs for at least seven days. The blood was anticoagulated with r-hirudin (200 U/ml). The neutrophils were isolated and counted using the same methodology as the O_2^- generation assay as discussed in section 2.4.3 of Chapter 2. After neutrophils were counted microscopically in a counting chamber, cells were resuspended at a concentration of 1.5×10^6 cells/ml in PBS containing Ca^{2+} and Mg^{2+}, and 5 μg/ml cytochalasin B and then divided into 4×8 ml aliquots.

Method

1. Neutrophils (450 μl) in hirudinised whole blood were treated with PBS or different concentrations of heparin to produce final concentrations of 0, 1, 4, 10 U/ml prior to the addition to tubes containing 50 μl of 3×10^{-8} to 10^{-5} M fMLP (3-1000 nM) and incubated for 10 minutes at 37°C.

2. At the end of incubation period, the reaction was terminated by immersing the tubes in ice for 5 minutes and the samples were centrifuged at 320×g, at 4°C for 10 minutes, to sediment the cells.

3. Aliquots of supernatant (30 μl) were dispensed in duplicate into a 96 well plate.

4. 200 μl of reaction mixture [0.1 mg/ml 3,3',5,5'- Tetramethylbenzidine (TMB) in 0.05 M citrate phosphate buffer (pH 5.0) supplemented with 0.012% (v/v) hydrogen peroxide] was immediately added. The reaction was allowed to develop for 7 minutes where the product from the MPO-dependent reaction of TMB and H_2O_2 appeared as a bright blue colour.

5. The reaction was then stopped by adding 50 μl of 4M H_2SO_4, and the colour of the product turned from a blue colour to a yellow colour.

4. The absorbance was read in a spectrophotometer (DYNATECH MR7000) at 460 nm. MPO release has been expressed as a percentage of the maximum response

observed with control cells stimulated with fMLP. In this assay the final percentage of DMSO was 0.09%.

4.4.4 Statistical analysis

All data except those of the *in vitro* study of neutrophil superoxide anion generation and MPO production are expressed as medians [interquartile range] unless otherwise stated. Analysis of the data used Arcus Quickstat Biomedical software (Addison Wesley Longman trading as Research Solutions). Dependent upon distribution, paired comparisions were made using Paired t-test or Wilcoxon signed rank tests. Data were analysed for multiple comparisons by either Kruskal-Wallis or Friedman distribution-free analyses of variance (ANOVA), using appropriate corrections for multiple comparisions or ties.

The results from the *in vitro* study of neutrophil superoxide anion generation and MPO production were analysed using Microsoft Excel software. Data are expressed as the mean ± s.e. mean of the averaged result taken from a minimum of five separate experiments. EC_{50} values were calculated (concentration of fMLP required to produce 50% of the maximal O_2^- produced by fMLP) for fMLP in the absence and presence of different concentrations of heparin. Statistical analysis was performed by two-tailed paired Student's *t* test for comparing O_2^- generation and MPO production between control and treatment groups. In addition, data involving multiple comparisons between groups were analysed by ANOVA (Two factors with replication). A value of $P<0.05$ was considered to be statistically significant.

4.5 RESULTS

4.5.1 Platelet macroaggregation in whole blood by impedance aggregometry

4.5.1.1 Patients

The macroaggregatory response to collagen (0.6 μg/ml) in whole blood was significantly reduced by heparinisation (before the onset of CPB) from 18.0 [13.9-21.6] to 2.5 [1.1-6.1] ohms ($P<0.0001$), n=14), and remained diminished at 1.7 [0.6-3.6] ohms after CPB (Table 4.1). The administration of heparinase to the blood samples before collagen stimulation did not in itself affect aggregation and did not restore the macroaggregatory response to collagen (Table 4.1). The macroaggregatory response to collagen (0.6 μg/ml) in whole blood in the presence of heparinase was 16.5 [10.8-21.6] and 3.1[1.3-4.8] ohms, before and after heparinisation ($P<0.0001$, n=14), and remained diminished at 1.3 [0.0-2.1] ohms after CPB.

Table 4.1 *Collagen-induced macroaggregation of platelets in whole blood before and 5 minutes after heparin and at end CPB in the presence and absence of heparinase as determined by impedance aggregometry.*

Intra-operative Sampling time	Without heparinase Collagen (0.6 μg/ml)	Heparinase Collagen (0.6 μg/ml)
Pre-heparin	18 [13.9-21.6]	16.5 [10.8-21.6]
Heparinised	2.5*** [1.1-6.1]	3.1*** [1.3-4.8]
End CPB	1.7*** [0.6-4.0]	1.3*** [0.0-2.1]

*Data are medians [interquartile range] of macroaggregation in ohms in whole blood from patients, with collagen (0.6 μg/ml). ***$P<0.0001$ indicates significantly different from pre-heparin.*

Examples of traces of the macroaggregatory response to collagen (0.6 μg/ml) in the presence and absence of heparinase in whole blood samples taken before heparinisation, at heparinisation and at end of CPB are shown in Figure 4.1.

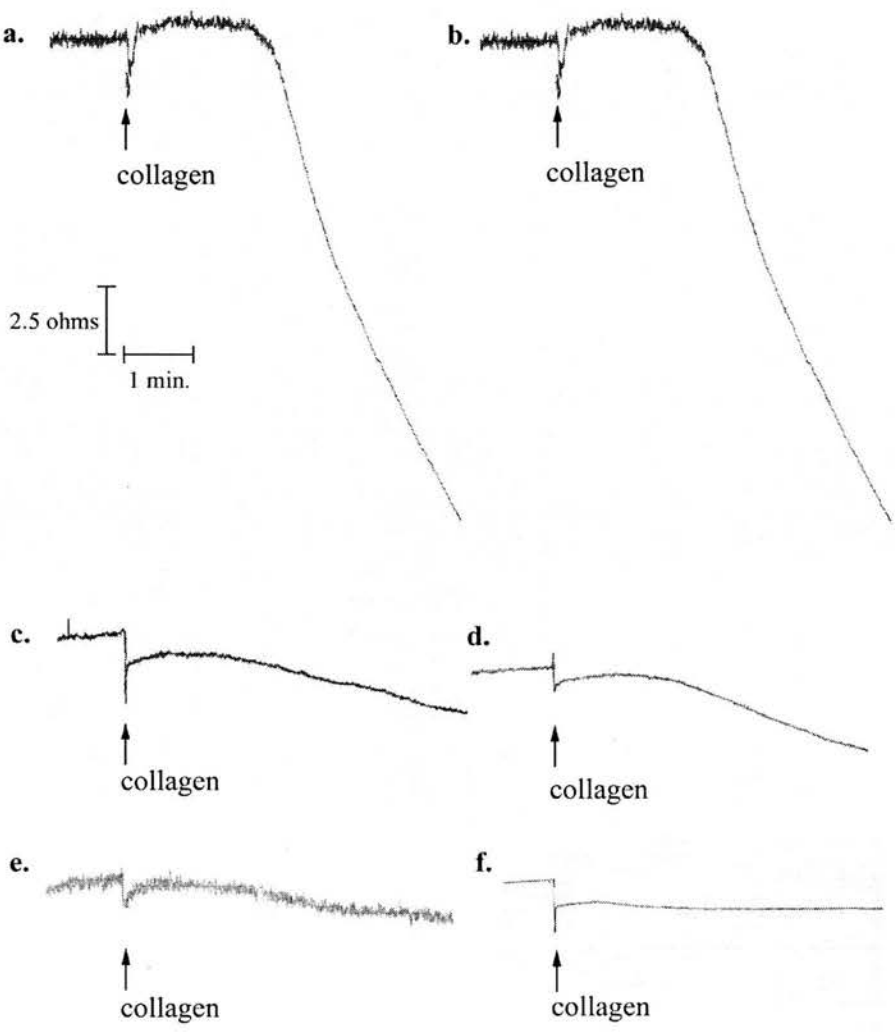


Figure 4.1 *Example of traces showing collagen-induced platelet macroaggregation in whole blood from patients, before heparin. (a) without heparinase, (b) with heparinase; 5 minutes after heparin (c) without heparinase, (d) with heparinase; at the end of CPB (e) without heparinase, (f) with heparinase. Arrows indicate point at which collagen (0.6 μg/ml) was added.*

The neutrophil stimulant fMLP at concentration of 10^{-7} M caused a platelet macroaggregatory response that was less marked and slower than that seen following

collagen stimulation. The response was measured at 15 minutes. Before heparinisation, the response was 3.6 [1.7-5.6] ohms (n=14). After heparinisation for CPB, this response was completely abolished and remained absent at the end of CPB. (Table 4.2). The administration of heparinase to the blood sample before stimulation with fMLP did not restore the macroaggregatory response to fMLP (Table 4.2).

Table 4.2 *fMLP-induced macroaggregation of platelets in whole blood before and 5 minutes after heparin and at end CPB in the presence and absence of heparinase as determined by impedance aggregometry.*

Intra-operative Sampling time	**Without heparinase fMLP(10^{-7} M)**	**Heparinase fMLP (10^{-7} M)**
Pre-heparin	3.6 [1.7-5.6]	2.8 [1.8-6.0]
Heparinised	0.0^{***} [0.0-0.0]	0.0^{***} [0.0-0.0]
End CPB	0.0^{***} [0.0-0.0]	0.0^{***} [0.0-0.0]

Data are medians [interquartile range] of macroaggregation in ohms in whole blood from patients, with fMLP (10^{-7} M). $^{***}P<0.0001$ *indicates significantly different from pre-heparin.*

Examples of traces from the platelet macroaggregatory response to fMLP (10^{-7} M) in the presence and absence of heparinase in whole blood samples taken from each stage are shown in Figure 4.2.

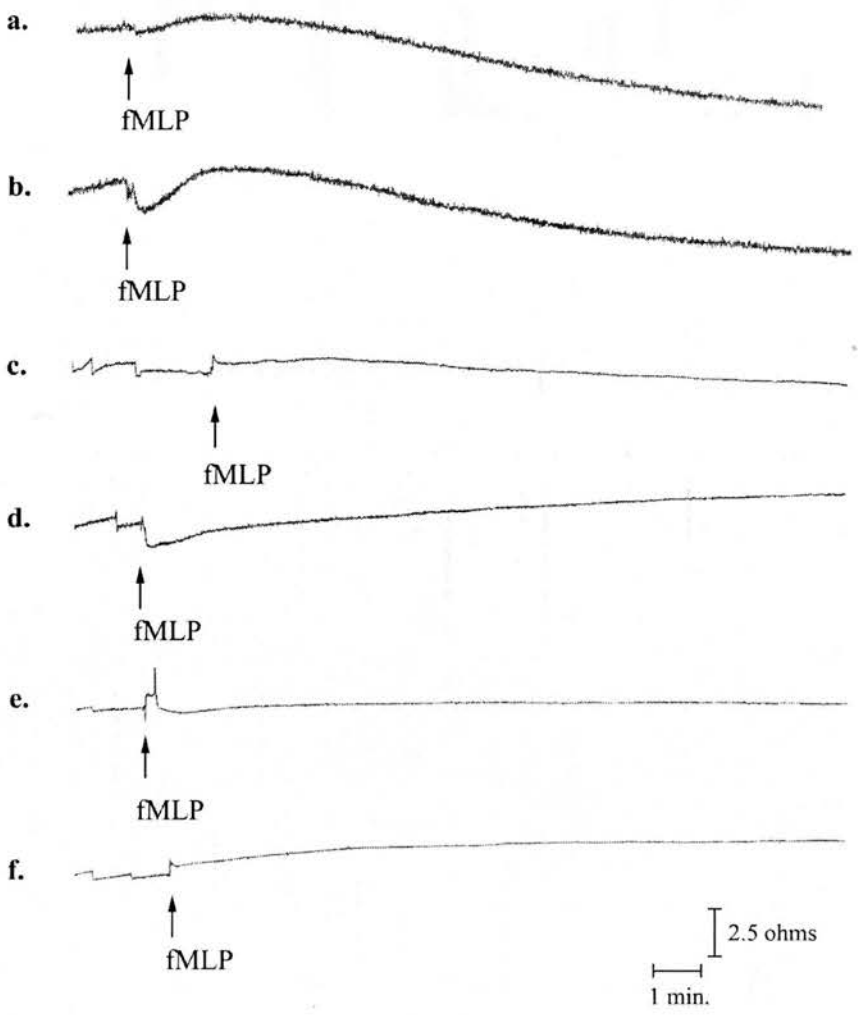


Figure 4.2 *Example of traces showing fMLP-induced platelet macroaggregation in whole blood from patients, before heparin. (a) without heparinase, (b) with heparinase; 5 minutes after heparin (c) without heparinase, (d) with heparinase; at the end of CPB (e) without heparinase, (f) with heparinase. Arrows indicate point at which fMLP (10^{-7} M) was added.*

4.5.1.2 Volunteers

In this study the effects of heparin concentrations (0.1-10.0 U/ml) upon collagen induced platelet macroaggregation were examined *in vitro*. In unheparinised blood, the macroaggregatory response to collagen (0.6 μg/ml) was 18.1 [17.3-19.9] ohms. Heparin concentrations at 0.1, 0.4 and 1.0 U/ml did not affect collagen-induced macroaggregation. However, heparin at concentrations of 4.0 and 10.0 U/ml caused a small but significant inhibition of collagen-induced macroaggregation and their

macroaggregatory responses were 13.8 [9.4-16.2] and 13.5 [6.5-14.7] respectively ($P<0.0001$, n=11) (Table 4.3). The addition of heparinase to blood samples with a heparin concentration of 4 U/ml before collagen stimulation prevented the inhibition of macroaggregation by heparin ($P<0.05$, n=11) (Table 4.3)

Table 4.3. *Collagen-induced macroaggregation of platelets in whole blood from volunteers in the presence and absence of heparinase as determined by impedance aggregometry.*

Heparin (u/ml)	Without heparinase Collagen (0.6 μg/ml)	With heparinase Collagen (0.6 μg/ml)
0	18.1 [17.3-19.9]	19.3 [17.8-21.4]
0.1	20.3 [19.1-22.6]	-
0.4	18.9 [16.5-21.8]	20.1 [16.8-22.0]
1	16.6 [13.2-19.3]	-
4	13.8*** [9.4-16.2]	18.7# [11.3-20.0]
10	13.5*** [6.5-14.7]	-

Data are medians [interquartile range] of macroaggregation in ohms in whole blood from volunteers, with collagen (0.6 g/ml). ***$P<0.005$ *indicates significantly different from heparin 0 u/ml;* #$P<0.05$ *indicates significantly different from the absence of heparinase.*

Examples of traces of the effect of different concentrations of heparin on macroaggregatory response to collagen (0.6 μg/ml) in the presence and absence of heparinase in whole blood samples from volunteers are shown in Figure 4.3.

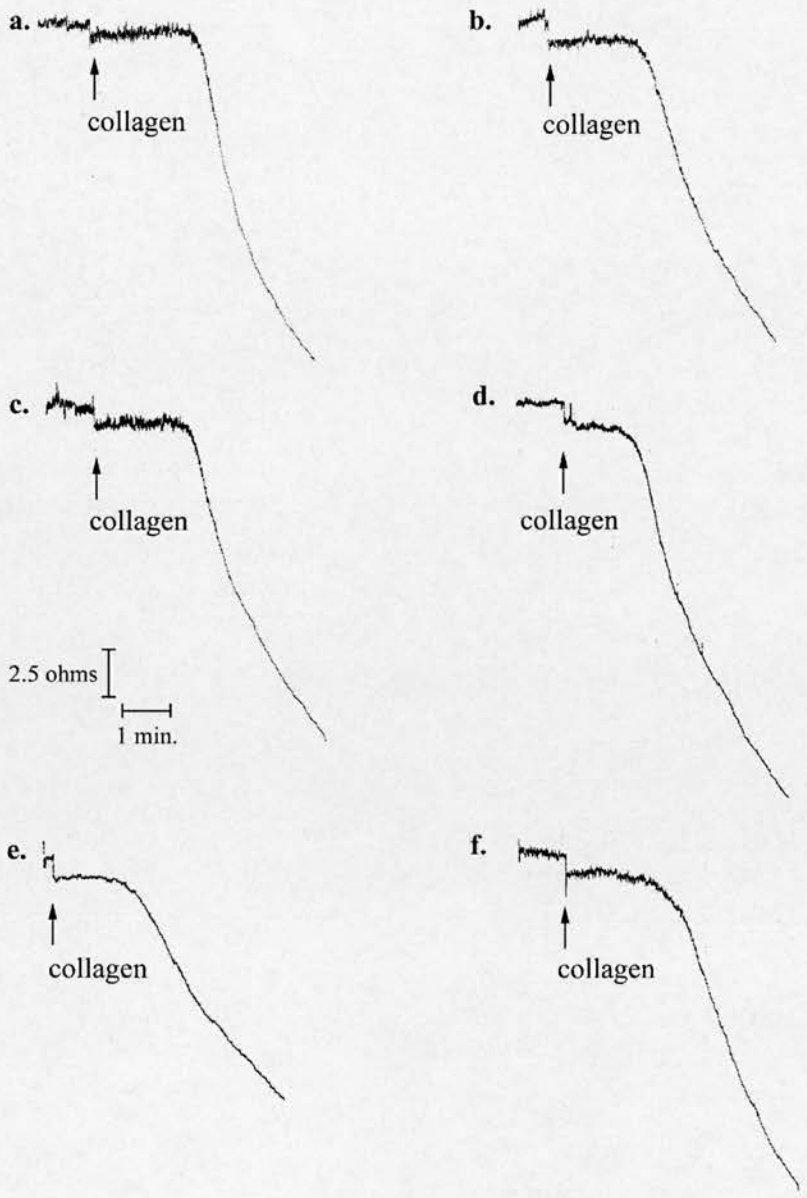


Figure 4.3 *Example of traces showing the effect of heparin 0 U/ml (a, b), 0.4 U/ml (c, d) and 4 U/ml (e, f) on collagen-induced platelet macroaggregation in whole blood from volunteers without heparinase (a, c and e) and with heparinase (b, d and f). Arrows indicate point at which collagen (0.6 μg/ml) was added.*

In control blood, the macroaggregatory response to fMLP (10^{-7} M) was 3.3 [1.9-5.7] ohms. In contrast to the effects of collagen, fMLP-induced macroaggregation was significantly potentiated by heparin concentrations 0.1, 0.4, 1, 4 U/ml ($P<0.0001$, n=11) but the potentiative effect of heparin was reduced to 4.4 [2.9-6.3] ohms at 10

U/ml of heparin (Table 4.4). The potentiative effects of heparin at the concentrations of 0.4 and 4 U/ml on fMLP-induced macroaggregation were significantly reversed by the addition of heparinase before fMLP stimulation ($P<0.05$ and $P<0.0001$, respectively) (Table 4.4).

Table 4.4 *fMLP-induced macroaggregation of platelets in whole blood from volunteers in the presence and absence of heparinase as determined by impedance aggregometry.*

Heparin (u/ml)	Without heparinase fMLP(10^{-7} M)	With heparinase fMLP(10^{-7} M)
0	3.3 [1.9-5.7]	3.2 [2.5-5.0]
0.1	8.0^{***} [5.9-13.3]	-
0.4	6.6^{***} [5.5-8.7]	$4.8^{\#}$ [4.3-6.3]
1	7.7^{***} [4.5-11.1]	-
4	7.5^{***} [4.6-8.0]	$3.1^{\#\#\#}$ [1.5-4.7]
10	4.4 [2.9-6.3]	-

Data are medians [interquartile range] of macroaggregation in ohms in whole blood from volunteers, with fMLP (10^{-7} M). $^{***}P<0.0001$ *indicates significantly different from heparin 0 u/ml;* $^{\#}P<0.05$ *and* $^{\#\#\#}P<0.0001$ *indicate significantly different from the absence of heparinase.*

Examples of traces of the potentiative effect of different concentrations of heparin on macroaggregatory response to fMLP (10^{-7} M) in the presence and absence of heparinase in whole blood samples from volunteers are shown in Figure 4.4.

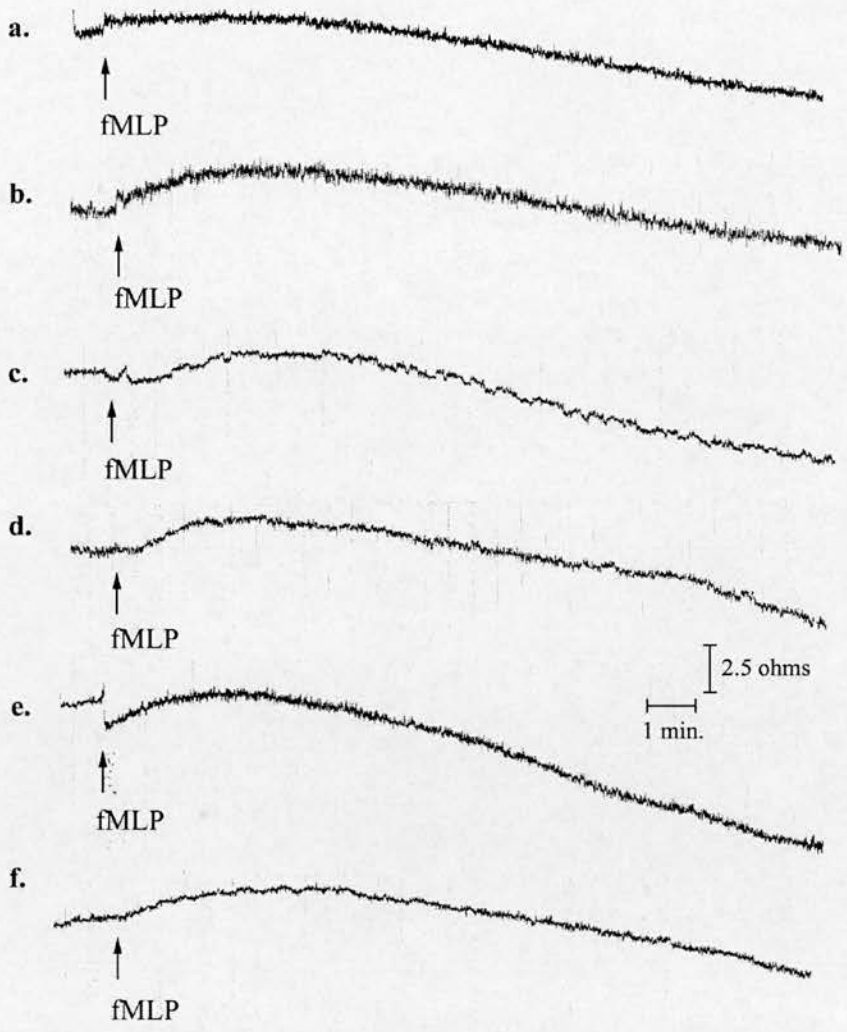


Figure 4.4 *Example of traces showing the effect of heparin 0 U/ml (a, b), 0.4 U/ml (c, d) and 4 U/ml (e, f) on fMLP-induced platelet macroaggregation in whole blood from volunteers without heparinase (a, c and e) and with heparinase (b, d and f). Arrows indicate point at which fMLP (10^{-7} M) was added.*

4.5.1.3 Platelet macroaggregation in citrated blood by impedance aggregometry

The macroaggregatory response to the direct platelet stimulant, collagen (0.6 μg/ml) in citrated blood was 16.0 [12.7-18] ohms (n=6) which was not significantly different from the macroaggregatory response in blood anticoagulated with r-hirudin (18.1 [17.3-19.9] ohms, n=11). As collagen acts through the thromboxane system by stimulating TXA_2 production and low Ca^{2+} concentration favours TXA_2 production (Abbate *et al.*, 1986; Schneider *et al.*, 1997), the platelet macroaggregation induced

by collagen can be seen in whole blood anticoagulated with citrate with chelation of Ca^{2+}.

In contrast fMLP, a direct neutrophil stimulant, (10^{-7} M) induced platelet macroaggrgation in r-hirudinised blood (3.3 [1.9-5.7], n=11) but did not induce platelet macroaggregation in whole blood anticoagulated with citrate. The neutrophil stimulant fMLP was found to induce platelet aggregation in whole blood only when extracellular Ca^{2+} was present because neutrophil activation is dependent on the level of cytosolic free Ca^{2+} concentration, which increases rapidly as cells become activated. As such, the aggregation induced by fMLP was not observed when citrate was used.

4.5.2 Platelet microaggregation in whole blood by single platelet counting

4.5.2.1 Patients

Heparinisation, before CPB and before addition of agonists (collagen or fMLP), significantly reduced the platelet count in stirred blood to 75% of control (from 120 [101-173]$\times 10^9$ cells/l to 90 [70-120]$\times 10^9$ cells/l ($P<0.005$, n=14)). Since the platelet count at end of CPB and before addition of collagen was 90 [86-135]$\times 10^9$ cells/l, CPB had no additional effect after correction for haemodilution (Table 4.5). The addition of heparinase to the sample taken before heparinisation and before addition of agonists (collagen or fMLP) caused a significant reduction in platelet count from 120 [101-173]$\times 10^9$ cells/l to 103 [84-131]$\times 10^9$ cells/l ($P<0.01$, n=14).

Addition of collagen (0.6 μg/ml) to stirred blood samples taken before heparinisation significantly reduced the platelet count from 120 [101-173]$\times 10^9$ cells/l to 36 [27-43]$\times 10^9$ cells/l ($P<0.005$, n=14). In heparinised blood, collagen significantly reduced the platelet count from the reduced value of 90 [70-120]$\times 10^9$ cells/l to 36 [25-57]$\times 10^9$ cells/l ($P<0.005$, n=14). The platelet count in stirred blood taken from at end of CPB was similarly reduced from 90 [86-135]$\times 10^9$ cells/l to 37 [31-48]$\times 10^9$ cells/l ($P<0.005$) with the addition of collagen. At each stage, collagen-induced

microaggregation was preserved with 70%, 60%, and 59 % of the unaggregated platelet count (all $p<0.0001$, n=14), respectively (Table 4.5).

The addition of heparinase to the sample taken before heparinisation and before addition of agonists (collagen or fMLP) caused a significant platelet loss from 120 [101-173]$\times 10^9$ cells/l to 103 [84-132]$\times 10^9$ cells/l ($P<0.01$, n=14). With the addition of heparinase, heparinisation, before CPB and before addition of agonists (collagen or fMLP), did not cause a significant change in platelet count in stirred blood (109 [90-117]$\times 10^9$ cells/l (n=14)). CPB had no additional effect after correction for haemodilution. The platelet count at end of CPB and before addition of collagen was 97 [77-121]$\times 10^9$ cells/l. Therefore, CPB had no additional effect after heparinisation. In the presence of heparinase, collagen-induced platelet microaggregation was preserved with 60%, 59% and 59% of the unaggregated platelet count at before and after heparinisation and at end of CPB (all $p<0.0001$, n=14) (Table 4.5).

Table 4.5. *Collagen-induced microaggregation of platelets in whole blood before and 5 minutes after heparin and at end CPB in the presence and absence of heparinase as determined by single platelet counting.*

Intra-operative Sampling time	**Platelet count ($\times 10^9$ cells/ml) Without heparinase**		**With heparinase**	
	Vehicle alone	**Collagen (0.6μg/ml)**	**Vehicle alone**	**Collagen (0.6 μg/ml)**
Pre-heparin	120 [101-173]	36^{***} [27-43]	103^{Ψ} [84-131]	41^{***} [31-66]
Heparinised	$90^{\#\#\#}$ [70-120]	36^{***} [25-57]	109 [90-117]	45^{***} [27-59]
End CPB	$90^{\#\#\#}$ [86-135]	37^{***} [31-48]	97 [77-121]	40^{***} [31-61]

Data are medians [interquartile range] of microaggregation in whole blood from patients, following 3 minutes stirring with vehicle alone and 5 minutes stirring with collagen (0.6 g/ml). $^{***}P<0.0001$ *indicates significantly different from vehicle alone,* $^{\#\#\#}P<0.05$ *indicates significantly different from pre-heparin and* $^{\Psi}P<0.01$ *indicates significantly different from the absence of heparinase.*

fMLP at a concentration of 10^{-7} M caused significant reduction in the platelet count of the sample taken before heparinisation and in the absence of heparinase from 120 [101-173]$\times10^9$ to 87 [50-116]$\times10^9$ cells/l ($P<0.0001$, n=14). In heparinised blood, fMLP did not cause a significant reduction in the platelet count in stirred blood, the platelet count was 86 [58-102]$\times10^9$ cells/l compared with 90 [70-120]$\times10^9$ cells/l in the heparinised blood with vehicle alone. At the end of CPB the platelet microaggregatory response to fMLP remained absent with a platelet count of 99 [63-133]$\times10^9$ cells/l (Table 4.6). However, after the addition of heparinase to the heparinised sample before CPB, fMLP caused a significant reduction in the platelet count in stirred blood from (109 [90-117]$\times10^9$ cells/l to 70 [63-105]$\times10^9$ cells/l) ($P<0.005$, n=14).

Table 4.6 *fMLP-induced microaggregation of platelets in whole blood before and 5 minutes after heparin and at end CPB in the presence and absence of heparinase as determined by single platelet counting.*

	Platelet count ($\times10^9$ cells/ml)			
Intra-operative	**Without heparinase**		**With heparinase**	
Sampling time	**Vehicle alone**	**fMLP (10^{-7} M)**	**Vehicle alone**	**fMLP (10^{-7} M)**
Pre-Heparin	120 [101-173]	87^{***} [50-116]	103^{Ψ} [84-131]	101 [82-128]
Heparinised	$90^{\#\#\#}$ [70-120]	86 [58-102]	109 [90-117]	$70^{***,\#\#\#}$ [63-105]
End CPB	$90^{\#\#\#}$ [86-135]	99 [63-133]	97 [77-121]	76^{*} [60-105]

Data are medians [interquartile range] of microaggregation in whole blood from patients, following 3 minutes stirring with vehicle alone and 15 minutes stirring with fMLP (10^{-7} M). $^{*}P<0.05$ and $^{***}P<0.0001$ indicates significantly different from vehicle alone, $^{\#}P<0.05$, $^{\#\#\#}P<0.005$ indicate significantly different from pre-heparin and $^{\Psi}P<0.01$ indicates significantly different from the absence of the heparinase.

4.5.2.2. Volunteers

The effects of heparin at 0.4, 4.0, 10.0 U/ml upon collagen-induced microaggregation and platelet count were examined. In the absence of both heparin and heparinase, collagen (0.6 μg/ml) caused microaggregation of about 90% of the unaggregated platelet count and reducing the platelet count from 214 [188-237]$\times 10^9$ cells/l to 21[20-29]$\times 10^9$ cells/l ($P<0.0001$, n=11) (Table 4.7). Heparin at a concentration of 0.4 U/ml did not affect collagen-induced microaggregation, but the microaggregatory response to collagen (0.6 μg/ml) was significantly reduced by 4 and 10 U/ml of heparin ($P<0.05$ and $P<0.01$, respectively, n=11) when compared with the response induced by collagen in the absence of heparin. These two concentrations of heparin caused significant reduction in platelet count from 178 [154-221]$\times 10^9$ cells/l to 35.2 [20-94]$\times 10^9$ cells/l ($P<0.0001$, n=11) and from 168 [137-243] $\times 10^9$ cells/l to 29.8 [22-71]$\times 10^9$ cells/l, ($P<0.0001$, n=11) respectively (Table 4.7). With the heparin concentration at 4 U/ml, the addition of heparinase to blood samples before collagen stimulation prevented the inhibition of microaggregation by heparin when compared with the response induced by collagen in the absence of heparin.

The same concentrations of heparin were used to examine the effects of heparin upon fMLP-induced microaggregation and platelet count in volunteers (n=11). In the absence of heparin, fMLP (10^{-7} M) did not significantly induce microaggregation. However, in the presence of different concentrations of heparin (0.4-1.0 U/ml), fMLP significantly induced platelet microaggregation, seen as significant decreases in platelet count ($P<0.005$, n=11). The platelet counts at 0.4, 4.0, and 10.0 U/ml of heparin were significantly lower than the count with zero heparin (139 [102-177] $\times 10^9$ cells/l, 133 [126-199] $\times 10^9$ cells/l, 132 [103-222] $\times 10^9$ cells/l, respectively) ($P<0.01$ and $P<0.05$) (Table 4.7). Addition of heparinase prevented the potentiation effect of 0.4 and 4U/ml of heparin and caused an increase in platelet count to 190 [127-222] $\times 10^9$ cells/l, 188 [132-197] $\times 10^9$ cells/l, respectively.

Table 4.7 *Collagen- and fMLP-induced microaggregation of platelets in whole blood from volunteers in the presence and absence of heparinase as determined by single platelet counting.*

Heparin (u/ml)	Platelet count ($\times 10^9$ cells/l) Without heparinase: Vehicle alone	Collagen (0.6 μg/ml)	fMLP (10^{-7} M)	Platelet count ($\times 10^9$ cells/l) With heparinase: Vehicle alone	Collagen (0.6 μg/ml)	fMLP (10^{-7} M)
0	214 [188-237]	21^{***} [20-29]	185 [174-201]	198 [180-245]	31^{***} [20-39]	187 [144-217]
0.4	201 [144-241]	31^{***} [16-41]	$139^{**,\#\#}$ [102-177]	208 [146-267]	31^{***} [14-31]	190 [127-222]
4	178 [154-221]	$35.2^{***,\#}$ [20-94]	$133^{**,\#}$ [126-199]	211 [165-228]	32.0^{***} [22-44]	188 [132-197]
10	$168^{\#}$ [137-243]	$29.8^{***,\#}$ [22-71]	132^{**} [103-222]	-	-	-

Data are medians [interquartile range] of microaggregation in whole blood from volunteers, following 3 minutes stirring with vehicle alone, 5 minutes stirring with collagen (0.6 g/ml) and 15 minutes stirring with fMLP (10^{-7} M). $^{**}P<0.005$ *and* $^{***}P<0.0001$ *indicate significantly different from vehicle alone;* $^{\#}P<0.05$ *and* $^{\#\#}P<0.01$ *indicate significantly different from no heparin.*

4.5.3 Macroaggregation in PRP

Collagen at concentrations of 0.2, 0.6, 0.8 and 1.0 μg/ml induced a concentration-related platelet macroaggregation in PRP by optical aggregometry. The median aggregatory responses were 4.25, 8.1, 9.5 and 9.3 cm, respectively (n=9). In contrast, fMLP (10^{-7} M) caused no response in PRP. The presence of heparin (4 U/ml) caused significant reduction in the macroaggregatory response to all concentrations of collagen used (P<0.0001 ANOVA) (Table 4.8).

Table 4.8 *Macroaggregation in PRP from volunteers' blood as determined by optical aggregometry.*

Heparin (u/ml)	Collagen(μg/ml)				fMLP (M)	
	0.2	0.6	0.8	1.0	10^{-7}	10^{-6}
0	4.25 [1.5-6.0]	8.1 [5.1-10.4]	9.5 [7.9-11.5]	9.3 [7.9-10.7]	0 [0.0]	0 [0.0]
4.0	1.8* [0.0-2.0]	5.2* [1.3-7.5]	6.3* [3.1-7.4]	7.0* [2.9-8.6]	0.3 [0-0.63]	0 [0.0]

Data are medians [interquatile range] for macroaggregation in PRP from volunteers' blood following stimulation for 3 minutes and 15 minutes with collagen and fMLP, respectively in the presence and absence of heparin 4 U/ml. $^{*}P<0.05$ *indicates significantly different from no heparin.*

4.5.4 The effects of heparin on neutrophil superoxide anion generation and MPO production

To evaluate the effect of heparin on neutrophil O_2^- generation, superoxide dismutase-inhibitable cytochrome C reduction was measured in human neutrophils treated with different concentrations of heparin. Figure 4.5 illustrates the dose-response curve of fMLP stimulated neutrophil O_2^- generation in nmol/10^6 cells/10 minutes and the effect of different concentrations of heparin on O_2^- generation.

In the control group (heparin 0 U/ml), fMLP at the concentration of 1000 nM induced the O_2^- generation in human neutrophils of 20.2 ± 2.2 nmol O_2^-/10^6 cells/10 minutes (Figure 4.5). As shown in Figure 4.5 heparin at a concentration of 1 U/ml caused no significant inhibition ($P>0.05$, ANOVA) of fMLP-induced O_2^- generation in human neutrophils, giving EC_{50} for fMLP of 84.1 ± 13.1; 54.0 ± 18.6 nM, n=6 ($P>0.05$) in its absence and presence, respectively. This concentration of heparin reduced the maximal effect of fMLP (1000 nM) from 20.2 ± 2.2 to 13.6 ± 2.1 nmol O_2^-/10^6 cells/10 minutes ($P>0.05$).

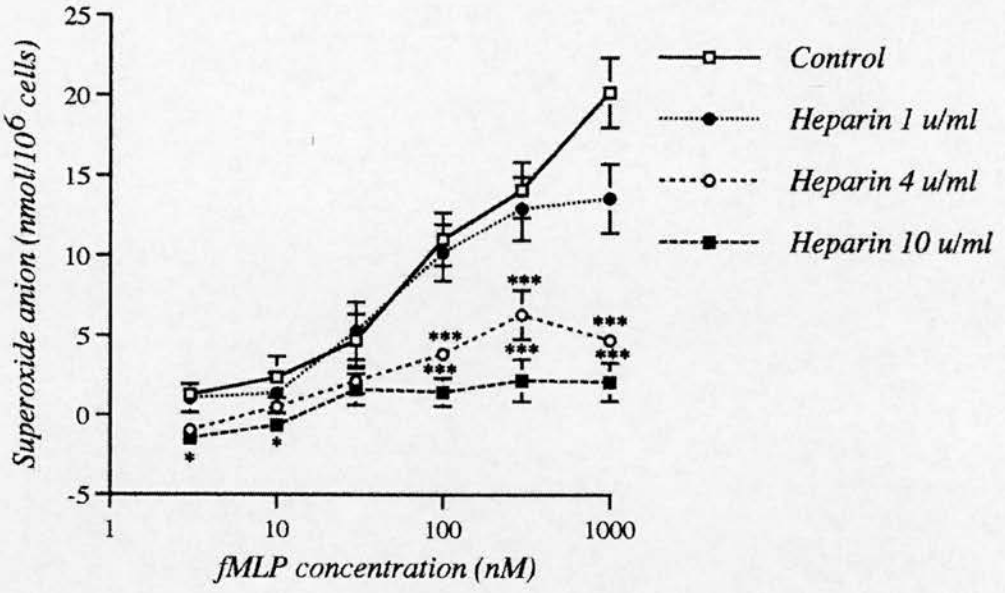


Figure 4.5 *Log concentration-effect curve for fMLP-induced superoxide anion generation in control neutrophils and neutrophils treated with heparin (1 U/ml, 4 U/ml and 10 U/ml). The values are the mean ± s.e. mean of 6 different volunteers. Statistically significant differences* $^{*}P< 0.05$ *and* $^{***}P<0.005$.

Heparin at a concentration of 4 U/ml caused significant inhibition ($P<0.05$, ANOVA) of fMLP-induced O_2^- generation in human neutrophils (Figure 4.5), giving EC_{50} for fMLP of 84.1 ± 13.1; 41.4 ± 10.9 nM, n=6 ($P<0.05$) in its absence and presence, respectively. This concentration of heparin significantly reduced the maximal effect of fMLP (1000 nM) from 20.2 ± 2.2 to 4.7 ± 0.6 nmol $O_2^-/10^6$ cells/10 minutes ($P<0.005$).

At the highest concentration (10 U/ml), heparin caused significant inhibition ($P<0.05$, ANOVA) of fMLP-induced O_2^- generation in human neutrophils (Figure 4.5), giving EC_{50} for fMLP of 84.1 ± 13.1; 245.6 ± 136.1 nM, n=6 ($P>0.05$) in its absence and presence, respectively. The maximal response of fMLP was significantly reduced by this concentration of heparin from 20.2 ± 2.2 to 2.1 ± 1.2 nmol $O_2^-/10^6$ cells/10 minutes at 1000 nM ($P<0.0005$).

Neutrophils were incubated with fMLP (3×10^{-8} to 10^{-5} M), causing a concentration-related production of MPO. Figure 4.6 illustrates the percentage of MPO produced in response to fMLP stimulated neutrophils in the presence of increasing concentrations of heparin. The maximum production of MPO by fMLP at 1000 nM in the control group was taken to be 100%.

Heparin at a concentration of 1 U/ml caused a significant inhibition ($P<0.005$, ANOVA) of fMLP-induced MPO production in human neutrophils (Figure 4.6), giving EC_{50} for fMLP of 30.3 ± 1.3; 29.1 ± 4.7 nM, n=5 ($P>0.05$) in its absence and presence, respectively. The maximal effect of fMLP (1000 nM) was significantly reduced by this concentration of heparin to 85.3 ± 1.2% ($P<0.01$).

Heparin at a concentration of 4 U/ml caused a significant inhibition ($P<0.005$, ANOVA) of fMLP-induced MPO production in human neutrophils (Figure 4.6), giving EC_{50} for fMLP of 30.3 ± 1.3; 27.1 ± 4.3 nM, n=5 ($P>0.05$) in its absence and presence, respectively. With the addition of 4 U/ml of heparin the maximal effect of fMLP (1000 nM) was significantly reduced to 73.7 ± 3.9% ($P<0.0005$).

The addition of 10 U/ml of heparin caused a significant inhibition ($P<0.005$, ANOVA) of fMLP-induced MPO production in human neutrophils (Figure 4.6), giving EC_{50} for fMLP of 30.3 ± 1.3; 23.4 ± 5.6 nM, n=5 ($P>0.05$) in its absence and presence, respectively. With the addition of 10 U/ml of heparin the maximal effect of fMLP (1000 nM) was significantly reduced to 58.6 ± 6.9% ($P<0.001$).

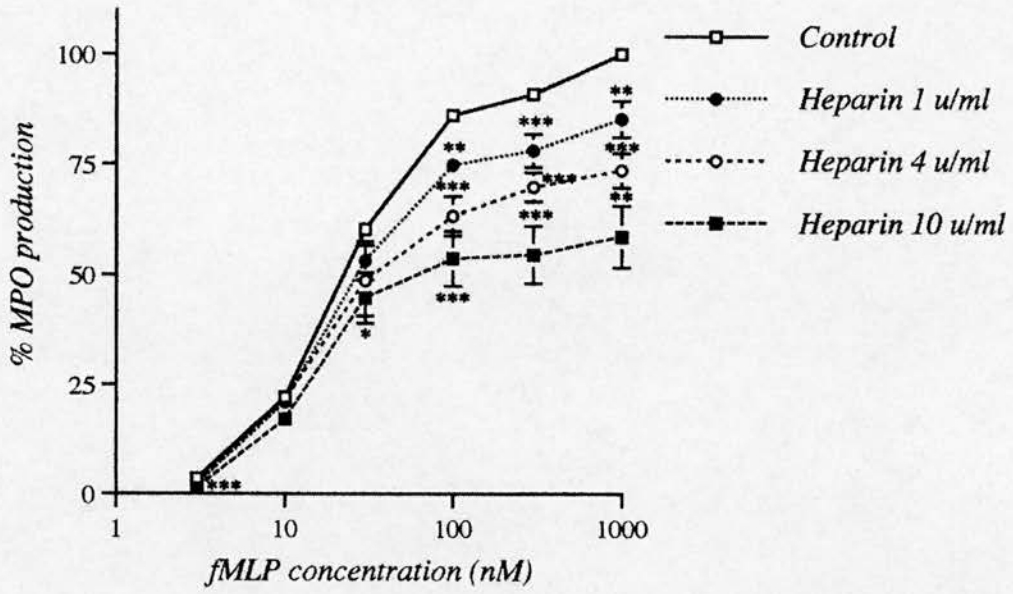


Figure 4.6 *Log concentration–effect curve for fMLP induced MPO production expressed as a percentage of MPO production in control neutrophils and neutrophils treated with heparin (1 U/ml, 4 U/ml and 10 U/ml). The values are the mean ± s.e. mean of 5 different volunteers. Statistically significant differences* $^{*}P<0.05$, $^{**}P<0.01$ *and* $^{***}P<0.005$.

4.6 DISCUSSION

CPB is associated with both impaired platelet macroaggregation and neutrophil activation. Previous studies have demonstrated that the bleeding diathesis associated with CPB is at least in part due to a defect in platelet macroaggregation, a problem which persists after the end of CPB with slow recovery (Menys *et al.*, 1994; 1995a; 1995b). Much research has been undertaken to determine the mechanism whereby platelets fail to form stable aggregates during CPB surgery. Some investigators suggested that platelet functional changes have been attributed to initial contact with the extracorporeal circuit (van Oeveren *et al.*, 1990; Jestice *et al.*, 1990). Recent research suggested that platelet dysfunction is evident before the initiation of CPB surgery but after the administration of heparin (Kestin *et al.*, 1993; Khuri *et al.*, 1995), suggesting heparin's involvement in the pathophysiology of platelet aggregation. Heparin may cause some platelet dysfunction (manifested by a

prolongation of bleeding time and a reduction in the production of TXB_2) in patients undergoing CPB (Kestin *et al*, 1993; Khuri *et al.*, 1995), although its specific action upon the aggregation process is unclear. Khuri *et al.* (1995) suggested that heparin contributes to the haemostatic defect observed in patients undergoing CPB not only by inhibiting coagulation through its effect on antithrombin III, but also by eliciting direct adverse effects on the platelet and the fibrinolytic system. Heparin has been suggested to affect platelet aggregation and accumulation along the endothelium by disrupting interactions among vWF with collagen and platelets (Savage *et al.*, 1996). Previous investigators showed that while macroaggregation in PRP measured by optical aggregometry is impaired by CPB, microaggregation in whole blood and PRP as determined by platelet counting is not (Menys *et al.*, 1994; Kawahito *et al.*, 1999).

The present study was designed to elucidate the effects of heparin, used as an anticoagulant in CPB surgery, on platelet aggregation both in patients undergoing CPB and in volunteers. The possibilities were: either that heparin had a specific but as yet unknown effect upon the platelet, or that heparin had an inhibitory effect upon neutrophil-platelet interactions that may stimulate platelet aggregation.

Whole blood was used to enable interactions between different cell types (eg. platelet-neutrophil) to occur. r-hirudin was used as anticoagulant in all studies namely: studies in whole blood both from patients and volunteers, *in vitro* studies in platelet-rich plasma (PRP) and *in vitro* studies of neutrophil $O_2^{\cdot}$ generation and MPO production. r-hirudin is a suitable anticoagulant for studying platelet functions because it does not produce any alterations in platelet reactions and does not provoke any changes in the ionised calcium concentration in blood (Glusa & Markwardt, 1990). In addition, r-hirudin can better preserve platelet function and may reduce the risk of postoperative bleeding, therefore it can be used successfully as an alternative anticoagulant to heparin during cardiac operations including CPB (Riess *et al.*, 1997). Neutrophil lactoferrin secretion is affected by the anticoagulant used and is best preserved by r-hirudin (Engstad *et al.*, 1997). Similarly r-hirudin does not interfere with the neutrophil response to fMLP (Karlsson *et al.*, 1996).

The effects of heparin on platelet macroaggregation in whole blood from patients were investigated. CPB resulted in haemodilution, but this was previously found not sufficient to interfere with aggregometry (Menys *et al.*, 1994). Collagen-induced macroaggregation *ex vivo* was found to be abolished by heparinisation *per se* rather than by CPB. As collagen-induced macroaggregation is mediated through TXA_2 generation it either suggests an action by heparin upon eicosanoid metabolism or a specific heparin effect upon an, as yet unknown, mechanism by which large stable aggregates are formed. The inhibitory effect of heparin on collagen-induced macroaggregation was not restored by adding heparinase, suggesting an indirect action of heparin in macroaggregation induced by collagen.

It was demonstrated, *ex vivo* in whole blood that fMLP-induced neutrophil stimulation could cause platelet macroaggregation; it was also shown that this was a neutrophil-mediated mechanism because of the absence of macroaggregatory responses to fMLP in platelet-rich plasma. Furthermore, the macroaggregatory response to fMLP in whole blood was much slower than that observed with a direct platelet stimulant, collagen. This also tends to indicate that macroaggregation induced by fMLP is an indirect action and being secondary to neutrophil activation as fMLP did not directly activate platelets. The macroaggregatory response of fMLP was measured at 15 minutes after the starting of macroaggregation. Although the responses were already clear at 15 minutes, this is probably too short a period for observation of the full fMLP effect, which takes about 30 minutes (Armstrong, R.A. personal communication). Heparinisation for CPB completely abolished fMLP-induced platelet macroaggregation and the abolition remained at the end of CPB. This showed that fMLP-induced macroaggregation *ex vivo* was abolished by heparinisation *per se* rather than by CPB. However, r-hirudin does not interfere with the neutrophil response to fMLP (Karlsson *et al.*, 1996). The abolition of fMLP-induced macroaggregation by heparinisation remained even when the sample was subsequently treated with heparinase (Table 4.2). This suggests that this effect of heparin may be irreversible or heparin may not exert a direct effect to inhibit fMLP-induced macroaggregation.

The findings of the present study, that neutrophil stimulation by fMLP caused macroaggregation of platelets in patients' blood, which was blocked by heparin, suggested release of mediators from the neutrophil, which stimulate platelet macroaggregation. A likely cause of the platelet macroaggregatory response was initially considered to be the release of cathepsin G from neutrophils. Cathepsin G has been shown, in washed cell systems, to promote platelet aggregation which can be inhibited by heparin as it forms heparin-cathepsin G complexes (Ferrer-Lopez *et al.*, 1992). However, in the present study, cathepsin G (200 and 250 nM) did not induce macroaggregation in whole blood (data not shown) and therefore this mechanism cannot be confirmed.

In contrast to the study in whole blood from patients, when volunteers' blood was examined with *in vitro* heparin concentration of 4 U/ml (i.e. the concentration measured in heparinised patients), the macroaggregatory response to collagen was only slightly inhibited. Lower concentrations of heparin (0.1, 0.4, and 1 U/ml) had no effect. The addition of heparinase abolished the *in vitro* inhibition of macroaggregation by 4 and 10 U/ml heparin thus suggesting that after *in vitro* heparinisation, digestion (neutralisation) of heparin by heparinase leaves no fragments of heparin that are inhibitory to platelet aggregation. The inhibition of macroaggregatory response to collagen of 4 and 10 U/ml heparin appears to be a direct heparin effect because it was inhibited by heparinase.

In contrast to the present study Chen *et al.* (1992a) showed that in an *in vitro* study in whole blood, heparin (0.5-5.0 U/ml) enhanced collagen-induced platelet aggregation both in calcium-chelated blood and in blood anticoagulated with hirudin, and that the heparin potentiating effect on platelet aggregation seemed to be independent of extracellular ionised calcium, and could be operative at physiological calcium concentration. The heparin effects were found to be related to activation of the platelet GPIIb/IIIa receptor complex (Chen *et al.*, 1992b). The discrepancy between these contradictory findings remains unexplained.

In contrast to the *ex vivo* findings, *in vitro* addition of heparin at concentration of 0.1-4.0 U/ml enhanced the macroaggregatory responses to fMLP. The potentiation of the macroaggregatory response to fMLP *in vitro* was inhibited by heparinase suggesting that this was a direct heparin effect.

The findings that : 1) *in vivo* heparinisation inhibits platelet macroaggregation to a much greater extent than *in vitro* heparinisation of whole blood, 2) that *ex vivo* heparinase failed to neutralise the inhibitory effects of heparin, whereas *in vitro* heparinase blocked the inhibitory effect of heparin, suggest that heparinisation for CPB causes the release of a mediator(s) from endothelium or other vascular cells, that can inhibit platelet macroaggregation. Intravenous administration of relatively low doses of heparin rapidly releases platelet factor 4 (PF_4) (Dawes *et al.*, 1982; O'Brien *et al.*, 1984), extracellular superoxide dismutase (Karlsson *et al.*, 1987), lipoprotein lipase and hepatic lipase from endothelium (Malmstrom, 1999), with maximum levels being achieved within 10 minutes. An association of increased bleeding time with lipase release has been seen in rabbits given unfractionated heparin with lesser effects seen with low molecular weight heparin (Barrowcliffe *et al.*, 1988). The release of hepatic lipase and lipoprotein lipase causes acute changes in plasma lipid profile (Malmstrom, 1999). Plasma lipid fatty acid composition has a variety of effects on platelet aggregation (MacIntyre *et al.*, 1984). Intravenous administration of heparin and other sulphated glycosaminoglycans releases numerous endothelial proteins into the plasma (Novotny *et al.*, 1993). Low doses of continuous heparin infusions maintain the peak levels of these proteins in plasma for several hours (Malmstrom *et al.*, 1999). During CPB, after large loading doses, plasma heparin levels remain elevated (Despostis *et al.*, 1995). It is therefore reasonable to assume that the levels of these heparin-released endothelial proteins remain elevated. Thus, it is possible that the inhibition of platelet macroaggregation may be secondary to a factor(s) released *in vivo* by heparin.

Heparinisation caused a significant loss of platelets before CPB. Heparinisation for CPB caused a loss of approximately 25% of the unaggregated platelet count before administration of agonist. This raises two concerns: the first is that the loss of this

amount of platelets may contribute to postoperative bleeding, but there is little evidence that the loss is large enough to cause this; the second concern is that heparin caused spontaneous *in vivo* platelet aggregation which may stimulate further aggregation. However, the evidence from this study and previous investigations has shown that large stable aggregate formation (macroaggregation) is inhibited by the presence of heparin (Menys 1994, 1995a, 1995b), rather than stimulated.

Collagen-induced microaggregation was not affected by heparinisation or CPB, thus confirming previous findings in which the microaggregation response was maintained despite the macroaggregatory response being inhibited (Menys *et al.*, 1994, 1995a, 1995b). fMLP-induced microaggregation was observed in patients before, but not after, heparinisation. The addition of heparinase to the sample taken before heparinisation and before addition of agonists (collagen or fMLP) caused a significant platelet loss, this effect was too small to be detected by impedance aggregometry. In the presence of heparinase, collagen-induced platelet microaggregation was preserved at each stage. The definite effect of heparinase on microaggregatory response to collagen remains unexplained. Addition of heparinase reversed the inhibitory effect of both heparinisation and CPB on fMLP-induced microaggregation suggesting that digestion of heparin by heparinase left no fragments that are inhibitory to microaggregation of platelets.

In contrast to the *ex vivo* effect, addition of heparin promotes both collagen- and fMLP-induced microaggregation *in vitro*; indeed, microaggregation was lacking in response to fMLP *in vitro* until heparin was added. This is consistent with a mild, direct stimulatory effect of heparin. The addition of heparinase prevented the potentiating effect of heparin on fMLP-induced platelet microaggregation. The definite microaggregatory response to heparin remains unexplained.

The mechanism of the decreased platelet counts (microaggregation) and the small but definite change in electrical impedance (macroaggregation) seen following fMLP stimulation in the present study may be complex. Platelet-neutrophil conjugates have been demonstrated in whole blood and their formation has been shown to be

P-selectin and divalent cation-dependent, thus suggesting that platelet activation is required for their formation. In one study, fMLP-induced neutrophil stimulation in citrated blood did not increase the number of platelet-neutrophil conjugates (Peters *et al.*, 1997), whereas another study, using flow cytometry with citrated blood, found a dose-dependent increase in conjugates in response to fMLP, PAF, ADP and thrombin (Li *et al.*, 1997). In the present study, fMLP was found not to induce platelet macroaggregation in whole blood anticoagulated with citrate. fMLP, a neutrophil stimulant, was found to induce platelet aggregation in whole blood only when extracellular Ca^{2+} was present. Certain aspects of neutrophil activation such as elastase release and O_2^- generation require the influx of extracellular calcium (Norgaer *et al.*, 1994; Khalfi *et al.*, 1996). As such, the platelet aggregation induced by fMLP was not observed once the Ca^{2+} had been removed by citrate. This finding provides further evidence to support the role of neutrophil activation in platelet macroaggregation. In addition, fMLP added to PRP did not cause platelet macroaggregation. It can therefore be postulated that the fMLP effect was neutrophil-mediated.

To support the hypothesis that heparin contributed to platelet dysfunction during CPB by interfering with a possible neutrophil-platelet interaction that contributes to platelet macroaggregation, the *in vitro* effects of heparin on neutrophil activation were investigated by measuring markers of neutrophil activation (O_2^- generation and MPO production). The results showed that heparin at all concentrations had an inhibitory effect on O_2^- generation. O_2^- generation in fMLP-stimulated neutrophils was dose-dependently reduced by heparin. With the addition of 4 U/ml and 10 U/ml of heparin, O_2^- generation was almost completely abolished. These findings are consistent with previous studies that showed the inhibitory effects of heparin on respiratory burst of neutrophils, stimulated with fMLP in a concentration-dependent manner (Pasini *et al.,* 1984; Bazzoni *et al.*, 1993; Cerletti *et al.*, 1994; Risenberg *et al.*, 1995). Others have observed a biphasic dose-response curve for inhibition of O_2^- production *in vitro* following fMLP stimulation, at low concentrations, heparin enhanced neutrophil O_2^- generation but was suppressed at higher concentrations. (Itoh *et al.*, 1995). These studies suggested that heparin has an inhibitory effect on

O_2^- generation, although the concentrations of heparin required to cause significant inhibition varied greatly.

Heparin *in vivo* has been implicated in the indirect neutralisation of O_2^- through its association with superoxide dismutase (SOD) (Oyanagui & Sato, 1990). Several studies have focused on the effects of heparin on other markers of neutrophil activation such as SOD and MPO. A study by Karlsson *et al.* (1987), suggested that extracellular superoxide dismutase (ecSOD), induced by heparin, may play a protective role against O_2^-, an event which would not be seen *in vitro*. Other studies have focused on lactoferrin and myeloperoxidase turnover (Larson *et al.*, 1996). In this study, the *in vitro* effects of different concentrations of heparin on MPO production in neutrophils from volunteers were measured. It was found that inhibition of MPO production was gradual and dose-dependent with increasing concentrations of heparin. The results that different concentrations of heparin inhibited fMLP-induced O_2^- generation and MPO production in neutrophils, illustrate the *in vitro* inhibition of neutrophil activation by heparin. This could be indirect evidence to support the hypothesis that heparin contributes to platelet dysfunction during CPB by interfering with a possible neutrophil-platelet interaction.

4.7 CONCLUSIONS

The present study demonstrated that *in vivo* heparinisation, *per se* rather than CPB abolished *ex vivo* macroaggregation induced by collagen or fMLP. This *ex vivo* inhibitory effect of heparin on platelet macroaggregation is suggested to be an indirect effect of *in vivo* heparinisation because heparinase cannot restore *ex vivo* collagen- or fMLP-induced platelet macroaggregation. This is further supported by the results from the *in vitro* study, which showed that inhibition of collagen-induced macroaggregation was far less marked and heparin enhanced the aggregatory response to fMLP. As heparinase could inhibit these effects of heparin on macroaggregation, the phenomenon is likely to be a direct heparin effect. These findings suggest either that, heparin is irreversibly bound and indigestible, or that

heparin exerts its *in vivo* effects indirectly on platelet macroaggregation. In addition, other factors such as endothelial/other cell types or plasma proteins, might also be involved.

Heparinisation caused a significant loss of platelets before CPB. *In vivo* heparinisation or CPB did not affect the collagen-induced microaggregation response, but affected the fMLP-induced microaggregation response. *Ex vivo* heparinase caused a fall in platelet count and was therefore not helpful in the investigation of microaggregation. In contrast to the *ex vivo* effect, heparin promoted both collagen-and fMLP-induced microaggregation *in vitro* and heparinase prevented the potentiation effect of heparin on platelet microaggregation.

The findings that different concentrations of heparin inhibited both O_2^- generation and MPO production *in vitro* in neutrophils, suggest that neutrophil activation was inhibited by heparin *in vitro* and this may be attributed in part, to its inhibitory effect on platelet macroaggregation *in vivo*.

CHAPTER 5

GENERAL DISCUSSION

The results from this study confirm that neutrophil activation results from the stimulation of several transduction systems. NO, cyclic GMP and PLD have been shown to play a role in the activation of human neutrophils since neutrophil chemotaxis and O_2^- generation, were attenuated by agents that inhibit these pathways. However, the increase in cyclic GMP and NO induced by fMLP, which are associated with neutrophil activation, are very small. In contrast, the NO donors, GEA 3162, GEA 5024 and SIN-1, which produced large amounts of NO (measured as total nitrate/nitrite) and cyclic GMP compared with fMLP, inhibit neutrophil chemotaxis.

Neutrophil activation has been suggested to have an important role in MI-R injury (Lefer *et al.*, 1993; Forman *et al.*, 1993). A number of studies have shown a very strong correlation between the extent of neutrophil accumulation in the ischaemic-reperfused myocardium and infarct size (Mullane *et al.*, 1985; Chatelain *et al.*, 1987). As it was clearly shown in Chapter 2 that the NO donor, GEA 3162 inhibits human neutrophil chemotaxis *in vitro*, it was reasonable to investigate the ability of this drug to inhibit neutrophil infiltration in an *in vivo* rat model of MI-R. GEA 3162 was compared with a potent selective A_{2A} receptor agonist 2-HE-NECA and the stable PGI_2 analogue cicaprost (ZK 96 480). All three drugs have been shown to be able to inhibit neutrophil infiltration and attenuate myocardial necrosis in the ischaemic myocardium. Although, these drugs, at the concentrations used in this study caused a significant attenuation in neutrophil accumulation and myocardial necrosis, they also caused a significant decrease in blood pressure and increase in heart rate. Thus, further investigation using lower doses of these drugs to infuse the ischaemic myocardium for a period of less than 2 hours are required to achieve the cardioprotective effect of these drugs without causing hypotension and reflex tachycardia (haemodynamic changes). In addition, if possible, the measurement of coronary blood flow (transmural blood flow) should be monitored. However, it is not known whether these drugs can preserve the ischaemic myocardium over longer periods of time. Therefore, a further investigation with a reperfusion period longer than 2 hours (e.g. 42-72 hours) in a closed-chest rat model of MI-R injury may be of

value to perform. The results from such studies could add to the support for the beneficial effects of these drugs in the long term.

GEA 3162, 2-HE-NECA and cicaprost have been shown to inhibit human neutrophil chemotaxis. The effects of these drugs on fMLP-induced rat neutrophil chemotaxis were also investigated in this study. Unfortunately, no results were obtained, even with fMLP-induced neutrophil chemotaxis in the control group. This is likely to be the result of the conditions used to measure chemotaxis being optimised for human rather than rat neutrophils. Indeed rat neutrophils were shown to behave differently from human neutrophils in terms of their sedimentation using polymorphoprep™. Unfortunately, this investigation could not be repeated because of time constraints and the cost of animals, but the effects of these drugs on fMLP-induced rat neutrophil chemotaxis need to be investigated further.

The results from neutrophil accumulation and measurement of myocardial injury cannot pinpoint the exact mechanisms by which these drugs achieve their beneficial effects, but the evidence from this study points to an inhibitory effect of these drugs on neutrophil infiltration. However, there are a number of other possible mechanisms by which these drugs could provide cardioprotection. Their effects may be related to inhibition of adherence of neutrophils to the coronary endothelium; inhibition of direct activation of neutrophils; inhibition of the release of cytotoxic metabolites (superoxide anion, hypochlorous acid, proteases, PAF etc.) from neutrophils; or a reduction in adherence-dependent injury to myocytes.

In order to identify the exact mechanisms for the cardioprotective effects of these drugs in MI-R injury, the following *in vitro* assays are required. Firstly, an *in vitro* neutrophil adherence assay could be used for assessing the effect of the test drugs on the adherence of activated fluorescent neutrophils to the endothelial surfaces of isolated coronary artery. Adherence can be determined by counting the number of neutrophils adhering to the endothelial surface/mm^2 of endothelium. Secondly, an *in vitro* assay of O_2^- production by isolated neutrophils could be used to investigate the effects of the test drugs on O_2^- production by activated neutrophils in suspension

(i.e., adherence-independent generation) which can be determined by measuring the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome C to ferrocytochrome C. Cytochrome C reduction can be monitored spectrophotometrically by determining the optical density of ferrocytochrome C, as discussed in Chapter 2. Thirdly, isolated coronary arterial rings can be used for evaluation of the effects of the test drugs on activated neutrophil-mediated coronary endothelial dysfunction. Endothelial function can be assessed by comparing vasorelaxation to an endothelium-dependent with an endothelium-independent vasodilator. The results from these assays should provide additional evidence to help reveal the exact cardioprotective mechanisms of these drugs in this rat model of MI-R injury.

Neutrophil activation is associated with cardiopulmonary bypass as well as failure of platelets to form large stable aggregates. The present study demonstrated that heparinisation contributes to the platelet dysfunction by inhibiting platelet macroaggregation before the onset of extracorporeal circulation. This finding is in agreement with that of Kestin *et al.* (1993) and Khuri *et al.* (1995) who demonstrated that platelet dysfunction is evident before the initiation of CPB but after the administration of heparin. It was suggested from this study that heparin may cause the release of some mediator(s) from endothelium or other cell types, that could inhibit platelet macroaggregation. The release of various plasma proteins from the endothelium into the plasma after administration of heparin (as discussed in Chapter 4) has been clearly demonstrated. It has subsequently been hypothesised by our group that if *in vivo* heparinisation inhibits platelet macroaggregation through release of one or more endothelial-derived factors into the plasma, then platelet poor plasma obtained from blood with dysfunctional platelets should impair the function of normal platelets. Recent results have demonstrated that platelet dysfunction is secondary to such a plasma change which is transferable *in vitro* to normal platelets (Dr E Murithi, personal communication) to impair their aggregation. The nature of this transferable factor still needs to be elucidated but these results support the contention that heparin effects result from an endogenous inhibitor rather than the lack of an *in vivo* agonist as previously suggested (Kestin *et al.*, 1993).

As the effects of heparin on platelet macroaggregation were so profound, it was not possible to identify whether CPB had any additional effects on aggregation. The use of alternative anticoagulants for CPB would help to clarify this. This study also did not address the mechanism by which heparin inhibited platelet macroaggregation. Since no measurements of adhesion molecule expression or production of cytokines were performed in this study, the effects of heparin in relation to these in whole blood, remain unclear. Assessment of platelet reactivity by measuring the expression of adhesion molecules such as P-selectin using flow cytometry might clarify the mechanism by which heparin exerts platelet dysfunction.

Recent *in vivo* research suggests that the complications associated with CPB are a result of multicellular activity, and are particularly associated with the respiratory burst, which initiates the production of oxygen-dependent free radicals from stimulated neutrophils. Although, *in vitro* studies have shown that heparin has an inhibitory effect on the respiratory burst, further investigations of the *in vivo* effects of heparinisation and CPB on neutrophil activation in patients undergoing CPB are required. This would help to identify the role of neutrophils in relation to the defect in platelet macroaggregation. Neutrophil activation could be measured *ex vivo* using O_2^- generation or MPO production.

One of the aims of this study was to identify the role of neutrophils in platelet macroaggregation. This was very difficult, mainly because of the techniques used in this study. Macroaggregation was induced by collagen, which is a platelet stimulant. However without looking at the composition of the aggregate, either by microscopy or flow cytometry, it is not possible to determine if neutrophils are recruited into the platelet macroaggregate secondary to platelet activation. Aggregation induced by fMLP was used to measure neutrophil-driven platelet aggregation. However because heparin inhibited both collagen and fMLP-induced macroaggregation it is not clear as yet, whether this is a result of inhibition of platelet activation, neutrophil activation or both. As can be seen from the aggregation traces, the aggregatory responses to fMLP are slow and much smaller than the collagen response. The size of the fMLP response varies considerably between donors, possibly as a result of

different neutrophil counts in whole blood. Although this fMLP response is a new finding, because it is only evident in blood anticoagulated with hirudin or heparin and is not observed when calcium chelators are used, it is a crude response. Clearly, a more sophisticated method of assessing neutrophil-platelet aggregation such as flow cytometric analysis is required to identify the role that this might play in platelet macroaggregation.

References

Abbate, R., Favilla, S., Boddi, M., Costanzo, G. and Prisco, D. Factors influencing platelet aggregation in whole blood. Am J Clin Pathol (1986); **86**: 91-96.

Achterberg, P.W., de Tombe, P.P., Harmsen, E. and de Jong, J.W. Myocardial S-adenosylhomocysteine hydrolase is important for adenosine production during normoxia. Biochim Biophys Acta (1985); **840**: 393-400.

Agwu, D.E., McPhail, L.C., Chabot, M.C., Daniel, L.W., Wykle, R.L. and McCall, C.E. Choline-linked phosphoglycerides. A source of phosphatidic acid and diglycerides in stimulated neutrophils. J Biol Chem (1989); **264**: 1405-1413.

Agwu, D.E., McPhail, L.C., Sozzani, S., Bass, D.A. and McCall, C.E. Phosphatidic acid as a second messenger in human polymorphonuclear leukocytes. Effects on activation of NADPH oxidase. J Clin Invest (1991); **88**: 531-539.

Agwu, D.W., McCall, C.E. and McPhail, L.C. Regulation of phospholipase D-induced hydrolysis of choline-containing phosphoglycerides by cyclic AMP in human neutrophils. J Immunol (1991a); **146**: 3895-3903.

Aitchison, K.A. and Coker, S.J. Potential interactions between iloprost and SIN-1 on platelet aggregation and myocardial infarct size *in vivo*. Eur J Pharmacol (1999); **374**: 59-69.

Akaike, T., Yoshida, M., Miyamoto, Y., Sato, K., Kohno, M., Sasamoto, K., Miyazaki, K., Ueda, S. and Maeda, H. Antagonistic action of imidazolineoxyl N-oxides against endothelium-derived relaxing factor/NO through a radical reaction. Biochemistry (1993); **32**: 827-832.

Akimaru, K., Utsumi, T., Sato, E.F., Klostergaard, J., Inoue, M. and Utsumi, K. Role of tyrosyl phosphorylation in neutrophil priming by tumor necrosis factor-alpha and granulocyte colony stimulating factor. Arch Biochem Biophys (1992); **298**: 703-709.

Allan, G., Bhattacherjee, P., Brook, C.D., Read, N.G. and Parke, A.J. Myeloperoxidase activity as a quantitative marker of polymorphonuclear leukocyte accumulation into an experimental myocardial infarct: the effect of ibuprofen on infarct size and polymorphonuclear leukocyte accumulation. J Cardiovasc Pharmacol (1985); **7**: 1154-1160.

Alon, R., Feizi, T., Yuen, C.T., Fuhlbrigge, R.C. and Springer, T.A. Glycolipid ligands for selectins support leukocyte tethering and rolling under physiologic flow conditions. J Immunol (1995); **154**: 5356-5366.

Alon, R., Hammer, D.A. and Springer, T.A. Lifetime of the P-selectin-carbohydrate bond and its response to tensile force in hydrodynamic flow [published erratum appears in Nature 1995 Sep 7; 376 (6544): 86]. Nature (1995); **374**: 539-542.

Altieri, D.C., Agbanyo, F.R., Plescia, J., Ginsberg, M.H., Edgington, T.S. and Plow, E.F. A unique recognition site mediates the interaction of fibrinogen with the leukocyte integrin Mac-1 (CD11b/CD18). J Biol Chem (1990); **265**: 12119-12122.

Amatruda, T.T., Gerard, N.P., Gerard, C. and Simon, M.I. Specific interactions of chemoattractant factor receptors with G-proteins. J Biol Chem (1993); **268**: 10139-10144.

Ambrosio, G., Weisfeldt, M.L., Jacobus, W.E. and Flaherty, J.T. Evidence for a reversible oxygen radical-mediated component of reperfusion injury: reduction by recombinant human superoxide dismutase administered at the time of reflow. Circulation (1987); **75**: 282-291.

Ammar, T. and Fisher, C.F. The effects of heparinase 1 and protamine on platelet reactivity. Anesthesiology (1997); **86**: 1382-1386.

Anderson, R., Glover, A., Koornhof, H.J. and Rabson, A.R. *In vitro* stimulation of neutrophil motility by levamisole: maintenance of cyclic GMP levels in chemotactically stimulated levamisole-treated neutrophils. J Immunol (1976); **117**: 428-432.

Aoki, N., Bitterman, H., Brezinski, M.E. and Lefer, A.M. Cardioprotective actions of human superoxide dismutase in two reperfusion models of myocardial ischaemia in the rat. Br J Pharmacol (1988); **95**: 735-740.

Armstrong, J.M., Chapple, D., Dusting, G.J., Hughes, R., Moncada, S. and Vane, J.R. Cardiovascular actions of prostacyclin (PGI_2) in chloralose anesthetized dogs. Br J Pharmacol (1977); **61**: 136P.

Armstrong, R.A. and Talpain, E. Comparision of the prostaglandin E (EP) receptor of human neutrophils and HL-60 cells differentiated with DMSO. Prostaglandin (1994); **48**: 221-234.

Armstrong, R.A. Investigation of the inhibitory effects of PGE_2 and selective EP agonists on chemotaxis of human neutrophils. Br J Pharmacol (1995); **116**: 2903-2908.

Arnaout, M.A., Pitt, J., Cohen, H.J., Melamed, J., Rosen, F.S. and Colten, H.R. Deficiency of a granulocyte-membrane glycoprotein (gp150) in a boy with recurrent bacterial infections. N Engl J Med (1982); **306**: 693-699.

Arnaout, M.A. Structure and function of the leukocyte adhesion molecules CD11/CD18. Blood (1990); **75**: 1037-1050.

Arnheim, N., Inouye, M., Law, L. and Laudin, A. Chemical studies on the enzymatic specificty of goose egg white lysozyme. J Biol Chem (1973); **248**: 233-236.

Aruoma, O.I. and Halliwell, B. Superoxide-dependent and ascorbate-dependent formation of hydroxyl radicals from hydrogen peroxide in the presence of iron: Are lactoferrin and transferrin promoters of hydroxyl-radical generation? Biochem J (1987); **241**: 273-278.

Asch, A.S., Barnwell, J., Silverstein, R.L. and Nachman, R.L. Isolation of the thrombospondin membrane receptor. J Clin Invest (1987); **79**: 1054-1061.

Aziz, K.A., Cawley, J.C., Kamiguti, A.S. and Zuzel, M. Degradation of platelet glycoprotein Ib by elastase released from primed neutrophils. Br J Haematol (1995a); **91**: 46-54.

Aziz, K.A., Cawley, J.C. and Zuzel, M. Platelets prime PMN via released PF4: mechanism of priming and synergy with GM-CSF. Br J Haematol (1995b); **91**: 846-853.

Babior, B.M., Kipnes, R.S. and Curnutte, J.T. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. J Clin Invest (1973); **52**: 741-743.

Babior, B.M. The respiratory burst of phagocytes. J Clin Invest (1984); **73**: 599-601.

Barbier, A.J. and Lefebvre, R.A. Effect of LY 83583 on relaxation induced by non-adrenergic non-cholinergic nerve stimulation and exogenous nitric oxide in the rat gastric fundus. Eur J Pharmacol (1992); **219**: 331-334.

Baggiolini, M. and Dewald, B. The neutrophil. Int Arch Allergy Appl Immunol (1985); **76**: 13-20.

Baggiolini, M., Dewald, B. and Thelen, M. Effects of PAF on neutrophils and mononuclear phagocytes. Prog Biochem Pharmacol (1988); **22**: 90-105.

Baggiolini, M., Kernen, P., Deranleau, D.A. and Dewald, B. Control of motility, exocytosis and the respiratory burst in human neutrophils. Biochem Soc Trans (1991); **19**: 55-59.

Baggiolini, M. and Clark-Lewis, I. Interleukin-8, a chemotactic and inflammatory cytokine. FEBS Lett (1992); **307**: 97-101.

Baggiolini, M., Boulay, F., Badwey, J.A. and Curnutte, J.T. Activation of neutrophil leukocytes: chemoattractant receptors and respiratory burst. FASEB J (1993); **7**: 1004-1010.

Baggiolini, M. Chemotactic and inflammatory cytokines CXC and CC proteins. Adv Exp Med Biol (1993); **351**: 1-11.

Baggiolini, M., Dewald, B. and Moser, B. Interleukin-8 (IL-8) and related chemotactic cytokines-C-X-C and C-C chemokines. Adv Immunol (1994); **55**: 97-179.

Barrowcliffe, T.W., Merton, R.E., Gray, E. and Thomas, D.P. Heparin and bleeding: an association with lipase release. Thromb Haemost (1988); **60**: 434-436.

Barten, A.D., Ryder, K., Lourenco, R.V., Dralle, W. and Weiss, S.G. Inflammatory reaction and airway damage in cystic fibrosis. J Lab Clin Med (1976); **88**: 423-426.

Bassenge, E. and Mulsch, A. Anti-ischaemic actions of molsidomine by venous and large coronary dilatation in combination with anti-platelet effects. J Cardiovasc Pharmacol (1989); **14**: S23-S28.

Bauldry, S.A., Bass, D.A., Cousart, S.L. and McCall, C.E. Tumor necrosis factor alpha priming of phospholipase D in human neutrophils. Correlation between phosphatidic acid production and superoxide generation. J Biol Chem (1991); **266**: 4173-4179.

Bauldry, S.A., Elsey, K.L. and Bass, D.A. Activation of NADPH oxidase and phospholipase D in permeabilized human neutrophils. Correlation between oxidase activation and phosphatidic acid production. J Biol Chem (1992); **267**: 25141-25152.

Baum, J. *et al.* A simplified method for the measurement of chemotaxis of polymorphonuclear leukocytes from human blood. J Lab Clin Med (1971); **77**: 501.

Baumheter, S., Singer, M.S., Henzel, W., Hemmerich, S., Renz, M., Rosen, S.D. and Lasky, L.A. Binding of L-selectin to the vascular sialomucin CD34. Science (1993); **262**: 436-438.

Bazzoni, G., Beltran Nunez, A., Mascellani, G., Bianchini, P., Dejana, E. and Del Maschio, A. Effect of heparin, dermatan sulfate, and related oligo-derivatives on human polymorphonuclear leukocyte functions [see comments]. J Lab Clin Med (1993); **121**: 268-275.

Bazzono, G., Dejana, E. and Del maschio, A. Platelet-neutophil interactions. Possible relevance in the pathogenesis of thrombosis and inflammation. Haematologica (1991); **76**: 491-499.

Beauvais, F., Michel, L. and Dubertret, L. Exogenous nitric oxide elicits chemotaxis of neutrophils *in vitro*. J Cell Physiol (1995); **165**: 610-614.

Becker, E.L., Kermode, J.C., Naccache, P.H., Yassin, R., Marsh, M.L., Munoz, J.J. and Sha'afi, R.I. The inhibition of neutrophil granule enzyme secretion and chemotaxis by pertussis toxin. J Cell Biol (1985); **100**: 1641-1646.

Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A. and Freeman, B.A. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci U S A (1990); **87**: 1620-1624.

Bednar, M.M., Balazy, M., Murphy, M., Booth, C., Fuller, S.P., Barton, A., Bingham, J., Golding, L. and Gross, C.E. Peroxynitrite augments fMLP-stimulated chemiluminescence by neutrophils in human whole blood. J Leukoc Biol (1996); **60**: 619-624.

Belenky, S.N., Robbins, R.A., Rennard, S.I., Gossman, G.L., Nelson, K.J. and Rubinstein, I. Inhibitors of nitric oxide synthase attenuate human neutrophil chemotaxis *in vitro*. J Lab Clin Med (1993); **122**: 388-394.

Belisle, S. and Hardy, J.F. Hemorrhage and the use of blood products after adult cardiac operations: myths and realities. Ann Thorac Surg (1996); **62**: 1908-1917.

Bennett, J.S. The molecular biology of platelet membrane proteins. Semin Hematol (1990); **27**: 186-204.

Berkow, R.L. and Dodson, R.W. Tyrosine-specific protein phosphorylation during activation of human neutrophils. Blood (1990); **75**: 2445-2452.

Berkow, R.L. and Dodson, R.W. Alterations in tyrosine protein kinase activities upon activation of human neutrophils. J Leukoc Biol (1991); **49**: 599-604.

Bern, R.M. The role of adenosine in the regulation of coronary blood flow. Circ Res (1980); **47**: 807-813.

Berndt, M.C., Ward, C.M., De Luca, M., Facey, D.A., Castaldi, P.A., Harris, S.J. and Andrews, R.K. The molecular mechanism of platelet adhesion. Aust N Z J Med (1995); **25**: 822-830.

Berneir, M., Hearse, D. and Manning, A. Ability of 6 anti-free radical interventions to reduce reperfusion-induced arrythmias caused by addition of a free radical generating system. J Mol Cell Cardiol (1986); **18**: 100-.

Bernier, M., Manning, A.S. and Hearse, D.J. Reperfusion arrhythmias: dose-related protection by anti-free radical interventions. Am J Physiol (1989); **256**: H1344-1352.

Berti, F., Mantovani, M., Niada, R., Omini, C., Pescador, R., Porta, R., Prino, G., Rossoni, G. and Tettamanti, R. Cardiovascular pharmacology of defibrotide. Semin Thromb Hemost (1988); **14 Suppl**, 38-42.

Berti, F., Rossoni, G., Magni, F., Caruso, D., Omini, C., Puglisi, L. and Galli, G. Nonsteroidal antiinflammatory drugs aggravate acute myocardial ischemia in the perfused rabbit heart: a role for prostacyclin. J Cardiovasc Pharmacol (1988); **12**: 438-444.

Bevilacqua, M.P., Pober, J.S., Wheeler, M.E., Cotran, R.S. and Gimbrone, M.A.J. Interleukin I acts on cultures human vascular endothelium to increase the adhesion of polymorphonuclear leukocytes, monocytes and related leukocyte cell lines. J Clin Invest (1985); **76**: 2005-2011.

Bevilacqua, M.P., Pober, J.S., Mendrick, D.L., Cotran, R.S. and Gimbrone, M.A.J. Identification of an inducible endothelial-leukocyte adhesion molecule. Proc Natl Acad Sci U S A (1987); **84**: 9238-9242.

Bevilacqua, M.P., Stenglin, S., Gimbrone, M. and Seed, B. An inducible receptor for neutrophils related to complement regulatory proteins and lactins. Science (1989); **243**: 1160-1165.

Bevilacqua, M., Butcher, E., Furie, B., Gallatin, M., Gimbrone, M., Harlan, J., Kishimoto, K., Lasky, L., McEver, R. *et al.* Selectins: a family of adhesion receptors [letter]. Cell (1991); **67**: 233.

Bevilacqua, M.P. and Nelson, R.M. Selectins. J Clin Invest (1993); **91**: 379-387.

Bevilacqua, M.P. Endothelial-leukocyte adhesion molecules. Annu Rev Immunol (1993); **11**: 767-804.

Billah, M.M., Eckel, S., Mullmann, T.J., Ergan, R.W. and Siegel, M.I. Phosphatidylcholine hydrolysis by PLD determines the phophatidate and diglyceride levels in chemotactic peptide stimulated neutrophils. J Biol Chem (1989); **264**: 17069-17077.

Billah, M.M., Pai, J.K., Mullmann, T.J., Egan, R.W. and Siegel, M.I. Regulation of phospholipase D in HL-60 granulocytes. J Biol Chem (1989b); **264**: 9069-9076.

Billah, M.M. and Anthes, J.C. The regulation and cellular functions of phosphatidylcholine hydrolysis. Biochem J (1990); **269**: 281-291.

Billah, M.M. Phospholipase D and cell signaling. Curr Opin Immunol (1993); **5**: 114-123.

Bitterman, H., Lefer, D.J. and Lefer, A.M. Novel mechanism of action of a prostacyclin enhancing agent in haemorrhagic shock. Naunyn Schmiedebergs Arch Pharmacol (1988); **337**: 679-686.

Blough, N.V. and Zafiriou, O.C. Reaction of superoxide with nitric oxide to form peroxynitrite in alkaline aqueous solution. Inorg Chem (1985); **24**: 3502-3504.

Bokoch, G.M. and Knaus, U.G. The role of small GTP-binding proteins in leukocyte function. Curr Opin Immunol (1994); **6**: 98-105.

Bolen, J.B. *et al.* The Src family of tyrosine protein kinases in hemopoietic signal transduction. FASEB J (1992); **6**: 3403-3409. Review.

Bolli, R., Patel, B.S. and Jeroudi, M.O. *et al.* Demonstration of free radical generation in "stunned myocardium" of intact dogs with the used of the spin trap alpha-phenyl N-tert butyl niytrone. J Clin Invest (1988); **82**: 476-485.

Bolli, R. Oxygen-derived free radicals and myocardial reperfusion injury: an overview. Cardiovasc Drugs Ther (1991); **5 Suppl 2**: 249-268.

Bonser, R.W., Thompson, N.T., Randall, R.W. and Garland, L.G. Phospholipase D activation is functionally linked to superoxide generation in the human neutrophil. [published erratum appears in Biochem J 1990 Feb 1; 265(3):932], Biochem J (1989); **264**: 617-620.

Bos, E.S., van der Doelen, A.A., van Rooy, N. and Schuurs, A.H. 3,3',5,5'-tetramethylbenzidine as an Ames test negative chromogen for horse-radish peroxidase in enzyme-immunoassay. J Immunoassay (1981); **2**: 187-204.

Bouma, M.G., van den Wildenberg, F.A. and Buurman, W.A. The anti-inflammatory potential of adenosine in ischemia-reperfusion injury: established and putative beneficial actions of a retaliatory metabolite. Shock (1997); **8**: 313-320.

Bouma, M.G., Jeunhomme, T.M., Boyle, D.L., Dentener, M.A., Voitenok, N.N., van den Wildenberg, F.A. and Buurman, W.A. Adenosine inhibits neutrophil degranulation in activated human whole blood: involvement of adenosine A_2 and A_3 receptors. J Immunol (1997); **158**: 5400-5408.

Bourin, M.C. and Lindahl, U. Glycosaminoglycans and the regulation of blood coagulation. Biochem J (1993); **289(2)**: 313-330.

Boxer, L.A., Haak, R.A. and Yang, H.H. *et al.* Membrane-bound lactoferrin alters the surface properties of polymorphonuclear leukocytes. J Clin Invest (1982); **70**: 1049-1057.

Boyden, S.V. The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leukocytes. J Exp Med (1962); **115**: 453-466.

Boyle, D.L., Sajjadi, F.G. and Firestein, G.S. Inhibition of synoviocyte collagenase gene expression by adenosine receptor stimulation. Arthritis Rheum (1996); **39**: 923-930.

Bradley, P.P., Priebat, D.A., Christensen, R.D. and Rothstein, G. Measurement of Cutaneous Inflammation: Estimation of Neutrophil Content with an Enzyme Marker. J Invest Dermatol (1982); **78**: 206-209.

Braunwald, E. and Kloner, R.A. The stunned myocardium: prolonged-postischaemic ventricular dysfunction. Circulation (1982); **66**: 1146-1149.

Braunwald, E. and Kloner, R.A. Myocardial reperfusion: a double-edged sword? J Clin Invest (1985); **76**: 1713-1719.

Bredt, D.S. and Synder, S.H. Isolation of nitric oxide synthase, a calmodulin-requiring enzyme. Proc Natl Acad Sci USA (1990); **87**: 6682-6685.

Brower, M.S., Levin, R.I. and Garry, K. Human neutrophil elastase modulates platelet function by limited proteolysis of membrane glycoproteins. J Clin Invest (1985); **75**: 657-666.

Brown, G.C. Nitric oxide regulates mitochondrial respiration and cell functions by inhibiting cytochrome oxidase. FEBS Letters (1995); **369**: 136-139.

Brown, K.K., Henson, P.M., Maclouf, J., Moyle, M., Ely, J.A. and Worthen, G.S. Neutrophil-platelet adhesion: relative roles of platelet P-selectin and neutrophil beta 2 (DC18) integrins. Am J Respir Cell Mol Biol (1998); **18**: 100-110.

Brune, B. and Lapetina, E.G. Activation of a cytosolic ADP-ribosyltransferase by nitric oxide generating agents. J Biol Chem (1989); **264**: 8455-8458.

Buerke, M., Weyrich, A.S., Zheng, Z., Gaeta, F.C., Forrest, M.J. and Lefer, A.M. Sialyl Lewis^{x}-containing oligosaccharide attenuates myocardial reperfusion injury in cats. J Clin Invest (1994); **3**: 1140-1148.

Bulkley, G.B., 1989. Mediators of splanchnic organ injury: overview and perspective. In: Splanchnic Ischaemia and Multiple Organ Failure. ed. A. Marston, G.B. Bulkey, R.G. Fiddian-Green, U.H. Haglund (Edward Arnold Publisher., London) pp. 191-193.

Bullough, D.A., Zhang, C., Montag, A., Mullane, K.M. and Young, M.A. Adenosine-mediated inhibition of platelet aggregation by acadesine. A novel antithrombotic mechanism *in vitro* and *in vivo*. J Clin Invest (1994); **94**: 1524-1532.

Bullough, D.A., Magill, M.J., Firestein, G.S. and Mullane, K.M. Adenosine activates A_2 receptors to inhibit neutrophil adhesion and injury to isolated cardiac myocytes. J Immunol (1995); **155**: 2579-2586.

Burkey, T.H. and Webster, R.O. Adenosine inhibits fMLP-stimulated adherence and superoxide anion generation by human neutrophils at an early step in signal transduction. Biochem Biophys Acta (1993); **1175**: 312-318.

Bush, L.R., Buja, L.M., Samowitz, W., Rude, R.E., Wathen, M., Tilton, G.D. and Willerson, J.T. Recovery of left ventricular segmental function after long-term reperfusion following temporary coronary occlusion in conscious dogs. Comparison of 2- and 4-hour occlusions. Circ Res (1983); **53**: 248-263.

Bush, P.A., Gonzalez, N.E., Griscavage, J.M. and Ignarro, L.J. Nitric oxide synthase from cerebellum catalyzes the formation of equimolar quantities of nitric oxide and citrulline from L-arginine. Biochem Biophys Res Commun (1992); **185**: 960-966.

Busse, R., Luckhoff, A. and Bassenge, E. Endothelium-derived relaxant factor inhibits platelet activation. Naunyn Schmeidebergs Arch Pharmacol (1987); **336**: 566-571.

Butcher, E.C. Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. Cell (1991); **67**: 1033-1036.

Butler, J., Parker, D., Pillai, R., Westaby, S., Shale, D.J. and Rocker, G.M. Effect of cardiopulmonary bypass on systemic release of neutrophil elastase and tumor necrosis factor. [published erratum appears in J Thorac Cardiovasc Surg 1993 June; 105 (6): 1056] [see comments], J Thorac Cardiovasc Surg (1993); **105**: 25-30.

Butt, E., Eigenthaler, M. and Genieser, H.G. (Rp)-8-pCPT-cGMPS, a novel Cyclic GMP-dependent protein kinase inhibitor. Eur J Pharmacol (Molecular Pharmacology Section) (1994); **269**: 265-268.

Buttrum, S.M., Hatton, R. and Nash, G.B. Selectin-mediated rolling of neutrophils on immobilized platelets. Blood (1993); **82**: 1165-1174.

Cai, T.Q. and Wright, S.D. Human leukocyte elastase is an endogenous ligand for the integrin CR3 (CD11b/CD18, Mac-1, alpha M beta 2) and modulates polymorphonuclear leukocyte adhesion. J Exp Med (1996); **184**: 1213-1223.

Camps, M., Carozzi, A., Schnabel, P., Scheer, A., Parker, P.J. and Gierschik, P. Isozyme-selective stimulation of phospholipase C-beta 2 by G protein beta gamma-subunits. Nature (1992); **360**: 684-686.

Carlos, M.T. and Harlan, M.J. Leukocyte-endothelial adhesion molecules. Blood (1994); **84**: 2068-2101.

Carp, H. Mitochondrial N-formylmethionyl proteins as chemoattractants for neutrophils. J Exp Med (1982); **155**: 264-275.

Casado, V., Casillas, T., Mallol, J., Canela, E.I., Lluis, C. and Franco, R. The adenosine receptors present on the plasma membrane of chromaffin cells are of the A_{2b} subtype. J Neurochem (1992); **59**: 425-431.

Cassimeris, L. and Zigmond, S.H. Chemoattractant stimulation of polymorphonuclear leukocyte locomotion. Semin Cell Biol (1990); **1**: 125-134.

Caterina, M.J. and Devreotes, P.N. Molecular insights into eukariotic chemotaxis. FASEB J, (1991); **5**: 3078-3085.

Cerletti, C., Rajtar, G., Marchi, E. and De Gaetano, G. Interaction between glycosaminoglycans, platelets, and leukocytes. SeminThrombo Hemost (1994); **20**: 245-253.

Cerletti, C., Evangelista, V., Molino, M. and de Gaetano, G. Platelet activation by polymorphonuclear leukocytes: role of cathepsin G and P-selectin. Thromb Haemost (1995); **74**: 218-223.

Chatelain, P., Latour, J.G., Tran, D., de Lorgeril, M., Dupras, G. and Bourassa, M. Neutrophil accumulation in experimental myocardial infarcts: relation with extent of injury and effect of reperfusion. Circulation (1987); **75**: 1083-1090.

Chen, J., Karlberg, K.E. and Sylven, C. Heparin enhances platelet aggregation irrespective of anticoagulation with citrate or with hirudin. Thromb Res (1992a); **67**: 253-262.

Chen, J. and Sylven, C. Heparin potentiation of collagen-induced platelet aggregation is related to the GPIIb/GPIIIa receptor and not to the GPIb receptor, as tested by whole blood aggregometry. Thromb Res (1992b); **66**: 111-120.

Cherry, P.D., Omar, H.A., Farrell, K.A., Stuart, J.S. and Wolin, M.S. Superoxide anion inhibits cGMP-associated bovine pulmonary arterial relaxation. Am J Physiol (1990); **259**: H1056-1062.

Chiang, P.H., Wu, S.N., Tsai, E.M., Wu, C.C., Shen, M.R. and Huang, C.H. Adenosine modulation of neurotransmission in penile erection. Br J Clin Pharmacol (1994); **38**: 357-362.

Chiariello, M., Golino, P., Cappelli-Bigazzi, H., Ambrosio, G., Tritto, I. and Salvatore, M. Reduction in infarct size by the prostacyclin analogue iloprost (ZK363Y4) after experimental coronary artery occlusion-reperfusion. Am Heart J (1988); **115**: 499-504.

Chignard, M., Selak, M.A. and Smith, J.B. Direct evidence for the existence of a neutrophil-derived platelet activator (neutrophilin). Proc Natl Acad Sci U S A (1986); **83**: 8609-8613.

Choay, J. Chemically synthesized heparin-derived oligosaccharides. Ann N Y Acad Sci (1989); **556**: 61-74.

Clancy, R.M., Leszczynska-Piziak, J. and Abramson, S.B. Nitric oxide, an endothelial cell relaxation factor, inhibits neutrophil superoxide anion production via a direct action on the NADPH oxidase. J Clin Invest (1992); **90**: 1116-1121.

Clancy, R.M., Leszczynska-Piziak, J. and Abramson, S.B. Nitric oxide stimulates the ADP-ribosylation of actin in human neutrophils. Biochem Biophys Res Commun (1993); **191**: 847-852.

Clancy, R., Leszczynsky, J., Amin, A., Levartovsky, D. and Abramson, S.B. Nitric oxide stimulates ADP ribosylation of actin in association with the inhibition of actin polymerization in human neutrophils. J Leukoc Biol (1995); **58**:196-202.

Clark, C., Forman, K.I., Kane, K.A., McDonald, F.M. and Parrat, J.R. Coronary artery ligation in anesthetised rats as a method for the production of experimental arrythmias and for the determination of infarct size. J Pharmacol Method (1980); **3**: 357-368.

Clark, R.A. The human neutrophil respiratory burst oxidase. J Infect Dis (1990); **161**: 1140-1147.

Cockroft, S. G protein regulated phospholipases C, D, and A_2-mediated signaling in neutrophils. Biochem Biophys Acta (1992); **1113**: 135-160.

Coetzee, W.A., Owen, P., Dennis, S.C., Saman, S. and Opie, L.H. Reperfusion damage: free radicals mediate delayed membrane changes rather than early ventricular arryhtmias. Cardiovas Res (1990); **24**: 156-164.

Cohen, A.B., Stevens, M.D., Miller, E.J., Atkinson, M.A. and Mullenbach, G. Generation of the neutrophil-activating peptide-2 by cathepsin G and cathepsin G-treated human platelets. Am J Physiol (1992); **263**: L249-256.

Coleman, R.A., Smith, W.L. and Narumiya, S. International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. Pharmacol Rev (1994); **46**: 205-229.

Corell, T., Pedersen, S.B., Lissau, B., Moilanen, E., Metsa-Ketela, T., Kankaanranta, H., Vuorinen, P., Vapaatalo, H., Rydell, E., Andersson, R. *et al.* Pharmacology of mesoionic oxatriazole derivatives in blood, cardiovascular and respiratory systems. Pol J Pharmacol (1994); **46**: 553-566.

Corretti, M.C., Koretsune, Y., Kusuoka, H., Chacko, V.P., Zweier, J.L. and Marban, E. Glycolytic inhibition and calcium overload as consequences of exogenously generated free radicals in rabbit hearts. J Clin Invest (1991); **88**: 1014-1025.

Corsini, A., Folco, G.C., Fumagalli, R., Nicosia, S., Noe, M.A. and Oliva, D. (5Z)-carbacyclin discriminates between prostacyclin-receptors coupled to adenylate cyclase in vascular smooth muscle and platelets. Br J Pharmacol (1987); **90**: 255-261.

Cramer, E.M., Meyer, D., le Menn, R. and Breton-Gorius, J. Eccentric localization of von Willebrand factor in an internal structure of platelet alpha-granule resembling that of Weibel-Palade bodies. Blood (1985); **66**: 710-713.

Cramer, E.M., Savidge, G.F., Vainchenker, W., Berndt, M.C., Pidard, D., Caen, J.P., Masse, J.M. and Breton-Gorius, J. Alpha-granule pool of glycoprotein IIb-IIIa in normal and pathologic platelets and megakaryocytes. Blood (1990); **75**: 1220-1227.

Cramer, E.M., Lu, H., Caen, J.P., Soria, C., Berndt, M.C. and Tenza, D. Differential redistribution of platelet glycoproteins Ib and IIb-IIIa after plasmin stimulation. [published erratum appears in Blood 1991 Jul 15; 78 (2): 545], Blood (1991); **77**: 694-699.

Cronstein, B.N., Kramer, S.B., Weissmann, G. and Hirschhorn, R. Adenosine: a physiological modulator of superoxide anion generation by human neutrophils. J Exp Med (1983); **158**: 1160-1177.

Cronstein, B.N., Rosenstein, E.D., Kramer, S.B., Weissmann, G. and Hirschhorn, R. Adenosine, a physiologic modulator of superoxide anion generation by human neutrophils. Adenosine acts via an A_2 receptor on human neutrophils. J Immunol (1985); **135**: 1366-1371.

Cronstein, B.N., Kramer, S.B., Rosenstein, E.D., Weissmann, G. and Hirschhorn, R. Adenosine modulates the generation of superoxide anion by stimulated human neutrophils via interaction with a specific cell surface receptor. Ann N Y Acad Sci (1985); **451**: 291-301.

Cronstein, B.N., Levin, R.I., Belanoff, J., Weissmann, G. and Hirschhorn, R. Adenosine: an endogenous inhibitor of neutrophil-mediated injury to endothelial cells. J Clin Invest (1986); **78**: 760-770.

Cronstein, B.N., Kramer, S.B., Rosenstein, E.D., Korchak, H.M., Weissmann, G. and Hirschhorn, R. Occupancy of adenosine receptors raises cyclic AMP alone and in synergy with occupancy of chemoattractant receptors and inhibits membrane depolarization. Biochem J (1988); **252**: 709-715.

Cronstein, B.N., Daguma, L., Nichols, D., Hutchinson, A.J. and Williams, M. The adenosine/neutrophil paradox resolved: Human neutrophils possess both A_1 and A_2 receptors that promote chemotaxis and inhibit O_2^- generation, respectively. J Clin Invest (1990); **85**: 1150-1157.

Cronstein, B.N., 1991. Role of adenosine and adenine nucleotides in the biological system. eds. S. Imai and M. Nakazawi (Elsevier, Amsterdam) pp. 515.

Cronstein, B.N., Levin, R.I., Philips, M., Hirschhorn, R., Abramson, S.B. and Weissmann, G. Neutrophil adherence to endothelium is enhanced via adenosine A_1 receptors and inhibited via adenosine A_2 receptors. J Immunol (1992); **148**: 2201-2206.

Cronstein, B.N. and Weissmann, G. The adhesion molecules of inflammation. Arthritis Rheum (1993); **36**: 147-157.

Cronstein, B.N. Adenosine, an endogenous anti-inflammatory agent. J Applied Physiol: Respiratory, Environmental & Exercise Physiology (1994); **76**: 5-13.

Cronstein, B.N., Naime, D. and Firestein, G. The antiinflammatory effects of an adenosine kinase inhibitor are mediated by adenosine. Arthritis Rheum (1995); **38**: 1040-1045.

Crooke, S.T., Monia, B.P., Chiang, M.Y. and Bennett, C.F. Leukotriene receptors and mechanisms of signal transduction. Ann N Y Acad Sci (1991); **629**: 120-124.

Cunningham, F.M., Smith, M.J.H., Ford-Hutchinson, A.W. and Walker, J.R. Migration of peritoneal polymorphonuclear leukocytes in the rat. J Pathol (1979); **128**: 1979.

Curie, D.C., S.H., S. and Peters, A.M. *et al.* Indium-111-labeled granulocyte accumulation in respiratory tract of patients with bronciectasis. Lancet (1987); **1**: 1335-1339

Cutler, L., Rodan, G. and Feinstein, M.B. Cytochemical localization of adenylate cyclase and of calcium ion, magnesium ion-activated ATPases in the dense tubular system of human blood platelets. Biochim Biophys Acta (1978); **542**: 357-371.

Daniel, J.L., Dangelmaier, C. and Smith, J.B. Evidence for a role for tyrosine phosphorylation of phospholipase C gamma 2 in collagen-induced platelet cytosolic calcium mobilization. Biochem J (1994); **302**: 617-622.

Dawes, J., Pumphrey, C.W., McLaren, K.M., Prowse, C.V. and Pepper, D.S. The *in vivo* release of human platelet factor 4 by heparin. Thromb Res (1982); **27**: 65-76.

De Duve, C. Biochemical studies on the occurrence, biogenesis and life history of mammalian peroxisomes. J Histochem Cytochem (1973); **21**: 941-948.

de Fougerolles, A.R. and Springer, T.A. Intercellular adhesion molecule 3, a third adhesion counter-receptor for lymphocyte function-associated molecule 1 on resting lymphocytes. J Exp Med (1992); **175**: 185-190.

de Fougerolles, A.R., Qin, X. and Springer, T.A. Characterization of the function of intercellular adhesion molecule (ICAM)-3 and comparison with ICAM-1 and ICAM-2 in immune responses. J Exp Med (1994); **179**: 619-629.

de Lorgeril, M., Basmadjian, A., Lavallee, M., Clement, R., Millette, D., Rousseau, G. and Latour, J.G. Influence of leukopenia on collateral flow, reperfusion flow, reflow ventricular fibrillation, and infarct size in dogs. Am Heart J (1989); **117**: 523-532.

Dehmer, G.J., Fisher, M., Tate, D.A., Teo, S. and Bonnem, E.M. Reversal of heparin anticoagulation by recombinant platelet factor 4 in humans. Circulation (1995); **91**: 2188-2194.

Dehmer, G.J., Lange, R.A., Tate, D.A., Pirwitz, M., Daniel, W., Fisher, M. and Bonnem, E.M. Randomized trial of recombinant platelet factor 4 versus protamine for the reversal of heparin anticoagulation in humans. Circulation (1996); **94**: II347-352.

Del Maschio, A., Evangelista, V., Rajtar, G., Chen, Z.M., Cerletti, C. and De Gaetano, G. Platelet activation by polymorphonuclear leukocytes exposed to chemotactic agents. Am J Physiol (1990); **258**: H870-879.

Del Maschio, A., Bazzoni, G. and Bertele, V. Investigation of platelet/leukocyte interaction by whole blood aggregometry. [letter], Thromb Haemost (1990); **64**: 487.

Desai, U.R., Wang, H.M. and Linhardt, R.J. Substrate specificity of the heparin lyases from Flavobacterium heparinum. Arch Biochem Biophys (1993); **306**: 461-468.

Despotis, G.J., Summerfield, A.L., Joist, J.H., Goodnough, L.T., Santoro, S.A., Zimmermann, J.J. and Lappas, D.G. *In vitro* reversal of heparin effect with heparinase: evaluation with whole blood prothrombin time and activated partial thromboplastin time in cardiac surgical patients. Anesth Analg (1994); **79(4)**: 670-674.

Despotis, G.J., Joist, J.H., Hogue, C.W.J., Alsoufiev, A., Kater, K., Goodnough, L.T., Santoro, S.A., Spitznagel, E., Rosenblum, M. and Lappas, D.G. The impact of heparin concentration and activated clotting time monitoring on blood conservation. A prospective, randomized evaluation in patients undergoing cardiac operation [see comments]. J Thorac Cardiovasc Surg (1995); **110**: 46-54.

Devreotes, P.N. and Zigmond, S.H. Chemotaxis in eukaryotic cells: a focus on leukocytes and Dictyostelium. Annu Rev Cell Biol (1988); **4**: 649-686.

Dewald, B., Thelen, M. and Baggiolini, M. Two transduction sequences are necessary for neutrophil activation by receptor agonists. J Biom Chem (1988); **263**: 16179-16184.

Diacovo, T.G., Roth, S.J., Buccola, J.M., Bainton, D.F. and Springer, T.A. Neutrophil rolling, arrest, and transmigration across activated, surface-adherent platelets via sequential action of P-selectin and the beta 2-integrin CD11b/CD18. Blood (1996); **88**: 146-157.

Diamond, J. and Chu, E.B. A novel cyclic GMP-lowering agent, LY 83583, blocks carbachol-induced cyclic GMP elevation in rabbit atrial strips without blocking the negative inotropic effects of carbachol. Can J Physiol Pharmacol (1985); **63**: 908-911.

Diamond, M.S., Staunton, D.E., Marlin, S.D. and Springer, T.A. Binding of the integrin Mac-1 (CD11b/CD18) to the third immunoglobulin-like domain of ICAM-1 (CD54) and its regulation by glycosylation. Cell, (1991); **65**: 961-971.

Diamond, M.S. and Springer, T.A. A subpopulation of Mac-1 (CD11b/CD18) molecules mediates neutrophil adhesion to ICAM-1 and fibrinogen. J Cell Biol (1993); **120**: 545-556.

DiPierro, F.V., Neely, C.F., Kong, M. and Gardner, T.J. A_1 Adenosine receptor antagonists reduce ischemia reperfusion injury in a feline heart regional infarct model. Circulation (1995); **92**: I-513 (Abstr. No. 2446).

Dong, Y.J., Jones, R.L. and Wilson, N.H. Prostaglandin E receptor subtypes in smooth muscle: agonist activities of stable prostacyclin analogues. Br J Pharmacol (1986); **87**: 97-107.

Downey, J.M. Free radicals and their involvement during long-term myocardial ischaemia and reperfusion. Annu Rev Physiol (1990); **52**: 487-504.

Downey, G.P. Mechanisms of leukocyte motility and chemotaxis. Curr Opin Immunol (1994); **6**: 113-124.

Doyle, M.P. and Hoekstra, J.W. Oxidation of nitrogen oxides by bound dioxygen in hemoproteins. J Inorg Biochem (1981); **14**: 351-358.

Dreyer, W.J., Michal, L.H., West, S., Smith, C.W., Rothlein, R., Rosbein, R.D., Anderson, D.C. and Entman, E.L. Neutrophil accumulation in ischaemic canine myocardium: insights into time course, distribution and mechanism of localization during early reperfusion. Circulation (1991); **84**: 400-411.

Dubey, R.K., Gillespie, D.G., Osaka, K., Suzuki, F. and Jackson, E.K. Adenosine inhibits growth of rat aortic smooth muscle cells. Possible role of A_{2b} receptor. Hypertension (1996); **27**: 786-793.

Dusi, S., Della-Bianca, V., Grzeskowiak, M. and Rossi, F. Relationship between phosphorylation and translocation to the plasma membrane of p47phox and p67phox and activation of the NADPH oxidase in normal and Ca^{2+} depleted human neutrophils. Biochem J (1993); **290**: 173-178.

Eberle, M., Traynor-Kaplan, A.E., Sklar, L.A. and Norgauer, J. Is there a relationship between phosphatidylinositol trisphosphate and F-actin polymerization in human neutrophils? J Biol Chem (1990); **265**: 16725-16728.

El Benna, J., Faust, L.P. and Babior, B.M. The phosphorylation of the respiratoy burst oxidase component p47phox during neutrophil activation: phosphorylation of sites recognized by protein kinase C and by proline-directed kinases. J Biol Chem (1994); **269**: 23431-23436.

Eliseev, V.V., Krylova, I.B. and Evdokimova, N.R. Effect of adenosine on the size of experimental myocardial infarction and "no re-flow" areas. Kardiologiia (1988); **28**: 98-99.

Engelberg, H. Heparin and the atherosclerotic process. Pharmacol Rev (1984); **36**: 91-110.

Engelberg, H. Update on the relationship of heparin to atherosclerotic and its thrombotic complications. Semin Throm Hemost (1988); **14(suppl.)**: 88-100005.

Engelberg, H., 1990. Heparin and the prevention of atherosclerotis. (Wiley-Liss, New York) p. 217pp.

Engler, R., Schmid-Schonbein, G.W. and Pravilec, R.S. Leukocyte capillary plugging in myocardial ischaemia and reperfusion in the dogs. Am J Pathol (1983); **111**: 98-111.

Engler, R.L., Dahlgren, M.D., Peterson, M.A., Dobbs, A. and Schmid-Schoenbein, G. Accumulation of polymorphonuclear leukocytes during 3 hour experimental myocardial ischaemia. Am J Physiol (1986); **251**: H93-H100.

Engler, R.L. and Covell, J.W. Granulocytes cause reperfusion ventricular dysfunction after 15 minutes after ischaemia in the dogs heart. Circ Res (1987); **61**: 20-28.

Engler, R.L. and Gruber, H.E., 1991. Adenosine: An Autocoid in the heart and cardiovascular system. ed. H.A. Fozzard (Raven, New York) pp. 1745-1764.

English, D. Involvement of phosphatidic acid, phosphatidate phosphohydrolase, and inositide-specific phospholipase D in neutrophil stimulus-response pathways. J Lab Clin Med (1992); **120**: 520-526.

Engstad, C.S., Gutteberg, T.J. and Osterud, B. Modulation of blood cell activation by four commonly used anticoagulants. Thromb Haemost (1997); **77**: 690-696.

Entman, M.L., Michael, L., Rossen, R.D., Dreyer, W.J., Anderson, D.C., Taylor, A.A. and Smith, C.W. Inflammation in the course of early myocardial ischemia. FASEB J (1991); **5**: 2529-2537.

Evangelista, V., Rajtar, G., de Gaetano, G., White, J.G. and Cerletti, C. Platelet activation by fMLP-stimulated polymorphonuclear leukocytes: the activity of cathepsin G is not prevented by antiproteinases. Blood (1991); **77**: 2379-2388.

Evangelista, V., Piccardoni, P., Maugeri, N., De Gaetano, G. and Cerletti, C. Inhibition by heparin of platelet activation induced by neutrophil-derived cathepsin G. Eur J Pharmacol (1992); **216**: 401-405.

Evangelista, V., Manarini, S., Rotondo, S., Martelli, N., Polischuk, R., McGregor, J.L., de Gaetano, G. and Cerletti, C. Platelet/polymorphonuclear leukocyte interaction in dynamic conditions: evidence of adhesion cascade and cross talk between P-selectin and the beta 2 integrin CD11b/CD18. Blood (1996); **88**: 4183-4194.

Falcon, C., Arnout, J. and Vermylen, J. Platelet aggregation in whole blood-studies with a platelet counting technique-methodological aspects and some applications. [see comments]. Thromb Haemost (1989); **61**: 423-428.

Fallman, M., Andersson, R. and Andersson, T. Signaling properties of CR3 (CD11b/CD18) and CR1 (CD35) in relation to phagocytosis of complement-opsonized particles. J Immunol (1993); **151**: 330-338.

Fantone, J.C. and Kinnes, D.A. Prostaglandin E_1 and prostaglandin I_2 modulation of superoxide production by human neutrophils. Biochem Biophys Res Commun (1983); **113**: 506-512.

Farber, N.E., Pieper, G.M., Thomas, J.P. and Gross, G.J. Beneficial effects of iloprost in the stunned canine myocardium. Circ Res (1988); **62**: 204-215.

Faull, R.J., Kovach, N.L., Harlan, J.M. and Ginsberg, M.H. Stimulation of integrin-mediated adhesion of T lymphocytes and monocytes: two mechanisms with divergent biological consequences. J Exp Med (1994); **179**: 1307-1316.

Feelisch, M. and Noack, E.A. Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. Eur J Pharmacol (1987); **139**: 19-30.

Feelisch, M., Noack, E. and Schroder, H. Explanation of the discrepancy between the degree of organic nitrate decomposition nitrite formation and guanylate cyclase stimulation. Eur Heart J (1988); **9 (Suppl A)**: 57-62.

Feelisch, M., Ostrowski, J. and Noack, E. On the mechanism of NO release from sydnonimines. J Cardiovasc Pharmacol (1989); **14 (suppl. 11)**: s13-s22.

Feelisch, M. The biochemical pathways of nitric oxide formation from nitrovasodilators: appropriate choice of exogenous NO donors and aspects of preparation and handling of aqueous NO solutions. J Cardiovasc Pharmacol (1991); **17 (suppl. 3)**: s25-s33.

Felding-Habermann, B. and Cheresh, D.A. Vitronectin and its receptors. Curr Opin Cell Biol (1993); **5**: 864-868.

Feoktistov, I.A., Pol, S., Khollister, A.S., Robertson, D. and Biadzhoni, I. Mechanism of action of adenosine on intracellular calcium and platelet aggregation. Kardiologia (1991); **31**: 76-79.

Feoktistov, I. and Biaggioni, I. Adenosine A_{2b} receptors evoke interleukin-8 secretion in human mast cells. An enprofylline-sensitive mechanism with implications for asthma. J Clin Invest (1995); **96**: 1979-1986.

Feoktistov, I., Sheller, J.R. and Biaggioni, I. Adenosine A_{2b} receptors in human lung mast cells as a target for antiasthmatic methylxanthines. FASEB J (1996); **10**: A1232.

Fernandez, H.N., Henson, P.M., Otani, A. and Hugli, T.E. Chemotactic response to human C3a and C5a anaphylatoxins. Evaluation of C3a and C5a leukotaxis *in vitro* and under stimulated *in vivo* conditions. J Immunol (1978); **120**: 109-115.

Ferrari, R. and Hearse, D.J. Reperfusion injury: Does it exist and does it have clinical relevance? Journal of Thrombosis & Thrombolysis (1997); **4**: 25-34.

Ferrer-Lopez, P., Renesto, P., Schattner, M., Bassot, S., Laurent, P. and Chignard, M. Activation of human platelets by C5a-stimulated neutrophils: a role for cathepsin G. Am J Physiol (1990); **258**: C1100-1107.

Ferrer-Lopez, P., Renesto, P., Prevost, M.C., Gounon, P. and Chignard, M. Heparin inhibits neutrophil-induced platelet activation via cathepsin G. J Lab Clin Med (1992); **119**: 231-239.

Firestein, G.S., Bullough, D.A., Erion, M.D., Jimenez, R., Ramirez-Weinhouse, M., Barankiewicz, J., Smith, C.W., Gruber, H.E. and Mullane, K.M. Inhibition of neutrophil adhesion by adenosine and an adenosine kinase inhibitor. The role of selectins. J Immunol (1995); **154**: 326-334.

Fitzpatrick, T.M., Alter, I., Corey, E.J., Ramwell, P.W., Rose, J.C. and Kot, P.A. Cardiovascular responses to PGI_2 (prostacyclin) in the dog. Circ Res (1978); **42**: 192-194.

Flaherty, J.T., Weisfeldt, M.L., Bulkley, B.H., Gardner, T.J., Gott, V.L. and Jacobus, W.E. Mechanisms of ischemic myocardial cell damage assessed by phosphorus-31 nuclear magnetic resonance. Circulation (1982); **65**: 561-570.

Flaherty, J.T., Becker, L.C., Bulkley, B.H., Weiss, J.L., Gerstenblith, G., Kallman, C.H., Silverman, K.J., Wei, J.Y., Pitt, B. and Weisfeldt, M.L. A randomized prospective trial of intravenous nitroglycerin in patients with acute myocardial infarction. Circulation (1983); **68**: 576-588.

Forman, M.B., Puett, D.W., Cates, C.U., McCroskey, D.E., Beckman, J.K., Geen, H.I. and Virmani, R. Glutathione redox pathway and reperfusion injury: Effect of N-acetylcysteine on infarct size and ventricular function. Circulation (1988); **78**: 202-213.

Forman, M.B., Puett, D.W. and Virmani, R. Endothelial and myocardial injury during ischemia and reperfusion: pathogenesis and therapeutic implications. J Am Coll Cardiol (1989); **13**: 450-459.

Forman, M.B., Virmani, R. and Puett, D.W. Mechanisms and therapy of myocardial reperfusion injury. Circulation (1990); **81 (Suppl IV)**: IV69-IV78.

Forman, M.B., Velasco, C.E. and Jackson, E.K. Adenosine attenuates reperfusion injury following regional myocardial ischaemia. Cardiovas Res (1993); **27**: 9-17.

Forsberg, E.J., Feuerstein, G., Shohami, E. and Pollard, H.B. Adenosine triphosphate stimulates inositol phospholipid metabolism and prostacyclin formation in adrenal medullary endothelial cells by means of P_2-purinergic receptors. Proc Natl Acad Sci U S A (1987); **84**: 5630-5634.

Fox, J.E., Boyles, J.K., Berndt, M.C., Steffen, P.K. and Anderson, L.K. Identification of a membrane skeleton in platelets. J Cell Biol (1988); **106**: 1525-1538.

Fredholm, B.B., Abbracchio, M.P., Burnstock, G., Daly, J.W., Harden, T.K., Jacobson, K.A., Leff, P. and Williams, M. Nomenclature and classification of purinoceptors. Pharmacol Rev (1994); **46**: 143-156.

Fredholm, B.B., Zhang, Y. and van der Ploeg, I. Adenosine A_{2A} receptors mediate the inhibitory effect of adenosine on formyl-Met-Leu-Phe-stimulated respiratory burst in neutrophil leucocytes. Naunyn Schmiedebergs Arch Pharmacol (1996); **354**: 262-267.

Frick, G.P. and Lowenstein, J.M. Studies of 5'-nucleotidases in the perfused rat heart, including measurements of the enzyme in perfused skeletal muscle and liver. J Biol Chem (1976); **251**: 6372-6378.

Fuji, C., Yanagi, S., Sada, K., Nagai, K., Taniguchi, T. and Yamamura, H. Involvement of protein-tyrosine kinase p72syk in collagen-induced signal transduction in platelets. Eur J Biochem (1994); **226**: 243-248.

Fujiwara, H., Ashraf, M., Sato, S. and Millard, R.W. Transmural cellular damage and blood flow distribution in early ischemia in pig hearts. Circ Res (1982); **51**: 683-693.

Fung, K.P., Wu, T.W., Zeng, L.H. and Wu, J. The opposing effects of an inhibition of nitric oxide synthesis and a donor of nitric oxide in rabbits undergoing myocardial ischaemic reperfusion. Life Sci (1994); **54(26)**: PL491-6.

Furchgott, R.F. and Zawadzki, J.V. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature (1980); **288**: 373-376.

Furchgott, R.F., Carvalho, M., Khan, M.T. and Matsunaga, K. Evidence for endothelium-dependent vasodilation of resistance vessels by acetylcholine. Blood Vessels (1987); **24**: 145-149.

Furchgott, R.F., 1988. Studies on relaxation of rabbit aorta by sodium nitrite: the basis for the proposal that acid-activatable inhibitory factor from retractor penis is inorganic nitrite and the endothelium-derived relaxing factor is nitric oxide, in: Vasodilatation: Vascular smooth muscle, Peptides, Autonomic Nerves and Endothelium, ed. P.M. Vanhoutte (Raven Press, New York) pp. 401-414.

Furchgott, R.F., Khan, M.T. and Jothianandan, K.D., 1990. Comparison of properties of nitric oxide and endothelium-derived relaxing factors: some cautionary findings, in: Endothelium-Derived Relaxing Factors, eds. G.M. Rubanyi and P.M. Vanhoutte (Karger, Basal).

Furie, M.B., Tancinco, M.C. and Smith, C.W. Monoclonal antibodies to leukocyte integrins CD11a/CD18 and CD11b/CD18 or intercellular adhesion molecule-1 inhibit chemoattractant-stimulated neutrophil transendothelial migration *in vitro*. Blood, (1991); **78**: 2089-2097.

Furie, M.B., Burns, M.J., Tancinco, M.C., Benjamin, C.D. and Lobb, R.R. E-selectin (endothelial-leukocyte adhesion molecule-1) is not required for the migration of neutrophils across IL-1-stimulated endothelium *in vitro*. J Immunol (1992); **148**: 2395-2404.

Furlong, B., Henderson, A.H., Lewis, M.J. and Smith, J.A. Endothelium-derived relaxing factor inhibits *in vitro* platelet aggregation. Br J Pharmacol (1987); **90**: 687-692.

Gaboury, J., Woodman, R.C., Granger, D.N., P., R. and Kubes, P. Nitric oxide prevents leukocyte adherence: role of superoxide. Am J Physiol (1993); **265: (**Heart Circ**.** Physiol. **34);** H862-867.

Gallagher, K.P., Buda, A.J., Pace, D., Gerren, R.A. and Shlafer, M. Failure of superoxide dismutase and catalase to alter size of infarction in conscious dogs after 3 hours of occlusion followed by reperfusion. Circulation (1986); **73**: 1065-1076.

Gallin, J. Interferon-gamma in the management of chronic granulomatous disease. Rev Infect Dis (1991); **13**: 973-978.

Ganz, T. and Lehrer, R.I. Defensins. Pharmacol Ther (1995); **66**: 191-205.

Garber, D.L. Guanylyl cyclase receptors and their endocrine, paracrine, and autocrine ligands. Cell (1992); **74**: 1-4.

Garg, U.C. and Hassid, A. Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured vascular smooth muscle cells. J Clin Invest (1989); **83**: 1774-1777.

Garthwaite, J. and Boulton, C.L. Nitric oxide signaling in the central nervous system. Annu Rev Physiol (1995); **57**: 683-706.

Gaudry, M., Caon, A.C., Gilbert, C., Lille, S. and Naccache, P.H. Evidence for the involvement of tyrosine kinases in the locomotory responses of human neutrophils. J Leukoc Biol (1992); **51**: 103-108.

Gawaz, M.P. *et al.* Ligand bridging mediates integrin alpha IIb beta 3 (platelet GPIIB-IIIA) dependent homotypic and heterotypic cell-cell interactions. J Clin Invest (1991); **88**: 1128-1134.

Gawaz, M., Reininger, A. and Neumann, F.J. Platelet function and platelet-leukocyte adhesion in symptomatic coronary heart disease. Effects of intravenous magnesium. Thromb Res (1996); **83**: 341-349.

Gelas, P., Von Tscharner, V., Record, M., Baggiolini, M. and Chap, H. Human neutrophil phospholipase D activation by N-formylmethionyl-leucylphenylalanine reveals a two-step process for the control of phosphatidylcholine breakdown and oxidative burst. Biochem J (1992); **287**: 67-72.

George, J.N., Pickett, E.B., Saucerman, S., McEver, R.P., Kunicki, T.J., Kieffer, N. and Newman, P.J. Platelet surface glycoproteins. Studies on resting and activated platelets and platelet membrane microparticles in normal subjects, and observations in patients during adult respiratory distress syndrome and cardiac surgery. J Clin Invest (1986); **78**: 340-348.

Gerard, C. and Gerard, N.P. The pro-inflammatory seven-transmembrane segment receptors of the leukocyte. Curr Opin Immunol (1994); **6**: 140-145.

Glusa, E. and Markwardt, F. Platelet functions in recombinant hirudin-anticoagulated blood. Haemostasis (1990); **20**: 112-118.

Goldstein, I., Hoffstein, S., Gallin, J. and Weissmann, G. Mechanisms of lysosomal enzyme release from human leukocytes: microtubule assembly and membrane fusion induced by a component of complement. Proc Natl Acad Sci U S A (1973); **70**: 2916-2920.

Goldstein, I.M., 1992. Complement: biologically active products. Vol. second edition (Raven, New York) pp. 55-74.

Gomez-Cambronero, J., Huang, C.K., Bonak, V.A., Wang, E., Casnellie, J.E., Shiraishi, T. and Sha'afi, R.I. Tyrosine phosphorylation in human neutrophils. Biochem Biophys Res Commun (1989); **162**: 1478-1485.

Gorman, R.R., Fitzpatrick, F.A. and Miller, O.V. A selective thromboxane synthetase inhibitor blocks the cAMP lowering activity of PGH_2. Biochem Biophys Res Commun (1977); **79**: 305-313.

Goto, M., Miura, T., Iliodoromitis, E.K., O'Leary, E.L., Ishimoto, R., Yellon, D.M. and Iimura, O. Adenosine infusion during early reperfusion failed to limit myocardial infarct size in a collateral deficient species. Cardiovasc Res (1991); **25**: 943-949.

Gotsch, U., Jager, U., Dominis, M. and Vestweber, D. Expression of P-selectin on endothelial cells is upregulated by LPS and TNF-alpha *in vivo*. Cell Adhes Commun (1994); **2**: 7-14.

Gravlee, G.P., Haddon, W.S., Rothberger, H.K., Mills, S.A., Rogers, A.T., Bean, V.E., Buss, D.H., Prough, D.S. and Cordell, A.R. Heparin dosing and monitoring for cardiopulmonary bypass. A comparison of techniques with measurement of subclinical plasma coagulation. J Thorac Cardiovasc Surg (1990); **99**: 518-527.

Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S. and Tannenbaum, S.R. Analysis of nitrate, nitrite, and [15 N] nitrate in biological fluids. Anal Biochem (1982); **126(1)**: 131-138.

Grenegard, M., Gustafsson, M.C., Andersson, R.G. and Bengtsson, T. Synergistic inhibition of thrombin-induced platelet aggregation by the novel nitric oxide-donor GEA 3175 and adenosine. Br J Pharmacol (1996); **118**: 2140-2144.

Gryglewski, R.J., Korbut, R., Kalecinska, A. and Zembowicz, A. Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. Nature (1986); **320**: 454-456.

Gryglewski, R.J., Szczeklik, A. and Wandzilak, M. The effect of six prostaglandins, prostacyclin and iloprost on generation of superoxide anions by human polymorphonuclear leukocytes stimulated by zymosan or formyl-methionyl-leucyl-phenylalanine. Biochem Pharmacol, (1987); **36**: 4209-4213.

Guerci, A.D., Gerstenblith, G., Brinker, J.A., Chandra, N.C., Gottlieb, S.O., Bahr, R.D., Weiss, J.L., Shapiro, E.P., Flaherty, J.T., Bush, D.E. *et al.* A randomized trial of intravenous tissue plasminogen activator for acute myocardial infarction with subsequent randomization to elective coronary angioplasty. N Engl J Med (1987); **317**: 1613-1618.

Gundry, S.R., Drongowski, R.A., Klein, M.D. and Coran, A.G. Postoperative bleeding in cardiovascular surgery. Does heparin rebound really exist? Am Surg (1989); **55**: 162-165.

Gunther, G.R. and Herring, M.B. Inhibition of neutrophil superoxide production by adenosine released from vascular endothelial cells. Ann Vasc Surg (1991); **5**: 325-330.

Ha, K.S. and Exton, J.H. Activation of actin polymerization by phosphatidic acid derived from phosphatidylcholine in IIC9 fibroblasts. J Cell Biol (1993); **123**: 1789-1796.

Hamberg, M., Svensson, J. and Samuelsson, B. Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides. Proc Natl Acad Sci U S A (1975); **72**: 2994-2998.

Hamburger, S.A. and McEver, R.P. GMP-140 mediates adhesion of stimulated platelets to neutrophils. Blood (1990); **75**: 550-554.

Handin, R.I., Karabin, R. and Boxer, G.J. Enhancement of platelet function by superoxide anion. J Clin Invest (1977); **59**: 959-965.

Harker, L.A., Malpass, T.W., Branson, H.E., Hessel, E.A. and Slichter, S.J. Mechanism of abnormal bleeding in patients undergoing cardiopulmonary bypass: acquired transient platelet dysfunction associated with selective alpha-granule release. Blood (1980); **56**: 824-834.

Harker, L.A. Bleeding after cardiopulmonary bypass [editorial]. N Engl J Med (1986); **314**: 1446-1448.

Harlan, M., Schwartz, B.R., Reidy, M.A., Schwartz, S.M., Ochs, H.D. and Harker, L.A. Activated neutrophils disrupt endothelial monolayer integrity by an oxygen radical-independent mechanism. Lab Invest (1985); **52**: 141-150.

Harlan, J.M. Neutrophil-mediated vascular injury. Acta Med Scand Suppl (1987); **715**: 123-129.

Harrison, J.E. and Schultz, J. Studies on the chlorinating activity of myeloperoxidase. J Biol Chem (1976); **251**: 1371-1374.

Harrison, P., Savidge, G.F. and Cramer, E.M. The origin and physiological relevance of alpha-granule adhesive proteins. Br J Haematol (1990); **74**: 125-130.

Hashimoto, H., Negishi, M. and Ichikawa, A. Identification of a prostacyclin receptor coupled to the adenylate cyclase system via a stimulatory GTP-binding protein in mouse mastocytoma P-815 cells. Prostaglandins (1990); **40**: 491-505.

Hashimoto, K., Pearson, P.J., Schaff, H.V. and Cartier, R. Endothelial cell dysfunction after ischemic arrest and reperfusion: a possible mechanism of myocardial injury during reflow. J Thorac Cardiovasc Surg (1991); **102**: 688-694.

Hattori, R., Hamilton, K.K., Fugate, R.D., McEver, R.P. and Sims, P.J. Stimulated secretion of endothelial von Willebrand factor is accompanied by rapid redistribution to the cell surface of the intracellular granule membrane protein GMP-140. J Biol Chem (1989); **264**: 7768-7771.

Hearse, D.J. and Bolli, R. Reperfusion-induced injury. Manifestations, mechanisms, and clinical relevance. Cardiovascular Research (1992); **26**: 101-108.

Hegyi, E. and Nakeff, A. Ultrastructural localization of platelet factor 4 in rat megakaryocytes and platelets by gold-labeled antibody detection. Exp Hematol (1989); **17**: 223-228.

Heinzel, B., John, M., Klatt, P., Bohme, E. and Mayer, B. Ca^{2+}/calmodulin-depenent formation of hydrogen peroxide by brain nitric oxide synthase. Biochem J (1992); **281**: 627-630.

Hempel, S.L., Haycraft, D.L., Hoak, J.C. and Spector, A.A. Reduced prostacyclin formation after reoxygenation of anoxic endothelium. Am J Physiol (1990); **259**: C738-745.

Henson, P.M. and Johnson, R.M.J. Tissue injury in inflammation. J Clin Invest (1987); **79**: 669-674.

Hess, M.L. and Kukreja, R.C. Free radicals, calcium homeostasis, heat shock proteins, and myocardial stunning. Ann Thorac Cardiovasc Surg (1995); **60**: 760-766.

Heyndrickx, G.R., Millard, R.W. and McRitchie, R.J. *et al.* Regional myocardial functional and electrophysiological alterations after brief coronary artery occlusion in conscious dogs. J Clin Invest (1975); **56**: 978-985.

Heyworth, P., Curnutte, J., Nauseef, W.M., *et al.* Neutrophil nicotinamide adenine dinucleotide phosphate oxidase assembly. J Clin Invest (1991); **87**: 352-356.

Hickey, M.J., Williams, S.A. and Roth, G.J. Human platelet glycoprotein IX: an adhesive prototype of leucine-rich glycoproteins with flank-center-flank structures. Proc Natl Acad Sci U S A (1989); **86**: 6773-6777.

Hickey, M.J., Hagen, F.S., Yagi, M. and Roth, G.J. Human platelet glycoprotein V: characterization of the polypeptide and the related Ib-V-IX receptor system of adhesive, leucine-rich glycoproteins. Proc Natl Acad Sci U S A (1993); **90**: 8327-8331.

Hieda, N., Toki, Y., Sugiyama, S., Ito, T., Satake, T. and Ozawa, T. Prostaglandin I_2 analogue and propranolol prevent ischaemia induced mitochondrial dysfunction through the stabilisation of lysosomal membranes. Cardiovasc Res (1988); **22**: 219-225.

Higgs, E.A., Moncada, S., Vane, J.R., Caen, J.P., Michel, H. and Tobelem, G. Effect of prostacyclin (PGI_2) on platelet adhesion to rabbit arterial subendothelium. Prostaglandins (1978); **16**: 17-22.

Hind, C.R., Griffin, J.F., Pack, S., Latchman, Y.E., Drake, H.F., Jones, H.M., Brostoff, J., Dormandy, T.L. and Treasures, T. Effect of cardiopulmonary bypass on circulating concentrations of leucocyte elastase and free radical activity. Cardiovasc Res, (1988); **22**: 37-41.

Hirata, M., Ushikubi, F. and Narumiya, S. Prostaglandin I receptor and prostaglandin D receptor. J Lipid Mediat Cell Signal (1995); **12**: 393-404.

Hitt, A.L. and Luna, E.J. Membrane interactions with the actin cytoskeleton. Curr Opin Cell Biol (1994); **6**: 120-130.

Hohlfeld, T., Strobach, H. and Schror, K. Stimulation of endogenous prostacyclin protects the reperfused pig myocardium from ischemic injury. J Pharmacol Exp Ther (1993); **264**: 397-405.

Holm, P., Kankaanranta, H., Metsa-Ketela, T. and Moilanen, E. Radical releasing properties of nitric oxide donors GEA 3162, SIN-1 and S-nitroso-N-acetylpenicillamine. Eur J Pharmacol (1998); **346**: 97-102.

Holmsen, H., 1994. Platelet secretion and energy metabolism, in: Hemostasis and Thrombosis: Basic Principles and Clinical Practice, ed. J.H. Colman RW, VJ Marder and EW Salzman (J B Lippincott Company, Philladelphia) pp. 524-545.

Holmsen, H. Significance of testing platelet functions *in vitro*. Eur J Clin Invest (1994); **24 Suppl 1**: 3-8.

Hook, M., Kjellen, L., Johanssen, S. and Robinson. Cell-surface glycosaminoglycans. Annu Rev Biochem (1984); **53**: 847-869.

Hori, M. and Kitakaze, M. Adenosine, the heart, and coronary circulation. Hypertension (1991); **18**: 565-574.

Hsu-Lin, S., Berman, C.L., Furie, B.C., August, D. and Furie, B. A platelet membrane protein expressed during platelet activation and secretion. Studies using a monoclonal antibody specific for thrombin-activated platelets. J Biol Chem (1984); **259**: 9121-9126.

Huang, C.K. Protein kinases in neutrophils: a review. Membr Biochem (1989); **8**: 61-79.

Huang, M.M., Indik, Z., Brass, L.F., Hoxie, J.A., Schreiber, A.D. and Brugge, J.S. Activation of Fc gamma RII induces tyrosine phosphorylation of multiple proteins including Fc gamma RII. J Biol Chem (1992); **267**: 5467-5473.

Hudson, K.F. A phenomenon of paradox: myocardial reperfusion injury. Heart Lung (1994); **23**, 384-393; quiz 394-386.

Huizinga, T.W., Dolman, K.M., van der Linden, N.J., Kleijer, M., Nuijens, J.H., von dem Borne, A.E. and Roos, D. Phosphatidylinositol-linked FcRIII mediates exocytosis of neutrophil granule proteins, but does not mediate initiation of the respiratory burst. J Immunol (1990); **144**: 1432-1437.

Hurst, J.K. and Barrette, W.C.J. Leukocytic oxygen activation and microbicidal oxidative toxins. Crit Rev Biochem Mol Biol (1989); **24**: 271-328.

Hussain, T. and Mustafa, S.J. Regulation of adenosine receptor system in coronary artery: functional studies and cAMP. Am J Physiol (1993); **264**, H441-447.

Hwang, S.B. Specific receptors of platelet-activating factor, receptor heterogeneity, and signal transduction mechanisms. J Lipid Med (1990); **2**: 123-158.

Ignarro, L.J., Lippton, H., Edwards, J., Baricos, W.H., Hyman, A.L., Kadowitz, P.J. and Gruetter, C.A. Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitropruside and nitric oxide: Evidence for the involvement of S-nitrosothilos as active intermediates. J Pharmacol Exp Ther (1981); **218**: 739-749.

Ignarro, L.J., Byrns, R.E., Buga, G.M. and Wood, K.S. Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical to those of nitric oxide radical. Circ Res (1987); **61**: 866-879.

Ignarro, L.J., Byrns, R.E. and Wood, K.S., 1988. Biological and pharmacological properties of endothelium-derived relaxing factor and its similarity to nitric oxide radicals, in: Vasodilatation: Vascular smooth muscle, Peptides, Autonomic Nerves and Endothelium, ed. P.M. Vanhoutte (Raven Press, New York) pp. 427-436.

Ignarro, L.J. Biological actions and properties of endothelium-derived nitric oxide formed and released from artery and vein. Circ Res (1989); **65**: 1-21.

Ignarro, L.J. Endothelium-derived nitric oxide: actions and properties. FASEB J (1989); **3**: 31-36.

Ignarro, L.J. Signal transduction mechanisms involving nitric oxide. Biochem Pharmacol (1991); **41**: 485-490.

Iha, S., Orita, S., Orita, K., Kanno, T., Utsumi, T., Sayo, E.F., Inoue, M. and Utsumi, K. Oxygen-dependent inhibition of neutrophil respiratory burst by nitic oxide. Free Radic Res (1996); **25**: 489-498.

Ikeda, Y., Handa, M., Kawano, K., Kamata, T., Murata, M., Araki, Y., Anbo, H., Kawai, Y., Watanabe, K., Itagaki, I. *et al.* The role of von Willebrand factor and fibrinogen in platelet aggregation under varying shear stress. J Clin Invest (1991); **87**: 1234-1240.

Ilton, M.K., Taylor, M.L., Misso, N.L., Thompson, P.J. and Hung, J. Neutrophil cathepsin G modulates platelet P-selectin expression and inhibits P-selectin-mediated platelet-neutrophil adhesion. Clin Sci (Colch) (1998); **94**: 437-445.

Imhof, B.A. and Dunon, D. Leukocyte migration and adhesion. Adv Immunol (1995); **58**: 345-416.

Ischiropoulos, H., Zhu, L., Chen, J., Tsai, M., Martin, J.C., Smith, C.D. and Beckman, J.S. Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. Arch Biochem Biophys (1992); **298**: 431-437.

Ito, B.R., Schmid-Schonbein, G. and Engler, R.L. Effects of leukocyte activation on myocardial vascular resistance. Blood Cells (1990); **16**: 145-163; discussion 163-146.

Itoh, K., Nakao, A., Kishimoto, W. and Takagi, H. Heparin effects on superoxide production by neutrophils. Eur Surg Res (1995); **27**: 184-188.

Iuliano, L., Colavita, A.R., Leo, R., Pratico, D. and Violi, F. Oxygen free radicals and platelet activation. Free Radic Biol Med (1997); **22**: 999-1006.

Iwamoto, T., Umemura, S., Toya, Y., Uchibori, T., Kogi, K., Takagi, N. and Ishii, M. Identification of adenosine A_2 receptor cAMP-system in human aortic endothelial cells. Biochem Biophys Res Commun (1994); **199**: 905-910.

Jackson, W.F., Konig, A., Dambacher, T. and Busse, R. Prostacyclin-induced vasodilation in rabbit heart is mediated by ATP- sensitive potassium channels. Am J Physiol (1993); **264**: H238-243.

Jacobson, K. Adenosine A_3 receptors: novel ligands and paradoxical effects. Trends Pharmacol Sci (1998); **19**: 184-192.

Jaconi, M.E., Theler, J.M., Schlegel, W., Appel, R.D., Wright, S.D. and Lew, P.D. Multiple elevations of cytosolic-free Ca^{2+} in human neutrophils: initiation by adherence receptors of the integrin family. J Cell Biol (1991); **112**: 1249-1257.

Jacques, L.B. Heparins. Anionic polyelectrolyte drugs. Pharmacol Rev (1979); **31**: 99-166.

Janero, D.R. Ischaemic heart disease and antioxidants: Mechanistic aspects of oxidative injury and its prevention. Cri Rev Food Sci Nut (1995); **35**: 65-81.

Jennings, R.B. and Reimer, K.A. Factors involved in salvaging ischaemic myocardium: Effect of reperfusion of arterial blood. Circulation (1983); **68**: I25-I36.

Jeroudi, M.O., Triana, F.J., Patel, B.S. and Bolli, R. Effect of superoxide dismutase and catalase, given separately, on myocardial "stunning". Am J Physiol (1990); **259**: H889-901.

Jeroudi, M.O., Hartley, C.J. and Bolli, R. Myocardial reperfusion injury: role of oxygen radicals and potential therapy with antioxidants. Am J Cardiol (1994); **73**: 2B-7B.

Jesaitis, A.J., Naemura, J.R., Sklar, L.A., Cochrane, C.G. and Painter, R.G. Rapid modulation of N-formyl chemotactic peptide receptors on the surface of human granulocytes: formation of high-affinity ligand-receptor complexes in transient association with cytoskeleton. J Cell Biol (1984); **98**: 1378-1387.

Jesaitis, A.J., Tolley, J.O. and Allen, R.A. Receptor-cytoskeleton interactions and membrane traffic may regulate chemoattractant-induced superoxide production in human granulocytes. J Biol Chem (1986); **261**: 13662-13669.

Jesaitis, A.J., Tolley, J.O., Bokoch, G.M. and Allen, R.A. Regulation of chemoattractant receptor interaction with transducing proteins by organizational control in the plasma membrane of human neutrophils. J Cell Biol, (1989); **109**: 2783-2790.

Jestice, H.K., Humphreys, J.E., English, T.A., Hoggarth, C.E. and Wells, F.C. A comparative study of prostacyclin infusion given before and during cardiopulmonary bypass to assess the first pass effect of the circuit on platelet number and function. Eur J Cardiothorac Surg (1990); **4**: 40-44.

Jin, J.G., Murthy, K.S., Grider, J.R. and Makhlouf, G.M. Activation of distinct cAMP-and cGMP-dependent pathways by relaxant agents in isolated gastric muscle cells. Am J Physiol (1993); **264**: G470-G477.

Johnson, G.I., Furlan, L.E., Aoki, N. and Lefer, A.M. Endothelium and myocardial protecting actions of taprostene, a stable prostacyclin analogue, after acute myocardial ischemia and reperfusion in cats. Circulation Research (1990); **66**: 1362-1370.

Johnson, I.G., Tsao, P.S. and Lefer, A.M. Cardioprotective effects of authentic nitric oxide in myocardial ischemia with reperfusion. Critical Care Medicine (1991); **19**: 244-252.

Jolly, S.R., Kane, W.J., Bailie, M.D., Abrams, G.D. and Lucchesi, B.R. Canine myocardial reperfusion injury: Its reduction by the combined administration of superoxide dismutase and catalase. Circ Res (1984); **54**: 277-285.

Jolly, S.R., Kane, W.J., Hook, B.G., Abrams, G.D., Kunkel, S.L. and Lucchesi, B.R. Reduction of myocardial infarct size by neutrophil depletion: effect of duration of occlusion. Am Heart J (1986); **112**: 682-690.

Jones, G. and Hurley, J.V. The effect of prostacyclin on the adhesion of leukocytes to injured vascular endothelium. J Pathol (1984); **142**: 51-59.

Jonzon, B., Karlberg, K.E., Querrol-Ferrer, V. and Sylven, C. Effects of adenosine on *ex vivo* filtragometry platelet aggregation in relation to plasma levels. Acta Physiol Scand (1997); **159**: 245-248.

Jordan, J.E., Zhao, Z.Q., Sato, H., Taft, S. and Vinten-Johansen, J. Adenosine A_2 receptor activation attenuates reperfusion injury by inhibiting neutrophil accumulation, superoxide generation and coronary endothelial adherence. J Pharmacol Exp Ther (1997); **280**: 301-309.

Juneau, C.F., Ito, B.R., del Balzo, U. and Engler, R.L. Severe neutrophil depletion by leucocyte filters or cytotoxic drug does not improve recovery of contractile function in stunned porcine myocardium [see comments]. Cardiovasc Res (1993); **27**: 720-727.

Jungi, T.W., Spycher, M.O., Nydegger, U.E. and Barandun, S. Platelet-leukocyte interaction: selective binding of thrombin-stimulated platelets to human monocytes, polymorphonuclear leukocytes, and related cell lines. Blood (1986); **67**: 629-636.

Kadota, L., Yui, Y., Hattori, R., Uchizumi, H. and Kawai, C. A new relaxing factor in supernatant of incubated rat peritoneal neutrophils. Am J Physiol (1991); **260**: H967-H972.

Kainoh, M., Imai, R., Umetsu, T., Hattori, M. and Nishio, S. Prostacyclin and beraprost sodium as suppressors of activated rat polymorphonuclear leukocytes. Biochem Pharmacol (1990); **39**: 477-484.

Kako, K.J. Free radical effects on membrane protein in myocardial ischemia/reperfusion injury. J Mol Cell Cardiol (1987); **19**: 209-211.

Kanaho, Y., Kanoh, H., Saitoh, K. and Nozawa, Y. Phospholipase D activation by platelet-activating factor, leukotriene B4, and formyl-methionyl-leucyl-phenylalanine in rabbit neutrophils. J Immunol (1991); **146**: 3536-3541.

Kanaho, Y., Nakai, Y., Katoh, M. and Nozawa, Y. The phosphatase inhibitor 2,3-diphosphoglycerate interferes with phospholipase D activation in rabbit peritoneal neutrophils. J Biol Chem (1993); **268**: 12492-12497.

Kankaanranta, H., Rydell, E., Petersson, A.S., Holm, P., Moilanen, E., Corell, T., Karup, G., Vuorinen, P., Pedersen, S.B., Wennmalm, A. and Metsa-Ketela, T. Nitric oxide-donating properties of mesoionic 3-aryl substituted oxatriazole-5-imine derivatives. Br J Pharmacol (1996); **117**: 401-406.

Kao, R.C.-C., Wehner, N.G. and Skubitz, K.M. *et al.* Proteinase 3: A distinct polymorphonuclear leukocyte protease, which produced emphysema. J Clin Invest (1988); **82**: 1693-1973.

Kaplan, D.R., Chao, F.C., Stiles, C.D., Antoniades, H.N. and Scher, C.D. Platelet alpha granules contain a growth factor for fibroblasts. Blood (1979); **53**: 1043-1052.

Kaplan, S.S., Billiar, T., Curran, R.D., Zdziarski, U.E., Simmons, R.L. and Basford, R.E. Inhibition of chemotaxis with N^G-Monomethyl-L-Arginine: A role for cyclic GMP. Blood (1989); **74**: 1885-1887.

Karlsson, K. and Marklund, S.L. Heparin-induced release of extracellular superoxide dismutase to human blood plasma. Biochem J (1987); **242**: 55-59.

Karlsson, C., Nygren, H. and Braide, M. Exposure of blood to biomaterial surfaces liberates substances that activate polymorphonuclear granulocytes. J Lab Clin Med (1996); **128**: 496-505.

Karnovski, M.L. Metabolic basis of phagocytic activity. Physiol Rev (1962); **42**: 143-168.

Karup, G., Preikschat, H., Wilhelmsen, E.S., Pederson, S.B., Marcinkiewicz, E., Cieslik, K. and Gryglewski, R.J. Mesoionic oxatriazole derivatives-a new group of NO-donors. Pol J Pharmacol (1994); **46**: 541-552.

Kase, H., Iwahashi, K., Nakaishi, S., Matsuda, Y., Yamada, K., Tkahashi, M., Murakata, C., Sato, A. and Kaneko, M. K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. Biochem Biosphy Res Commun (1987); **14**: 436-440.

Katsuyama, M., Sugimoto, Y., Namba, T., Irie, A., Negishi, M., Narumiya, S. and Ichikawa, A. Cloning and expression of a cDNA for the human prostacyclin receptor. FEBS Lett (1994); **344**: 74-78.

Kawahito, K., Kobayashi, E., Iwasa, H., Misawa, Y. and Fuse, K. Platelet aggregation during cardiopulmonary bypass evaluated by a laser light-scattering method. Ann Thorac Surg (1999); **67**: 79-84.

Keely, P.J. and Parise, L.V. The alpha2 beta1 integrin is a necessary co-receptor for collagen- induced activation of Syk and the subsequent phosphorylation of phospholipase C gamma 2 in platelets. J Biol Chem (1996); **271**: 26668-26676.

Kenakin, T.P. and Beek, D. Measurement of antagonist affinity for purine receptors of drugs producing concomitant phosphodiesterase blockade: the use of pharmacologic resultant analysis. J Pharmacol Exp Ther (1987); **243**: 482-486.

Kenakin, T.P. and Beek, D. The effects on Schild regressions of antagonist removal from the receptor compartment by a saturable process. Naunyn Schmiedebergs Arch Pharmacol (1987); **335**: 103-108.

Kenakin, 1987. Functional antagonism and synergism, in: Pharmacologic analysis of drug-receptor interaction (Raven Press); New York, pp. 245-257.

Kessels, G.C.R., Gervaix, A., Lew, P.D. and Verhoeven, A.J. The chymotrypsin inhibitor carbobenzyloxy-leucine-tyrosine-chloromethylketone interferes with phospholipase D activation induced by formyl-methionyl-leucyl-phenylalanine in human neutrophils. J Biol Chem (1991a); **266**: 15870-15875.

Kessels, G.C., Roos, D. and Verhoeven, A.J. fMet-Leu-Phe induced activation of phospholipase D in human neutrophils. J Biol Chem (1991b); **266**: 23152-23156.

Kestin, A.S., Valeri, C.R., Khuri, S.F., Loscalzo, J., Ellis, P.A., MacGregor, H., Birjiniuk, V., Ouimet, H., Pasche, B., Nelson, M.J. *et al.* The platelet function defect of cardiopulmonary bypass [see comments]. Blood (1993); **82**: 107-117.

Khalfi, F., Gressier, B., Brunet, C., Dine, T., Luyckx, M., Cazin, M. and Cazin, J.C. Involvement of the extracellular calcium in the release of elastase and the human neutrophils oxidative burst. Cell Mol Biol (Noisy-le-grand) (1996); **42**: 1211-1218.

Khuri, S.F., Wolfi, J.A. and Josa, M. *et al.* Hematologic changes during and after cardiopulmonary bypass and their relationship to the bleeding time and nonsurgical blood loss. J Thorac Cardiovasc Surg (1992); **104**. 94-107.

Khuri, S.F., Michelson, A.D. and Valeri, C.R., 1994. The effect of cardiopulmonary bypass on hemostasis and coagulation, in: Thrombosis and Haemorrhage, eds. J. LOscalzo and e. Schafer AI (MA: Blackwell Scientific, Cambridge) pp. 1051-1073.

Khuri, S.F., Valeri, C.R., Loscalzo, J., Weinstein, M.J., Birjiniuk, V., Healey, N.A., MacGregor, H., Doursounian, M. and Zolkewitz, M.A. Heparin causes platelet dysfunction and induces fibrinolysis before cardiopulmonary bypass [see comments]. Ann Thorac Surg (1995); **60**: 1008-1014.

Kim, M.S. and Akera, T. O_2 free radicals: cause of ischemia-reperfusion injury to cardiac Na^+- K^+-ATPase. Am J Physiol (1987); **252**: H252-257.

Kimberly, R.P., Ahlstrom, J.W., Click, M.E. and Edberg, J.C. The glycosyl phosphatidylinositol-linked FcRIII mediates transmembrane signaling events distinct from FcRII. J Exp Med (1990); **171**: 1239-1255.

Kirchhofer, D., Languino, L.R., Ruoslahti, E. and Pierschbacher, M.D. Alpha 2 beta 1 integrins from different cell types show different binding specificities. J Biol Chem (1990); **265**: 615-618.

Kishimoto, T., Warnock, R. and Jutila, M.A. *et al.* Antibodies against human neutrophil LECAM-1 (LAM-1/Leu-8/DREG-56 antigen) and endothelial cell ELAM-1 inhibit a common CD18-dependent adhesion pathway *in vitro*. Blood (1991); **78**: 805-811.

Kishimoto, T.K. and Anderson, D.C., 1992. The role of integrins in inflammation, in: Inflammation: Basic Principles and Clinical Correlates, eds. J.I. Gallin, I.M. Goldstien and R. Snyderman (Raven Press, New York) pp. 353-406.

Kjeldsen, L., Sengelov, H., Lollike, K., Nielsen, M.H. and Borregaard, N. Isolation and characterization of gelatinase granules from human neutrophils. Blood (1994); **83**: 1640-1649.

Kjeldsen, L., Bainton, D.F., Sengelov, H. and Borregaard, N. Identification of neutrophil gelatinase-associated lipocalin as a novel matrix protein of specific granules in human neutrophils. Blood (1994); **83**: 799-807.

Kloner, R.A., Ganobe, C.E. and Jennings, R.B. The "no-reflows phenomenon" after temporary coronary occlusion in the dog. J Clin Invest (1974); **54**: 1496-1508.

Kloner, R.A., Ellis, S.G., Lange, R. and Braunwald, E. Studies of experimental coronary artery reperfusion: effects on infarct size, myocardial function, biochemistry, ultrastructure and microvascular damage. Circulation (1983); **68 Suppl. 1**: 8-15.

Kloner, R.A., Przyklenk, K. and Whittaker, P. Deleterious effects of oxygen radicals in ischemia/reperfusion. Resolved and unresolved issues. Circulation (1989); **80**: 1115-1127.

Kloner, R.A. Does reperfusion injury exist in humans? J Am Coll Cardiol (1993); **21**: 537-545.

Knaus, U.G., Heyworth, P.G., Kinsella, B.T., Curnutte, J.T. and Bokock, G.M. Purification and characterization of Rac 2. A cytosolic GTP-binding protein that regulates human neutrophil NADPH oxidase. J Biol Chem (1992); **267**: 23575-23582.

Knezevic, I., Dieter, J.P. and Le Breton, G.C. Mechanism of inositol 1,4,5-trisphosphate-induced aggregation in saponin-permeabilized platelets. J Pharmacol Exp Ther (1992); **260**: 947-955.

Knowles, R.G., Palacios, M., Palmer, R.M. and Moncada, S. Formation of nitric oxide from L-arginine in the central nervous system: a transduction mechanism for stimulation of the soluble guanylate cyclase. Proc Natl Acad Sci U S A (1989); **86**: 5159-5162.

Knowles, R.G. and Moncada, S. Nitric oxide as a signal in blood vessels. Trends Biochem Sci (1992); **17**: 399-402.

Kobzir, L., Reid, M.B., Bredt, D.S. and Stamler, J.S. Nitric oxide in skeletal muscle. Nature (London) (1994); **372**: 546-548.

Kolb, h. and Kolb-Bachofen, V. Nitric oxide: a pathogenic factor in autoimmunity. Immunology Today (1992); **13**: 157-160.

Koltai, M. and Braquet, P.G. Involvement of PAF in atherogenesis. Brief review. Agents Actions Suppl, (1992); **37**: 333-339.

Konno, N., Yanagishita, T., Geshi, E. and Katagiri, T. Degradation of the cardiac sarcoplasmic reticulum in acute myocardial ischemia. Jpn Circ J (1987); **51**: 411-420.

Konya, L., Kekesi, V., Juhasz-Nagy, S. and Feher, J. The effect of superoxide dismutase in the myocardium during reperfusion in the dog. Free Radic Biol Med (1992); **13**: 527-532.

Krishnamurthi, S., Westwick, J. and Kakkar, V.V. Regulation of human platelet activation-analysis of cyclooxygenase and cyclic AMP-dependent pathways. Biochem Pharmacol (1984); **33**: 3025-3035.

Ku, D.D. Coronary vascular reactivity after acute myocardial ischemia. Science (1982); **218**: 576-578.

Kubes, P., Suzuki, M. and Granger, D.N. Nitric oxide: an endogenous modulator of leukocyte adhesion. Proc Natl Acad Sci U S A (1991); **88**: 4651-4655.

Kuna, P., Reddigari, S.R., Schall, T.J., Rucinski, D., Sadick, M. and Kaplan, A.P. Characterisation of the human basophil response to cytokines, growth factors and histamine releasing factors of the intercrine/chemokine family. J Immunol (1993); **150**: 1932-1943.

Kusunoki T, Higashi, Hosoi S, Hata D, Sugie K, Mayumi M and H., M. Tyrosine phosphorylation and its possible role in superoxide production by human neutrophils stimulated with FMLP and IgG. Biochem Biophys Res Commun (1992); **183**: 789-796.

Kwon, N.S., Nathan, C.F., Gilker, C., Griffith, O.W., Matthews, D.E. and Stuehr, D.J. L-citrulline production from L-arginine by macrophage nitric oxide synthase. The ureido oxygen derives from dioxygen. J Biol Chem (1990); **265**: 13442-13445.

Kwon, N.S., Stuehr, D.J. and Nathan, C.F. Inhibition of tumor cell ribonucleotide reductase by macrophage-derived nitric oxide. J Exp Med (1991); **17**: 761-768.

Lages, B. and Weiss, H.J. Dependence of human platelet functional responses on divalent cations: aggregation and secretion in heparin- and hirudin-anticoagulated platelet-rich plasma and the effects of chelating agents. Thromb Haemost (1981); **45**: 173-179.

Lalor, P. and Nash, G.B. Adhesion of flowing leucocytes to immobilized platelets. Br J Haematol (1995); **89**: 725-732.

LaRosa, C.A., Rohrer, M.J., Benoit, S.E., Rodino, L.J., Barnard, M.R. and Michelson, A.D. Human neutrophil cathepsin G is a potent platelet activator. J Vasc Surg (1994a); **19**: 306-318; discussion 318-309.

LaRosa, C.A., Rohrer, M.J., Benoit, S.E., Barnard, M.R. and Michelson, A.D. Neutrophil cathepsin G modulates the platelet surface expression of the glycoprotein (GP) Ib-IX complex by proteolysis of the von Willebrand factor binding site on GPIb alpha and by a cytoskeletal-mediated redistribution of the remainder of the complex. Blood (1994b); **84**: 158-168.

Larsen, E., Palabrica, T., Sajer, S., Gilbert, G.E., Wagner, D.D., Furie, B.C. and Furie, B. PADGEM-dependent adhesion of platelets to monocytes and neutrophils is mediated by a lineage-specific carbohydrate, LNF III (CD15). Cell (1990); **63**: 467-474.

Larson, R.S. and Springer, T.A. Structure and function of leukocyte integrins. Immunol Rev (1990); **114**: 181-217.

Larson, D.F., Bowers, M. and Schechner, H.W. Neutrophil activation during cardiopulmonary bypass in paediatric and adult patients. Perfusion (1996); **11**: 21-27.

Lasky, L.A. Selectins: interpreters of cell-specific carbohydrate information during inflammation. Science (1992); **258**: 964-969.

Lasley, R.D. and Mentzer, R.M.J. Protective effects of adenosine in the reversibly injured heart. Ann Thorac Surg (1995); **60**: 843-846.

Laudanna, C., Rossi, F. and Berton, G. Effect of inhibitors of distinct signalling pathways on neutrophil O_2^- generation in response to tumor necrosis factor-alpha, and antibodies against CD18 and CD11a: evidence for a common and unique pattern of sensitivity to wortmannin and protein tyrosine kinase inhibitors. Biochem Biophys Res Commun (1993); **190**: 935-940.

Laudanna, C., Campbell, J.J. and Butcher, E.C. Role of Rho in chemoattractant-activated leukocyte adhesion through integrins. Science (1996); **271**: 981-983.

Lawrence, M.B. and Springer, T.A. Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. Cell (1991); **65**: 859-873.

Lee, D.K.H., Faunce, D., Henry, D., Sturm, R.J. and Rimele, T. Neutrophil-derived relaxing factor relaxes vascular smooth muscle through a cGMP-mediated mechanism. Life Sci (1990); **46**: 1531-1538.

Lee, L.M., Sheu, J.R. and Yen, M.H. BN-063, a newly synthesized adenosine A_1 receptor agonist, attentuates myocardial reperfusion injury in rats. Eur J Pharmacol (1995); **279**: 251-256.

Lee, S.B. and Rhee, S.G. Significance of PIP_2 hydrolysis and regulation of phospholipase C isozymes. Curr Opin Cell Biol (1995); **7**: 183-189.

Lefer, A.M., Ogletree, M.L., Smith, J.B., Silver, M.J., Nicolaou, K.C., Barnette, W.E. and Gasic, G.P. Prostacyclin: a potentially valuable agent for preserving myocardial tissue in acute myocardial ischemia. Science (1978); **200**: 52-54.

Lefer, A.M. Physiologic and pathophysiologic role of cyclo-oxygenase metabolites of arachidonic acid in circulatory disease states. Cardiovasc Clin (1987); **18**: 85-99.

Lefer, A.M. and Darius, H. Taprostene (CG-4203). Cardiovascular Drug Reviews (1989); **7**: 39-51.

Lefer, A.M. and Aoki, N. Leukocyte-dependent and leukocyte-independent mechanisms of impairment of endothelium-mediated vasodilation. Blood Vessels (1990); **27**: 162-168.

Lefer, A.M., Tsao, P.S., Lefer, D.J. and Ma, X.L. Role of endothelial dysfunction in the pathogenesis of reperfusion injury after myocardial ischemia. FASEB J (1991); **5**: 2029-2034.

Lefer, A.M. and Lefer, D.J. Pharmacology of the endothelium in ischemia-reperfusion and circulatory shock. Annu Rev Pharmacol Toxicol (1993); **33**: 71-90.

Lefer, D.J., Nakanishi, K., Johnston, W.E. and Vinten-Johansen, J. Antineutrophil and myocardial protecting actions of a novel nitric oxide donor after acute myocardial ischemia and reperfusion in dogs. Circulation (1993); **88**: 2337-2350.

Lefer, A.M. and Lefer, D.J. Therapeutic role of nitric oxide donors in the treatment of cardiovascular disease. Drugs of the Future (1994); **19**: 665-672.

Lefer, A.M., Weyrich, A.S. and Buerke, M. Role of selectins, a new family of adhesion molecules, in ischaemia-reperfusion injury. Cardiovasc Res, (1994); **28**: 289-294.

Lefer, A.M., Murohara, T. *et al.* Effects of taprostene on neutrophil-endothelium interactions in isolated coronary arteries. Methods Find Exp Clin Pharmacol (1994); **16**: 623-631.

Lefer, D.J., Flynn, D.M., Jeffords, P.R., Vok, D. and Buda, A.J. A sialyl lewis^{x} -containing carbohydrate reduces infarct size following myocardial ischaemia and prolonged reperfusion. Circ, (1995); **92**:

Lefer, A.M. Attenuation of myocardial ischemia-reperfusion injury with nitric oxide replacement therapy. Ann Thorac Surg, (1995); **60**: 847-851.

Lefer, A.M. Role of selectins in myocardial ischemia-reperfusion injury. Ann Thorac Surg (1995); **60**: 773-777.

Lefer, D.J., Scalia, R., Campbell, B., Nossuli, T., Hayward, R., Salamon, M., Grayson, J. and Lefer, A.M. Peroxynitrite inhibits leukocyte-endothelial cell interactions and protects against ischemia-reperfusion injury in rats. J Clin Invest (1997); **99**: 684-691.

Lehrer, R.I., Ganz, T. and Selsted, M.E. Phagocytic defects. Abnormalities outside of the respiratory burst. Oxygen-independent bactericidal systems. Mechanism and disorders. Hematol Oncol Clin North Am (1988); **2**: 159-169.

Leijh, P.C., van den Barselaar, T., van Zwet, T., Daha, R. and van Furth, R. Requirement of extracellular complement and immunoglobulin for intracellular killing of microorganisms by human monocytes. J Clin Invest (1979); **63**: 772-784.

Leone, A.M., Palmer, R.M., Knowles, R.G., Francis, P.L., Ashton, D.S. and Moncada, S. Constitutive and inducible nitric oxide synthases incorporate molecular oxygen into both nitric oxide and citrulline. J Biol Chem (1991); **266**: 23790-23795.

Levine, P.H., Weinger, R.S., Simon, J., Scoon, K.L. and Krinsky, N.I. Leukocyte-platelet interaction. Release of hydrogen peroxide by granulocytes as a modulator of platelet reactions. J Clin Invest (1976); **57**: 955-963.

Levy, O., Ooi, C.E. and Elsbach, P. *et al.* Antibacteril proteins of granulocytes differ in interaction with endotoxin. Comparision of bactericidal/permeability-increasing protein, p15s, and defensins. J Immunol (1995); **154**: 5403-5410.

Levy, J.H., Cormack, J.G. and Morales, A. Heparin neutralization by recombinant platelet factor 4 and protamine. Anesth Analg (1995a); **81**: 35-37.

Lew, D.P. Receptor signalling and intracellular calcium in neutrophil activation. Eur J Clin Invest (1989); **19**: 338-346.

Lew, P.D. Receptors and intracellular signaling in human neutrophils. Am Rev Respir Dis (1990); **141**: 5127.

Li, N., Batzer, A., Daley, R., Yajnik, V., Skolink, E., Chardin, P., Bar-Sagi, D., Margolis, B. and Schlessinger, J. Gaunine-Nucleotide-Releasing Factor hSOS 1 Binds To Grb-1 and Links Receptor Tyrosine Kinases to Ras Signalling. Nature (1993); **363**: 85-88.

Li, N., Goodall, A.H. and Hjemdahl, P. A sensitive flow cytometric assay for circulating platelet-leucocyte aggregates. Br J Haematol (1997); **99**: 808-816.

Liang, B.T. Adenosine receptors and cardiovascular function. Trends Cardiovasc Med (1992); **2**:

Liang, B.T. and Haltiwanger, B. Adenosine A_{2a} and A_{2b} receptors in cultured fetal chick heart cells. High- and low-affinity coupling to stimulation of myocyte contractility and cAMP accumulation. Circ Res (1995); **76**: 242-251.

Liang, B.T. and Morley, J.F. A new cyclic AMP-independent, Gs-mediated stimulatory mechanism via the adenosine A_{2a} receptor in the intact cardiac cell. J Biol Chem (1996); **271**: 18678-18685.

Liew, F.Y. and Cox, F.E.G. Nonspecific defence mechanism: the role of nitric oxide. Immunoparasitology Today (1991); A17-A21.

Litt, M.R., Jeremy, R.W., Weisman, H.F., Winkelstein, J.A. and Becker, L.C. Neutrophil depletion limited to reperfusion reduces myocardial infarct size after 90 minutes of ischemia. Evidence for neutrophil-mediated reperfusion injury. Circulation (1989); **80**: 1816-1827.

Loftus, J.C., O'Toole, T.E., Plow, E.F., Glass, A., Frelinger, A.L.d. and Ginsberg, M.H. A beta 3 integrin mutation abolishes ligand binding and alters divalent cation-dependent conformation. Science (1990); **249**: 915-918.

Lopez Farre, A., Riesco, A., Moliz, M., Egido, J., Casado, S., Hernando, L. and Caramelo, C. Inhibition by L-arginine of the endothelium-mediated increase in cytosolic calcium in human neutrophils. Biochem Biophys Res Commun (1991); **178**: 884-891.

Lucchesi, B.R., Werns, S.W. and Fantone, J.C. The role of the neutrophil and free radicals in ischemic myocardial injury. J Mol Cell Cardiol (1989); **21**: 1241-1251.

Lucchesi, B.R. Modulation of leukocyte-mediated myocardial reperfusion injury. Annu Rev Physiol (1990); **52**: 561-576.

Luscinskas, F.W., Cybulsky, M.I., Kiely, J.M., Peckins, C.S., Davis, V.M. and Gimbrone, M.A.J. Cytokine activated human endothelial monolayers support enhanced neutrophil transmigration via a mechanism involving both endothelial-leukocyte adhesion molecule-1. J Immunol (1991); **146**: 1617-1625.

Ma, X.L., Tsao, P.S. and Lefer, A.M. Antibody to CD-18 exerts endothelial and cardiac protective effects in myocardial ischemia and reperfusion. J Clin Invest (1991); **88**,: 1237-1243.

Ma, X.L., Johnson, G.d. and Lefer, A.M. Low doses of superoxide dismutase and a stable prostacyclin analogue protect in myocardial ischemia and reperfusion. J Am Coll Cardiol (1992); **19**: 197-204.

Ma, X.L., Lefer, D.J. and Lefer, A.M. *et al.* Coronary endothelial and cardiac protective effects of a monoclonal antibody to intercellular adhesion molecule-1 in myocardial ischemia and reperfusion. Circulation (1992); **86**: 937-946.

Ma, X.L., Weyrich, A.S. and Lefer, D.J. *et al.* Diminished basal nitric oxide release after mycardial ischemia and reperfusion promotes neutrophil adherence to coronary endothelium. Circ Res (1993); **72**: 403-412.

Ma, X.I., Weyrich, A.S. and Lefer, D.J. *et al.* Monoclonal antibody to L-selectin attenuates neutrophil accumulation and protects ischemic reperfused cat myocardium. Circulation (1993); **88**: 649-658.

MacIntyre, D.E., Hoover, R.L., Smith, M., Steer, M., Lynch, C., Karnovsky, M.J. and Salzman, E.W. Inhibition of platelet function by cis-unsaturated fatty acids. Blood (1984); **63**: 848-857.

MacKenzie, W.M., Hoskin, D.W. and Blay, J. Adenosine inhibits the adhesion of anti-CD3-activated killer lymphocytes to adenocarcinoma cells through an A_3 receptor. Cancer Res (1994); **54**: 3521-3526.

Mackie, I.J., Jones, R. and Machin, S.J. Platelet impedance aggregation in whole blood and its inhibition by antiplatelet drugs. J Clin Pathol (1984); **37**: 874-878.

Majumdar, S., Rossi, M.W., Fujiki, T., Phillips, W.A., Disa, S., Queen, C.F., Johnston, R.B.J., Rosen, O.M., Corkey, B.E. and Korchak, H.M. Protein kinase C isotypes and signalling in neutrophils. Differential substrate specificities of a translocatable calcium- and phospholipid- dependent beta-protein kinase C and a phospholipid-dependent protein kinase which is inhibited by long chain fatty acyl coenzyme A. J Biol Chem (1991); **266**: 9285-9294.

Malo-Ranta, U., Yla-Herttuala, S., Metsa-Ketela, T., Jaakkola, O., Moilanen, E., Vuorinen, P. and Nikkari, T. Nitric oxide donor GEA 3162 inhibits endothelial cell-mediated oxidation of low density lipoprotein. FEBS Lett (1994); **337**: 179-183.

Malstrom. R. Acute regulation of VLDL Apo B metabolism in healthy subjects and in patients with type 2 diabetes impact of insulin and free fatty acids. University of Helsinki (1999); p 52.

Manning, A.S. and Hearse, D.J. Reperfusion-induced arrhythmias: mechanisms and prevention. J Mol Cell Cardiol (1984); **16**: 497-518.

Mannion, B.A., Weiss, J. and Elsbach, P. Separation of sublethal and lethal effects of polymorphonuclear leukocytes on *Escherichia coli*. J Clin Invest (1990); **86**: 631-641.

Maras, P., Della Grazie, D., Klugmann, S., Mogera, T., Salvi, A., Pandullo, C and Camerini, F. Reperfusion ventricular arrythmias during intracoronary thrombolysis. Eur. Heart J (1986); 7: 23-30.

Marcus, A.J., Safier, L.B., Broekman, M.J., Islam, N., Fliessbach, J.H., Hajjar, K.A., Kaminski, W.E., Jendraschak, E., Silverstein, R.L. and von Schacky, C. Thrombosis and inflammation as multicellular processes: significance of cell-cell interactions. Thromb Haemost (1995); **74**: 213-217.

Marks, P.W. and Maxfield, F.R. Transient increases in cytosolic free calcium appear to be required for the migration of adherent human neutrophils. [published erratum appears in J Cell Biol 1990 Mar; 110(3):861], J Cell Biol (1990); **110**: 43-52.

Marquardt, D.L., Walker, L.L. and Heinemann, S. Inhibition of protein kinase A fails to alter mast cell adenosine responsiveness. Agents Actions (1994); **43**: 7-12.

Martin, J.F. and Levine, R.F., 1991. Evidence in favour of the lungs as the site of platelet production, in: The Platelet in Health and Disease, ed. C.P. Page (Blackwell Scientific Press, Oxford) pp. 1-9.

Martin, P.L., Ueeda, M. and Olsson, R.A. 2-Phenylethoxy-9-methyladenine: an adenosine receptor antagonist that discriminates between A_2 adenosine receptors in the aorta and the coronary vessels from the guinea pig. J Pharmacol Exp Ther (1993); **265**: 248-253.

Martin, E., Ganz, T. and Lehrer, R.I. Defensins and other endogenous antibiotics of vertebrates. J Leuk Biol (1995); **58**: 128-136.

Mateo, J., Castro, E., Zwiller, J., Aunis, D. and Miras-Portugal, M.T. 5' (n-ethylcarboxamido) adenosine inhibits Ca^{2+} influx and activates a protein phosphatase in bovine adrenal chromaffin cells. J Neurochem (1995); **64**: 77-84.

Matheis, G., Sherman, M.P., Buckberg, G.D., Haybron, D.M., Young, H.H. and Ignarro, L.J. Role of L-arginine-nitric oxide pathway in myocardial reoxygenation injury. Am J Physiol (1992); **262**: H616-620.

Maugeri, N., Evangelista, V., Celardo, A., Dell'Elba, G., Martelli, N., Piccardoni, P., de Gaetano, G. and Cerletti, C. Polymorphonuclear leukocyte-platelet interaction: role of P-selection in thromboxane B_2 and leukotriene C_4 cooperative synthesis. Thromb Haemost (1994); **72**: 450-456.

Maxwell, M.P., Hearse, D.J. and Yellon, D.M. Inability of desferrioxamine to limit tissue injury in the ischaemic and reperfused rabbit heart. J Cardiovasc Pharmacol (1989); **13**: 608-615.

Mayer, B., John, M. and Bohme, E. Purification of a Ca^{2+}/calmodulin-dependent nitric oxide synthase from porcine cerebellum. Cofactor-role of tetrahydrobiopterin. FEBS Lett (1990); **277**: 215-219.

Mayer, B., John, M., Heinzel, B., Werner, E.R., Wachter, H., Schultz, G. and Bohme, E. Brain nitric oxide synthase is a biopterin- and flavin-containing multi-functional oxido-reductase. FEBS Lett (1991); **288**: 187-191.

McCall, T., Whittle, B.J.R., Boughton-Smith, N.K. and Moncada, S. Inhibition of fMLP-induced aggregation of rabbit neutrophils by nitric oxide. Br J Pharmacol (1988); **95**: 517p.

McCall, T.B., Boughton-Smith, N.K., Palmer, R.M., Whittle, B.J. and Moncada, S. Synthesis of nitric oxide from L-arginine by neutrophils. Release and interaction with superoxide anion. Biochem J (1989); **261**: 293-296.

McColl, S.R., DiPersio, J.F., Caon, A.C., Ho, P. and Naccache, P.H. Involvement of tyrosine kinases in the activation of human peripheral blood neutrophils by granulocyte-macrophage colony-stimulating factor. Blood (1991); **78**: 1842-1852.

McCord, J.M. Oxygen-derived free radicals in postischemic tissue injury. N Engl J Med (1985); **312**: 159-163.

McEver, R.P. Leukocyte interactions mediated by selectins. Thromb Haemost (1991); **66**: 80-87.

McGarrity, S.T., Stephenson, A.H. and Webster, R.O. Regulation of human neutrophil functions by adenosine nucleotides. J Immunol (1989); **142**: 1986-1994.

McPhail, L., Clayton, C. and Snyderman, R. The NADPH oxidase of human polymorphonuclear leukocytes: Evidence for regulation by multiple signals. J Biol Chem (1984); **259**: 5768-5775.

Mehta, P., Mehta, J., Lawson, D., Krop, I. and Letts, L.G. Leukotrienes potentiate the effects of epinephrine and thrombin on human platelet aggregation. Thromb Res (1986); **41**: 731-738.

Mehta, J.L., Nichols, W.W. and Mehta, P. Neutrophils as potential participants in acute myocardial ischaemia: relevance to reperfusion. J Am Coll Cardiol (1988); **11**: 1309-1316.

Mehta, J.L., NIchols, W.W., Donnelly, W.M., Lawson, D.L. and Saldeen, T.G.P. Impaired canine responses to acetylcholine and bradykinin after occlusion-reperfusion. Circ Res (1989); **64**: 43-54.

Mehta, J.L., Lawson, D.L., Nicolini, F.A., Cain, D.A., Metha, P. and Schreier, H. Evidence for the generation of a large amount of nitic oxide-like vascular smooth muscle relaxant by cholesterol-rich neutrophils. Biochem Biophys Res Commun (1990); **173**: 438-442.

Menys, V.C. Collagen induced human platelet aggregation: serotonin receptor antagonism retards aggregate growth *in vitro*. Cardiovasc Res (1993); **27**: 1916-1919.

Menys, V.C., Belcher, P.R., Noble, M.I., Evans, R.D., Drossos, G.E., Pillai, R. and Westaby, S. Macroaggregation of platelets in plasma, as distinct from microaggregation in whole blood (and plasma); as determined using optical aggregometry and platelet counting respectively, is specifically impaired following cardiopulmonary bypass in man. Thromb Haemost (1994); **72**: 511-518.

Menys, V.C., Belcher, P.R., Noble, M.I., Drossos, G.E., Pillai, R. and Westaby, S. Impaired platelet aggregation after cardiopulmonary bypass in man: enhancement of collagen-induced aggregation in whole blood and plasma by adrenaline *ex vivo*. Clin Sci (Colch) (1995a); **88**: 269-275.

Menys, V.C., Smith, C.C.T., and Pillai, R. The influence of aspirin on the proaggregatory action of adrenaline after cardiopulmonary bypass in man. Platelets (1995b); 6377-6380.

Messmore, H.L., Griffin, B., Fareed, J., Cotne, E. and Seghatchian, J. *In vitro* studies of the interaction of heparin, low molecular weight heparin and heparinoids with platelets. Ann NY Acad Sci (1989); **556**: 217-232.

Michelsen, L.G., Kikura, M., Levy, J.H., Lee, M.K., Lee, K.C., Zimmermann, J.J. and Szlam, F. Heparinase I (neutralase) reversal of systemic anticoagulation. Anesthesiology (1996); **85**: 339-346.

Michelson, A.D. Flow cytometry: a clinical test of platelet function. Blood (1996); **87**: 4925-4936.

Miki, S., Ashraf, M., Salka, S. and Sperelakis, N. Myocardial dysfunction and ultrastructural alterations mediated by oxygen metabolites. J Mol Cell Cardiol (1988); **20**: 1009-1024.

Miller, M.D. and Krangel, M.S. Biology and biochemistry of the chemokines: a family of chemotactic and inflammatory cytokines. CRC Crit Rev Immunol (1992); **12**: 17-46.

Miura, T., Downey, J.M., Hotta, D. and Iimura, O. Effect of superoxide dismutase plus catalase on myocardial infarct size in rabbits. Can J Cardiol (1988); **4**: 407-411.

Moilanen, E., Vuroinen, P., Kankaanranta, H., Metsa-Ketela, T. and Vapaatalo, H. Inhibition by nitric oxide-donors of human polymorphonuclear leucocyte functions. Br J Pharmacol (1993); **109**: 852-858.

Moilanen, E., Arola, O., Malo-ranta, U., Yla-herttuala, S., Vuorinen, P., Metsa-ketela, T. and Vapaatalo, H., 1994. Nitric oxide donors inhibit neutrophil adhesion to endothelial cells, in: Nitric oxide, Enzymology and Biochemistry, Vol. IV, eds. S. Moncada, M. Feelisch, R. Busse and E.A. Higgs (Portland Press, London) pp. 271-275.

Moilanen, E. and Vapaatalo, H. Nitric oxide in inflammation and immune response. Ann Med (1995); **27**: 359-367.

Molino, M., Blanchard, N., Belmonte, E., Tarver, A.P., Abrams, C., Hoxie, J.A., Cerletti, C. and Brass, L.F. Proteolysis of the human platelet and endothelial cell thrombin receptor by neutrophil-derived cathepsin G. J Biol Chem (1995); **270**: 11168-11175.

Moncada, S., Gryglewski, R.J., Bunting, S. and Vane, J.R. A lipid peroxide inhibits the enzyme in blood vessel microsomes that generates from prostaglandin endoperoxides the substance (prostaglandin X) which prevents platelet aggregation. Prostaglandins (1976); **12**: 715-737.

Moncada, S., Gryglewski, R., Bunting, S. and Vane, J.R. An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. Nature, (1976); **263**: 663-665.

Moncada, S. and Vane, J.R. Arachidonic acid metabolites and the interactions between platelets and blood-vessel walls. N Engl J Med, (1979); **300**: 1142-1147.

Moncada, S., Palmer, R.M. and Higgs, E.A. The biological significance of nitric oxide formation from L-arginine. Biochem Soc Trans (1989); **17**: 642-644.

Moncada, S., Richard, M.J., Palmer and Higgs, E.A. Biosynthesis of nitric oxide from L-arginine: A pathway for the regulation of cell function and communication. Biochem Pharmacol (1989); **38**: 1709-1715.

Moncada, S. and Higgs, E.A. Endogenous nitric oxide: physiology, pathology and clinical relevance. Eur J Clin Invest (1991); **21**: 361-374.

Moncada, S., Palmer, R.M. and Higgs, E.A. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev (1991); **43**: 109-142.

Moncada, S. The 1991 Ulf von euler Lecture. The L-arginine: nitric oxide pathway. Acta Physiologica Scandanavica (1991b); **145**: 201-227.

Monopoli, A., Conti, A., Zocchi, C., Casati, R., Volpini, R., Cristalli, G. and Ongini, E. Pharmacology of the new selective A_{2a} adenosine receptor agonist 2-Hexynl-5'-N-ethyl-carboxamidoadenosine. Arzneim-Forsch/Drug Res (1994); **44**: 1296-1304.

Moore, K.L., Stults, N.L., Diaz, S., Smith, D.F., Cummings, R.D., Varki, A. and McEver, R.P. Identification of a specific glycoprotein ligand for P-selectin (CD62) on myeloid cells. J Cell Biol (1992); **118**: 445-456.

Morse, D.S., Adams, D. and Magnani, B. Platelet and neutrophil activation during cardiac surgical procedures: impact of cardiopulmonary bypass. Ann Thorac Surg (1998); **65**: 691-695.

Mullane, K.M. and Moncada, S. The salvage of ischaemic myocardium by BW755C in anaesthetised dogs. Prostaglandins (1982); **24**: 255-266.

Mullane, K.M., Read, N., Salmon, J.A. and Moncada, S. Role of leukocytes in acute myocardial infarction in anesthetized dogs: Relationship to myocardial salvage by anti-inflammatory drugs. J Pharmacol Exp Ther (1984); **228**: 510-522.

Mullane, K.M., Kraemer, R. and Smith, B. Myeloperoxidase activity as a quantitative assessment of neutrophil infiltration into ischemic myocardium. J Pharmacol Meth, (1985); **14**: 157-167.

Mullane, K.M., 1988. Myocardial ischaemia-reperfusion injury: role of neutrophils and neutrophil-derived mediators, in: In Human Inflammatory Disease (Decker Inc., Toronto).

Muller, W.A., Weigl, S.A., Deng, X. and Phillips, D.M. PECAM-1 is required for transendothelial migration of leukocytes. J Exp Med (1993); **178**: 449-460.

Mulsch, A.R., Busse, R., Liebau, S. and Forstermann, U. LY 83583 interferes with the release of endothelium-derived relaxing factor and inhibits soluble guanylate cyclase. J Pharmacol Exp Ther (1988); **247**: 283-288.

Murad, F., Waldman, S.A., Fiscus, R.R. and Rapoport, R.M. Regulation of cyclic GMP synthesis and the interactions with calcium. J Cardiovasc Pharmacol (1986); **8 Suppl 8**, S57-60.

Murad, F. Cyclic guanosine monophosphate as a mediator of vasodilation. J Clin Invest (1986); **78**, 1-5.

Murohara, T., Guo, J.P. and Lefer, A.M. Cardioprotection by a novel recombinant serine protease inhibitor in myocardial ischemia and reperfusion injury. J Pharmacol Exp Ther (1995); **274**: 1246-1253.

Murphy, P.M. The molecular biology of leukocyte chemoattractant receptors. Annu Rev Immunol (1994); **12**: 593-633.

Myers, M.L., Bolli, R., Lekich, R.F., Hartley, C.J. and Roberts, R. Enhancement of recovery of myocardial function by oxygen free-radical scavengers after reversible regional ischemia. Circulation (1985); **72**: 915-921.

Myers, P.R., Minor, R.L., Guerra, R.J., Bates, J.N. and Harrison, D.G. Vasorelaxant properties of the endothelium-derived relaxing factor more closely resemble S-nitrosocysteine than nitric oxide. Nature (1990); **345**: 161-163.

Naccache, P.H., Gilbert, C., Caon, A.C., Gaudry, M., Huang, C.K., Bonak, V.A., Umezawa, K. and McColl, S.R. Selective inhibition of human neutrophil functional responsiveness by erbstatin, an inhibitor of tyrosine protein kinase. Blood (1990); **76**: 2098-2104.

Naef, A., Damerau, B. and Keller, H.U. Relationship between the transient cAMP increase, exocytosis from specific and azurophil granules and chemotaxis in neutrophil granulocytes. Agents Actions (1984); **14**: 63-71.

Nakanishi, K., Vinten-Johansen, J., Lefer, D.J., Zhao, Z., Fowler, I.W., McGee, D.S. and Johnston, W.E. Intracoronary L-arginine during reperfusion improves endothelial function and reduces infarct size. Am J Physiol (1992); **263**: H1650-H1658.

Naseem, S.A., Kontos, M.C., Rao, P.S., Jesse, R.L., Hess, M.L. and Kukreja, R.C. Sustained inhibition of nitric oxide by N^G-nitro-L-arginine improve myocardial function following ischaemia/reperfusion in isolated rat heart. J Mol Cell Cardiol (1995); **27**: 419-426.

Nathan, C. Nitric oxide as a secretory product of mammalian cells. FASEB J (1992); **6**: 3051-3064.

Nejima, J., Knight, D.R., Fallon, J.T., Uemura, N., Manders, W.T., Canfield, D.R., Cohen, M.V. and Vatner, S.F. Superoxide dismutase reduces reperfusion arrhythmias but fails to salvage regional function or myocardium at risk in conscious dogs. Circulation (1989); **79**: 143-153.

Neumann, F.J., Ott, I., Wilhelm, A., Katus, H., Tillmanns, H. and Schomig, A. Release of chemoattractants and neutrophil activation in acute myocardial infarction immediately after successful recanalization of the infarct-related vessel by angioplasty. Eur Heart J (1994); **15**: 171-178.

Ney, P., Schroder, H. and Schror, K. Nitrovasodilator-induced inhibition of LTB_4 release from human PMN may be mediated by cyclic GMP. Eicosanoids (1990); **3**, 243-245.

Nguyen, T., Nrunson, D., Crespi, C.L., Penman, B.W., Wishnok, J.S. and Tannenbaum, S.R. DNA damage and mutation in human cells exposed to nitric oxide. Proc Natl Acad Sci USA (1992); **89**: 3030-3034.

Nicholls, J., Brownhill, V.R. and Hourani, S.M. Characterization of P1-purinoceptors on rat isolated duodenum longitudinal muscle and muscularis mucosae. Br J Pharmacol (1996); **117**: 170-174.

Nichols, W.W., Mehta, J.L., Donnelly, W.H., Lawson, D., Thompson, L. and Terriet, M. Reduction in coronary vasodilator reserve following coronary occlusion and reperfusion in anesthetized dog: role of endothelium-derived relaxing factor, myocardial neutrophil infiltration and prostaglandins. J Mol Cell Cardiol (1988); **20**: 943-954.

Noak, E.A., Sandrock, K. and Hutter, J., 1992. Sulfhydryl-containing organic nitrates: a new class of nitrovasodilators with direct nitric oxide (NO)-donating properties (Portland Press, London).

Nolte, D., Lehr, H.A. and Messmer, K. Adenosine inhibits postischemic leukocyte-endothelium interaction in postcapillary venules of the hamster. Am J Physiol (1991); **261**: H651-655.

Nolte, D., Lorenzen, A., Lehr, H.A., Zimmer, F.J., Klotz, K.N. and Messmer, K. Reduction of postischemic leukocyte-endothelium interaction by adenosine via A_2 receptor. Naunyn Schmiedebergs Arch Pharmacol (1992); **346**: 234-237.

Norgauer, J., Krutmann, J., Dobos, G.J., Traynor-Kaplan, A.E., Oades, Z.G. and Schraufstatter, I.U. Actin polymerization, calcium-transients, and phospholipid metabolism in human neutrophils after stimulation with interleukin-8 and N-formyl peptide. J Invest Dermatol (1994); **102**: 310-314.

Norton, E.D., Jackson, E.K., Virmani, R. and Forman, H.B. Effect of intravenous adenosine on myocardial reperfusion injury in a model with low myocardial blood flows. Am Heart J (1991); **122**: 1283-1291.

Norton, E.D., Jackson, E.K., Turner, M.B., Virmani, R. and Forman, M.B. The effects of intravenous infusions of selective adenosine A_1-receptor and A_2-receptor agonists on myocardial reperfusion injury. Am Heart J (1992); **123**: 332-338.

Nossuli, T.O., Hayward, R., Scalia, R. and Lefer, A.M. Peroxynitrite reduces myocardial infarct size and preserves coronary endothelium after ischemia and reperfusion in cats. Circulation (1997); **96**: 2317-2324.

Nossuli, T.O., Hayward, R., Jensen, D., Scalia, R. and Lefer, A.M. Mechanisms of cardioprotection by peroxynitrite in myocardial ischemia and reperfusion injury. Am J Physiol (1998); **275**: H509-519.

Novotny, W.F., Maffi, T., Mehta, R.L. and Milner, P.G. Identification of novel heparin-releasable proteins, as well as the cytokines midkine and pleiotrophin, in human post-heparin plasma. Arterioscler Thromb (1993); **13**: 1798-1805.

Nurinen, M.L. and Vapaatalo, H. Effect of intracerebroventricular and intravenous administration of nitric oxide donors on blood pressure and heart rate in the anaesthetised rat. Br J Pharmacol (1996); **119**: 1422-1426.

Nussler, A.K. and Billiar, T.R. Inflammation, immunoregulation and inducible nitric oxide. J Leuk Biol (1993); **54**: 171-178.

O'Brien, J.R., Etherington, M.D. and Pashley, M. Intra-platelet platelet factor 4 (IP PF4) and the heparin-mobilisable pool of PF4 in health and atherosclerosis. Thromb Haemost (1984); **51**: 354-357.

O'Donnell, M.E. and Owen, N.E. Role of cyclic GMP in atrial natriuretic factor stimulationof Na^+, K^+, Cl^- cotransport in vascular smooth muscle cells. J Biol Chem (1986); **261**: 15461-15466.

O'Neill, P., Menendez, T., Hust, R., Howell, J., Espada, R. and Pacifico, A. Prolonged ventricular fibrillation-salvage using a new percutaneous cardiopulmonary support system. Am J Cardiol (1989); **64**: 545.

Ofek, I., Rest, R. and Sharon, N. Nonopsonic phagocytosis of microorganisms. ASM news (1992); **58**: 429-435.

Ogletree, M.L., Lefer, A.M., Smith, J.B. and Nicolaou, K.C. Studies on the protective effect of prostacyclin in acute myocardial ischemia. Eur J Pharmacol (1979); **56**: 95-103.

Ogura, R. Hydroxyl radical generation from heart mitochondria damaged by ischemia. J Nutr Sci Vitaminol (Tokyo) (1992); **Spec No**, 309-312.

Okada, M., Mizuno, K. and Kaneko, S. Adenosine A_1 and A_2 receptors modulate extracellular dopamine levels in rat striatum. Neurosci Lett (1996); **212**: 53-56.

Okuyama, M., Kambayashi, J., Sakon, M., Kawasaki, T. and Monden, M. PGI_2 analogue, sodium beraprost, suppresses superoxide generation in human neutrophils by inhibiting p47phox phosphorylation. Life Sci (1995); **57**: 1051-1059.

Olah, M.E. and Stiles, G.L. Adenosine receptor subtypes: characterization and therapeutic regulation. Annu Rev Pharmacol Toxicol (1995); **35**: 581-606.

Olson, S.C., Bowman, E.P. and Lambeth, J.D. Phospholipase D activation in a cell free system from human neutrophils by phorbol 12 myristate 13 acetate and guanosine 5'-*O*-(3-thiotriphosphate). J Biol Chem (1991); **266**: 17236-17242.

Omar, H.A. and Wolin, M.S. Endothelium-dependent and independent cGMP mechanisms appear to mediate O_2^- responses in calf pulmonary resistance arteries. Am J Physiol (1992); **262**: L560-565.

Oppenheim, J.J., Zachariae, C.O., Mukaida, N. and Matsushima, K. Properties of the novel pro-inflammatory supergene "intercrine" cytokine family. Ann Rev Immunol (1991); **9**: 617-648.

Oram, J.D. and Reiter, B. Inhibition of bacteria by lactoferrin and other iron-chelating agents. Biochem Biophys Acta (1968); **170**: 351-365.

Oseas, R., Yang, H.H., Baehner, R.L. and Boxer, L.A. Lactoferrin : A promoter of polymorphonuclear leukocye adhesiveness. Blood (1981); **57**: 939-945.

Ott, I., Neumann, F.J., Gawaz, M., Schmitt, M. and Schomig, A. Increased neutrophil-platelet adhesion in patients with unstable angina. [see comments], Circulation (1996); **94**: 1239-1246.

Oyanagui, Y. and Sato, S. Heparin, a potent releasing agent of extracellular superoxide dismutase (ecsod C); suppresses ischaemic paw oedema in mice. Free Rad Res Commun (1990); **9**: 87-99.

Packham, M.A., Bryant, N.L., Guccione, M.A., Kinlough-Rathbone, R.L. and Mustard, J.F. Effect of the concentration of Ca^{2+} in the suspending medium on the responses of human and rabbit platelets to aggregating agents. Thromb Haemost (1989); **62**: 968-976.

Pai, J.K., Siegel, M.I., Egan, R.W. and Billah, M.M. Phospholipase D catalyzes phospholipid metabolism in chemotactic peptide-stimulated HL-60 granulocytes. J Biol Chem (1988); **263**: 12472-12477.

Painter, R.G., Sklar, L.A., Jesaitis, A.J., Schmitt, M. and Cochrane, C.G. Activation of neutrophils by N-formyl chemotactic peptides. Fed Proc (1984); **43**: 2737-2742.

Palabrica, T., Lobb, R., Furie, B.C., Aronovitz, M., Benjamin, C., Hsu, Y.M., Sajer, S.A. and Furie, B. Leukocyte accumulation promoting fibrin deposition is mediated *in vivo* by P-selectin on adherent platelets. Nature (1992); **359**: 848-851.

Palacios, M., Knowles, R.G., Palmer, R.M. and Moncada, S. Nitric oxide from L-arginine stimulates the soluble guanylate cyclase in adrenal glands. Biochem Biophys Res Commun (1989); **165**: 802-809.

Pallandi, R.T., Perry, M.A. and Campbell, T.J. Proarrhythmic effects of an oxygen-derived free radical generating system on action potentials recorded from guinea pig ventricular myocardium: a possible cause of reperfusion-induced arrhythmias. Circ Res (1987); **61**: 50-54.

Palmer, R.M., Ferrige, A.G. and Moncada, S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature, (1987); **327**: 524-526.

Pasini, F.L., Pasqui, A.L., Ceccatelli, L., Caecchi, P.L., Orrico, A. and di Perri, T. Heparin inhibition of polymorphonuclear leukocyte activation *in vitro*: A possible pharmacological approach to granulocyte-mediated vascular damage. Thromb Res (1984); **35**: 527-537.

Patel, B.S., Jeroudi, M.O., O'Neill, P.G., Roberts, R. and Bolli, R. Effect of human recombinant superoxide dismutase on canine myocardial infarction. Am J Physiol (1990); **258**: H369-380.

Pedvis, L.G., Wong, T. and Frojmovic, M.M. Differential inhibition of the platelet activation sequence: shape change, micro- and macro-aggregation, by a stable prostacyclin analogue (Iloprost). Thromb Haemost (1988); **59**: 323-328.

Pernow, J., Uriuda, Y., Wang, Q.D., Li, X.S., Nordlander, R. and Rydeen, L. The protective effect of L-aginine on myocardial injury and endothelial function following ischaemia and reperfusion in the pig. Eur Heart J (1994); **15**: 1712-1719.

Perret, B., Chap, H.J. and Douste-Blazy, L. Asymmetric distribution of arachidonic acid in the plasma membrane of human platelets. A determination using purified phospholipases and a rapid method for membrane isolation. Biochim Biophys Acta (1979); **556**: 434-446.

Perry, D.K., Hand, W.L., Edmondson, D.E. and Lambeth, J.D. Role of phospholipase D-derived diradylglycerol in the activation of the human neutrophil respiratory burst oxidase. Inhibition by phosphatidic acid phosphohydrolase inhibitors. J Immunol (1992); **149**: 2749-2758.

Peters, M.J., Heyderman, R.S., Hatch, D.J. and Klein, N.J. Investigation of platelet-neutrophil interactions in whole blood by flow cytometry. J Immunol Methods (1997); **209**: 125-135.

Phillips, D.R., Charo, I.F., Parise, L.V. and Fitzgerald, L.A. The platelet membrane glycoprotein IIb-IIIa complex. Blood (1988); **71**: 831-843.

Phillips, M.L., Nudelman, E., Gaeta, F.C., Perez, M., Singhal.A.K., Hakomori, S. and Paulson, J.C. ELAM-1 mediateds cell adhesion by recognition of a carbohydrate ligand. sialyl-Lewis^{x}. Science (1990); **250**: 1130-1132.

Phillis, J.W., O'Regan, M.H. and Perkins, L.M. Effect of adenosine receptor agonists on spontaneous and K (+)-evoked acetylcholine release from the *in vivo* rat cerebral cortex. Brain Res (1993); **605**: 293-297.

Pidard, D., Renesto, P., Berndt, M.C., Rabhi, S., Clemetson, K.J. and Chignard, M. Neutrophil proteinase cathepsin G is proteolytically active on the human platelet glycoprotein Ib-IX receptor: characterization of the cleavage sites within the glycoprotein Ib alpha subunit. Biochem J (1994); **303**: 489-498.

Pidard, D., Renesto, P., Rabhi, S. and Chignard, M. Regulation of the structure and activity of platelet adhesion receptors by leukocyte proteinases. Nouv Rev Fr Hematol (1994); **36 Suppl 1**: S99-101.

Pitarys, C.J.d., Virmani, R., Vildibill, H.D.J., Jackson, E.K. and Forman, M.B. Reduction of myocardial reperfusion injury by intravenous adenosine administered during the early reperfusion period. Circulation (1991); **83**: 237-247.

Plow, E.F., McEver, R.P., Coller, B.S., Woods, V.L.J., Marguerie, G.A. and Ginsberg, M.H. Related binding mechanisms for fibrinogen, fibronectin, von Willebrand factor, and thrombospondin on thrombin-stimulated human platelets. Blood, (1985); **66**: 724-727.

Pober, J.S., Gimbrone, M.A.J., Lapierre, L.A., mendrick, D.L., Fiers, W., Rothlein, R. and Springer, T.A. Overlapping patterns of activation of human endothelial cells by interleukin 1, tumor necrosis factor, and immune interferon. J Immunol (1986); **137**: 1893-1896.

Pober, J.S. and Cotran, R.S. The role of endothelial cells in inflammation. Transplantation (1990); **50(4)**: 537-44.

Polakis, P.G., Weber, R.F., Nevins, B., Didsbury, J.R., Evans, T. and Snyderman, R. Identification of the ral and rac1 gene products, low molecular mass GTP-binding proteins from human platelets. J Biol Chem (1989); **264**: 16383-16389.

Pollock, J.S., Forstermann, U., Mitchell, J.A., Warner, T.D., Schmidt, H.H., Nakane, M. and Murad, F. Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. Proc Natl Acad Sci U S A (1991); **88**: 10480-10484.

Premaratue, S., Limm, W., Mugiishi, M.M., Piette, L.H. and McNamara, J.J. Quantitative assessment of free-radical generation during ischaemia and reperfusion in the isolated rabbit heart. Coron Artery Dis (1993); **4**: 1093-1096.

Prentice, D.J. and Hourani, S.M. Activation of multiple sites by adenosine analogues in the rat isolated aorta. Br J Pharmacol (1996); **118**: 1509-1517.

Price, T.H., Ochs, H.D., Gershoni-Baruch, R., Harlan, J.M. and Etzioni, A. *In vivo* neutrophil and lymphocyte function studies in a patient with leukocyte adhesion deficiency type II. Blood (1994); **84**: 1635-1639.

Probst, W.B., Snyder, L.A., Schuster, D.I., Brosius, J. and Sealfon, S.C. Sequence alignment of the G protein-coupled receptor superfamily. DNA Cell Biol (1992); **11**: 1-20.

Pryzwanski, K.B., Wyatt, T.A., Nichols, H. and Lincoln, T.M. Compartmentalization of cyclic GMP-dependent protein kinase in formyl-peptide stimulated neutrophils. Blood (1990); **76**: 612-618.

Przyklenk, K. and Kloner, R.A. Superoxide dismutase plus catalase improve contractile function in the canine model of the "stunned myocardium". Circ Res (1986); **58**: 148-156.

Przyklenk, K., Whittaker, P. and Kloner, R.A. *In vivo* infusion of oxygen free radical substrates causes myocardial systolic, but not diastolic dysfunction. Am Heart J (1990); **119**: 807-815.

Przyklenk, K. Lethal myocardial reperfusion injury. J Thromb Thrombol (1997); **4**: 1-154.

Pytela, R., Pierschbacher, M.D., Ginsberg, M.H., Plow, E.F. and Ruoslahti, E. Platelet membrane glycoprotein IIb/IIIa: member of a family of Arg-Gly-Asp-specific adhesion receptors. Science (1986); **231**: 1559-1562.

Quesenberry, P. and Levitt, L. Hematopoietic stem cells (third of three parts). N Engl J Med (1979); **301**: 868-872.

Quesenberry, P. and Levitt, L. Hematopoietic stem cells (second of three parts). N Engl J Med (1979); **301**: 819-823.

Quesenberry, P. and Levitt, L. Hematopoietic stem cells. N Engl J Med (1979); **301**: 755-761.

Baugh, R.F., Deemar, K.A. and Zimmermann, J.J. Heparinase in the Activated Clotting Time Assay: Monitoring heparin-independent alterations in coagulation function. Anesth Analg (1992); **74**: 201-205.

Radi, R., Beckman, J.S., Bush, K.M. and Freeman, B.A. Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. Arch Biochem Biophys (1991); **288**: 481-487.

Radi, R., Beckman, J.S., Bush, K.M. and Freeman, B.A. Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. J Biol Chem (1991); **266**: 4244-4250.

Radomski, M.W., Palmer, R.M.J. and Moncada, S. Comparative pharmacology of endothelium-derived relaxing factor, nitric oxide and prostacyclin in platelets. Br J Pharmacol (1987a); **92**: 1181-1187.

Radomski, M.W., Palmer, R.M.J. and Moncada, S. The anti-aggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide. Br J Pharmacol (1987b); **92**: 639-646.

Radomski, M.W., Palmer, R.M.J. and Moncada, S. The role of nitric oxide and cyclic GMP in platelet adhesion to vascular endothelium. Biochem Biophys Res Commun (1987c); **148**: 1482-1489.

Radomski, M.W., Palmer, R.M.J. and Moncada, S. Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. Lancet (1987d); **2**: 1057-1058.

Ramkumar, V. and Stiles, G.L. Reciprocal modulation of agonist and antagonist binding to A_1 adenosine receptors by guanine nucleotides is mediated via a pertussis toxin-sensitive G protein. J Pharmacol Exp Ther (1988); **246**: 1194-1200.

Ramos, C.L., Pou, S., Britigan, B.E., Cohen, M.S. and Rosen, G.M. Spin trapping evidence for myeloperoxidase-dependent hydroxyl radical formation by human neutrophils and monocytes. J Biol Chem (1992); **267**: 8307-8312.

Rapoport, R.M. and Murad, F. Endothelium-dependent and nitrovasodilator-induced relaxation of vascular smooth muscle: Role for cyclic GMP. J Cyclic Nucleotide Protein Phosphorylation Res (1983b); **9**: 281-296.

Reeves, J.P., Bailey, C.A. and Hale, C.C. Redox modification of sodium-calcium exchange activity in cardiac sarcolemmal vesicles. J Biol Chem (1986); **261**: 4948-4955.

Reimer, K.A. and Jennings, R.B. The "wavefront phenomenon" of myocardial ischemic cell death. Transmural progression of necrosis within the framework of ischemic bed size (myocardium at risk) and collateral flow. Lab Invest (1979); **40**: 633-644.

Reimer, K.A., Jennings, R.B., Cobb, F.R. *et al.* Animal Models for protecting ischemic myocardium: Results of the NHLBI cooperative study. Comparison of unconscious and conscious dog models. Circ Res (1985); **56**: 651-665.

Reimer, K.A., Murry, C.E. and Richard, V.J. The role of neutrophils and free radicals in the ischemic-reperfused heart: why the confusion and controversy? J Mol Cell Cardiol (1989); **21**: 1225-1239.

Renesto, P., Ferrer-Lopez, P. and Chignard, M. Interference of recombinant eglin C, a proteinase inhibitor extracted from leeches, with neutrophil-mediated platelet activation. Lab Invest (1990); **62**: 409-416.

Renesto, P. and Chignard, M. Enhancement of cathepsin G-induced platelet activation by leukocyte elastase: consequence for the neutrophil-mediated platelet activation. Blood (1993); **82**: 139-144.

Renesto, P. and Chignard, M. Neutrophil-mediated platelet activation: a key role for serine proteinases. Gen Pharmacol (1995); **26**: 905-910.

Rengasamy, A. and Johns, R.A. Regulation of nitric oxide synthase by nitric oxide. Mol Pharmacol (1993); **44**: 124-128.

Revan, S., Montesinos, M.C., Naime, D., Landau, S. and Cronstein, B.N. Adenosine A_2 receptor occupancy regulates stimulated neutrophil function via activation of a serine/threonine protein phosphatase. J Biol Chem (1996); **271**: 17114-17118.

Richter, J., Olsson, I. and Andersson, T. Correlation between spontaneous oscillations of cytosolic free Ca^{2+} and tumor necrosis factor-induced degranulation in adherent human neutrophils. J Biol Chem (1990); **265**: 14358-14363.

Richter, J. Effect of adenosine analogues, and cAMP-raising agents on TNF-, GM-CSF-, and chemotactic peptide-induced degranulation in single adherent neutrophils. J Leukoc Biol (1992); **51**: 270-275.

Rider, L.G. and Niedel, J.E. Diacylglycerol accumulation and superoxide anion production in stimulated human neutrophils. J Biol Chem (1987); **262**: 5603-5608.

Ridley, A.J., Paterson, H.F., Johnston, C.L., Diekmann, D. and Hall, A. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell (1992); **70**: 401-410.

Ridley, A.J. and Hall, A. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell (1992); **70**: 389-399.

Ridley, A.J. and Hall, A. Distinct patterns of actin organization regulated by the small GTP- binding proteins Rac and Rho. Cold Spring Harb Symp Quant Biol (1992); **57**: 661-671.

Riesenberg, K., Schlaeffer, F., Katz, A. and Levy, R. Inhibition of superoxide production in human neutrophils by combinations of heparin and thrombolytic agents. Br Heart J (1995); **73**: 14-19.

Riess, F.C., Bleese, N., Kormann, J., Potzsch, B., Madlener, K., Muller-Berghaus, G. and Greinacher, A. Recombinant hirudin is a heparin alternative in cardiac surgery [letter; comment]. J Cardiothorac Vasc Anesth (1997); **11**: 538-539.

Rimele, T.J., Sturm, R.J., Adams, L.M., Henry, D.E., Heaslip, R.J., Weichman, B.M. and Grimes, D. Interaction of neutrophils with vascular smooth muscle: identification of neutrophil-derived relaxing factor. J Pharmacol Exp Ther (1988); **245**: 102-111.

Rimele, T.J., Armstrong, S.J., Grimes, D. and Sturm, R.J. Rat peritoneal neutrophils selectively relax vascular smooth muscle. J Pharmacol Exp Ther (1991); **258**: 963-971.

Rinder, C.S., Mathew, J.P., Rinder, H.M., Bonan, J., Ault, K.A. and Smith, B.R. Modulation of platelet surface adhesion receptors during cardiopulmonary bypass. Anesthesiology (1991); **75**: 563-570.

Rinder, H.M., Bonan, J.L., Rinder, C.S., Ault, K.A. and Smith, B.R. Dynamics of leukocyte-platelet adhesion in whole blood. Blood (1991); **78**: 1730-1737.

Rinder, C.S., Bohnert, J., Rinder, H.M., Mitchell, J., Ault, K. and Hillman, R. Platelet activation and aggregation during cardiopulmonary bypass. Anesthesiology (1991); **75**: 388-393.

Rinder, C.S., Bonan, J.L., Rinder, H.M., Mathew, J., Hines, R. and Smith, B.R. Cardiopulmonary bypass induces leukocyte-platelet adhesion. Blood (1992); **79**: 1201-1205.

Rinder, H.M., Tracey, J.L., Rinder, C.S., Leitenberg, D. and Smith, B.R. Neutrophil but not monocyte activation inhibits P-selectin-mediated platelet adhesion. Thromb Haemost (1994); **72**: 750-756.

Rinder, C.S., Gaal, D., Student, L.A. and Smith, B.R. Platelet-leukocyte activation and modulation of adhesion receptors in pediatric patients with congenital heart disease undergoing cardiopulmonary bypass. J Thorac Cardiovasc Surg (1994); **107**: 280-288.

Roberts, P.A., Newby, A.C., Hallett, M.B. and Campbell, A.K. Inhibition by adenosine of reactive oxygen metabolite production by human polymorphonuclear leucocytes. Biochem J (1985); **227**: 669-674.

Rollet, E., Caon, A.C., Roberge, C.J., Liao, N.W., Malawista, S.E., McColl, S.R. and Naccache, P.H. Tyrosine phosphorylation in activated human neutrophils. Comparison of the effects of different classes of agonists and identification of the signaling pathways involved. J Immunol (1994); **153**: 353-363.

Romson, J.L., Hook, B.G., Kunkel, S.L., Abrams, G.D., Schork, M.A. and Lucchesi, B.R. Reduction of the extent of ischemic myocardial injury by neutrophil depletion in the dog. Circulation (1983); **67**: 1016-1023.

Root, R.K. and Cohen, M.S. The microbicidal mechanisms of human neutrophils and eosinophils. Rev Infect Dis (1981); **3**: 565-598.

Rose, F., Hirschorn, R., Weissman, G. and Cronstein, B. Adenosine promotes neutrophil chemotaxis. J Exp Med (1988); **167**: 1186-1194.

Rosen, S.D. Cell surface lectins in the immune system. Semin Immunol (1993); **5**: 237-247.

Rossi, F. The O_2^--forming NADPH oxidase of the phagocytes: Nature, mechanisms of activation and function. Biochim Biophys Acta (1986); **853**, 65.

Rossi, F., Grzeskowiak, M., Della Bianca, V., Calzetti, F. and Gandini, G. Phosphatidic acid and not diacylglycerol generated by phospholipase D is functionally linked to the activation of the NADPH oxidase by fMLP in human neutrophils. Biochem Biophys Res Commun (1990); **168**: 320-327.

Rotrosen, D., Yeung, C.L., Leto, T.L., Malech, H.L. and Kwong, C.H. Cytochrom b_{558}: the flavin-binding component of the phagocyte NADPH oxidase. Science (1992); **256**: 1459-1462.

Rubanyi, G. and Vanhoutte, P.S. Oxygen-derived free radicals and endothelial responsiveness vascular smooth muscle. Am J Physiol (Heart Circ Physiol 19) (1987); **250**: H815-H821.

Rubanyi, G.M., Ho, E.H., Cantor, E.H., Lumma, W.C. and Botelho, L.H. Cytoprotective function of nitric oxide: inactivation of superoxide radicals produced by human leukocytes. Biochem Biophys Res Commun (1991); **181**: 1392-1397.

Rubiera, C., Velasco, G., Michell, R.H., Lazo, P.S. and Shears, S.B. 1D-myo-inositol 1,4,5-trisphosphate dephosphorylation by rat enterocytes involves an intracellular 5-phosphatase and non-specific phosphatase activity at the cell surface. Biochem J (1988); **255**: 131-137.

Rudolphi, K.A., Schubert, P., Parkinson, F.E. and Fredholm, B.B. Neuroprotective role of adenosine in cerebral ischaemia. Trends Pharmacol Sci (1992); **13**: 439-445.

Ruf, A., Schlenk, R.F., Maras, A., Morgenstern, E. and Patscheke, H. Contact-induced neutrophil activation by platelets in human cell suspensions and whole blood. Blood (1992); **80**: 1238-1246.

Ruf, A. and Patscheke, H. Platelet-induced neutrophil activation: platelet-expressed fibrinogen induces the oxidative burst in neutrophils by an interaction with CD11C/CD18. Br J Haematol (1995); **90**: 791-796.

Ruocco, N.A., Most, A.S., Sasken, H., Steiner, M. and Gewirtz, H. Role of endogenous prostacyclin in myocardial blood flow regulation distal to a severe coronary stenosis. Cardiovasc Res (1988); **22**: 511-519.

Saelman, E.U., Nieuwenhuis, H.K., Hese, K.M., de Groot, P.G., Heijnen, H.F., Sage, E.H., Williams, S., McKeown, L., Gralnick, H.R. and Sixma, J.J. Platelet adhesion to collagen types I through VIII under conditions of stasis and flow is mediated by GPIa/IIa (alpha 2 beta 1-integrin). Blood (1994); **83**: 1244-1250.

Sako, D., Chang, X.J., Barone, K.M., Vachino, G., White, H.M., Shaw, G., Veldman, G.M., Bean, K.M., Ahern, T.J., Furie, B. *et al.* Expression cloning of a functional glycoprotein ligand for P-selectin. Cell (1993); **75**: 1179-1186.

Salmon, J.E. and Cronstein, B.N. Fc gamma receptor-mediated functions in neutrophils are modulated by adenosine receptor occupancy: A_1 receptors are stimulatory and A_2 receptors are inhibitory. Journal of Immunology (1990); **145**: 2235-2240.

Salvemini, D., Denucci, G., Gryglewski, R.J. and Vane, J.R. Human neutrophils and mononuclear cells inhibit platelet aggregation by releasing a nitric-oxide like factor. Proc Natl Acad Sci USA (1989); **86**: 6328-6332.

Sander, H.J., Slot, J.W., Bouma, B.N., Bolhuis, P.A., Pepper, D.S. and Sixma, J.J. Immunocytochemical localization of fibrinogen, platelet factor 4, and beta thromboglobulin in thin frozen sections of human blood platelets. J Clin Invest (1983); **72**: 1277-1287.

Sandler, J.A., Gallin, J.I. and Vaughn, M. Effect of serotonin, carbomylcholine and ascorbic acid on leukocyte cyclic GMP and chemotaxis. J Cell Biol (1975); **67**: 480-484.

Sandoli, D., Chiu, P.J. *et al.* *In vivo* and *ex vivo* effects of adenosine A_1 and A_2 receptor agonists on platelet aggregation in the rabbit. Eur J Pharmacol, (1994); **259**: 43-49.

Santoro, S.A. Identification of a 160,000 dalton platelet membrane protein that mediates the initial divalent cation-dependent adhesion of platelets to collagen. Cell (1986); **46**: 913-920.

Savage, B., Saldivar, E. and Ruggeri, Z.M. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. Cell (1996); **84**: 289-297.

Sawyer, D.W., Donowitz, G.R. and Mandel, G.L. Polymorphonuclear neutrophils; an effective antimicrobial force. Rev Infect Dis (1989); **11(suppl 7)**: S1532-S1544.

Schaper, W. Experimental coronary artery occlusion measurement of infarct size. Basic Res Cardiol (1979); **74**: 46-53.

Schaper, W., 1984. Therapeutic Approaches to Myocardial Infarct Size Limitation, in: Experimental infarcts and the microcirculation, ed. D.J. Hearse, Yellow, D.M. (Raven, New York) pp. 79-90.

Schiffmann, E., Corcoran, B.A. and Wahl, S.M. N-formylmethionyl peptides as chemoattractants for leucocytes. Proc Natl Acad Sci U S A, (1975); **72**: 1059-1062.

Schlack, W., Schafer, M., Uebing, A., Schafer, S., Borchard, U. and Thamer, V. Adenosine A_2-receptor activation at reperfusion reduces infarct size and improves myocardial wall function in dog heart. J Cardiovasc Pharmacol (1993); **22**: 89-96.

Schmeichel, C.J. and Thomas, L.L. Methylxanthine broncodilators potentiate multiple human neutrophil functions. J Immunol (1987); **138**: 1896-1903.

Schmid-Schonbein, G.W. and Engler, R.L. Granulocytes as active participants in acute myocardial ischemia and infarction. Am J Cardiovasc Pathol (1987); **1**: 15-30.

Schmid-Schonbein, G.W. Capillary plugging by granulocytes and the no-reflow phenomenon in the microcirculation. Fed Proc (1987); **46**: 2397-2401.

Schmidt, M.J., Sawyer, B.D., Truex, L.L., Marshall, W.S. and Fleisch, J.H. An agent that lowers intracellular levels of cyclic guanosine 3',5'-monophosphate. J Pharmacol Exp Ther (1985); **232**: 764-769.

Schmidt, H.W., Seifeert, R. and Bohme, E. Formation and release of nitric oxide from human neutrophils and HL-60 cells induced by a chemotactic peptide, platelet activating factor and leukotriene B_4. FEBS Lett (1989); **244**: 357-360.

Schmidt, H.H., Pollock, J.S., Nakane, M., Gorsky, L.D., Forstermann, U. and Murad, F. Purification of a soluble isoform of guanylyl cyclase-activating-factor synthase. Proc Natl Acad Sci U S A (1991); **88**: 365-369.

Schmidt, H.H.W., Lohmann, S.M. and Walter, U. The nitric oxide and cyclic GMP signal transductiion system: regulation and mechanism of action. Biochim Biophys Acta (1993); **1178**: 153-175.

Schneider, D.J., Tracy, P.B., Mann, K.G. and Sobel, B.E. Differential effects of anticoagulants on the activation of platelets *ex vivo*. Circulation (1997); **96**: 2877-2883.

Schoenwaelder, S.M., Jackson, S.P., Yuan, Y., Teasdale, M.S., Salem, H.H. and Mitchell, C.A. Tyrosine kinases regulate the cytoskeletal attachment of integrin alpha IIb beta 3 (platelet glycoprotein IIb/IIIa) and the cellular retraction of fibrin polymers. J Biol Chem (1994); **269**: 32479-32487.

Schrier, D.J.and Imre, K. M. The effects of adenosine agonists on human neutrophil function. J Immunol (1986); **137**: 3284-3289.

Schroder, H., Ney, P., Woditsch, I. and Schror, K. Cyclic GMP mediated SIN-1-induced inhibition of human polymorphonuclear leukocytes. Eur J Pharmacol (1990); **182**: 211-218.

Schror, K., Darius, H., Matzky, R. and Ohlendorf, R. The antiplatelet and cardiovascular actions of a new carbacyclin derivative (ZK 36 374)-equipotent to PGI_2 *in vitro*. Naunyn Schmiedebergs Arch Pharmacol (1981); **316**: 252-255.

Schror, K., Thiemermann, C. and Ney, P. Protection of the ischemic myocardium from reperfusion injury by prostaglandin E_1 inhibition of ischemia-induced neutrophil activation. Naunyn Schmiedebergs Arch Pharmacol (1988); **338**: 268-274.

Schultz, J. and Kaminker, K. Myeloperoxidase of the leukocyte of normal human blood: content and localization. Arch Biochem (1962); **96**: 465-467.

Schwaner, I., Offermanns, S., Spicher, K., Seifert, R. and Schultz, G. Differential activation of Gi and Gs proteins by E- and I-type prostaglandins in membranes from the human erythroleukaemia cell line, HEL. Biochim Biophys Acta, (1995); **1265**: 8-14.

Schwarz, F., Schuler, G., Katus, H., Hofmann, M., Manthey, J., Tillmanns, H., Mehmel, H.C. and Kubler, W. Intracoronary thrombolysis in acute myocardial infarction: duration of ischemia as a major determinant of late results after recanalization. Am J Cardiol (1982); **50**: 933-937.

Segal, A.W. The electron transport chain of the microbicidal oxidase of phagocytic cells and its involvement in the molecular pathology of chronic granulomatous disease. J Clin Invest (1989); **83**: 1785-1793.

Segal, A.W., West, I., Wientjes, F., Nugent, J.H.A., Chavan, A.J., Haley, B., Garcia, R.C., Rosen, H. and Scrace, G. Cytochrome b_{245} is a flavocytochrome containing FAD and the NADPH-binding site of the microbial oxidase of phagocytes. Biochem J (1992); **284**: 781-788.

Sekili, S., Li, X.Y., Zyg, H., Sun, J.Z. and Bolli, R. Evidence for a major pathogenic role of hydroxyl radical in myocardial "stunning" in the concious dogs. Circulation (1991); **84**: Suppl.2, II-656.

Selak, M.A., Chignard, M. and Smith, J.B. Cathepsin G is a strong platelet agonist released by neutrophils. Biochem J (1988); **251**, 293-299.

Selak, M.A. Cathepsin G and thrombin: evidence for two different platelet receptors. Biochem J (1994); **297**: 269-275.

Selak, M.A. Neutrophil-platelet interactions in inflammation. Receptor (1994); **4**: 3-7.

Sengelov, H., Kjeldsen, L., Kroeze, W., Berger, M. and Borregaard, N. Secretory vesicles are the intracellular reservoir of complement receptor 1 in human neutrophils. J Immunol (1994); **153**: 804-810.

Shanberge, J.N., Murato, M., Quattrociocchi-Longe, T. and van Neste, L. Heparin-protamine complexes in the production of heparin rebound and other complications of extracorporeal bypass procedures. Am J Clin Pathol (1987); **87**: 210-217.

Shariff, A. and Luna, E.J. Diacylglycerol-stimulated formation of actin nucleation sites at plasma membranes [see comments]. Science (1992); **256**: 245-247.

Shattil, S.J., Ginsberg, M.H. and Brugge, J.S. Adhesive signaling in platelets. Curr Opin Cell Biol (1994); **6**: 695-704.

Shimizu, Y., Newman, W. and Tanaka, Y. *et al.* Lymphocyte interactions with endothelial cells. Immunol Today (1992); **13**: 93-99.

Siedlecki, C.A., Lestini, B.J., Kottke-Marchant, K.K., Eppell, S.J., Wilson, D.L. and Marchant, R.E. Shear-dependent changes in the three-dimensional structure of human von Willebrand factor. Blood (1996); **88**: 2939-2950.

Siegfried, M.R., Erhardt, J., Rider, T., Ma, X.L. and Lefer, A.M. Cardioprotection and attenuation of endothelial dysfunction by organic nitric oxide donors in myocardial ischemia-reperfusion. J Pharmacol Exp Ther (1992a); **260**: 668-675.

Siegfried, M.R., Carey, C., Ma, X.L. and Lefer, A.M. Beneficial effects of SPM-5185, a cysteine-containing NO donor in myocardal ischaemia-reperfusion. Am J Physiol (1992b); **263**: H771-H777.

Siess, W., Cuatrecasas, P. and Lapetina, E.G. A role for cyclooxygenase products in the formation of phosphatidic acid in stimulated human platelets. Differential mechanisms of action of thrombin and collagen. J Biol Chem, (1983); **258**: 4683-4686.

Silver, M.J., Sutton, J.M., Hook, S., Lee, P., Malycky, J.L., Phillips, M.L., Ellis, S.G., Topol, E.J. and Nicolini, F.A. Adjunctive selectin blockade successfully reduces infarct size beyond thrombolysis in the electrolytic canine coronary artery model. Circulation (1995); **92**: 492-499.

Siminiak, T., Zozulinska, D. and Wysocki, H. Nitric oxide donor SIN-1 inhibits neutrophil activation both directly and via platelet-mediated mechanism. J Molec Cell Cardiol (Suppl. 1) (1992); **24**:

Simpson, P.A., Mickelson, J.K. and Lucchesi, B.R. Free radical scavengers in myocardial ischaemia. Fed Proc (1987); **46**: 2413-2421.

Simpson, P.J., Mickelson, J., Fantone, J.C., Gallagher, K.P. and Lucchesi, B.R. Iloprost inhibits neutrophil function *in vitro* and *in vivo* and limits experimental infarct size in canine heart. Circ Res (1987a); **60**: 666-673.

Simpson, P.J., Mitsos, S.E., Ventura, A., Gallagher, K.P., Fantone, J.C., Abrams, G.D., Schork, M.A. and Lucchesi, B.R. Prostacyclin protects ischemic reperfused myocardium in the dog by inhibition of neutrophil activation. Am Heart J (1987b); **113**: 129-137.

Simpson, P.J., Todd, R.F.d., Fantone, J.C., Mickelson, J.K., Griffin, J.D. and Lucchesi, B.R. Reduction of experimental canine myocardial reperfusion injury by a monoclonal antibody (anti-Mo1, anti-CD11b) that inhibits leukocyte adhesion. J Clin Invest (1988); **81**: 624-629.

Sims, P.J., Ginsberg, M.H., Plow, E.F. and Shattil, S.J. Effect of platelet activation on the conformation of the plasma membrane glycoprotein IIb-IIIa complex. J Biol Chem (1991); **266**: 7345-7352.

Skuballa, W., Schillinger, E., Sturzebecher, E.S. and Vorbruggen, H. Synthesis of a new chemically and metabolically stable prostacyclin analogue with high and long-lasting oral activity. J Med Chem (1986); **29**: 313-315.

Skubitz, K.M., Wickham, N.W. and Hammerschmidt, D.E. Endogenous and exogenous adenosine inhibit granulocyte aggregation without altering the associated rise in intracellular calcium concentration. Blood (1988); **72**: 29-33.

Smedly, L.A., Tonnesen, M.G., Sandhaus, R.A., Haslett, C., Guthrie, L.A., Johnston, R.B.J., Henson, P.M. and Worthen, G.S. Neutrophil-mediated injury to endothelial cells. Enhancement by endotoxin and essential role of neutrophil elastase. J Clin Invest (1986); **77**: 1233-1243.

Smith, J.B., Ingerman, C., Kocsis, J.J. and Silver, M.J. Formation of prostaglandins during the aggregation of human blood platelets. J Clin Invest (1973); **52**: 965-969.

Smith, R.J. and Ignarro, L.J. Bioregulation of enzyme secretion from human neutrophils: roles of guanosine 3':5'-monophosphate and calcium in stimulus-secretion coupling. Proc Natl Acad Sci USA (1975); **72**: 108-112.

Smith, G.M. and Duncan, G.G. A study of intravascular platelet aggregation by continuous platelet counting. Throm Res (1981); **23**: 275-288.

Smith, C.W., Marlin, S.D., Rothlein, R., Toman, C. and Anderson, D.C. Cooperative interactions of LFA-1 and MAC-1 with intercellular adhesiion molecule-1 in facilitating adherence and transendothelial migration of human neutrophils *in vitro*. J Clin Invest (1989); **83**: 2008-2017.

Smith, C.W., Entman, M.L., Lane, C.L., Beaudet, A.L., Ty, T.I., Youker, K., Hawkins, H.K. and Anderson, D.C. Adherence of neutrophils to canine cardiac myocytes *in vitro* is dependent on intercellular adhesion molecule-1. J Clin Invest (1991); **88**: 1216-1223.

Smith, J.B., Dangelmaier, C., Selak, M.A., Ashby, B. and Daniel, J. Cyclic AMP does not inhibit collagen-induced platelet signal transduction. Biochem J (1992); **283**: 889-892.

Smolen, J.E., Stoehr, S.J. and Bartone, D. Protein kinase C is not involved in secretion by permeabilized human neutrophils. Cell Signal (1989); **1**: 471-481.

Smyth, S.S., Joneckis, C.C. and Parise, L.V. Regulation of vascular integrins [published erratum appears in Blood 1994 Apr 1; 83(7):2013]. Blood (1993); **81**: 2827-2843.

Snyderman, R., Pike, M.C., Edge, S. and Lane, B. A chemoattractant receptor on macrophages exists in two affinity states regulated by guanine nucleotides. J Cell Biol (1984); **98**: 444-448.

Snyderman, R. Regulatory mechanisms of a chemoattractant receptor on human polymorphonuclear leukocytes. Rev Infect Dis (1985); **7**: 390-394.

Snyderman, R., Smith, C.D. and Verghese, M.W. Model for leukocyte regulation by chemoattractant receptors: roles of a guanine nucleotide regulatory protein and polyphosphoinositide metabolism. J Leukoc Biol (1986); **40**: 785-800.

Snydermann, R. and Uhing, R.J., 1992. Chemoattractant stimulus-response coupling, in: Inflammation: Basic Principles and Clinical Correlates, second edition, ed. I.G. ed. JI Gallin, R Snydermann (Raven, New York) pp. 421-439.

Southwick, F.S. *et al.* Contractile proteins in leukocyte function. Semin Hematol (1983); **20**: 305-321. Review.

Spitznagel, J.K. Antibiotic proteins of human neutrophils. J Clin Invest (1990); **86**: 1381-1386.

Springer, T. Adhesion receptors of the immune system. Nature, (1990); **346**: 425-434.

Springer, T.A. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell (1994); **76**: 301-314.

Stadler, J., Billar, T.R., Curran, R.D., Stuehr, D.J., Ochoa, J.B. and Simmons, R.L. Effect of endogenous and exogenous nitric oxide on mitochondrial respiration of rat hepatocytes. Am J Physiol (1991); **260**: C910-C916.

Stambaugh, K., Jacobson, K.A., Jiang, J.L. and Liang, B.T. A novel cardioprotective function of adenosine A_1 and A_3 receptors during prolonged simulated ischemia. Am J Physiol (1997); **273**: H501-505.

Stamler, J.S., Jakari, O., Osborne, J., Simon, D.I., Keaney, J., Vita, J., Valeri, C.R. and Loscalzo, J. Nitric oxide circulates in mamalian plasma primarily as an S-nitroso adduct of serum albumin. Proc Natl Acad Sci USA (1992); **89**: 7674-7677.

Stehle, J.H., Rivkees, S.A., Lee, J.J., Weaver, D.R., Deeds, J.D. and Reppert, S.M. Molecular cloning and expression of the cDNA for a novel A_2-adenosine receptor subtype. Mol Endocrinol (1992); **6**: 384-393.

Stein, C., Classen, H.G. and Schwedt, G. Kinetic studies on nitrite and nitrate in rats by ion-pair chromatography. Clin Chim Acta (1988); **175**: 167-173.

Steiner, A.L., Ferrendelli, J.A. and Kipnis, D.M. Radioimmunoassay for cyclic nucleotides. Effect of ischemia, changes during development and regional distribution of adenosine 3',5'- monophosphate and guanosine 3',5'-monophosphate in mouse brain. J Biol Chem (1972); **247**: 1121-1124.

Steiner, A.L., Pagliara, A.S., Chase, L.R. and Kipnis, D.M. Radioimmunoassay for cyclic nucleotides. Adenosine 3',5'- monophosphate and guanosine 3',5'-monophosphate in mammalian tissues and body fluids. J Biol Chem (1972); **247**: 1114-1120.

Steiner, A.L., Parker, C.W. and Kipnis, D.M. Radioimmunoassay for cyclic nucleotides. Preparation of antibodies and iodinated cyclic nucleotides. J Biol Chem (1972); **247**: 1106-1113.

Stenberg, P.E., Shuman, M.A., Levine, S.P. and Bainton, D.F. Redistribution of alpha-granules and their contents in thrombin- stimulated platelets. J Cell Biol (1984); **98**: 748-760.

Stenberg, P.E., McEver, R.P., Shuman, M.A., Jacques, Y.V. and Bainton, D.F. A platelet alpha-granule membrane protein (GMP-140) is expressed on the plasma membrane after activation. J Cell Biol (1985); **101**: 880-886.

Stephens, R.J. and Snyderman, R. Cyclic nucleotides regulate the morphologic alterations required for chemotaxis in monocytes. J Immunol (1982); **28**: 1192-1197.

Stossel, T.P. The machinery of blood cell movements. Blood (1994); **84**: 367-369.

Strickler, J., Jacobson, K.A. and Liang, B.T. Direct preconditioning of cultured chick ventricular myocytes. Novel functions of cardiac adenosine A_{2a} and A_3 receptors. J Clin Invest (1996); **98**: 1773-1779.

Stuehr, D.J., Won, N.S., Gross, S.S., Thiel, B.A., Levi, R. and Nathan, C.F. Synthesis of nitrogen oxides from L-arginine by macrophage cytosol: requirement for inducible and constitutive components. Biochem Biophys Res Commun (1989); **161(2)**: 420-426.

Stuehr, D.J. and Nathan, C.F. Nitric oxide: a macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. J Exp Med (1989); **169**: 1543-1545.

Stuehr, D.J., Kwon, N.S., Nathan, C.F., Griffith, O.W., Feldman, P.L. and Wiseman, J. N omega-hydroxy-L-arginine is an intermediate in the biosynthesis of nitric oxide from L-arginine. J Biol Chem (1991); **266**: 6259-6263.

Stuehr, D.J., Cho, H.J., Kwon, N.S., Weise, M.F. and Nathan, C.F. Purification and characterisation of a cytokine-induced macrophage nitric oxide synthase. Proc Natl Acad Sci USA (1991); **88**: 365-369.

Stuehr, D.J. and Griffith, O.W. Mammalian nitric oxide synthases. Adv Enzymol Relat Areas Mol Biol (1992); **65**: 287-346.

Sturzebecher, S., Haberey, M., Muller, B., Schillinger, E., Schroder, G., Skuballa, W., Stock, G., Vorbruggen, H. and Witt, W. Pharmacological profile of model carbacyclin derivative with high metabolic stability and oral activity in the rat. Prostaglandins (1986); **31**: 95-109.

Sturzebecher, C.S., Loge, O., Schroder, G., Muller, B. and Witt, W. Cardiovascular and antithrombotic effects of ZK 96 480, a novel metabolically stable carbacyclin derivative with oral activity. Prog Clin Biol Res (1987); **242**: 425-431.

Sturzebecher, C.S., Haberey, M., Muller, B., Schillinger, E., Schroder, G., Skuballa, W. and Stock, G., 1985. Pharmacological profile of ZK 96 480, a new chemically and metabolically stable prostacyclin analogue with oral availability and high PGI_2 intrinsic activity, in: 2^{nd} Int. Symp. on prostagladins and other eicosanoids in the cardiovascular system, ed. K. SchrorKarger, Basel pp. 485-491.

Suzuki, H., Kinlough-Rathbone, R.L., Packham, M.A., Tanoue, K., Yamazaki, H. and Mustard, J.F. Immunocytochemical localization of fibrinogen during thrombin-induced aggregation of washed human platelets. Blood (1988); **71**: 1310-1320.

Suzuki, H., Katagiri, Y., Tsukita, S., Tanoue, K. and Yamazaki, H. Localization of adhesive proteins in two newly subdivided zones in electron-lucent matrix of human platelet alpha-granules. Histochemistry (1990); **94**: 337-344.

Suzuki, H., Nakamura, S., Itoh, Y., Tanaka, T., Yamazaki, H. and Tanoue, K. Immunocytochemical evidence for the translocation of alpha-granule membrane glycoprotein IIb/IIIa (integrin alpha IIb beta 3) of human platelets to the surface membrane during the release reaction. Histochemistry (1992); **97**: 381-388.

Synderman, R. Regulatory mechanisms of a chemoattractant receptor on human polymorphonuclear leukocytes. Rev Infect Dis (1985); **7**: 390-394.

Talpain, E., Armstrong, R.A., Coleman, R.A. and Vardey, C.J. Characterisation of the PGE receptor subtype mediating inhibition of superoxide production in human neutrophils. Br J Pharmacol (1995); **114**: 1459-1465.

Tanaka, T., Minematsu, Y. and Reilly, C.F. *et al.* Human leukocyte cathepsin G. Subsite mapping with 4-nitroanilides, chemical modification and effect of possible cofactors. Biochemistry (1985); **24**: 2040-2047.

Tanaka, M., Brooks, S.E., Richard, V.J., FitzHarris, G.P., Stoler, R.C., Jennings, R.B., Arfors, K.E. and Reimer, K.A. Effect of anti-CD18 antibody on myocardial neutrophil accumulation and infarct size after ischemia and reperfusion in dogs. Circulation (1993); **87**: 526-535.

Tandon, N.N., Kralisz, U. and Jamieson, G.A. Identification of glycoprotein IV (CD36) as a primary receptor for platelet-collagen adhesion. J Biol Chem (1989); **264**: 7576-7583.

Tateson, J.E., Moncada, S. and Vane, J.R. Effects of prostacyclin (PGx) on cyclic AMP concentrations in human platelets. Prostaglandins (1977); **13**: 389-397.

Tedder, T.F., Steeber, D.A., Chen, A. and Engel, P. The selectins: vascular adhesion molecules. FASEB J (1995); **9**: 866-873.

Tesmafarium, B., Halpern, W. and Osol, G. Effect of perfusion and endothelium on the reactivity of isolated resistance arteries. Blood Vessels (1985); **22**: 301-305.

Thelen, M., Dewald, B. and Baggiolini, M. Neutrophil signal transduction and activation of the respiratory burst. Physiol Rev (1993); **73**: 797-821.

Thelen, M. and U, W. Phospholipases and protein kinases during phagocyte activation. Curr Opin Immunol (1994); **6**: 106-112.

Thiel, M. and Bardenheuer, H. Regulation of oxygen radical production of human polymorphonuclear leukocytes by adenosine: the role of calcium. Pfluegers Arch (1992); **420**: 522-528.

Thiemermann, C., Lobel, P. and Schror, K. Usefulness of defibrotide in protecting ischemic myocardium from early reperfusion damage. Am J Cardiol (1985); **56**: 978-982.

Thiemermann, C., Ney, P. and Schror, K. Protection of the ischaemic myocardium from reperfusion injury by defibrotide and its possible mechanism of action. Biomed Biochim Acta (1988); **47**: S19-22.

Toda, N., Kitamura, Y. and Okamra, T. Neural mechanism of hypertension by nitric oxide synthase inhibitor in dogs. Hypertension (1993); **21**: 3-8.

Toombs, C.F., McGee, S., Johnston, W.E. and Vinten-Johansen, J. Myocardial protective effects of adenosine. Infarct size reduction with pretreatment and continued receptor stimulation during ischemia. Circulation (1992); **86**: 986-994.

Tracey, W.R., Magee, W., Masamune, H., Kennedy, S.P., Knight, D.R., Buchholz, R.A. and Hill, R.J. Selective adenosine A_3 receptor stimulation reduces ischemic myocardial injury in the rabbit heart. Cardiovasc Res (1997); **33**: 410-415.

Triana, J.F., Li, X.Y., Jamaluddin, U., Thornby, J.I. and Bolli, R. Postischemic myocardial "stunning". Identification of major differences between the open-chest and the conscious dog and evaluation of the oxygen radical hypothesis in the conscious dog. Circ Res (1991); **69**: 731-747.

Truett, A.P., III, V., M.W., Dillon, S.B. and Snyderman, R. Calcium influx stimulates a second pathway for sustained diacylglycerol production in leukocytes activated by chemoattractants. Proc Natl Acad Sci USA (1988); **85**: 1549-1553.

Truett, A.P.d., Snyderman, R. and Murray, J.J. Stimulation of phosphorylcholine turnover and diacylglycerol production in human polymorphonuclear leukocytes. Novel assay for phosphorylcholine. Biochem J (1989); **260**: 909-913.

Tsao, P.S., Aoki, N., Lefer, D.J., Johnson, I.G. and Lefer, A.M. Time course of endothelial dysfunction and myocardial injury during myocardial ischemia and reperfusion in the cat. Circulation (1990); **82**: 1402-1412.

Tsao, P.S. and Lefer, A.M. Time course and mechanism of endothelial dysfunction in isolated ischaemic and hypoxic perfused rat hearts. Am J Physiol (1990); **259**: H1660-H1666.

Tsuji, T., Nagata, K., Koiki, J., Todoroki, N. and Irimura, T. Induction of superoxide anion production from monocytes and neutrophils by activated platelets through the P-selectin-sialyl Lewis ^{x} interaction. J Leukocyte Biol (1994); **56**: 583-587.

Uchiyama, S., Yamazaki, M. and Maruyama, S. Role of platelet-activating factor in aggregation of leukocytes and platelets in cerebral ischemia. Lipids (1991); **26**: 1247-1249.

Ueno, Y., Miyauchi, Y. and Nishio, S. Beraprost sodium protects occlusion/reperfusion injury in the dog by inhibition of neutrophil migration, Gen Pharmacol (1994); **25**, 427-432.

Uings, I.J., Thompson, N.T., Randell, R.W., Spacey, G.D., Bonser, R.W., Hudson, A.T. and Garland, L.G. Tyrosine phosphorylation is involved in receptor coupling to phospholipase D but not phospholipase C in the human neutrophils. Biochem J (1992); **281**: 597-600.

Uzan, G., Frachet, P., Lajmanovich, A., Prandini, M.H., Denarier, E., Duperray, A., Loftus, J., Ginsberg, M., Plow, E. and Marguerie, G. cDNA clones for human platelet GPIIb corresponding to mRNA from megakaryocytes and HEL cells. Evidence for an extensive homology to other Arg-Gly-Asp adhesion receptors. Eur J Biochem (1988); **171**: 87-93.

Valeri, C.R., Khabbaz, K., Khuri, S.F. *et al.* Effect of skin temperature on platelet function in patients undergoing extracorporeal bypass. J Thorac Cardiovasc Surg (1992); **104**: 108-116.

Vallance, P., Leone, A., Calver, A., Collier, J. and Moncada, S. Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. Lancet (1992); **339**: 572-575.

Vallance, P. and Moncada, S. Role of endogenous nitric oxide in septic shock. New Horizons (1993); **1**: 77-87.

Valles, J., Santos, M.T., Marcus, A.J., Safier, L.B., Broekman, M.J., Islam, N., Ullman, H.L. and Aznar, J. Down regulation of human platelet reactivity by neutrophils. Participation of lipoxygenase derivatives and adhesive proteins. J Clin Invest (1993); **92**: 1357-1365.

Van Der Meulen, J. and Haslam, R.J. Phorbol ester treatment of intact rabbit plaelets greatly enhances both the basal and guanosine 5'-[- thio] triphosphate-stimulated phospholipase D activities of isolated platelet membrane. Biochem J (1990); **271**: 693-700.

van Oeveren, W., Harder, M.P., Roozendaal, K.J., Eijsman, L. and Wildevuur, C.R. Aprotinin protects platelets against the initial effect of cardiopulmonary bypass. J Thorac Cardiovasc Surg (1990); **99**: 788-796; discussion 796-787.

Van Ufflelen, B.E., De Koster, B.M., Van Der Broek, P.J., Vansteveninck, J. and Elferink, J.G. Modulation of neutrophil migration by exogenous gaseous nitric oxide. J Leuk Biol (1996); **60**: 94-100.

Van Benthuysen, K.M., McMurtry, I.F. and Horwitz, L.D. Reperfusion after acute coronary occlusion in dogs impairs endothelium-dependent relaxation to acetylcholine and augments contractile reactivity *in vitro*. J Clin Invest (1987); **79**: 265-274.

Vanin, A.F. Endothelial-derived relaxing factor is a nitrosyl iron complex with thiol ligands. FEBS Letters (1991); **289**: 1-3.

Vaporciyan, A.A., DeLisser, H.M., Yan, H.C., Mendiguren, I.I., Thom, S.R., Jones, M.L., Ward, P.A. and Albelda, S.M. Involement of platelet-endothelial cell adhesion molecule-1 in neutrophil recruitment *in vivo*. Science (1993); **262**: 1581-1582.

Verghese, M.W., Charles, L., Jakoi, L., Dillon, S.B. and Snyderman, R. Role of a guanine nucleotide regulatory protein in the activation of phospholipase C by different chemoattractants. J Immunol (1987); **138**: 4374-4380.

Vinten-Johansen, J., Lefer, D.J., Nakanishi, K., Johnson, W.E., Brian, C.A. and Cordell, A.R. Controlled coronary hydrodynamics at the time of reperfusion reduces postischaemic injury. Coron Artey Dis (1992); **3**: 1081-1093.

Vinten-Johansen, J., Zhao, Z.Q., Sato, H., Jordan, J.E., Williams, M.W. and Sarvotham, S.S. Reduction in infarct size but not neutrophil accumulation by controlled haemodynamics of reperfusion. FASEB J (1995b); **9**: A420.

von Andrian, U.H., Chambers, J.D., McEvoy, L.M., Bargatze, R.F., Arfors, K.E. and Butcher, E.C. Two-step model of leukocyte-endothelial cell interaction in inflammation: distinct roles for LECAM-1 and the leukocyte beta 2 integrins *in vivo*. Proc Natl Acad Sci U S A (1991); **88**: 7538-7542.

Walday, P. and Aas, P. Prejunctional stimulation of cholinergic nerves in rat airway smooth muscle by an adenosine analogue. Pulm Pharmacol (1991); **4**: 114-119.

Waldman, S.A. and Murad, F. Cyclic GMP synthesis and function. Pharmacol Rev (1987); **39**: 163-196.

Walker, B.A., Cunningham, T.W., Freyer, D.R., Todd, R.F., Johnson, K.J. and Ward, P.A. Regulation of superoxide responses of human neutrophils by adenine compounds. Independence of requirement for cytoplasmic granules. Lab Invest (1989); **61**: 515-521.

Wallen, N.H., Held, C., Rehnqvist, N. and Hjemdahl, P. Impact of treatment with acetylsalicylic acid on the proaggregatory effects of adrenaline *in vitro* in patients with stable angina pectoris: influence of the anticoagulant. Clin Sci (Colch) (1993); **85**: 577-583.

Walsh, C.E., Dechatelet, L.R., Chilton, F.H., Wykle, L. and Waite, M. Mechanism of arachidonic acid release in human polymorphonuclar leukocytes. Biochim Biophys Acta (1983); **750**: 32-40.

Walz, G., Aruffo, A., Kolanus, W., Belvilacqua, M. and Seed, B. Recognition by ELAM-1 of the sialyl-Lex determinant on myeloid and tumor cells. Science (1990); **250**: 1132-1135.

Wanikiat, P., Woodward, D.F. and Armstrong, R.A. Investigation of the role of nitric oxide and cyclic GMP in both the activation and inhibition of human neutrophils. Br J Pharmacol, (1997); **122**: 1135-1145.

Watson, F., Robinson J and SW., E. Protein kinase C-dependent and -independent activation of the NADPH oxidase of human neutrophils. J Biol Chem (1991); **266**: 7432-7439.

Webb, R.L., Sills, M.A., Chovan, J.P., Balwierczak, J.L. and Francis, J.E. CGS 21680: a potent selective adenosine A_2 receptor agonist. Cardiovasc Drug Rev (1992); **10**: 26-53.

Weber, T. and Springer, T. Neutrophil accumulation on activated, surface-adherent platelets in flow is mediated by interaction of mac-1 with fibrinogen bound to aIIb3 and stimulated by platelet-activating factor. J Clin Invest (1997); **100**: 2085-2093.

Weiss, H.J., Baumgartner, H.R., Tschopp, T.B., Turitto, V.T. and Cohen, D. Correction by factor VIII of the impaired platelet adhesion to subendothelium in von Willebrand disease. Blood (1978); **51**: 267-279.

Weiss, S.J. and Lampert, M.B.T., S.T. Long-lived oxidants generated by human neutrophls: characterization and bioactivity. Science (1983); **222**: 625-628.

Weiss, S.J. Tissue destruction by neutrophils [see comments]. N Engl J Med (1989); **320**: 365-376.

Weiss, H.J., Hawiger, J., Ruggeri, Z.M., Turitto, V.T., Thiagarajan, P. and Hoffmann, T. Fibrinogen-independent platelet adhesion and thrombus formation on subendothelium mediated by glycoprotein IIb-IIIa complex at high shear rate. J Clin Invest (1989); **83**: 288-297.

Weksler, B.B., 1988. Platelets, in: Inflammation: Basic Principles and Clinical Correlates, eds. J.I. Gallin, G. I.M. and R. Synderman (Raven Press, New York) pp. 543-557.

Wencel-Drake, J.D., Painter, R.G., Zimmerman, T.S. and Ginsberg, M.H. Ultrastructural localization of human platelet thrombospondin, fibrinogen, fibronectin, and von Willebrand factor in frozen thin section. Blood (1985); **65**: 929-938.

Wenger, R.K., Lukasiewicz, H., Mikuta, B.S., Niewiarowski, S. and Edmunds, L.H.J. Loss of platelet fibrinogen receptors during clinical cardiopulmonary bypass. J Thorac Cardiovasc Surg (1989); **97**: 235-239.

Wenzel-Seifert, K., Ervens, J. and Seifert, R. Differential inhibition and potentiation by cell-permeant analogues of cylic AMP and cyclic GMP and NO-containing compounds of exocytosis in human neutrophils. Naunyn-Schmied Arch Pharmacol (1991); **344**: 396-402.

Werns, S.W. and Lucchesi, B.R. Leukocytes, oxygen radicals, and myocardial injury due to ischemia and reperfusion. Free Radic Biol Med (1988); **4**: 31-37.

Werns, S., 1994. Free radical scavengers and leukocyte inhibitors, in: Text book of Interventional Cardiology, Vol. 2, ed. E.J. Topol (Saunders, Philadelphia) pp. 137-160.

Westlin, W. and Mullane, K.M. Alleviation of myocardial stunning by leukocyte and platelet depletion. Circulation (1989); **80**: 1828-1836.

Weyrich, A.S., Ma, X.L. and Lefer, A.M. The role of L-arginine in ameliorating reperfusion injury after myocardial ischemia in the cat. Circulation (1992); **86**: 279-288.

Weyrich, A.S., Ma, X. and Lefer, D.J. *et al.* *In vivo* neutralization of P-selectin protects feline heart and endothelium in myocardial ischemia and reperfusion injury. J Clin Invest (1993); **91**: 2620-2629.

White, J.G. Exocytosis of secretory organelles from blood platelets incubated with cationic polypeptides. Am J Path (1972); **69**: 41-53.

White, J.G. Identification of platelet secretion in the electron microscope. Ser Haematol (1973); **6**: 429-459.

White, J.G. Views of the platelet cytoskeleton at rest and at work. Ann N Y Acad Sci (1987); **509**: 156-176.

White, J.G. An overview of platelet structural physiology. Scanning Microsc (1987); **1**: 1677-1700.

White, K.A. and Marletta, M.A. Nitric oxide synthase is a cytochrome P-450 type hemoprotein. Biochemistry (1992); **31**: 6627-6630.

Wiklund, N.P., Leone, A.M., Gustafsson, L.E. and Moncada, S. Release of nitric oxide evoked by nerve stimulation in guinea-pig intestine. Neuroscience (1993); **53**: 607-611.

Willerson, J.T., Watson, J.T., Hutton, I., Templeton, G.H. and Fixler, D.E. Reduced myocardial reflow and increased coronary vascular resistance following prolonged myocardial ischemia in the dog. Circ Res (1975); **36**: 771-781.

Williams, F.M., Kus, M., Tanda, K. and Williams, T.J. Effect of duration of ischaemia on reduction of myocardial infarct size by inhibition of neutrophil accumulation using an anti-CD18 monoclonal antibody. Br J Pharmacol (1994); **111**: 1123-1128.

Williams, M.W., Taft, C.S., Ramnauth, S., Zhao, Z.Q. and Vinten-Johansen Endogenous nitric oxide (NO) protects against ischaemia-reperfusion injury in the rabbit. Cardiovasc Res (1995); **30**: 79-86.

Wise, H. The inhibitory effects of non-prostanoid prostacyclin mimetics on rat neutrophil function. Prostaglandins Leukoc Essent Fatty Acids (1996); **54**: 351-360.

Wollner, A., Wollner, S. and Smith, J.B. Acting via A_2 receptors, adenosine inhibit the upregulation of Mac-1 (Cd11b/CD18) expression on fMLP-stimulated neutrophils. Am J Respir Cell Mol Biol (1993); **9(2)**: 179-85.

Woodman, R.C. and Harker, L.A. Bleeding complications associated with cardiopulmonary bypass. Blood (1990); **76**: 1680-1697.

Woodward, B. and Zakaria, M.N. Effect of some free radical scavengers on reperfusion induced arrhythmias in the isolated rat heart. J Mol Cell Cardiol (1985); **17**: 485-493.

Worku, Y. and Newby, A.C. The mechanism of adenosine production in rat polymorphonuclear leukocytes. Biochem J (1983); **214**: 1-6.

Wright, D.G. and Gallin, J.I. A functional differentiation of human neutrophil granules: Generation of C5a by a specific (secondary) granule product and inactivation of C5a by azurophil (primary) granule products. J Immunol (1977); **119**: 1068-1076.

Wright, S.D., Weitz, J.I., Huang, A.J., Levin, S.M., Silverstein, S.C. and Loike, J.D. Complement receptor type three (CD11b/CD18) of human polymorphonuclear leukocytes recognizes fibrinogen. Proc Natl Acad Sci U S A (1988); **85**: 7734-7738.

Wright, C.D., Mulsch, A., Busse, R. and Osswald, H. Generation of nitric oxide by human neutrophils. Biochem Biophys Res Commun (1989); **160**: 813-819.

Wyatt, T.T., Lincoln, T.M. and Pryzwansky, K.B. Compartmentalization of cyclic GMP-dependent protein kinase (G-kinase) in calcium inophore and phorbol ester stimulated human neutrophils. FASEB J (1990); **4**: A872.

Wyatt, T.A., Lincoln, T.M. and Pryzwansky, K.B. Vimentin is transiently localized with and phosphorylated by cyclic GMP-dependent protein kinase in formyl-peptide stimulated neutrophils. J Biol Chem (1991); **266**: 21274-80.

Wyatt, T.A., Lincoln, T.M. and Pryzwansky, K.B. KT 5823 activates human neutrophils and fails to inhibit cGMP-dependent protein kinase phosphorylation of vimentin. Res Commun Chem Pathol Pharm, (1991); **74**: 3-13.

Wyatt, T.A., Lincoln, T.M. and Pryzwansky, K.B. Regulation of human neutrophil degredation by LY-83583 and L-arginine: role of cGMP-dependent protein kinase. Am J Physiol (1993); **265**: C201-C211.

Yamazaki, T., Seko, Y. and Tamatani, T. *et al.* Expression of intercellular adhesion molecule-1 in rat heart with ischemia/reperfusion and limitation of infarct size by treatment with antibodies against cell adhesion molecules. Am J Pathol (1993); **143**: 410-418.

Yasui, K., Masuda, M., Matsuoka, T., Yamazaki, M., Komiyama, A., Akabane, T., Hasui, M., Kobayashi, Y. and Murata, K. Abnormal membrane fluidity as a cause of impaired functional dynamics of chemoattractant receptors on neonatal polymorphonuclear leukocytes: lack of modulation of the receptors by a membrane fluidizer. Pediatr Res (1988); **24**: 442-446.

Yeo, E.L., Sheppard, J.A. and Feuerstein, I.A. Role of P-selectin and leukocyte activation in polymorphonuclear cell adhesion to surface adherent activated platelets under physiologic shear conditions (an injury vessel wall model). Blood (1994); **83**: 2498-2507.

Yoshimura, K., Nakagawa, S., Koyama, S., Kobayashi, T. and Homma, T. Roles of neutrophil elastase and superoxide anion in leukotriene B_4-induced lung injury in rabbit. J Appl Physiol (1994); **76**: 91-96.

Ytrehus, K., Liu, Y.G., Tsuchida, A., Miura, T., Liu, G.S., Yang, X.M., Herbert, D., Cohen, M.V. and Downey, J.M. Rat and rabbit heart infarction: Effects of anesthesia, perfusate, risk zone, and method of infarct sizing. Am J Physio (1994); **36**: H2383-H2390.

Yui, Y., Hattori, R., Kosuga, K., Eizawa, H., Hiki, K., Ohkawa, S., Ohnishi, K., Terao, S. and Kawai, C. Calmodulin-independent nitric oxide synthase from rat polymorphonuclear neutrophils. J Biol Chem (1991); **266**: 3369-3371.

Zhang, J., King, W.G., Dillon, S., Hall, A., Feig, L. and Rittenhouse, S.E. Activation of platelet phosphatidylinositide 3-kinase requires the small GTP-binding protein Rho. J Biol Chem (1993); **268**: 22251-22254.

Zhao, Z.Q., Bato, H. and Williams, M.W. Adenosine A_2-receptor activation inhibits neutrophil-mediated injury to coronary endothelium. Am J Physiol (1996); **271**: H1456-H1464.

Zilla, P., Fasol, R., Groscurth, P., Klepetko, W., Reichenspurner, H. and Wolner, E. Blood platelets in cardiopulmonary bypass operations. Recovery occurs after initial stimulation, rather than continual activation [see comments]. J Thorac Cardiovasc Surg (1989); **97**: 379-388.

Zucker, M.B. The functioning of blood platelets. Sci Am (1980); **242**:86-103.

Zurier, R.B., Weissmann, G., Hoffstein, S., Kammerman, S. and Tai, H.H. Mechanisms of lysosomal enzyme release from human leukocytes. II. Effects of cAMP and cGMP, autonomic agonists, and agents which affect microtubule function. J Clin Invest (1974); **53**: 297-309.

APPENDIX I

Buffers and Solutions used in this study

Buffers and solutions used for isolation of human neutrophils

Saline solution	
0.2% saline	0.2g NaCl in 100 ml dist.H_2O
0.9% saline	4.5g NaCl in 500 ml dist.H_2O
9% saline	9g NaCl in 500 ml dist.H_2O
1.6% saline	1.6g NaCl in 100 ml dist.H_2O
3% Dextran solution	12 g dextran in 400 ml 0.9% saline
Percoll solution	
81% percoll	40.5 ml percoll, 5ml 0.9% saline and 4.5 ml 9% saline
70% percoll	35.5 ml percoll, 10.5ml 0.9% saline and 4 ml 9% saline
55% percoll	27.5 ml percoll, 19.5ml 0.9% saline and 3 ml 9% saline
Phosphate buffer (pH 7.4)	Made from commercial tablets Dissolved 1 tablet in 200ml dist.H_2O; Autoclaved at 115°C for 15 minutes

Buffer and solutions used for measurement of superoxide anion generation

PBS/cytochrome C/ cytochalasin B	0.125g cytochrome C and 50µl cytochalasinB (stock solution 5mg/ml in DMSO) added to 50 ml PBS

Buffers and solutions used for measurement of cyclic GMP

50mM Acetate buffer (pH 5.0)	500 ml 0.1M acetic acid added to 500 ml 0.1 M sodium acetate anhydrous ; adjusted to pH 5.0 using NaOH. Stored at 4°C
50mM Phosphate buffer (pH 7.4)	250ml 50mM $NaH_2PO_4.2H_2O$ added to 500ml 50mM Na_2HPO_4; adjusted to pH 7.4 using $NaH_2PO_4.2H_2O$; sodium azide added as a preservative. Stored at 4°C
Trimethylamine/ acetic anhydride solution (2:1)	10ml acetic anhydride added to 20ml triethylamine

Buffers and solutions used for extraction of MPO from heart tissue and rat neutrophils

Homogenisation Buffer I (pH 4.7) (0.02M Phosphate buffer)	1.168g NaCl (0.1M), 0.624g NaH_2PO_4 (0.02M) and 1.116g NaEDTA (0.05M) in 200 ml dist. H_2O; adjusted to pH 4.7 using NaOH
Homogenisation Buffer II (pH 5.4) (0.05M Phosphate buffer)	4 ml 0.05M Na_2HPO_4 added to 196ml 0.05M NaH_2PO_4; adjusted to pH 5.4.

Buffers and solutions used for measurement of MPO

0.05M Citrate Phosphate Buffer (pH5)	7ml 0.05M citric acid added to 13ml 0.05M Na_2HPO_4; adjusted to pH 5.0.
Reaction Buffer	2mg 3,3'5,5'-tetramethylbenzidine and 8 µl H_2O_2. (0.012%v/v) added to20ml 0.05M citrate phosphate buffer

Solutions used for measurement of myocardial infarct size

4% Evan's blue	0.4g Evan's blue in 10ml dist.H_2O
1% w/v 2,3,5-triphenyltetrazolium chloride (TTC)	1g TTC in 0.9% saline
10% formalin	10ml of 40% formaldehyde and 0.9g NaCl added to 90ml dist.H_2O

APPENDIX II

Stock solutions

Solvents for stock solutions

fMLP	Dissolved in DMSO at concentration of 10^{-2} M, aliquoted stored at -20°C.
L-nMMA	Dissolved in 0.9%saline at concentration of 10^{-2} M, aliquoted stored at -20°C.
L-Canavanine	Dissolved in distilled H_2O at concentration of 10^{-1} M, aliquoted stored at -20°C.
Carboxy-PTIO	Dissolved in distilled H_2O at concentration of 10^{-2} M, aliquoted stored at -20°C.
LY 83583	Dissolved in DMSO at concentration of 10^{-2} M, aliquoted stored at -20°C.
KT 5823	Dissolved in DMSO at concentration of 10^{-2} M, aliquoted stored at -20°C.
Rp-8-cpCPT-cGMPS	Dissolved in 0.9% saline at concentration of 10^{-2} M, aliquoted stored at -20°C.
2,3 DPG	Dissolved in 0.9%saline at concentration of 10^{-2} M, aliquoted stored at -20°C.
GEA 3162, GEA 5024 and SIN-1	Dissolved in 0.9%saline at concentration of 10^{-2} M, aliquoted stored at -20°C.
Cytochalasin B	Dissolved in DMSO at concentration of 5 µg/ml aliquoted stored at -20°C.
Cyclic GMP	Dissolved in acetate buffer at 3.2 mM
Cicaprost	Stock 50µg/ml in 0.9% saline
2-HE-NECA	Dissolved in DMSO at concentration 10^{-3} M
r-hirudin	Dissolved in PBS (pH 7.4) containing 0.1% Polyethylene glycol 600, stored at -20°C.
Heparinase I	Dissolved in 62.5 mM Na-Phosphate, 125 mM NaCl pH7.

APPENDIX III

Published Papers

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Investigation of the role of nitric oxide and cyclic GMP in both the activation and inhibition of human neutrophils

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1 The aim of this study was to establish the role of nitric oxide (NO) and cyclic GMP in chemotaxis and superoxide anion generation (SAG) by human neutrophils, by use of selective inhibitors of NO and cyclic GMP pathways. In addition, inhibition of neutrophil chemotaxis by NO releasing compounds and increases in neutrophil nitrate/nitrite and cyclic GMP levels were examined. The ultimate aim of this work was to resolve the paradox that NO both activates and inhibits human neutrophils.

2 A role for NO as a mediator of N-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced chemotaxis was supported by the finding that the NO synthase (NOS) inhibitor L-NMMA (500 μM) inhibited chemotaxis; EC_{50} for fMLP 28.76 ± 5.62 and 41.13 ± 4.77 pmol/10^6 cells with and without L-NMMA, respectively. Similarly the NO scavenger carboxy-PTIO (100 μM) inhibited chemotaxis; EC_{50} for fMLP 19.71 ± 4.23 and 31.68 ± 8.50 pmol/10^6 cells with and without carboxy-PTIO, respectively.

3 A role for cyclic GMP as a mediator of chemotaxis was supported by the finding that the guanylyl cyclase inhibitor LY 83583 (100 μM) completely inhibited chemotaxis and suppressed the maximal response; EC_{50} for fMLP 32.53 ± 11.18 and 85.21 ± 15.14 pmol/10^6 cells with and without LY 83583, respectively. The same pattern of inhibition was observed with the G-kinase inhibitor KT 5823 (10 μM); EC_{50} for fMLP 32.16 ± 11.35 and >135 pmol/10^6 cells with and without KT 5823, respectively.

4 The phosphatase inhibitor, 2,3-diphosphoglyceric acid (DPG) (100 μM) which inhibits phospholipase D, attenuated fMLP-induced chemotaxis; EC_{50} for fMLP 19.15 ± 4.36 and 61.52 ± 16.2 pmol/10^6 cells with and without DPG, respectively.

5 Although the NOS inhibitors L-NMMA and L-canavanine (500 μM) failed to inhibit fMLP-induced SAG, carboxy-PTIO caused significant inhibition (EC_{50} for fMLP 36.15 ± 7.43 and 86.31 ± 14.06 nM and reduced the maximal response from 22.14 ± 1.5 to 9.8 ± 1.6 nmol O_2^-/10^6 cells/10 min with and without carboxy-PTIO, respectively). This suggests NO is a mediator of fMLP-induced SAG.

6 A role for cyclic GMP as a mediator of SAG was supported by the effects of G-kinase inhibitors KT 5823 (10 μM) and Rp-8-pCPT-cGMPS (100 μM) which inhibited SAG giving EC_{50} for fMLP of 36.26 ± 8.77 and 200.01 ± 43.26 nM with and without KT 5823, and 28.35 ± 10.8 and 49.25 ± 16.79 nM with and without Rp-8-pCTP-cGMPS.

7 The phosphatase inhibitor DPG (500 μM) inhibited SAG; EC_{50} for fMLP 33.93 ± 4.23 and 61.12 ± 14.43 nM with and without DPG, respectively.

8 The NO releasing compounds inhibited fMLP-induced chemotaxis with a rank order of potency of GEA 3162 ($IC_{50} = 14.72 \pm 1.6$ μM) > GEA 5024 ($IC_{50} = 18.44 \pm 0.43$ μM) > SIN-1 ($IC_{50} > 1000$ μM). This order of potency correlated with their ability to increase cyclic GMP levels rather than the release of NO, where SIN-1 was most effective (SIN-1 ($EC_{50} = 37.62 \pm 0.9$ μM) > GEA 3162 ($EC_{50} = 39.7 \pm 0.53$ μM) > GEA 5024 ($EC_{50} = 89.86 \pm 1.62$ μM)).

9 In conclusion, chemotaxis and SAG induced by fMLP can be attenuated by inhibitors of phospholipase D, NO and cyclic GMP, suggesting a role for these agents in neutrophil activation. However, the increases in cyclic GMP and NO induced by fMLP, which are associated with neutrophil activation, are very small. In contrast much larger increases in NO and cyclic GMP, as observed with NO releasing compounds, inhibit chemotaxis.

Keywords: Neutrophils; nitric oxide; superoxide anion; chemotaxis; guanylyl cyclase; cyclic GMP; G-kinase; phospholipase D; nitric oxide synthase

Introduction

Neutrophils represent the first line of host defence against bacterial infection. They are recruited from the bloodstream by chemotactic factors generated and released locally in injured tissue (Barten *et al.*, 1976; Weiss, 1989). Once at the site of inflammation, neutrophils release toxic substances such as superoxide anion (O_2^-) and lysosomal enzymes, and ingest micro-organisms by phagocytosis.

Neutrophil locomotion to a specific chemoattractant is a complex, multi-step process requiring ligation of a cell surface receptor, transduction of a signal from the receptor to intracellular effectors, reorganisation of the cytoskeleton and finally a directed crawling movement towards the source of chemotaxin (Cassimeris & Zigmond, 1990). Several cellular pathways, as well as numerous specific macromolecules have been identified as being essential for the process of neutrophil movement (Cassimeris & Zigmond, 1990; Gaudry *et al.*, 1992; Amatruda *et al.*, 1993). However, the effector signalling pathways activated in neutrophils to promote chemotaxis are still poorly understood. Unstimulated and primed human and rat neutrophils have been shown to generate and release factors with the pharmacological characteristics of nitric oxide (NO) (Stephens & Snyderman, 1982; Rimele *et al.*, 1988; 1991; Wright *et al.*, 1989; Schmidt *et al.*, 1989; Salvemini *et al.*, 1989; McCall *et al.*, 1989; Myers *et al.*, 1990; Mehta *et al.*, 1990; Lee

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et al., 1990; Yui *et al.*, 1991; Moncada & Higgs, 1991; Moncada *et al.*, 1991; Lopez Farre *et al.*, 1991; Kadota *et al.*, 1991). The release of NO is regulated by nitric oxide synthase (NOS), a cytosolic enzyme that catalyses the conversion of L-arginine to L-citrulline and NO (Moncada *et al.*, 1991). Through the stimulation of guanylyl cyclase, nitric oxide increases guanosine 3′:5′-cyclic monophosphate (cyclic GMP) formation. Intracellular accumulation of cyclic GMP has been suggested to regulate neutrophil chemotaxis *in vitro* (Sandler *et al.*, 1975; Smith & Ignarro, 1975; Stephens & Snyderman, 1982; Anderson *et al.*, 1986; Kaplan *et al.*, 1989). Consistent with these concepts, it has been shown that fMLP-mediated chemotaxis was decreased by an inhibitor of NOS, N^G-monomethyl-L-arginine (L-NMMA), and that exogenous cyclic GMP reversed this inhibition (Kaplan *et al.*, 1989). Furthermore, it has been shown that NOS inhibitors significantly attenuate chemotaxis of unstimulated and primed human neutrophils *in vitro* and that these effects were specific and modulated by cyclic GMP (Belenky *et al.*, 1993). These two latter experiments suggest a role for NO as an intracellular messenger mediating neutrophil chemotaxis, possibly by increasing cell polarization (Caterina & Deureotes, 1991). This is supported by the recent demonstration that exogenous NO induced chemotaxis of neutrophils *in vitro* (Beauvais *et al.*, 1995).

However, in contradiction to this, there is also data suggesting that NO or NO-releasing compounds can inhibit aspects of neutrophil activation such as chemotaxis, degranulation, leukotriene (LT) production and O_2^- release (Clancy *et al.*, 1982). Some of these effects were suggested to be mediated, at least in part, by an increase of cyclic GMP from activation of soluble guanylyl cyclase (Ney *et al.*, 1990; Schroder *et al.*, 1990; Kubes *et al.*, 1991; Wenzel-Seifert *et al.*, 1991; Moilanen *et al.*, 1993).

The aim of this study was to clarify the role of NO and cyclic GMP in chemotaxis and superoxide anion generation (SAG) by human neutrophils. For this purpose, the inhibitory effects of the NOS inhibitors, L-NMMA and L-canavanine; the NO scavenger carboxy-PTIO; the guanylyl cyclase inhibitor LY 83583; the G-kinase inhibitors, KT 5823 and Rp-8-cCPT-cGMPS and the phosphatase inhibitor, 2,3 diphosphoglycerate (DPG) have been investigated. In addition the NO-releasing compounds, 3-morpholinosydnonimine (SIN-1) and 4-aryl-substituted oxatriazol derivatives (GEA 3162 and GEA 5024) have been tested for inhibition of neutrophil chemotaxis as well as for their ability to increase neutrophil nitrate/nitrite and cyclic GMP levels. The ultimate aim of this work was to resolve the paradox that NO appears to be able to both activate and inhibit human neutrophils.

Methods

Isolation of human neutrophils

Human neutrophils were isolated as described previously (Talpain *et al.*, 1995). Any contaminating red cells were removed by hypotonic lysis with ice-cold NaCl (0.2% w/v) and the cells returned to isotonic conditions with NaCl (1.6%). The cells were > 95% viable as determined by trypan blue exclusion and were resuspended as required below.

Chemotaxis procedure

Cells were resuspended at a concentration of 3×10^6 cells ml^{-1} in RPMI 1640 medium (HEPES buffered, without glutamine, Gibco) and chemotaxis measured in a 96 well chemotaxis chamber (Neuroprobe, Cabin John, Md). The bottom wells of the chamber were filled with chemoattractant, N-formyl-methionyl-lencyl-phenylalanine (fMLP; 0.1–3 μM) in 30 μl RPMI medium which had been warmed to 37°C. The top plate with the filter (3 μm) installed was then inverted onto the filled bottom plate, and the upper wells filled with cells (225 μl) which had been treated with inhibitor or RPMI medium. In the case of L-NMMA, SIN-1, GEA 3162 and GEA 5024, the cells were pre-incubated with these drugs for 10–45 min, as appropriate. With carboxy-PTIO, LY 83583, KT 5823 and Rp-8-pCPT-cGMPS, no preincubation was required. The chamber was then incubated for 45 min at 37°C in a moist, 5% CO_2 atmosphere. At the end of the incubation period, the filter was removed, washed, fixed and stained with Diff Quick (Baxter Diagnostics AG; fixative-fast green in methanol for 5 min, eosin G in phosphate buffer for 5 min, thiazine dye in phosphate buffer for 5 min). Chemotaxis was quantified spectrophotometrically by measuring absorbance at 550 nm and the magnitude of the absorbance taken as directly proportional to the number of cells which have migrated and are trapped in the filter. Basal absorbance was taken as cells without fMLP. Each incubation was carried out in triplicate and the values were averaged.

Superoxide anion generation

Neutrophil SAG was assayed by spectrophotometric evaluation of the reduction of ferricytochrome C to ferrocytochrome C (A 550 nm) as described previously (Armstrong, 1995). Briefly, cells (1.5×10^6 cells ml^{-1}) were resuspended in PBS containing cytochrome C (2.5 mg ml^{-1}) and cytochalasin B (5 μg ml^{-1}). Cells were treated with PBS or inhibitor, immediately before the addition to the tubes containing fMLP (3–300 nM) and incubated for 10 min at 37°C. With L-NMMA, L-canavanine and DPG, cells were pre-incubated for 10–45 min, as appropriate. The reaction was terminated by immersing the tubes in ice for 5 min and the samples were centrifuged at 300 *g*, at 4°C for 10 min, to sediment the cells. Aliquots (200 μl) from each tube were dispensed into a 96 well plate and the absorbance at 550 nm was measured. Basal absorbance was taken as cells without fMLP. Each incubation was carried out in triplicate and the values were averaged.

Cyclic GMP measurement

Neutrophils were resuspended to give 5×10^6 cells ml^{-1} in PBS containing 0.25 mM isobutylmethylxanthine (IBMX). Cells (450 μl) were incubated for 10 min at 37°C, with buffer, fMLP or NO releasing compounds, and the reaction stopped by the addition of ethanol (1.0 ml). Five minutes later, the samples were centrifuged at 650 *g*, 20°C for 20 min, the ethanolic supernatants removed and evaporated to dryness at 55°C. The residue was dissolved in assay buffer (0.5 ml) and centrifuged at 1900 *g*, 4°C, for 30 min to remove insoluble material. Two samples (50 μl) of the supernatant were assayed. Both samples and standards were acetylated with a mixture of triethylamine/acetic anhydride (2:1) before measurement of cyclic GMP by radioimmunoassay. Each assay tube contained the following substances: unlabelled cyclic GMP (Sigma) (50 μl of 0.0625–32 nM) or samples to be measured and sodium acetate buffer (pH 6.0) (200 μl of 0.05 mM) containing specific antibody and guanosine 3′,5′-cyclic phosphoric acid 2′-O′-succinyl-3-[^{125}I]-iodotyrosine methyl ester (5000 c.p.m./tube, Amersham). The assay tubes were kept at 4°C for 1 h, then donkey anti-rabbit serum (50 μl of 1:10 dilution in phosphate buffer) and normal rabbit serum (50 μl of 1:100 dilution in phosphate buffer) were added and the assay tubes were incubated overnight. The assay tubes were washed with 6% polyethylene glycol in deionized water (1.0 ml) and then centrifuged at 1900 *g* for 30 min at 4°C. The supernatant was aspirated and the residue counted with a gamma counter.

Total nitrate and nitrite production

Total nitrate and nitrite production was measured by use of Cayman's nitrate/nitrite assay kit (Alexis Corporation). The measurement is a simple two-step process where nitrate is converted to nitrite with nitrate reductase, and nitrite is measured spectrophotometrically at 540 nm by use of the Griess reagents.

Cells (1.5×10^6 cells ml^{-1}) were resuspended in PBS and treated with NO-releasing compounds (GEA 3162, GEA 5024 and SIN-1) for 10 min at 37°C before addition to the tubes containing PBS and incubated for 30 min at 37°C. With fMLP, cells were treated with PBS for 10 min at 37°C before addition to the tubes containing fMLP (3–300 nM) and incubated for 30 min at 37°C. The reaction was terminated by immersing the tubes in ice for 5 min and the samples were centrifuged at 300 *g*, at 4°C for 10 min to sediment the cells. Aliquots (80 μl) were dispensed into a microtitre plate and assayed spectrophotometrically.

Data analysis

Chemotaxis and SAG EC_{50} values were calculated as the concentration of fMLP required to produce 50% of the maximal response obtained in each experiment with fMLP (300 nM). As such, when drug treatment suppressed the fMLP maximum response, this observed maximum (i.e. determined in the presence of drug) was used for the purpose of determining the EC_{50} value. Each concentration-effect curve was illustrated by use of the Apple Macintosh programme 'Kaleidagraph' and the EC_{50} value determined.

Effects of the NO-releasing compounds, GEA 3162, GEA 5024 and SIN-1, on fMLP-induced chemotaxis were expressed as the percentage inhibition of the response produced by a submaximally effective concentration of fMLP (100 nM). The EC_{50} values for GEA 5024 and SIN-1 were determined relative to the maximum effect achieved with GEA 3162. From EC_{50} values, equieffective concentration-ratios (EEC) were calculated relative to the standard inhibitor, GEA 3162 (EEC = 1).

Statistical analysis

Data are expressed as the mean ± s.e.mean, of the averaged result taken from a minimum of four separate experiments. Data were analysed with Student's paired two-tailed *t* test. In addition, data involving multiple comparisons were analysed by ANOVA (two factor with replication) by use of microsoft Excel. A value of $P < 0.05$ was taken as significant.

Materials

The following compounds were gifts which are gratefully acknowledged: 4-aryl-substituted oxatriazol derivatives GEA 3162 (3-(3′,4′-dichlorophenyl)-1,2,3,4-oxatriazol-5-imine) and GEA 5024 (3-(3′-chloro-2′-methylphenyl)-1,2,3,4-oxatriazol-5-imine) from Dr S.B. Pedersoen (GEA Ltd, Copenhagen, Denmark) specific antibody against acetylated cyclic GMP from Dr I. Gow (Department of Physiology, University of Edinburgh).

N-formyl-methionyl-leucyl-phenylalanine (fMLP), L-canavanine, PBS (containing Ca^{2+} and Mg^{2+}), 2,3-diphosphoglycerate (DPG), trypan blue, guanosine 3′:5′-cyclic monophosphate, polyethylene glycol, cytochrome C and cytochalasin B were purchased from Sigma; RPMI 1640 from Gibco; N^G-monomethyl-L-arginine (L-NMMA), 6-anilinoquinoline-5-8-quinone (LY 83583), (8**R**,9**S**,11**S**)-(−)-9-methoxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7b, 11a-triazadibenzo (a,g) cycloocta (cde)-trinden-1-one (KT 5823) from Calbiochem; Rp-8-(4-chlorophenylthio)-guanosine-3′-5′-cyclic monophosphorothioate (Rp-8pCPT-cGMPS) from Biolog; Diff-Quick from Gamidor; 2-(4-carboxyphenyl)-4,4,5,5-tetra methylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) and 3-morpholinosydnonimine (SIN-1) from Tocris Cookson; Cayman's nitrate/nitrite assay kit from Alexis Corporation; quanosine 3′,5′-cyclic phosphoric acid, 2′-O′-succinyl-3-[^{125}I]-iodo tyrosine methyl ester from Amersham; triethylamine and acetic anhydride from BDH. Donkey-anti-rabbit serum and normal rabbit serum were supplied by the Scottish Antibody Production Unit (Carluke).

Results

Neutrophil chemotaxis

Effect of NOS inhibition When neutrophils were preincubated with the NOS inhibitor, L-NMMA (500 μM) for 45 min at 37°C, significant attenuation ($P < 0.001$, ANOVA) of fMLP-induced neutrophil chemotaxis occurred (Figure 1a), EC_{50} for fMLP 28.76 ± 5.62 and 41.13 ± 4.77 pmol/10^6 cells, $n = 5$ ($P < 0.05$) in the absence and presence of L-NMMA, respectively. The maximum effect of fMLP was reduced from 1.1 ± 0.09 to 0.72 ± 0.09, $n = 5$ ($P < 0.05$). Similarly, L-NMMA at a concentration of 100 μM induced a significant but less

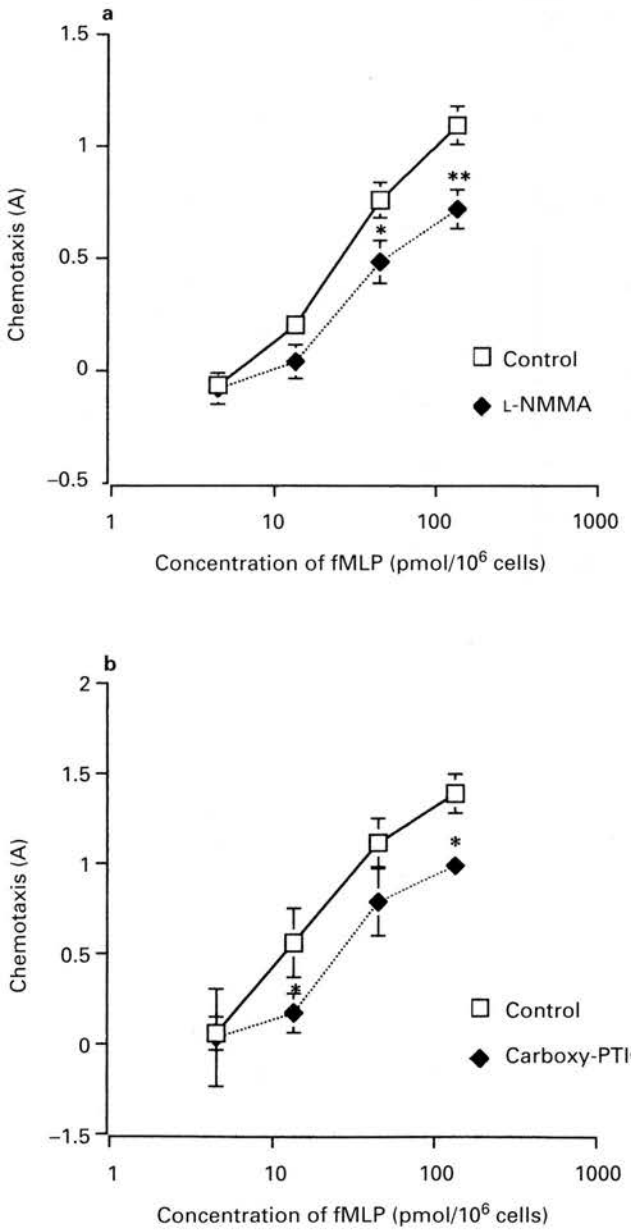


Figure 1 Log concentration-effect curves for fMLP induced neutrophil chemotaxis in control cells and cells treated with (a) L-NMMA (500 μM) and (b) carboxy-PTIO (100 μM). Cells were preincubated with L-NMMA for 45 min at 37°C. No preincubation was required with carboxy-PTIO. The values are the mean, and vertical lines show s.e.mean, of 5 different donors. Statistically significant difference of $*P < 0.05$ and $**P < 0.01$.

pronounced attenuation of neutrophil chemotaxis (data not shown; $P<0.05$).

Effect of a NO scavenger Carboxy-PTIO, a NO scavenger, at a concentration of 100 μM caused slight attenuation ($P<0.05$, ANOVA) of fMLP-induced neutrophil chemotaxis (Figure 1b); EC_{50} for fMLP 19.71 ± 4.23 and 31.68 ± 8.50 pmol/10^6 cells, $n=4$, ($P=0.052$) in the absence and presence of carboxy-PTIO, respectively.

Effect of guanylyl cyclase inhibition LY 83583, an inhibitor of guanylyl cyclase, at concentrations of 10 μM ($P<0.01$, ANOVA) and 100 μM ($P<0.001$, ANOVA) caused significant attenuation of fMLP-induced neutrophil chemotaxis (Figure 2a and b); EC_{50} for fMLP 19.07 ± 4.3 and 47.04 ± 7.52, $n=4$ ($P<0.05$) and 32.53 ± 11.18 and >135 pmol/10^6 cells, $n=4$ ($P<0.05$) in the absence and presence of LY-83583 at the two concentrations, respectively. LY 83583 at a concentration of 100 μM caused a significant reduction in maximal effect of fMLP from 1.65 ± 0.01 to 0.32 ± 0.05, $n=4$ ($P<0.005$).

Effect of G-kinase inhibition KT 5823, a specific inhibitor of cyclic GMP-dependent protein kinase (G-kinase) at a concentration of 1 μM had no significant inhibitory effect ($P>0.05$, ANOVA) on fMLP-induced neutrophil chemotaxis (Figure 3a); EC_{50} for fMLP 19.07 ± 4.29 and 35.45 ± 13.54 pmol/10^6 cells, $n=4$ ($P>0.05$) in its absence and presence, respectively. However, at a concentration of 10 μM, KT 5823 completely inhibited ($P<0.001$, ANOVA) fMLP-induced chemotaxis (Figure 3b); EC_{50} for fMLP 32.16 ± 11.35 and >135 pmol/10^6 cells, $n=4$ ($P<0.005$) in the absence and presence of KT 5823, respectively.

Rp-8-pCPT-cGMPS, another inhibitor of cyclic GMP-dependent protein kinase G1α both at concentrations of 10 and 100 μM had no significant inhibitory effect ($P>0.05$, ANOVA) on fMLP-induced chemotaxis (figures not shown); EC_{50} for fMLP 19.07 ± 4.3 and 32.67 ± 14.81, $n=4$ ($P>0.05$) and 32.16 ± 11.35 and 21.67 ± 4.15 pmol/10^6 cells, $n=4$ ($P>0.05$) in its absence and presence at the two concentrations, respectively.

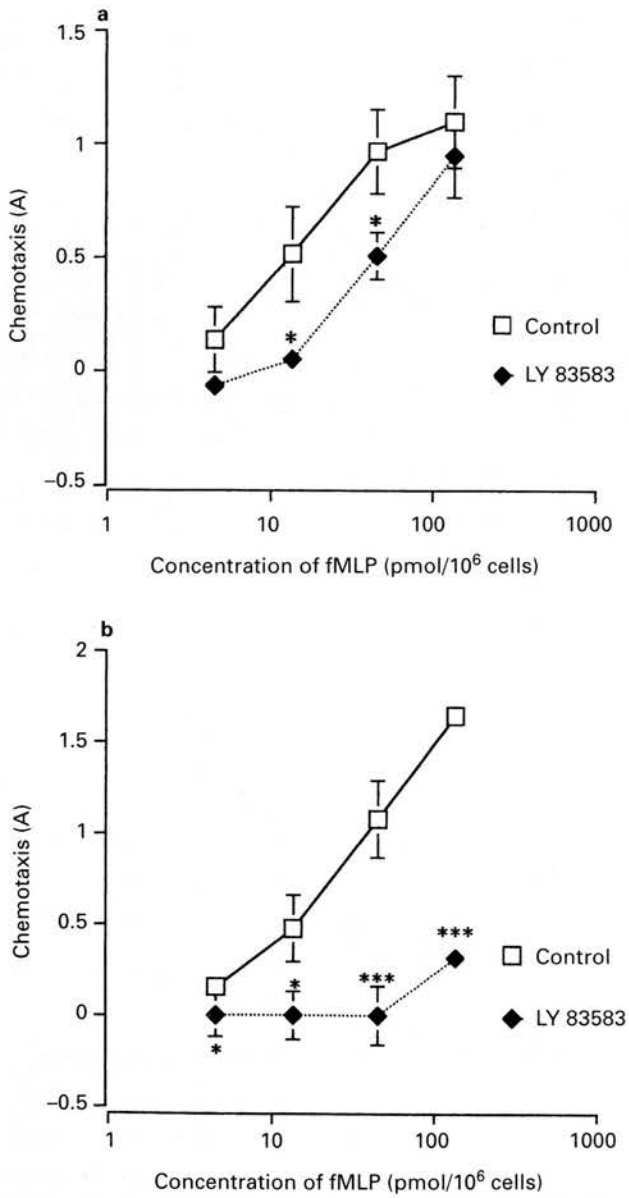


Figure 2 Log concentration-effect curves for fMLP induced neutrophil chemotaxis in control cells and cells treated with LY 83583 (a) 10 μM and (b) 100 μM. The values are the mean, and vertical lines show s.e.mean, of 4 different donors. Statistically significant difference, $*P<0.05$ and $***P<0.005$.

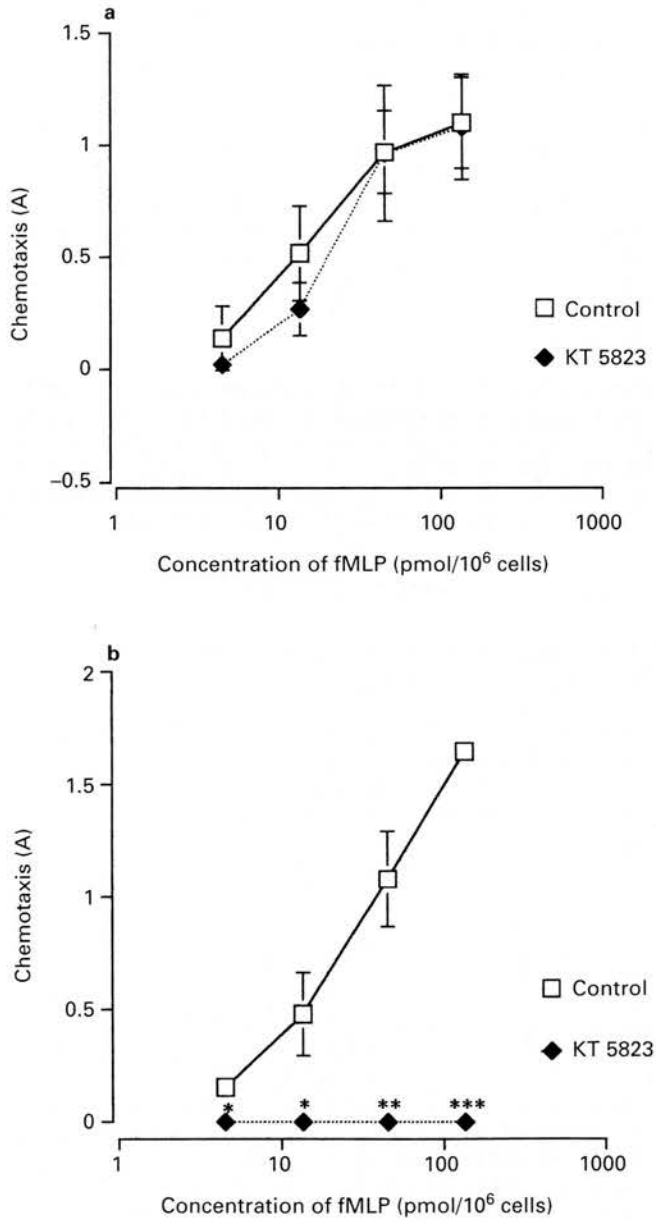


Figure 3 Log concentration-effect curves for fMLP induced neutrophil chemotaxis in control cells and cells treated with KT 5823 (a) 1 μM and (b) 10 μM. The values are the mean, and vertical lines show s.e.mean, of 5 different donors. Statistically significant difference, $*P<0.05$, $**P<0.01$ and $***P<0.005$.

Effect of phosphatase inhibition DPG, an inhibitor of inositol polyphosphate-5-phosphatase, at concentrations of 10 and 100 μM (both $P<0.001$, ANOVA) caused significant attenuation of fMLP-induced neutrophil chemotaxis (Figure 4a and b); EC_{50} for fMLP 25.97 ± 4.25 and 34.37 ± 4.04, $n=4$ ($P<0.05$) and 19.15 ± 4.36 and 61.52 ± 16.2 pmol/10^6 cells, $n=4$ ($P<0.05$) in its absence and presence at the two concentrations, respectively. Maximal effects of fMLP were reduced by 10 and 100 μM DPG from 1.38 ± 0.05 to 0.86 ± 0.14 and from 1.39 ± 0.11 to 0.72 ± 0.14, $n=4$ ($P<0.05$), respectively.

Neutrophil superoxide anion generation (SAG)

Effect of NOS inhibition When neutrophils were preincubated with L-NMMA at a concentration of 100 μM for 45 min at 37°C, L-NMMA caused no significant inhibition ($P<0.05$, ANOVA) of fMLP-induced SAG in human neutrophils (figure not shown); EC_{50} for fMLP 54.24 ± 11.5 and 61.36 ± 12.93 nM, $n=6$ ($P>0.05$) in its absence and presence, respectively. Even when the concentration of L-NMMA was increased to 500 μM, no significant inhibition of fMLP-induced SAG was observed (EC_{50} for fMLP 48.93 ± 12.51 and 57.13 ± 10.93 nM, respectively $n=6$ ($P>0.05$)).

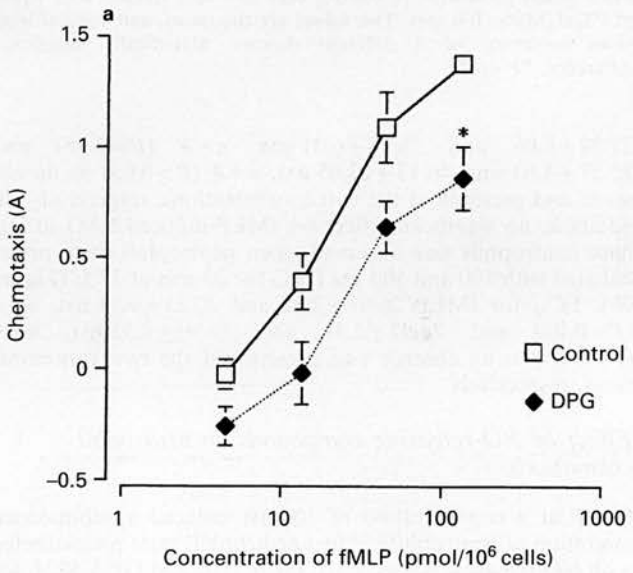
Similarly, L-canavanine at concentrations of 100 and 500 μM caused no significant inhibition (both $P<0.05$, ANOVA) of fMLP-induced SAG in human neutrophils (figure not shown); EC_{50} for fMLP 36.75 ± 7.87 and 32.9 ± 5.41, $n=5$ ($P>0.05$) and 36.75 ± 7.87 and 33.60 ± 8.28 nM, $n=5$ ($P>0.05$) in its absence and presence at the two concentrations, respectively.

Effect of a NO scavenger Carboxy-PTIO, a NO scavenger, at a concentration of 100 μM caused significant attenuation ($P<0.05$, ANOVA) of fMLP-induced SAG in human neutrophils (Figure 5); EC_{50} for fMLP 36.15 ± 7.43 and 86.31 ± 14.06 nM, $n=6$ ($P<0.05$) in its absence and presence, respectively. Maximal effects of fMLP were reduced from 22.14 ± 1.5 to 9.8 ± 1.6 nmol O_2^-/10^6 cells 10 min^{-1} at 300 nM.

Effect of guanylyl cyclase inhibition LY 83583, an inhibitor of guanylyl cyclase, at concentrations of 10 and 100 μM caused no significant inhibition of fMLP-induced SAG in human neutrophils (figure not shown); EC_{50} for fMLP 23.81 ± 1.76 and 18.96 ± 4.52, $n=4$ ($P>0.05$), and 26.27 ± 1.44 and 13.73 ± 3.33 nM, $n=4$ ($P>0.05$) in its absence and presence at the two concentrations, respectively. However, a significant enhancement in fMLP-induced SAG at the lowest concentrations of fMLP tested (3 – 10 nM) was observed with 100 μM LY 83583 ($P<0.05$).

Effect of G-kinase inhibition KT 5823, a specific inhibitor of G-kinase, at concentrations of 1 and 10 μM caused significant inhibition (both $P<0.001$, ANOVA) of fMLP-induced SAG in human neutrophils (Figure 6a and b); EC_{50} for fMLP 34.28 ± 8.9 and 52.59 ± 4.9, $n=5$ ($P=0.05$) and 36.26 ± 8.77 and >300 nM, $n=5$ ($P<0.05$) in its absence and presence at the two concentrations, respectively. Maximal effects of fMLP were reduced by 1 and 10 μM KT 5823 from 22.22 ± 0.68 to 12.17 ± 1.43, $n=5$, ($P<0.005$) and from 28.64 ± 4.15 to 6.59 ± 2.06 nmol O_2^-/10^6 cells/10 min at 300 nM, $n=5$ ($P<0.001$), respectively.

Rp-8-pCPT-cGMPS (100 μM), a moderately potent inhibitor of cyclic GMP-dependent protein kinase G1α, caused some



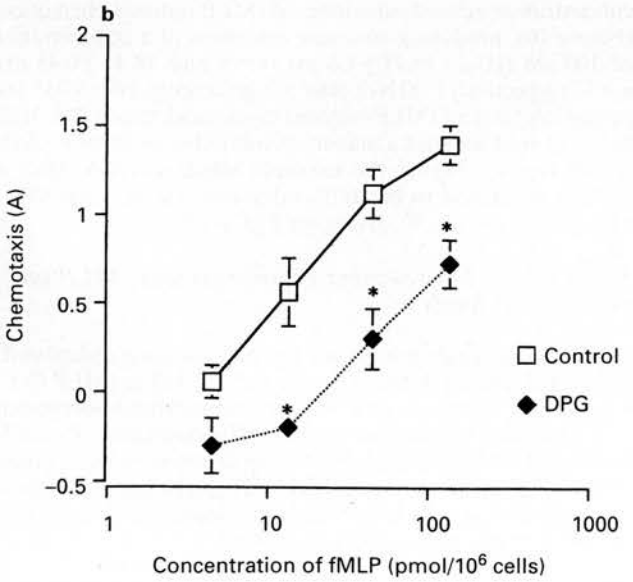


Figure 4 Log concentration-effect curves for fMLP induced neutrophil chemotaxis in control cells and cells treated with 2,3-diphosphoglyceric acid (DPG) (a) 10 μM and (b) 100 μM. The values are the mean, and vertical lines show s.e.mean, of 4 different donors. Statistically significant difference, $*P<0.05$.

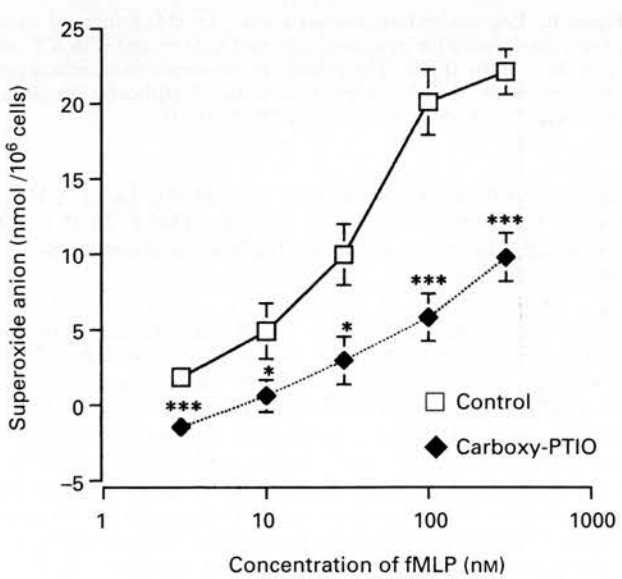


Figure 5 Log concentration-effect curves for fMLP induced superoxide anion generation in control cells and cells treated with carboxy-PTIO (100 μM). The values are the mean, and vertical lines show s.e.mean, of 6 different donors. Statistically significant difference, $*P<0.05$ and $***P<0.005$.

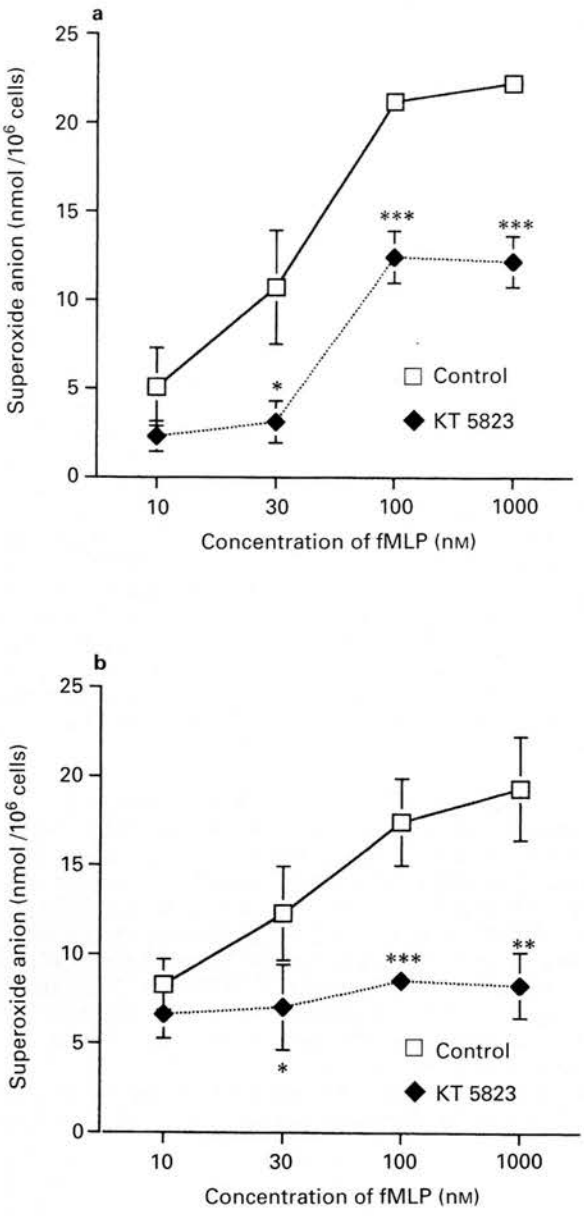


Figure 6 Log concentration-effect curves for fMLP-induced superoxide anion generation in control cells and cells treated with KT 5823 (a) 1 μM and (b) 10 μM. The values are the mean, and vertical lines show s.e.mean, of 5 different donors. Statistically significant difference, $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.005$.

inhibition ($P=0.058$, ANOVA) of fMLP-induced SAG in human neutrophils (Figure 7); EC_{50} for fMLP 28.35 ± 10.82 and 49.25 ± 16.79 nM, $n=4$ ($P<0.05$) in its absence and presence, respectively.

Effects of phosphatase inhibition DPG, an inhibitor of inositol polyphosphate-5-phosphatase, at a concentration of 500 ($P<0.005$, ANOVA) but not 100 μM caused significant inhibition of fMLP-induced SAG in human neutrophils (Figure 8a and b). EC_{50} for fMLP 36.23 ± 9.05 and 44.59 ± 8.88, $n=4$ ($P<0.05$, one-tailed test only) and 33.93 ± 4.23 and 61.12 ± 14.43 nM, $n=4$ ($P<0.05$) were obtained in the absence and presence of 100 and 500 μM DPG, respectively. Maximal effects of fMLP were reduced by 100 and 500 μM DPG from 25.64 ± 1.75 to 24.18 ± 2.15 ($n=4$) and from 26.17 ± 2.7 to 20.59 ± 3.0 nmol O_2^- 10^6 cells 10 min^{-1}, $n=4$ ($P<0.05$), respectively. In contrast, when neutrophils were preincubated with 100 and 500 μM DPG for 10 min at 37°C, neither concentration of DPG caused a significant effect on fMLP-induced SAG in human neutrophils (Figure 9a); EC_{50} for fMLP

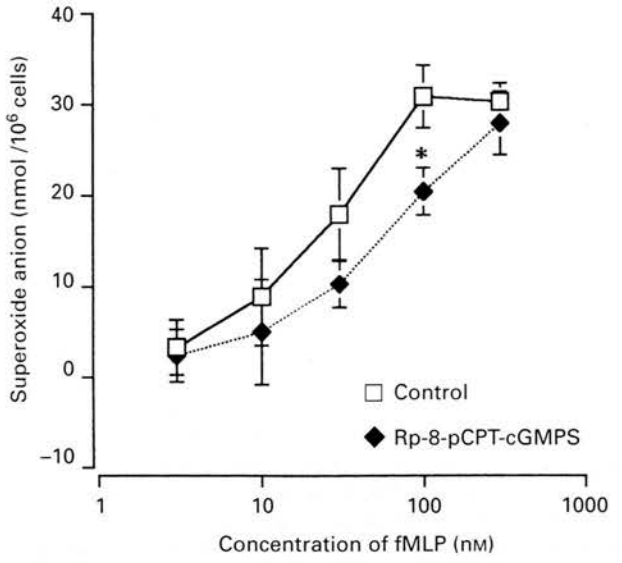


Figure 7 Log concentration-effect curves for fMLP-induced superoxide anion generation in control cells and cells treated with Rp-8-pCPT-cGMPS (100 μM). The values are the mean, and vertical lines show s.e.mean, of 4 different donors. Statistically significant difference, $^*P<0.05$.

27.32 ± 6.06 and 26.95 ± 6.31 nM, $n=4$ ($P>0.05$) and 25.57 ± 4.63 and 46.13 ± 23.05 nM, $n=4$ ($P>0.05$) in its absence and presence at the two concentrations, respectively. In addition, no significant effect on fMLP-induced SAG in human neutrophils was observed when neutrophils were preincubated with 100 and 500 μM DPG for 20 min at 37°C (Figure 9b); EC_{50} for fMLP 26.97 ± 2.32 and 30.23 ± 9.98 nM, $n=4$ ($P>0.05$) and 26.97 ± 2.31 and 26.90 ± 9.53 nM, $n=4$ ($P<0.05$) in its absence and presence at the two concentrations, respectively.

Effect of NO-releasing compounds on neutrophil chemotaxis

fMLP at a concentration of 100 nM induced a submaximal migration of neutrophils. When neutrophils were preincubated with NO-releasing compounds, GEA 3162 and GEA 5024, for 10 min at 37°C, these two compounds (1–100 μM) caused concentration-related inhibition of fMLP-induced chemotaxis (Figure 10), producing complete inhibition at a concentration of 100 μM ($IC_{50}=14.71\pm1.6$ μM, $n=5$ and 18.44 ± 0.43 μM, $n=5$, respectively). SIN-1 was a significantly ($P<0.05$) less potent inhibitor of fMLP-induced chemotaxis than GEA 3162; SIN-1 (1 mM induced a maximum inhibition of $24.99\pm7.64\%$ ($n=8$) (Figure 11). If the maximal effect of GEA 3162 at 100 μM was taken to be 100% inhibition, the IC_{50} for SIN-1 was >1000 μM ($n=8$), giving an EEC>62.7.

Effect of the NO-releasing compounds and fMLP on cyclic GMP levels

Incubation of neutrophils with the NO-releasing compounds, GEA 3162 and GEA 5024 (1–100 μM), as well as fMLP (0.1–0.3 μM), for 10 min at 37°C induced concentration-dependent and significant increases in cyclic GMP production ($P<0.05$). Both GEA 3162 and GEA 5024 were found to be more potent than fMLP at increasing cyclic GMP production in human neutrophils (Table 1). SIN-1 was considerably less potent than the GEA compounds (data not shown).

Effect of the NO-releasing compounds and fMLP on nitrate/nitrite

Incubation of neutrophils with the NO-releasing compounds, GEA 3162, GEA 5024 and SIN-1 (1–100 μM) for 30 min at

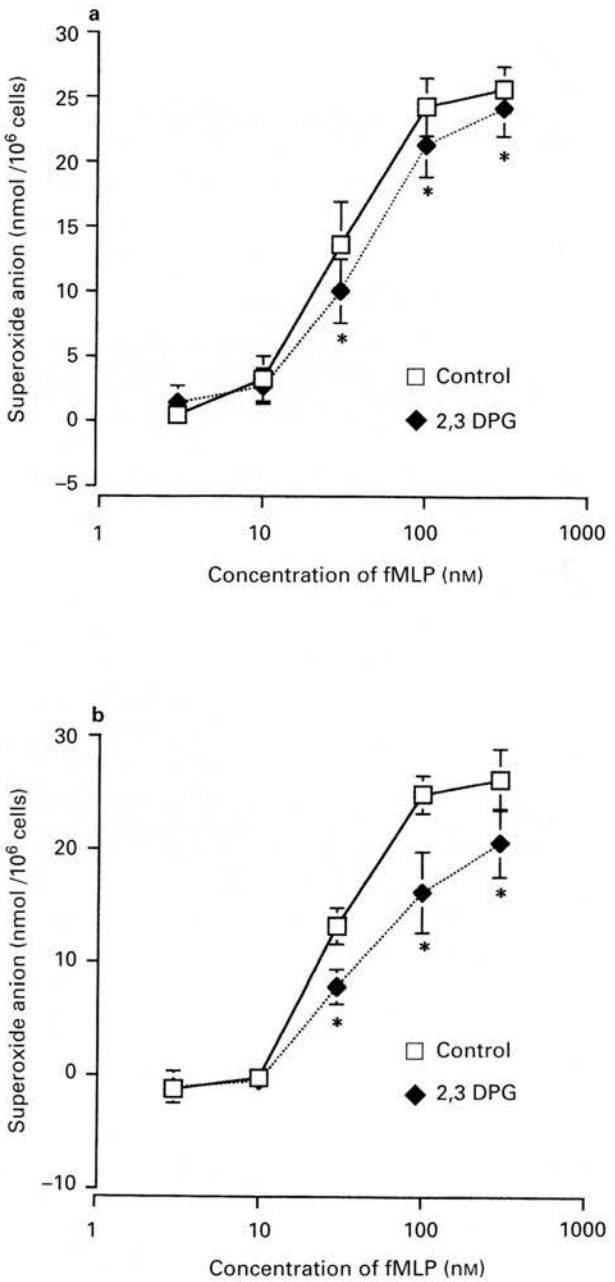


Figure 8 Log concentration-effect curves for fMLP induced superoxide anion generation in control cells and cells treated with DPG (a) 100 μM and (b) 500 μM. The values are the mean, and vertical lines show s.e.mean, of 4 different donors. Statistically significant difference, $*P<0.05$.

37°C induced concentration-related increases in total nitrate/nitrite production (Table 2). GEA 3162 and GEA 5024 were less potent than SIN-1. With GEA 3162 as the standard agonist ($EC_{50}=39.70\pm0.53$ μM), apparent EC_{50} values calculated for SIN-1 and GEA 5024 were 37.62 ± 0.9 ($n=4$; EEC of 0.95) and 89.86 ± 1.62 μM ($n=4$; EEC of 2.26), respectively. fMLP at concentrations of 3–300 nM caused no significant increase in total nitrate/nitrite.

Discussion

In the neutrophil, several second messenger/signal transduction systems can become activated, and these may be involved in the regulation of a variety of neutrophil effector functions. It has been shown that fMLP-induced chemotaxis in human neutrophils results from a rise in cyclic GMP levels subsequent

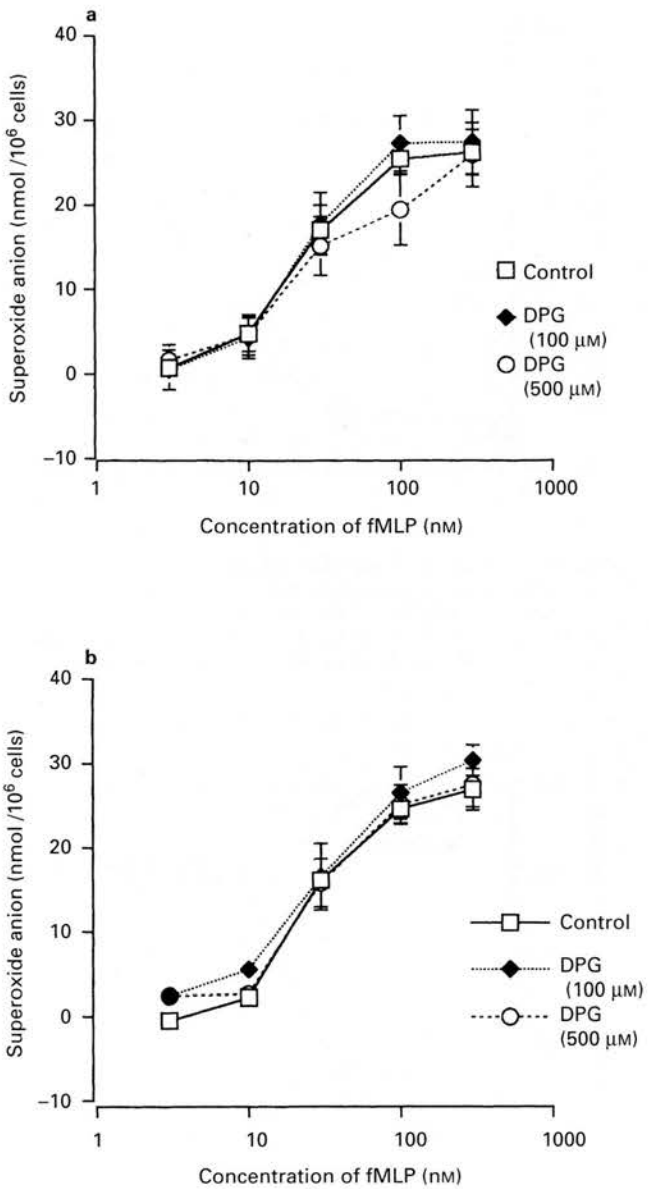


Figure 9 Log concentration-effect curves for fMLP induced superoxide anion generation in control cells and cells treated with 2,3-diphosphoglyceric acid (DPG) 100 μM and 500 μM. Unlike Figure 8, here cells were preincubated at 37°C with DPG for (a) 10 min and (b) 20 min. The values are the mean, and vertical lines show s.e.mean, of 4 different donors.

to the production of NO (Kaplan *et al.*, 1989; Belenky *et al.*, 1993). Such a role for NO has been supported by our results here where inhibition of NOS with L-NMMA (Figure 1a) and chemical antagonism of NO with the NO scavenger carboxy-PTIO (Akaike *et al.*, 1993) (Figure 1b) inhibited fMLP-induced chemotaxis. However, it must be noted that high concentrations of both of these agents were used (500 and 100 μM, respectively), suggesting that NO represents only one of the pathways by which chemotaxis is induced, as complete block of NO only partially blocked the chemotactic response of fMLP.

It has been shown that cyclic GMP and G-kinase regulate neutrophil activation in response to fMLP or A-23187 (Pryzwansky *et al.*, 1990; Wyatt *et al.*, 1990). After activation by fMLP, G-kinase transiently co-localizes with the intermediate filaments, resulting in the phosphorylation of its substrate protein, vimentin (Wyatt *et al.*, 1991). LY 83583 is an inhibitor of guanylyl cyclase and has been shown to inhibit the fMLP-stimulated increase in neutrophil cyclic GMP levels resulting in inhibition of the co-localization and subsequent phosphoryla-

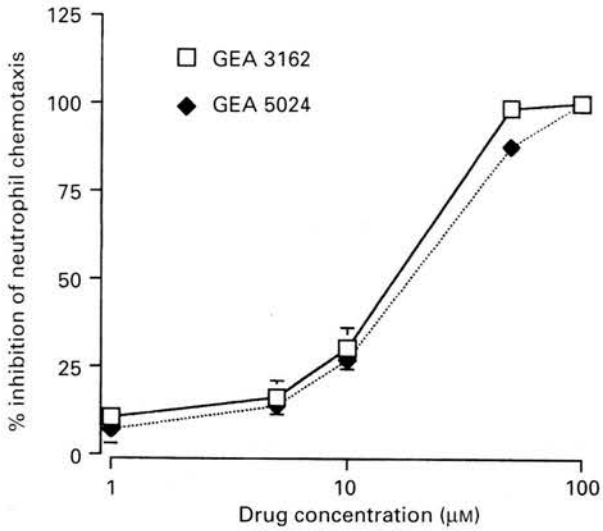


Figure 10 Log concentration-effect curves for inhibition of fMLP-induced chemotaxis, observed with GEA 3162 and GEA 5024. Cells were preincubated with GEA 3162 and GEA 5024 for 10 min at 37°C before being added to the chemotaxis chamber. The values are the mean, and vertical lines show s.e.mean, of 5 different donors.

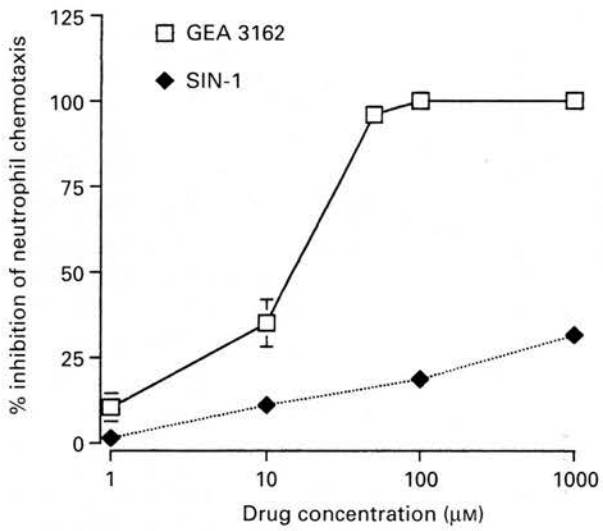


Figure 11 Log concentration-effect curves for inhibition of fMLP-induced chemotaxis, observed with GEA 3162 and SIN-1. Cells were preincubated with GEA 3162 and SIN-1 for 10 min at 37°C before being added to the chemotaxis chamber. The values are the mean, and vertical lines show s.e.mean, of 8 different donors.

Table 1 The effects of NO releasing compounds (GEA 3162 and GEA 5024) and fMLP on cyclic GMP levels in human neutrophils

Drug concentration (μM)	*Cyclic GMP levels* (pmol/10^6 cells) *GEA 3162*	*GEA 5024*	*fMLP*
0	0.1±0.02	0.1±0.01	0.09±0.01
0.1			0.18±0.03*
0.3			0.22±0.05*
10	0.87±0.15***	0.59±0.11**	
50	1.13±0.23***	0.73±0.14**	
100	1.32±0.24***	0.85±0.18**	

Data are expressed as mean±s.e.mean of 5 different donors. Significant increase in cyclic GMP above basal, $*P<0.05$, $**P<0.01$, $***P<0.005$.

Table 2 The effects of NO releasing compounds (GEA 3162, GEA 5024 and SIN-1) on total nitrate/nitrite production in human neutrophils

Drug concentration (μM)	*Total nitrate/nitrite* (μmol/10^6 cells) *GEA 3162*	*GEA 5024*	*SIN-1*
0	5.8±3.3	5.8±3.3	5.8±3.3
1	11.3±4.8	2.8±0.0*	9.4±4.6**
10	71.8±3.6	30.5±0.6**	55.4±1.6**
50	282.2±5.5	145.0±1.4**	$305.2\pm5.1^{\#}$
100	458.0±3.6	251.4±1.6**	$552.0\pm14.7^{\#\#}$

Data are expressed as mean±s.e.mean of 4 different donors. *Significantly less nitrate/nitrite production than the equivalent concentration of GEA 3162, $*P<0.05$, $**P<0.005$. $^{\#}$Significantly more nitrate/nitrite production than the equivalent concentration of GEA 3162, $^{\#}P<0.01$, $^{\#\#}P<0.005$. GEA 3162 significantly increased nitrate/nitrite levels at all concentrations tested.

tion of vimentin by G-kinase (Wyatt *et al.*, 1993). We found that LY 83583 significantly attenuated fMLP-induced chemotaxis (Figure 2a and b) confirming that the co-localization and phosphorylation of G-kinase and vimentin are involved in fMLP-induced neutrophil chemotaxis. Two inhibitors of G-kinase were used to substantiate such a role for cyclic GMP, KT 5823 (Kase *et al.*, 1987) and Rp-8-pCPT-cGMPS (Butt *et al.*, 1994). At a concentration of 10 μM (Figure 3a) KT 5823 completely inhibited fMLP-induced chemotaxis. The block observed with both LY 83583 (100 μM) and KT 5823 (10 μM) was greater than would be expected if the rise in cyclic GMP resulted only from NO. This suggests either that fMLP can increase cyclic GMP levels independently of NO or that these agents are not acting as selective inhibitors of guanylyl cyclase and G-kinase at the concentrations used. Rp-8-pCTP-cGMPS (10 and 100 μM) failed to block fMLP-induced chemotaxis, suggesting that the G-kinase activated in neutrophil chemotaxis is not type Glα. The inability of Rp-8-pCPT-cGMPS to block chemotaxis is unlikely to result from too low a concentration being used as a significant effect was observed with Rp-8-pCPT-cGMPS (100 μM) on SAG (Figure 7).

It has been shown that more than 90% of the diglyceride formed in neutrophils in response to fMLP occurs through the activation of phospholipase D (PLD)/phosphatidic acid (PA) phosphohydrolase (Billah *et al.*, 1989). A phosphatase inhibitor, DPG, significantly attenuated fMLP-induced chemotaxis (Figure 4a and b), suggesting that activation of PLD is a major signal in neutrophil chemotaxis. It is not clear from these results whether increased intracellular Ca^{2+} resulting from PLD activation is the trigger responsible for activation of NOS.

However the roles of NO and cyclic GMP are less clear in fMLP-induced SAG. Two NOS inhibitors were investigated and neither L-NMMA nor L-canavanine inhibited fMLP-induced SAG, even when used at the concentration (500 μM) required to inhibit neutrophil chemotaxis. However, the NO scavenger carboxy-PTIO (100 μM) significantly inhibited fMLP-induced SAG (Figure 5). The reason for this discrepancy is not clear, but these results suggest that NO may also play a role in SAG by fMLP.

Results with the guanylyl cyclase inhibitor LY 83583 suffer from the ability of LY 83583 to enhance significantly the amount of SAG by low concentrations of fMLP (3–10 nM) consistent with data showing that LY 83583 can itself stimulate SAG. However, both inhibitors of G-kinase, KT 5823 (Figure 6a and b) and Rp-8-pCPT-cGMPS (Figure 7) significantly inhibited fMLP-induced SAG. As with chemotaxis, the effect of KT 5823 was quite dramatic, suggesting that cyclic GMP may play an additional role to NO. However, the selectivity of the inhibitors used is crucial to this interpretation and experiments looking at phosphorylation of G-kinase are required to substantiate these findings. Furthermore, the dif

ferent sensitivity to these G-kinase inhibitors observed with chemotaxis and SAG suggests that the G-kinase activated in the two processes may be different. Consistent with this, KT 5823 has been found not to inhibit the neutrophil G-kinase which phosphorylates vimentin (Wyatt & Pryzwensky, 1991).

PLD is thought to play a major signalling role in SAG in the primed neutrophil (Bonser *et al.*, 1989; Kanaho *et al.*, 1993). In particular PLD is thought to ensure that diacylglycerol levels are sustained, which is a requirement for SAG (Billah & Anthes, 1990). Such a role for PLD has been confirmed in these experiments by use of the phosphatase inhibitor DPG, which significantly inhibited fMLP-induced SAG in these cytochalasin B-treated neutrophils (Figure 8a and b). This effect of DPG was lost if cells were pre-incubated with DPG for 10 or 20 min (Figure 9a and b). This result contrasts with those obtained by Kanaho *et al.* (1993) who observed greater block with preincubation. A greater degree of block was observed with chemotaxis than with SAG.

While these results suggest that endogenous NO plays a role in mediating neutrophil chemotaxis, other evidence has been presented indicating that NO releasing compounds can inhibit neutrophil activation (Ney *et al.*, 1990; Schroder *et al.*, 1990; Kubes *et al.*, 1991; Wenzen-Seifert *et al.*, 1991). To resolve this apparent paradox we have investigated the effects of GEA 3162, GEA 5024 and SIN-1 which have previously been shown to inhibit neutrophil chemotaxis (Moilanen *et al.*, 1993). GEA 3162 and GEA 5024 (1–100 μM) caused significant concentration-dependent inhibition of fMLP-induced chemotaxis (Figure 10). SIN-1 was less potent and caused significantly less inhibition of chemotaxis than GEA 3162 (Figure 11). The rank order of potency was GEA 3162 ($EC_{50} = 14.7 \pm 1.58$ μM) > GEA 5024 ($EC_{50} = 18.4 \pm 0.43$ μM) > SIN-1 ($EC_{50} = >$ 1000 μM). One possible explanation for the difference in potency of these agents as inhibitors of chemotaxis may relate to the ability of SIN-1 to release superoxide anion (Feelisch *et al.*, 1989; Feelisch, 1991). The concomitant release of NO and superoxide anion by SIN-1 may well attenuate the inhibitory effects of NO on chemotaxis as NO is inactivated by superoxide anions, to form peroxynitrite (Gryglewski *et al.*, 1986). Furthermore, peroxynitrite production by SIN-1 has been shown to enhance fMLP-induced neutrophil respiratory burst (measured as luminol-dependent chemiluminescence in whole blood) masking its otherwise inhibitory effects, such as a reduction in leukotriene B_4 production (Bednar *et al.*, 1996). In addition peroxynitrite formed from SIN-1 has been shown to stimulate phorbol ester-induced respiratory burst (Iha *et al.*, 1996). In contrast, GEA 3162 and GEA 5024 (in concentrations up to 100 mM) do not release significant amounts of superoxide anions to form peroxynitrite. If peroxynitrite augments chemotaxis as well as SAG, this could explain why GEA 3162 and GEA 5024 are more potent inhibitors of neutrophil chemotaxis than SIN-1.

The NO releasing compounds increased total nitrate/nitrite production (Table 2) with a rank order of potency of SIN-1 ($EC_{50} = 37.62 \pm 0.9$ μM) > GEA 3162 ($EC_{50} = 39.7 \pm 0.5$ μM) > GEA 5024 ($EC_{50} = 89.9 \pm 1.7$ μM). Taking GEA 3162 as the standard compound, this gives EEC values for SIN-1 and GEA 5024 of 0.95 and 2.26 for nitrate/nitrite production compared to >62.7 and 1.25 for inhibition of chemotaxis.

Clearly some reason is required to explain the lack of potency of SIN-1 at inhibiting chemotaxis compared with releasing NO. Interestingly, as found by Moilanen *et al.* (1993), SIN-1 was much weaker than GEA 3162 and GEA 5024 at increasing cyclic GMP levels, giving a maximal increase of 1.4 fold over basal (data not shown) compared with 13.2 and 8.3 fold for GEA 3162 and GEA 5024, respectively (Table 1). Consequently, there is a better correlation between effects on cyclic GMP and inhibition of chemotaxis, than for effects on NO and inhibition of chemotaxis. At first glance, this suggests that neutrophil inhibition is likely to be related to increased cyclic GMP levels rather than ADP ribosylation by NO (Clancy *et al.*, 1995), but the role of peroxynitrite formed by SIN-1 requires further clarification, particularly with respect to chemotaxis. At present it is not clear whether peroxynitrite augments chemotaxis induced by fMLP as is the case for SAG (Iha *et al.*, 1996).

These results do not prove that GEA 3162, GEA 5024 and SIN-1 inhibit neutrophil chemotaxis by a NO-dependent mechanism. However, this is quite difficult to test. A NO scavenger such as carboxy-PTIO will itself inhibit chemotaxis (Figure 1b), so that the NO donor would be tested against a smaller fMLP stimulus. Because of the nature of physiological antagonism, it is easier to inhibit a smaller stimulus than a larger one (Kenakin, 1987) making comparison difficult. The scavenger oxyhaemoglobin may prove useful in elucidating the role of NO, if this can be used at a low enough concentration not to affect endogenous NO and the control chemotactic response to fMLP.

In conclusion, these results confirm that neutrophil activation results from the stimulation of several signal transduction systems. We have shown that chemotaxis can be attenuated by inhibitors of PLD, NO and cyclic GMP. It appears that increases in cyclic GMP and activation of G-kinase resulting in chemotaxis can occur via a NO-dependent as well as NO-independent pathway. As such, small increases in cyclic GMP but not NO were detectable after neutrophil stimulation by fMLP. Similar pathways appear to operate in SAG. In contrast, the NO releasing compounds, GEA 3162, GEA 5024 and SIN-1, which produce large amounts of NO (measured as total nitrate/nitrite) compared to fMLP, inhibit neutrophil chemotaxis. This hypothesis, that low concentrations of NO activate while high concentrations inhibit neutrophils, has also been suggested recently by VanUfflen *et al.* (1996); they studied the effects of gaseous NO on rabbit peritoneal neutrophils.

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References

AKAIKE, T., YOSHIDA, M., MIYAMOTO, Y., SATO, K., KOHNO, M., SASAMOTO, K., MIYAZAKI, K., UEDA, S. & MAEDA, H. (1993). Antagonistic action of imidazolineoxyl N-oxides against endothelium-derived relaxing factor/NO through a radical reaction. *Biochemistry*, **32**, 827–832.

AMATRUDA, T.T., GERALD, N.P., GERALD, C. & SIMON, M.I. (1993). Specific interactions of chemoattractant factor receptors with G-proteins. *J. Biol. Chem.*, **268**, 10139–10144.

ANDERSON, R., GLOVER, A., KOORNHOF, H.J. & RABSON, A.R. (1986). In vitro stimulation of neutrophil motility by levamisole: maintenance of cyclic GMP levels in chemotactically stimulated levamisole-treated neutrophils. *J. Immunol.*, **117**, 28–432.

ARMSTRONG, R. (1995). Investigation of the inhibitory effects of PGE_2 and selective EP agonists on chemotaxis of human neutrophils. *Br. J. Pharmacol.*, **116**, 2903–2980.

BARTEN, A.D., RYDER, K., LOURENCO, R.V., DRALLE, W. & WEISS, S.G. (1976). Inflammatory reaction and airway damage in cystic fibrosis. *J. Lab. Clin. Med.*, **88**, 423–426.

BEAUVAIS, F., MICHEL, L. & DUBERTRET, L. (1995). Exogenous nitric oxide elicits chemotaxis of neutrophils in vitro. *J. Cell. Physiol.*, **165**, 610–614.

BEDNAR, M.M., BALAZY, M., MURPHY, M., BOOTH, C., FULLER, S.P., BARTON, A., BINGHAM, J., GOLDING, L. & GROSS, C.E. (1996). Peroxynitrite augments fMLP-stimulated chemiluminescence by neutrophils in human whole blood. *J. Leukocyte Biol.*, **60**, 619–624.

BELENKY, S.N., ROBBINS, R.A., RENNARD, S.I., GOSSMAN, G.L., NELSON, K.J. & RUBINSTEIN, I. (1993). Inhibitors of nitric oxide synthase attenuate human neutrophil chemotaxis in vitro. *J. Lab. Clin. Med.*, **122**, 388–394.

BILLAH, M.M., ECKEL, S., MULLMANN, T.J., ERGAN, R.W. & SIEGEL, M.I. (1989). Phosphatidylcholine hydrolysis by PLD determines the phophatidate and diglyceride levels in chemotactic peptide stimulated neutrophils. *J. Biol. Chem.*, **264,** 17069–17077.

BILLAH, M.M. & ANTHES, J.C. (1990). The regulation and cellular functions of phosphatidylcholine hydrolysis. *Biochem. J.*, **269,** 281–291.

BONSER, R.W., THOMSON, N.T., RANDALL, R.W. & GARLAND, L.G. (1989). Phospholipase D activation is functionally linked to superoxide generation in the human neutrophil. *Biochem. J.*, **264,** 617–620.

BUTT, E., EIGENTHALER, M. & GENIESER, H.G. (1994). (RP)-8-pCPT-cGMPS, a novel cGMP-dependent protein kinase inhibitor. *Eur. J. Pharmacol.*, **269,** 265–268.

CASSIMERIS, L. & ZIGMOND, S.H. (1990). Chemotractant stimulation of polymorpho-leukocyte locomotion. *Semin. Cell. Biol.*, **1,** 125–134.

CATERINA, M.J. & DEVREOTES, P.N. (1991). Molecular insights into eukariotic chemotaxis. *FASEB J.*, **5,** 3078–3085.

CLANCY, R.M., LESZCZYNSKA-PIZIAK, J. & AMBRASON, S.B. (1992). Nitric oxide, an endothelial cell relaxation factor, inhibits neutrophil superoxide anion production via a direct action on the NADPH oxidase. *J. Clin. Invest.*, **90,** 1116–1121.

CLANCY, R., LESZCZYNSKY, J., AMIN, A., LEVARTOVSKY, D. & ABRAMSON, S.B. (1995). Nitric oxide stimulates ADP ribosylation of actin in association with the inhibition of actin polymerization in human neutrophils. *J. Leukocyte Biol.*, **58,** 196–202.

FEELISCH, M., OSTROWSKI, J. & NOACK, E. (1989). On the mechanism of NO release from sydnonimines. *J. Cardiovasc. Pharmacol.*, **14,** (Suppl. 11), S13–S22.

FEELISCH, M. (1991). The biochemical pathways of nitric oxide formation from nitrovasodilators: appropriate choice of exogenous NO donors and aspects of preparation and handling of aqueous NO solutions. *J. Cardiovasc. Pharmacol.*, **17,** (Suppl. 3), S25–S33.

GAUDRY, M., CAON, A.C., GILBERT, C., LILLE, S. & NACCACHE, P.H. (1992). Evidence for the involvement of tyrosine kinases in the locomotory response in human neutrophils. *J. Leukocyte Biol.*, **51,** 103–108.

GRYGLEWSKI, R.J., KORBUT, R., KALECINSKA, A. & ZEMBOWICZ, A. (1986). Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. *Nature*, **320,** 454–456.

IHA, S., ORITA, K., KANNO, T., UTSUMI, T., SATO, E.F., INOUE, M. & UTSUMI, K. (1996). Oxygen-dependent inhibition of neutrophil respiratory burst by nitric oxide. *Free Radical Res.*, **25,** 489–498.

KADOTA, K., YUI, Y., HATTORI, R., UCHIZUMI, H. & KAWAI, C. (1991). A new relaxing factor in supernatant of incubated rat peritoneal neutrophils. *Am. J. Physiol.*, **260,** H967–H972.

KANAHO, Y., NAKAI, Y., KATOH, M. & NOZAWA, Y. (1993). The phosphatase inhibitor 2,3-diphosphoglycerate interferes with phospholipase D activation in rabbit peritoneal neutrophils. *J. Biol. Chem.*, **268,** 12492–12497.

KAPLAN, S.S., BILLIAR, T., CURRAN, R.D., ZDZIARSKI, U.E., SIMMONS, R.L. & BASFORD, R.E. (1989). Inhibition of chemotaxis with N^G-monomethyl-L-arginine: A role for cyclic GMP. *Blood*, **74,** 1885–1887.

KASE, H., IWAHASHI, K., NAKAISHI, S., MATSUDA, Y., YAMADA, K., TAKAHASHI, M., MURAKATA, C., SATO, A. & KANEKO, M. (1987). K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. *Biochem. Biophys. Res. Commun.*, **142,** 436–440.

KENAKIN, T.P. (1987). Functional antagonism and synergism. In *Pharmacologic Analysis of Drug Receptor Interaction*, pp. 245–257. New York: Raven Press.

KUBES, P., SUZUKI, M. & GRANDER, D.N. (1991). Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc. Natl. Acad. Sci. U.S.A.*, **88,** 4651–4655.

LEE, D.K.H., FAUNCE, D., HENRY, D., STURM, R.J. & RIMELE, T. (1990). Neutrophil-derived relaxing factor relaxes vascular smooth muscle through a cGMP-mediated mechanism. *Life Sci.*, **46,** 1531–1538.

LOPEZ FARRE, A., RIESCO, A., MOLIZ, M., EGIDO, J., CASADO, S., HERNANDO, L. & CARAMELO, C. (1991). Inhibition of L-arginine of the endothelin-mediated increase in cytosolic calcium in human neutrophils. *Biochem. Biophys. Res. Commun.*, **178,** 884–891.

MCCALL, T.B., BOUGHTON-SMITH, N.K., PALMER, R.M.J. & MONCADA, S. (1989). Synthesis of nitric oxide from L-arginine by neutrophils: release and interaction with superoxide anion. *Biochem. J.*, **261,** 283–286.

MEHTA, J.L., LAWSON, D.L., NICOLINI, F.A., CAIN, D.A., METHA, P. & SCHREIER, H. (1990). Evidence for generation of a large amount of nitric oxide-like vascular smooth muscle relaxant by cholesterol-rich neutrophils. *Biochem. Biophys. Res. Commun.*, **173,** 438–442.

MOILANEN, E., VUORINEN, P., KANKAANRANTA, H., METSA-KETELA, T. & VAPAATALO, H. (1993). Inhibition by nitric oxide-donors of human polymorphonuclear leucocyte functions. *Br. J. Pharmacol.*, **109,** 852–858.

MONCADA, S. & HIGGS, E.A. (1991). Endogenous nitric oxide: physiology, pathology and clinical relevance. *Eur. J. Clin. Invest.*, **21,** 361–374.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.*, **43,** 109–142.

MYERS, P.R., MINOR, Jr, R.L., GUERRA, Jr, R., BATES, J.N. & HARRILSON, D.G. (1990). Vasorelaxant properties of the endothelium-derived relaxing factor more closely resemble S-nitrosocysteine than nitric oxide. *Nature*, **345,** 151–163.

NEY, P., SCHRODER, H. & SCHROR, K. (1990). Nitrovasodilator induced inhibition of LTB_4 release from human PMN may mediated by cyclic GMP. *Eicosanoids*, **3,** 243–245.

PRYZWANSKY, K.B., WYATT, T.A., NICHOLS, H. & LINCOLN, T.M. (1990). Compartmentalization of cyclic GMP-dependent protein kinase in formyl-peptide stimulated neutrophils. *Blood*, **76,** 612–618.

RIMELE, T.J., STURM, R.J., ADAMS, L.M., HENRY, D.E., HEASLIP, R.J., WEICHMAN, B.M. & GRIMES, D. (1988). Interaction of neutrophils with vascular smooth muscle: identification of a neutrophil-derived relaxing factor. *J. Pharmacol. Exp. Ther.*, **245,** 102–111.

RIMELE, T.J., ARMSTRONG, S.J., GRIMES, D. & STRUM, R.J. (1991). Rat peritoneal neutrophils selectively relax vascular smooth muscle. *J. Pharmacol. Exp. Ther.*, **258,** 963–971.

SALVEMINI, D., DENUCCI, G., GRYGLEWSKI, R.J. & VANE, J.R. (1989). Human neutrophils and mononeuclear cells inhibit platelet aggregation by releasing a nitric-oxide like factor. *Proc. Natl. Acad. Sci. U.S.A.*, **86,** 6328–6332.

SANDLER, J.A., GALLIN, J.I. & VAUGHN, M. (1975). Effect of serotonin, carbamylcholine, and ascorbic acid on leukocyte cyclic GMP and chemotaxis. *J. Cell. Biol.*, **67,** 480–484.

SCHMIDT, H.W., SEIFEERT, R. & BOHME, E. (1989). Formation and release of nitric oxide from human neutrophils and HL-60 cells induced by a chemotactic peptide, platelet activating factor and leukotriene B_4. *FEBS Lett.*, **244,** 357–360.

SCHRODER, H., NEY, P., WODITSCH, I. & SCHROR, K. (1990). Cyclic GMP mediated SIN-1-induced inhibition of human polymorphonuclear leukocytes. *Eur. J. Pharmacol.*, **182,** 211–218.

SMITH, R.J. & IGNARRO, L.J. (1975). Bioregulation of enzyme secretion from human neutrophils: roles of guanosine 3′:5′-monophosphate and calcium in stimulus-secretion coupling. *Proc. Natl. Acad. Sci. U.S.A.*, **72,** 108–112.

STEPHENS, R.J. & SNYDERMAN, R. (1982). Cyclic nucleotides regulate the morphologic alterations required for chemotaxis in monocytes. *J. Immunol.*, **28,** 1192–1197.

TALPAIN, E., ARMSTRONG, R.A., COLEMAN, R.A. & VARDEY, C.J. (1995). Characterisation of the PGE receptor subtype mediating inhibition of superoxide production in human neutrophils. *Br. J. Pharmacol.*, **114,** 1459–1465.

VAN UFFLELEN, B.E., DE KOSTER, B.M., VAN DEN BROEK, P.J., VANSTEVENINCK, J. & ELFERINK, J.G. (1996). Modulation of neutrophil migration by exogenous gaseous nitric oxide. *J. Leukocyte Biol.*, **60,** 94–100.

WEISS, S.J. (1989). Tissue destruction by neutrophils. *N. Engl. J. Med.*, **320,** 813–819.

WENZEL-SEIFERT, K., ERVENS, J. & SEIFERT, R. (1991). Differential inhibition and potentiation by cell-permeant analogues of cyclic AMP and cyclic GMP and NO-containing compounds of exocytosis in human neutrophils. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **344,** 396–402.

WRIGHT, C.D., MULSCH, A., BUSSE, R. & OSSWALD, H. (1989). Generation of nitric oxide by human neutrophils. *Biochem. Biophys. Res. Commun.*, **160,** 813–819.

WYATT, T.T., LINCOLN, T.M. & PRYZWANSKY, K.B. (1990). Compartmentalization of cyclic GMP-dependent protein kinase (G-kinase) in calcium ionophore and phorbol ester stimulated human neutrophils. *FASEB J.*, **4,** A872.

WYATT, T.A., LINCOLN, T.M. & PRYZWANSKY, K.B. (1991). Vimentin is transiently co-localized with and phosphorylated by cyclic GMP-dependent protein kinase in formyl-peptide stimulated neutrophils. *J. Biol. Chem.*, **266,** 21274–21280.

WYATT, T.A. & PRYZWANSKY, K.B. (1991). KT 5823 activates human neutrophils and fails to inhibit cGMP-dependent protein kinase phosphorylation of vimentin. *Res. Commun. Chem. Pathol. Pharmacol.*, **74,** 3–13.

WYATT, T.A., LINCOLN, T.M. & PRYZWANSKY, K.B. (1993). Regulation of human neutrophil degradation by LY-83583 and L-arginine: role of cGMP-dependent protein kinase. *Am. J. Physiol.*, **265,** C201–C211.

YUI, Y., HATTORI, R., KOSUGA, K., EIZAWA, H., HIKI, K., OHKAWA, S., OHNISHI, K., TERAO, S. & KAWAI, C. (1991). Calmodulin-independent nitric oxide synthase from rat polymorphonuclear neutrophils. *J. Biol. Chem.*, **266,** 3369–3371.

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