For my Father and in memory of my late mother

MAY ALLAH'S BLESSING BE UPON THEM

THE INFLUENCE OF LIPOPROTEINS AND PEROXIDES ON PLATELET ~~FUNCTION~~ activation and inhibition

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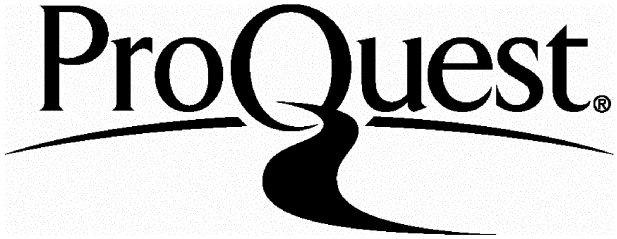

ABSTRACT.

Low density lipoproteins (LDL) have been implicated as possible pro-thrombotic factors by enhancing platelet responsiveness. The aim of this study was to assess how the degree of oxidative modification of LDL altered their effects on platelet function. The effects of native and modified LDL on platelet responsiveness and platelet sensitivity to nitric oxide (NO) *in vitro* were assessed by conventional aggregometry and flow cytometry combined with immunolabelling.

Native LDL (nLDL) inhibited activation in platelet rich plasma (PRP) and aggregation of washed platelets (WP). Oxidised LDL (oxLDL) also inhibited platelet activation in PRP, but was found to inhibit aggregation in both PRP and WP, suggesting that inhibition was induced by a different mechanism to that of nLDL. In contrast, minimally modified LDL (mmLDL) potentiated ADP-induced fibrinogen binding and aggregation, possibly by enhanced release of platelet granules, and induced primary aggregation of WP independently of other agonists. The major physicochemical difference between native and minimally modified LDL was a relatively small increase in the level of lipid hydroperoxides (LPO), these low levels of LPO may account for the contrasting effects of nLDL and mmLDL with respect to platelet function. The data suggest that mmLDL may be potentially important pro-thrombotic factors.

The influence of peroxides on platelet aggregation and the sensitivity of platelets to NO were investigated using hydrogen peroxide, cumene hydroperoxide and 15 (S)-hydroperoxyeicosatetraenoic acid as models for LPO. Hydrogen peroxide, and cumene hydroperoxide both potentiated agonist-

induced aggregation, but only when added post-agonist. These peroxides also attenuated the inhibition of platelet aggregation by NO when added post-agonist. 15 (S)-hydroperoxyeicosatetraenoic acid alone did not affect aggregation, but potentiated aggregation when used as a complex with nLDL. 15 (S)-hydroperoxyeicosatetraenoic acid antagonised the inhibitory actions of NO, but this only occurred when the peroxide was incubated with platelets before the addition of NO and thrombin.

In contrast, if hydrogen peroxide and NO were added to platelets before thrombin, aggregation was inhibited to a greater extent than by NO alone. The presence of hydrogen peroxide prolonged and enhanced the effects of NO when applied directly to WP. This was due in part to increased stimulation of guanylate cyclase activity, and could be reproduced with an NO-donor. Simultaneous addition of NO with cumene hydroperoxide or 15 (S)-hydroperoxyeicosatetraenoic acid did not produce the same effect. This suggested that hydrogen peroxide may have a physiological role in the enhancement of inhibition of platelets by NO.

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LIST OF ABBREVIATIONS.

ACD	acid citrate dextrose
Ach	acetylcholine
ADP	adenosine diphosphate
ATP	adenosine triphosphate
apo	apolipoprotein
BK	bradykinin
$[Ca^{2+}]_i$	intracellular free Ca^{2+} ion concentration
COX	cyclooxygenase
cum-OOH	cumene hydroperoxide
cAMP	cyclic adenosine 3', 5' -monophosphate
cGMP	cyclic guanosine 3', 5' -monophosphate
DAG	diacylglycerol
DTPA	diethylenetriaminepentaacteic acid
EC_{50}	effective concentration at 50%
FGN	fibrinogen
g	centrifugal force
G-protein	guanine-nucleotide binding protein
GPIIb-IIIa	Glycoprotein IIb-IIIa
GSH Px	glutathione peroxidase
HDL	high density lipoproteins
4-HNE	4-hydroxynonenal
H_2O_2	hydrogen peroxide
15 (S)-HpETE	15 (S)-hydroperoxyeicosatetraenoic acid
IC_{50}	inhibitory concentration at 50%
kDa	kilodaltons
LDL	low density lipoproteins
LPO	lipid hydroperoxide
MCP-1	Monocyte chemotactic protein-1
MDA	malondialdehyde
MLCK	Myosin light chain kinase
NO	nitric oxide
$ONOO^-$	peroxynitrite anion
PAF	platelet activating factor
PAF-AH	platelet activating factor acetylhydrolase
PL	phospholipid
PLA_2	phospholipase A_2
PLC	phospholipase C
PGI_2	prostacyclin
PMN	polymorphonuclear leukocytes
PUFA	polyunsaturated fatty acid
PRP	platelet rich plasma
REM	relative electrophoretic mobilty
ROS	reactive oxygen species
SOD	superoxide dismutase
SMC	smooth muscle cells
S-NOG	S-nitrosoglutathione

TBARs	thiobarbituric acid reactive subtances
TXA_2	thromboxane A_2
VLDL	very low density lipoproteins
WHHL	Watanabe heritable hyperlipidaemic
WP	washed platelets

1.0 INTRODUCTION

1.1 Atherosclerosis.

Atherosclerosis is a benign multifactoral disease which leads to a narrowing of the arteries [Ross, 1986]. The disease is characterised by the presence of plaques beneath the luminal surface of the arteries. As the disease progresses the plaques enlarge such that they protrude into the lumen of the artery and if sufficiently large can compromise blood flow. Fissuring of atherosclerotic plaques are associated with the formation of thrombi [Falk, 1983; Davies and Thomas, 1985]. Thrombosis is an associated mutifactoral disease which can cause a reduction and blockage of the flow of blood leading to ischaemic events [Fuster *et al.*, 1990]. Together these diseases of the cardiovascular system are the leading cause of mortality in industrialised nations.

Numerous risk factors contribute to the pathogenesis of these diseases, including smoking, hypertension, diabetes and elevated concentrations of low density lipoproteins (LDL). Atherosclerotic plaques contain lipid-laden macrophages, the major lipid present is cholesterol derived mostly from LDL [Hoff *et al.*, 1979; Hoff and Gavbatz, 1981]. Evidence implicates the free radical-mediated oxidation of LDL as a key event in the pathogenesis of atherosclerosis [Steinberg *et al.*, 1989]. Post-secretory oxidative modification of LDL renders the particles increasingly atherogenic, allowing their unregulated uptake by macrophages [Henriksen *et al.*, 1981]. Modified forms of LDL are thought to enhance the atherosclerotic process by inducing functional changes in local cells [Steinberg and Witztum, 1990]. Different cell types involved in this process include monocytes/macrophages, endothelial cells, smooth muscle cells (SMC), T-lymphocytes and platelets.

Platelets were originally proposed to be involved in the initiation of atherosclerosis, as described in the `response to injury hypothesis' [Ross and Harker, 1976] (*see section 1.4.2*). More recently, platelets have been relegated to a secondary role, whereby they contribute to the growth of the plaque, by being incorporated as a thrombus. Platelet activation and aggregation are intimately involved in the formation of thrombi [Heptinstall, 1980]. The formation of platelet aggregates at the plaque surface can have two outcomes depending on the extent of the fissure. If the fissure is small, then generally the subsequent aggregate would be modest and the thrombus incorporated into the plaque. If the plaque rupture is more extensive, then platelet aggregation is more substantial. If the coagulation cascade becomes activated, the subsequent thrombus is much larger and can lead to complete occlusion of the artery. Ultimately this leads to myocardial infarction and possibly death.

Therefore, both LDL and platelets are two of the key factors in the pathophysiology of arterial disease. In the following sections the properties and functions of lipoproteins and platelets will be discussed, followed by an outline of their known interactions under normal and pathological conditions.

1.2. Plasma lipoproteins.

Lipids are relatively insoluble in aqueous media, and as a result are transported *in vivo* as specialised particles termed lipoproteins. Plasma lipoproteins are complexes of lipid and protein structurally conserved by non-covalent bonds. Lipoproteins perform three major functions: 1) transport of dietary lipids from the intestinal mucosa, site of absorption, to other tissues. 2) transport of triglycerides (TG) from the liver to peripheral tissues where they are stored or

utilised for energy, and 3) transport of cholesterol to and from the peripheral tissues.

Plasma lipoproteins are divided into five functionally distinct subclasses: chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). The distinction between different lipoprotein classes is determined by their individual flotation densities in saline media [Hatch and Lees, 1968], which can be used to separate them by ultracentrifugation. However, other parameters such as electrophoretic mobility and flotation rate have also been used to classify lipoproteins.

All lipoproteins possess a common structure. The particle core is hydrophobic in nature and is composed of variable amounts of cholesteryl esters (CE) and TG. This core is surrounded by a phospholipid (PL) monolayer (mainly phosphatidylcholine), and free cholesterol. Each class of lipoprotein possesses one or more specific proteins, termed apolipoproteins, which are embedded in the phospholipid monolayer *[Table 1.1]*. Apolipoproteins remain associated with lipids by hydrophobic bonding between the fatty acyl chains of PLs and the non-polar regions of the apolipoprotein and, by ionic interactions of the polar head groups of the PLs and oppositely charged amino acids of the apolipoprotein [Thompson, 1989]. Apolipoproteins have three major functions with respect to lipid metabolism: 1) they stabilise lipoprotein structure allowing functional viability, 2) they act as cofactors for enzymes involved in lipid metabolism and 3) they function as specific ligands for the receptor-mediated uptake of lipoproteins [Mahley *et al.*, 1984].

property	CM	VLDL	IDL	LDL	HDL_2	HDL_3
major apoproteins	A- I B- 48 C- I C- II C - III	B- 100 C- I C- II C- III E	B- 1OO E	B- 100	A- I A- II C- I C- II C- III	A- I A- II C- I C- II C- III
lipid:protein ratio	98:2	91:9	80:20	77:23	62:38	48:52
chemical compostion (% by wt.)						
CE	5	12	25	39	26	20
C	2	7	9	9	5	3
TG	84	54	30	6	4	4
PL	7	18	16	23	27	21
protein	2	9	20	23	38	52

Table 1.1: The chemical composition of the five major classes of lipoproteins.

Source: Handbook of hyperlipidaemia, Thompson, [1989].

1.2.1. Low-density lipoproteins.

1.2.1.1 Structure.

LDL are spherical particles which have a plasma half-life of 2-3 days. They are the most abundant and major cholesterol carrying particle found in humans. The hydrophobic core contains a high proportion of CE (mainly cholesteryl oleate) with lower amounts of TG. The relative proportions of lipids found in LDL are summarised in *Table 1.1*. LDL show variation in composition and particle size and can be separated into two major subfractions, light and heavy, by ultracentrifugation [Krauss and Burke, 1982; Swinkels *et al.*, 1987].

LDL contain only one apolipoprotein, termed $apoB_{100}$, which is synthesised in the liver and is secreted as part of VLDL. $ApoB_{100}$ possesses 4563 amino acids corresponding to a molecular weight of 510kDa [Olofsson *et al.*, 1987]. The protein is highly glycosylated, containing 8-10% carbohydrate, which is mainly mannose [Mahley, 1984]. $ApoB_{100}$ is fundamental to the function of LDL as it contains a specific amino acid sequence which is recognised by the cellular receptor, termed apoB/E receptor [Goldstein *et al.*, 1985] (*see section 1.2.1.4*). LDL also possesses various lipid soluble antioxidants: the most abundant of these is α-tocopherol, 6 molecules per LDL particle [Esterbauer *et al.*, 1990]. Other antioxidants include β-carotene, 1 molecule per 3 LDL particles, γ-tocopherol, lycopene and ubiquinone [Esterbauer *et al.*, 1992] *[Table 1.2]*. The α-tocopherol content is positively correlated with the PUFA (linoleate and arachidonate) content of LDL [Esterbauer *et al.*, 1990]. A diagrammatic representation of a LDL particle is shown in *Figure 1.1*.

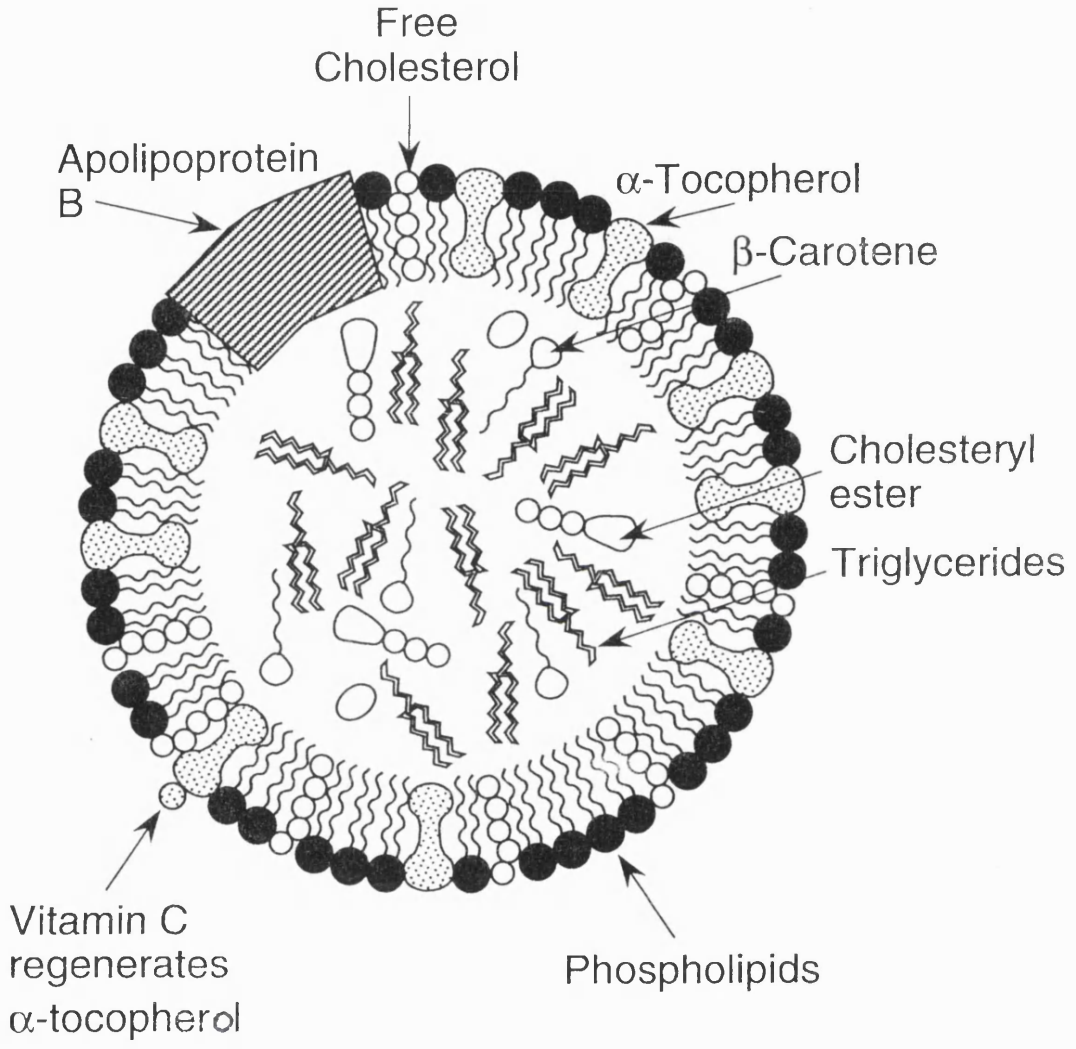


Figure 1.1: Schematic representation of the composition of low density lipoproteins.

	nmol/mg protein	n	mol/mol LDL
	mean ± sd		mean
Palmitic acid	1260 ± 375	19	693
Palmitoleic acid	80 ± 44	19	44
Stearic acid	260 ± 118	19	143
Oleic acid	825 ± 298	19	454
Linoleic acid	2000 ± 541	31	1100
Arachidonic acid	278 ± 100	31	153
Docosahexaenoic acid	53 ± 31	15	29
α-tocopherol	11.6 ± 3.3	87	6.37
δ-tocopherol	0.93 ± 0.36	88	0.51
ß-carotene	0.53 ± 0.47	122	0.29
carotene	0.22 ± 0.25	28	0.12
Lycopene	0.29 ± 0.23	136	0.16
Phytofluene	0.09 ± 0.05	10	0.05
Ubiquinol-10	0.18 ± 0.18	7	0.1
Total PUFAs (mean)	2332		1283
Total antioxidants (mean)	13.8		7.6

Table 1.2: Polyunsaturated fatty acids and antioxidant composition in low density lipoproteins.

Source: The role of lipid peroxidation and antioxidants in the oxidative modification of LDL, Esterbauer *et al.*, [1992].

1.2.1.2 Lipoprotein metabolism (synthesis of low density lipoproteins).

Lipoprotein metabolism can be divided into two pathways: endogenous and exogenous. LDL is formed by the endogenous pathway. Exogenous lipid transport begins with the incorporation of dietary fats into TG-rich chylomicrons in the intestine, which are subsequently secreted into the blood. Lipoprotein lipase (LPL), anchored to the luminal surface of the endothelium by glycosaminoglycans, is activated by apoC-II present on chylomicrons. The activated LPL hydrolyses the chylomicron core leading to the release of free fatty acids, which are absorbed into the local tissues. The remaining CE-rich 'chylomicron remnants' are taken out of circulation by the liver via specific apoE receptors present on its surface.

The endogenous pathway begins with the synthesis and secretion of TG-rich VLDL particles from the liver. VLDL is hydrolysed by LPL, via apoC-II, and the TG removed to form IDL with the loss of apoA, C-I, C-II and C-III to the HDL pool. The resultant IDL has two possible fates; it is removed by the liver via the apoE receptor or it is converted to LDL by the action of hepatic triglyceride lipase and with the loss of apoE. It has been suggested that each LDL particle is synthesised from a single VLDL particle [Soutar *et al.*, 1977]. LDL is subsequently removed from the circulation by a specific high affinity LDL receptor and undergoes cellular degradation (*see section 1.2.1.3.*).

Excess cellular cholesterol is removed from cells by HDL and is returned to the liver for excretion or recycling, by a process named reverse cholesterol transport [Oram, 1990]. The mechanism of this process remains unresolved, but may involve interactions between HDL and the cell surface. Free excess cholesterol

is esterified by the plasma enzyme lecithin:cholesterol acyl transferase (LCAT). The neutral CE are attracted to the hydrophobic core of HDL allowing further esterification at the cell surface. ApoA-I present on HDL is thought to activate LCAT. HDL are secreted by the liver as nascent HDL taking the form of disc like bilayers of PL with apoA-I and apoA-II, while other components are acquired during circulation. HDL are divided into two subclasses, HDL_2 and HDL_3: it is HDL_3 which are thought to accept cholesterol at the cell surface. HDL_2 formed by the accumulation of CE catalysed by the reaction of LCAT. HDL_2 transfer CE to VLDL in exchange for TG, a process which is facilitated by cholesterol ester transfer protein (CETP) [Barter *et al.*, 1987]. Therefore, through a process driven by LCAT, HDL helps maintain cholesterol homeostasis.

1.2.1.3. Cellular uptake of low density lipoproteins.

The cellular uptake of LDL occurs by a process referred to as receptor mediated endocytosis. The LDL receptor, termed apoB/E receptor, are found clustered in clathrin coated pits at the cell surface. LDL binds to the receptor via a specific interaction with $apoB_{100}$, which facilitates the endocytosis of LDL as a vesicle. Once inside the cell, several LDL vesicles fuse to form an endosome, a subsequent drop in pH causes the receptor to dissociate from the LDL particles. Receptors are then recycled to the coated pits at the cell surface. Lysosomes bind to the LDL endosome stimulating the degradation of $apoB_{100}$ to its constitutive amino acids and hydrolysis of CE to cholesterol. The re-esterification of cholesterol is catalysed by the enzyme acyl:cholesterol acyltransferase (ACAT). The resultant increase in free cholesterol acts to regulate its own cellular concentration by:-

[1] Down regulation of hydroxy methyl-glutaryl coenzyme A, which catalyses the rate limiting step in de novo cholesterol synthesis.

[2] Increased activity of ACAT.

[3] Down regulation of LDL receptor synthesis.

The process outlined above was The Nobel prize winning work of Brown and Goldstein, [review, 1986] and was originally completed using human skin fibroblasts.

LDL function to deliver cholesterol to cells, used in both cell membrane structures and for steroidogenesis. However, the other components of the particles should not be disregarded. LDL provides α-tocopherol for protection against oxidative stress and phospholipids utilised during mitosis.

1.2.1.4. The ApoB/E receptor.

The apoB/E receptor is a single chain transmembrane acidic glycoprotein with a relative molecular mass of 160kDa [Goldstein *et al.*, 1985; Schneider, 1989]. Levels of expression of the receptor varies with the cell type, but is particularly high in the adrenals and gonads where cholesterol is used in steroidogenesis. The ligand binding domains are characterised by seven repetitive cysteine-rich negatively charged sequences which interact specifically with positively charged arginine and lysine residues on $apoB_{100}$. LDL with modified arginine and lysine residues do not undergo endocytosis [Goldstein *et al.*, 1979].

The mature LDL receptor is divided into five distinguishable domains:

[1] The first domain (ligand binding), which contains the NH_2 terminal and

consists of 292 amino acids, houses the binding sites for the LDL particles. The domain consists of seven repeat sequences, which are extremely rich in cysteine residues. The cysteines form disulphide bonds and these are thought to confer stability to the binding sites. Housed in each of the repeating sequences is a cluster of negatively charged amino acids, which are thought to be complementary to positively charged sequences in both apoE and B, allowing lipoprotein particles to bind.

[2] The second domain consists of 400 amino acids and is homologous to a portion of the extracellular domain of the EGF precursor.

[3] The third domain is immediately external to the membrane spanning domain of the receptor. The region contains carbohydrate chains attached by an O-linkage. The function of these carbohydrate chains are unknown, but may keep the receptors extended from the membrane.

[4] The fourth domain is the membrane spanning region and consist of 22 hydrophobic amino acids.

[5] The fifth domain is the cytoplasmic tail, which contains 50 amino acids. This domain may play an important role in the clustering of the receptor in coated pits, either through an interaction with clathrin or another clathrin associated protein.

1.2.1.5. Familial hypercholesterolaemia.

The elucidation of LDL catabolism led to better understanding of the inheritable disease, familial hypercholesterolaemia (FH). FH (heterozygous) is caused by inheritance of one mutant gene coding for the apoB/E receptor. Inheritance of two mutant genes results in homozygous FH. Based on behaviour of the mutant protein, five classes of mutations at the apoB/E (LDL) receptor have been

elucidated [Goldstein *et al.*, 1985]:-

[1] Class 1 mutations fail to produce any immunoprecipitable protein.
[2] Class 2 mutations encode proteins that do not fold properly after synthesis and are blocked, either partially or completely, in transport between the endoplasmic reticulum and the Golgi apparatus.
[3] Class 3 mutations encode proteins that are synthesised and transported to the cell surface, but have a reduced capacity to bind LDL.
[4] Class 4 mutations encode proteins that move to the cell surface and bind LDL normally, but are unable to cluster in clathrin-coated pits and thus do not internalize the lipoprotein.
[5] Class 5 mutations encode receptors which bind and internalize ligand in the coated pits, but fail to discharge the ligand in the endosome and fail to recycle to the cell surface.

A wide variety of mutations occurs within these five basic classes, all producing slightly different behaviours in the mutant proteins. Ultimately, these conditions result in a large increase in plasma LDL concentrations, leading to an accelerated development of atherosclerosis and reduced longevity of the individual.

1.3. The oxidative modification of low density lipoproteins.

Increased plasma LDL concentrations are associated with an increased risk of atherosclerosis [Steinberg *et al.*, 1989]. Recently, it has been proposed that LDL becomes oxidatively modified *in vivo*, which renders the particles more atherogenic [Steinberg and Witztum, 1990]. Goldstein *et al.*, [1979] showed that

LDL modified by acetylation resulted in a reduced rate of uptake by fibroblasts via apoB/E, but an increased uptake by the macrophage scavenger receptor. This led to massive cholesterol accumulation in the macrophages. It was proposed that the lipid laden foam cells found in atherosclerotic plaques were derived from blood-borne monocytes and macrophages [Gerrity, 1981]. However, chemical acetylation of LDL was unlikely to occur *in vivo*. The key finding linking the two observations was made by Henriksen *et al.*, [1981], who demonstrated that incubation of LDL with bovine aortic endothelial cells in the presence of trace amounts of transition metal ions, altered the LDL to a form recognised by the macrophage scavenger receptor. It was subsequently shown that activated macrophages [Cathcart *et al.*, 1985], smooth muscle cells (SMC) [Morel *et al.*, 1984] and more recently platelets [Aviram *et al.*, 1990] could induce the same modification of LDL. The study by Morel *et al.*, [1984] showed oxidised LDL (oxLDL) to have an increased relative electrophoretic mobility (REM) and was the first to suggest the modification of LDL involved a free radical mechanism. In contrast to endothelial cells and SMC, cultured fibroblasts failed to modify LDL under identical conditions [Henriksen *et al.*, 1981; Morel *et al.*, 1984]. Steinbrecher *et al.*, [1984] suggested endothelial cells modification of LDL may involve lipid peroxidation of the PL, leading to the formation of lipid hydroperoxides.

An important observation was made by Heinecke *et al.*, [1984] who demonstrated that the incubation of LDL in media containing increased levels of transition metal ions enhanced the modifications induced by endothelial cells. This indicated that the concentrations of transition metal ions present played a key role in the oxidation of LDL, although there was an additional effect of

cellular activity. It was subsequently demonstrated that Cu^{2+} ions could oxidise LDL independently of cells. This key finding allowed the oxidation kinetics of LDL to be studied in great detail. Esterbauer *et al.*, [1987] modified LDL by exposure to oxygen-saturated buffer or $CuCl_2$ confirmed the involvement of lipid peroxidation. This study showed that oxidation of LDL was associated initially with the loss of α-tocopherol and secondly the loss of PUFA content. The products of oxidation included lipid hydroperoxy and hydroxy derivatives of linoleate and arachidonate [Lenz *et al.*, 1990] and various reactive aldehydes produced by the subsequent breakdown of the lipid hydroperoxides [Esterbauer *et al.*, 1990]. In addition, oxidation of LDL led to an increase in cholesterol oxides, although the prominence of individual oxides was dependent on the method of oxidation (endothelial cells or Cu^{2+}) [Bhadra *et al.*, 1991]. LDL is also thought to possess an intrinsic PLA_2 like activity due to the constant production lysolecithin during oxidation [Pathasarathy *et al.*, 1985]. A role for lipoxygenase in LDL modification has also been proposed. Sparrow *et al.*, [1988], demonstrated that co-incubation of soyabean lipoxygenase and PLA_2 with LDL mimicked cellular oxidation without the requirement of transition metal ions. Lipoxygenase enzymes catalyse the oxidation of arachidonic acid to hydperoxyeicosatetraenoic acids (HpETEs). The kinetics and products of LDL oxidation *in vitro* has been reviewed extensively by Esterbauer *et al.*, [1990].

The increase in REM associated with oxidation of LDL [Morel *et al.*, 1984] indicated that structural changes of $apoB_{100}$ had occurred in addition to modification of the lipid moiety. It has been proposed that the reactive aldehydes produced during oxidation, including malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), form adducts with $apoB_{100}$, leading to an increase in

net negative charge [Steinbrecher, 1987; Steinbrecher *et al.*, 1989]. The aldehydes form a Schiff's base with the ε- amino group on lysine residues of the $apoB_{100}$. Fragmentation of $apoB_{100}$ has also been shown to occur, arising from cleavage of the polypeptide chain by lipid peroxyl radicals. The structural changes of $apoB_{100}$ observed with modification of LDL only occur after extensive oxidation; mild or short term oxidation of LDL did not increase REM [Zhang *et al.*, 1989]. Esterbauer *et al.*, [1990] showed that a rapid rise in LPO during initial oxidation which was associated with a very small increase in TBARS. These studies suggested that oxidation of LDL may initially proceed without protein modification.

The oxidation of LDL *in vivo* is thought to occur in the subendothelial space immediately below the endothelium [Steinberg *et al.*, 1989], but the mechanism by which these changes occur still remains to be resolved. Evidence suggests the involvement of a free radical mechanism [Morel *et al.*, 1984; Steinbrecher *et al.*, 1984], although these studies also showed an absolute requirement for transition metal ions. The availability of free metal ions *in vivo* is limited, but may occur in areas of tissue damage [Halliwell and Gutteridge, 1989]. Subsequently, the production of free radicals *in vivo* was proposed to play a role in LDL modification. Superoxide anion ($O_2^{\bullet -}$) formed by endothelial cells, by activated macrophages during respiratory burst or SMC, was a proposed perpetrator. Heinecke *et al.*, [1986] and Steinbrecher, [1987] both showed that $O_2^{\bullet -}$ generated by cultured SMC and endothelial cells could be involved in oxidation of LDL. In contrast, VanHinsbergh *et al.*, [1986] showed that cell mediated oxidation of LDL did not involve $O_2^{\bullet -}$ or H_2O_2. Hydroxyl radicals have also been implicated in the initiation of LDL oxidation, but their production requires Fe^{2+},

as described by the Fenton reaction (reaction 1) which is unlikely to be available *in vivo*, at least under normal conditions.

$$Fe^{2+} + H_2O_2 \rightarrow OH^{\bullet} + OH^{-} + Fe^{3+} \quad (1)$$

However, both $O_2^{\bullet-}$ and $OH^{\bullet}$ may be involved in the oxidation of LDL by another mechanism. Beckman *et al.*, [1990] have shown $O_2^{\bullet-}$ reacts rapidly with NO to form the peroxynitrite anion ($ONOO^-$), which rapidly breaks down when protonated to form $OH^{\bullet}$. Since both $O_2^{\bullet-}$ and NO are produced by endothelial cells and macrophages in close proximity to LDL, they may form radicals which initiate oxidation of LDL *in vivo*. The effects and influence of oxLDL on the development and progression of atherosclerosis will be discussed in *section 1.4.3.*

1.3.1. Lipid peroxidation.

It is now accepted that oxidation of LDL probably occurs by a free radical chain reaction [Morel *et al.*, 1984; Steinbrecher *et al.*, 1984; Esterbauer *et al.*, 1987] *[Figure 1.2]*. This can be initiated by the abstraction of a H atom from the PUFA sidechains of the PL, to form a carbonyl radical ($-C^{\bullet}H-$). The carbonyl radical spontaneously rearranges its structure to form a conjugated diene. In the presence of molecular oxygen, the conjugated dienes reacts to produce a lipid peroxyl radical ($-COO^{\bullet}-$) (reaction 1).

$$-C^{\bullet}H- + O_2 \rightarrow -HCOO^{\bullet}- \quad (1)$$

Lipid peroxyl radicals propagate the chain reaction by abstracting another H atom, forming a lipid hydroperoxide (CHOOH) and another carbonyl radical. This part of the chain reaction only ceases when H atoms and/or O_2 are no longer available. The lipid hydroperoxides break down to form alkoxyl radicals $CO^{\bullet}$, which eventually produce aldehydic derivatives.

Oxidation mediated by Cu^{2+} has an absolute requirement for preformed lipid hydroperoxides [Thomas and Jackson, 1990]. Cu^{2+} facilitates the breakdown of lipid hydroperoxides by fission of O–O bond, to release alkoxyl radicals [Halliwell and Gutteridge, 1989] (reactions 2 and 3).

$$-H_2COOH + Cu^{2+} \rightarrow -H_2COO^{\bullet} + Cu^{+} + H^{+} \qquad (2)$$

$$-H_2COOH + Cu^{+} \rightarrow -H_2CO^{\bullet} + Cu^{2+} + OH^{-} \qquad (3)$$

Antioxidant protection found within LDL, such as α-tocopherol, are consumed before the chain reaction commences [Esterbauer *et al.*, 1987]. α-tocopherol protects against oxidation by scavenging lipid peroxyl radicals (chain breaking antioxidant) (reaction 4).

$$-H_2COO^{\bullet} + \alpha\text{-toc} \rightarrow -H_2COOH + \alpha\text{-toc}^{\bullet} \qquad (4)$$

α-tocopherol is thought to be replenished by electron transfer to α-tocopherol from ascorbic acid, a plasma (water) soluble antioxidant (reaction 5).

$$\alpha\text{-toc}^{\bullet} + asc \rightarrow \alpha\text{-toc} + asc^{\bullet} \qquad (5)$$

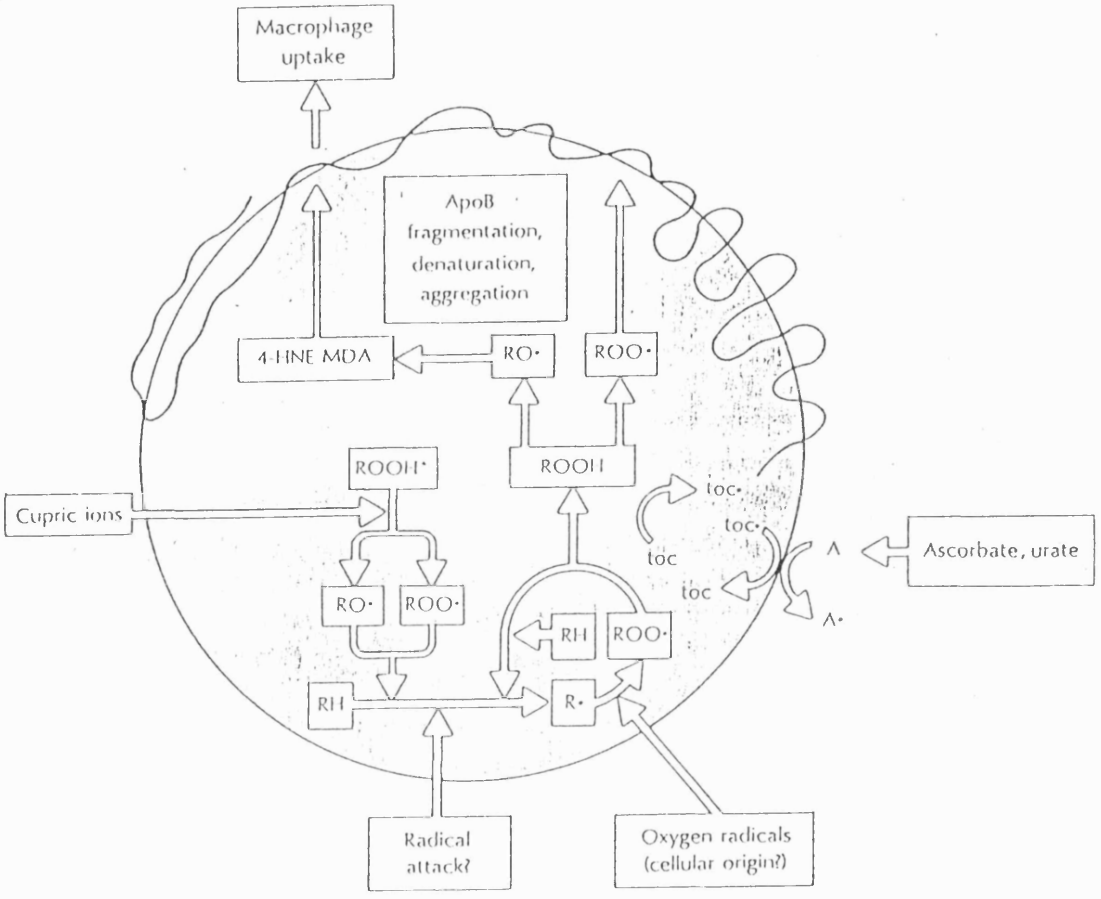


Figure 1.2: Schematic representation of mechanism of lipid peroxidation within an LDL particle.

Source: Free radicals, lipid peroxidation and atherosclerosis. Bruckdorfer, 1990.

This model of lipid peroxidation was proposed by Halliwell and Gutteridge [1989].

1.3.2. Do oxidised low density lipoproteins exist in vivo ?

If the oxidation of LDL occurs *in vivo*, it is unlikely to occur in the plasma, since this compartment contains high levels of antioxidants which would attenuate oxidative modification. In addition, the lipoproteins are unlikely to encounter high localised oxidative environments in the circulation as the blood flow would remove this very quickly. Modification requires LDL to be present in an environment where there is a sustained oxidative stress leading to the depletion of the antioxidant protection. The subendothelial space has been proposed to be such a compartment [Steinberg *et al.*, 1989]. LDL freely enters the sub-endothelium, although they may be retained here by interactions between apoB and the proteoglycans associated with connective tissue of the intima [Hurt and Camejo, 1987]. Here, the lipoproteins may be subjected to free radical attack, the radicals being produced by cellular metabolism.

It has been demonstrated that oxLDL is present in atherosclerotic plaques. Haberland *et al.*, [1988] demonstrated the presence of modified LDL using antibodies directed against MDA treated LDL. Using immunostaining it was found that these antibodies reacted with components of lesions in rabbit aortae, but not in other areas of the vessel. Material extracted from the aortic lesions of WHHL rabbits was found to react with antibodies against MDA-LDL, 4-HNE-LDL and oxLDL, suggesting the presence of these complexes *in vivo* [Palinski *et al.*, 1989]. In both of these studies the antibodies failed to show reactivity towards autologous nLDL. In addition Palinski *et al.*, [1989] demonstrated the presence

of autoantibodies to oxLDL in the plasma of WHHL rabbits, and these autoantibodies reacted with various forms of LDL *in vivo.* More importantly, Salonen *et al.*, [1992] showed that autoantibodies to oxLDL were present in human plasma. Finally, convincing evidence for the role of oxidative modification in lesion formation comes from studies demonstrating antioxidants such as BHT and probucol can inhibit lesion formation in WHHL rabbits and cholesterol fed rabbits respectively [Carew *et al.*, 1987; Kita *et al.*, 1987; Bjorkhem *et al.*, 1991].

The oxidation of LDL in the subendothelium is likely be a continuous process, with LDL at different stages of modification being present at any one time. While LDL are only mildly oxidised, with minimal protein modification, it is possible that they may escape into the plasma. Several reports have shown an LDL subfraction isolated from plasma which had a slightly increased negative charge [Avogaro *et al.*, 1988, Cazzolato *et al.*, 1991, Shimano *et al.*, 1991]. Several authors have investigated the actions of minimally modified LDL (mmLDL), defined as LDL with lipid, but no protein modification, as potentially important atherogenic factors. Their results show mmLDL to exert several cellular responses *in vitro*, which are similar to events proposed to occur in the early pathogenesis of the disease. These studies suggest that mmLDL are more biologically relevant than oxLDL and are discussed in greater detail in *sections 1.4.3 , 3.7.1 and 3.7.3.*

1.4 BIOCHEMICAL PATHOGENESIS OF ATHEROSCLEROSIS.

Atherosclerosis is a multifactoral disease, which is characterised by the presence of lesions on the intima of the arterial wall. The pathogenesis and progession of atherosclerosis involves various cells, a large number of growth

factors, cytokines and vasoregulatory molecules. The interactions between cells via different mediators is a highly complex process which has been reviewed extensively by Ross, [1993]. The following section provides a general overview of the biochemical pathogenesis of atherosclerosis and the theories proposed for disease development.

1.4.1. Atherosclerotic plaques.

The earliest lesions of atherosclerosis, the fatty streak, can be found in young children whereas the more advanced lesion, the fibromuscular plaque, generally appears in early adulthood and progresses with age.

1.4.1.1. Fatty streaks.

Macroscopically fatty streaks appear as a yellowish discolouration beneath the lining of the coronary arteries. Principally they consist of lipid-laden macrophages along with smaller numbers of lipid filled SMC [Stary, 1989]. Some evidence exists to suggest that fatty streaks were localised at anatomical sites which later developed into more advanced lesions, although this is not completely certain.

1.4.1.2. Fibromuscular plaques.

Fibrous plaques are white in appearance and are usually large enough to protrude into the artery, if sufficiently large they can compromise blood flow. The lesions consist of large numbers of lipid-laden macrophages, T-lymphoctes and proliferated SMC which are surrounded by large amounts of connective tissue (collagen, elastic fibres and proteoglycans). If the lesion has become unstable, it is prone to haemorrhage and thrombosis, if this has occurred the structure is

referred to as a complicated lesion.

1.4.2. The `Response to injury' hypothesis.

The `response to injury' hypothesis was first proposed by Ross and Glomset, [1976] and was based on the similarity in appearance between early lesions of atherosclerosis and the response of arteries to experimentally induced injury. It was proposed that alterations to endothelial cells affects their function as a protective barrier. The insult to the endothelium can be induced mechanically, by the use of a balloon catheter to cause abrasion, or chemically by conditions such as hyperlipidaemia, homocysteineuria, diabetes or hypertension. For example, hypercholesterolaemic baboons were shown to have lost almost 10% of the endothelial lining of the thoracic and abdominal aorta [Ross and Harker, 1976]. The loss of endothelial cells resulted in adhesion of platelets to underlying collagen fibrils. In vessels denuded of their endothelium platelets adhere to the subendothelium within seconds and continued for upto 48hours [Stemerman and Ross, 1972]. Adherent platelets degranulate thereby releasing various growth factors and active amines (*see section 1.5.6*). PDGF, which is stored in platelet alpha granules, induces the migration of SMC from the media to the intima and their subsequent proliferation. Proliferating SMC secrete large amounts of connective tissue, including collagen and proteoglycans [Opsahl *et al.*, 1987]. At the same time, there is an accumulation of cellular lipid deposits into plaques, which also contributes to the bulk of the plaque. The lipid laden cells have been shown to be of monocytic origin [Aqel *et al.*, 1984].

The response to injury hypothesis implicates platelets as the key cell in the induction of atherosclerosis by their ability to induce themigration and proliferation of SMC.

1.4.3. Lipid infiltration theory.

This hypothesis places more importance on LDL in the initiation and subsequent development of atherosclerosis. LDL can freely enter and leave the subendothelial space during its normal functioning and metabolism. Once present in the subendothelial space LDL may become trapped, possibly by binding to proteoglycans of the internal elastic lamina [Hurt and Camejo., 1987]. The retention of LDL in a microenviroment may lead to an exhaustion of antioxidant protection and therefore increases the potential for oxidation. Several lines of evidence point to the existence of oxLDL *in vivo* (*see section 1.3.2*).

One of the earliest events in experimentally induced atherosclerosis is the adherence of monocytes to the endothelium [Gerrity *et al.*, 1979]. MmLDL has been proposed to play a key role in this event. In cell culture studies mmLDL induced the release of MCP-1, a chemoattractant specific for monocytes, from endothelial cells [Cushing *et al.*, 1990], potentiated the release of MCSF and GSCF [Rajavashisth *et al.*, 1990] and induced the expression of glycoprotein adhesion molecules on the surface of endothelial cells [Berliner *et al.*, 1990]. Monocytes enter the subendothelium where they undergo phenotypic transformation to macrophages. Activated macrophages may enhance monocyte entry, by releasing interleukin-1 which induced increased monocyte adhesion [Bevilacqua *et al.*, 1985].

Fully oxidised LDL is thought to contribute heavily to the formation of the plaque. They decrease the effectiveness of nitric oxide (NO) disrupting vascular tone [Andrews *et al.*, 1987, Jacobs *et al.*, 1990, Tanner *et al.*, 1991], increase Ca^{2+}

entry of SMC allowing potential proliferation [Weisser *et al.*, 1992], and exert cytotoxic effects on endothelial cells possibly leading to increased entry of LDL and monocytes [Quinn *et al.*, 1987]. Macrophages accumulate oxLDL via the scavenger receptor, possibly as a protective mechanism against the cytotoxic effects of oxLDL, to form lipid-laden foam cells. The metabolic activity of macrophages is thought to contribute to oxidation of LDL leading to greater accumulation within the plaque. Foam cells along with proliferated SMC constitute the bulk of the plaque. Eventually the macrophages necrose, leaving a core of lipid and cell debris in the plaque which is surrounded by alternating layers of SMC and connective tissue.

1.4.4. Unification of theories.

The response to injury hypothesis suggests the key event is the initial damage to the endothelium and the subsequent activation of platelets. However, several studies have shown fatty streaks can develop under an intact endothelium [Faggiotto *et al.*, 1984]. This points to an alteration in endothelial function rather than damage. The cytotoxic nature of oxLDL implicates it as a possible mediator of a dysfunctional endothelium. However, mmLDL have also been shown to increase adhesion molecule expression on cultured endothelial cells, without inducing cytotoxicity [Berliner *et al.*, 1990]. The retention of LDL would allow intimate contact between LDL and endothelial cells which could lead to lipid exchange, leading to subtle alterations in the membrane and associated functions. Macrophages laden with oxLDL may also be involved with this process: in attempting to return to the plasma compartment they may cause physical pressures which lead to the loss over overlying endothelial cells. At this point the sequence of events proposed by the original response to injury

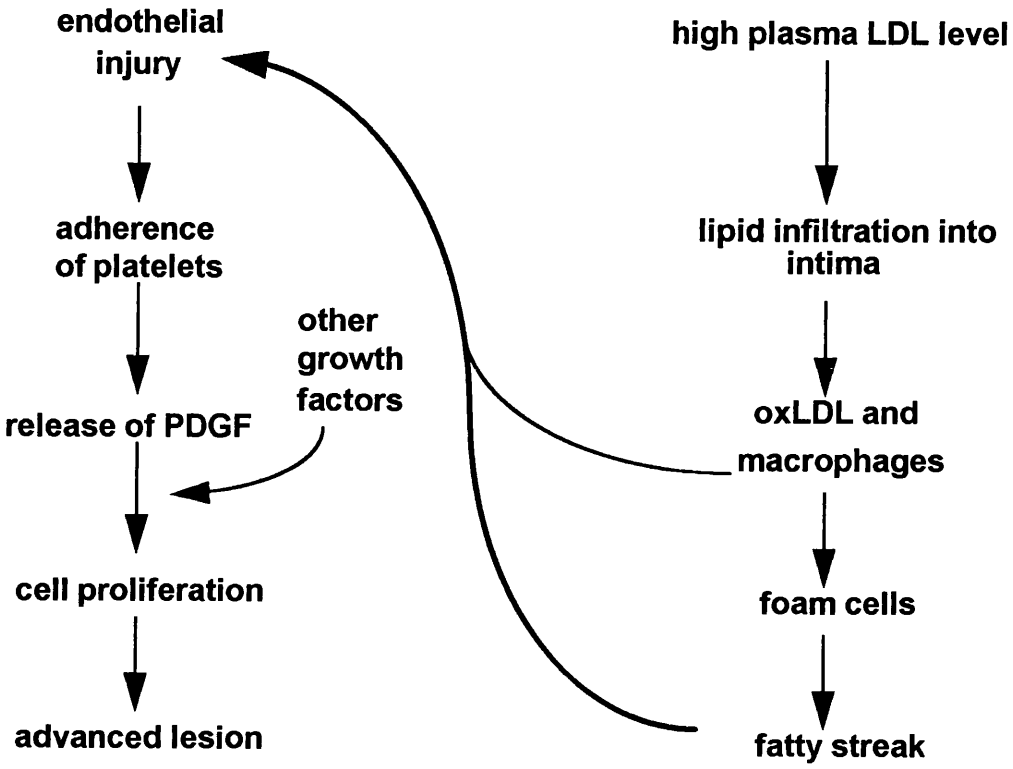


Figure 1.3: Scheme showing the possible unification of the `response to injury' and `lipid infiltration' theories.

Source: Beyond cholesterol: modification of LDL that increase its atherogenicity. Steinberg *et al.*, [1989].

hypothesis would be applicable *[Figure 1.3]*. The role of platelets is of much more importance in the clinical sequelae of atherosclerosis, in particular thrombosis.

1.5 PLATELETS

Platelets are circulating anucleate discoid cells (2 - 4μm in diameter) which when activated form a haemostatic plug at sites of vascular injury thereby preventing excessive blood loss. Platelets are released as fully mature cells from bone marrow megakaryocytes. The cells have a plasma density of 2 - 4 x 10^8 per ml and circulate in the blood for 7 - 10days before removal by the spleen.

1.5.1 Platelet ultrastructure.

The platelet possesses a standard phospholipid bilayer membrane which has a highly glycosylated outer surface. This exterior coat or 'glycocalyx' is composed of several different glycoproteins which function as receptors for the platelet. The membrane serves to protect the interior of the cell, but when the cell is activated acts as a specific phospholipid surface for amplification of the coagulation cascade (*see section 1.5.9*). Immediately below the cytoplasmic surface of the membrane is a network of overlapping actin filaments which are thought to be important in pseudopodia formation. Beneath the filaments is a single microtubule coiled on itself several times. The microtubule is found along the greatest circumference of the equatorial plane of the cell and helps maintain the resting discoid shape [White, 1986].

Within the cytoplasmic zone are several structures and organelles, these

include, mitochondria, peroxisomes, lysosomes, glycogen granules, molecular actin and two types of storage granules, α-granules and dense granules *[Figure 1.4]*. In addition the platelet possesses two membrane systems, the open canalicular system (OCS) and the dense tubular system (DTS). The OCS is composed of numerous invaginations continuous with the plasma membrane and reaches into the furthest recesses of the cell. Ultimately this acts to greatly increase the surface area of the cell. Platelet DTS is randomly dispersed in the cell and is thought to originate from the rough endoplasmic reticulum of the parent megakaryocyte. The DTS selectively takes up Ca^{2+} ions by a Ca^{2+}/Mg^{2+}ATPase system and is the major storage site for Ca^{2+} ions in the cell. The two membrane systems are not completely isolated and are found in close association with each other at several sites in most platelets [White, 1986] [*Figure 1.4*].

1.5.2 Physiology and biochemistry of the platelet.

Platelets circulate in the blood as quiescent cells. However, when confronted with a damaged endothelium they quickly adhere to the injured surface and form a haemostatic plug. The plug becomes re-enforced by the generation of thrombin at the platelet surface and the subsequent formation of fibrin. The functional response of platelets involves a series of marked morphological and biochemical changes which result in the accumulation of platelets at sites of vascular injury. The process involves the following principal events: a) activation, b) shape change, c) adhesion, d) aggregation, e) secretion, and f) metabolism of arachidonic acid (AA) and subsequent release of thromboxane A_2 (TXA_2).

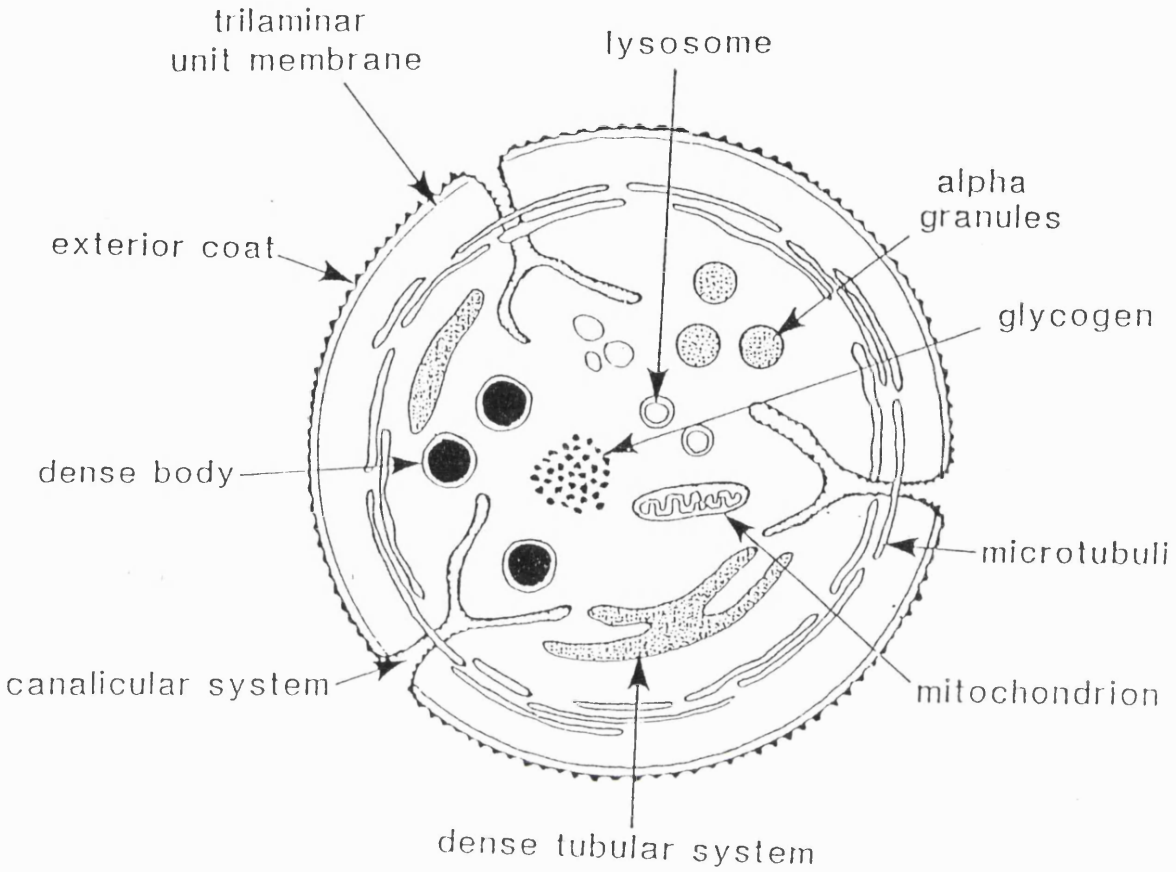


Figure 1.4: Diagrammatic representation of the ultrastructure of the human blood platelet (equatorial plane).

1.5.2.1 Platelet activation.

The physiological activation of platelets involves the binding of specific agonists to their individual receptors on the platelet surface. These agonists include adenosine diphosphate (ADP), adrenaline, collagen, platelet activating factor (PAF), serotonin, thrombin and TXA_2. Agonist-receptor coupling leads to a cascade of biochemical events resulting in adhesion and aggregation [*summarised in Figure 1.5*].

1.5.2.2 Guanosine triphosphate (G) - binding proteins.

G-proteins are regulatory binding proteins which link receptor-agonist (or antagonist) interactions to secondary messenger production [Negata and Nozawa, 1990]. Each G-protein is composed of three subunits, α, β and γ, with GDP tightly bound to the α subunit. Agonist receptor coupling causes the dissociation of GDP from the alpha-subunit and in the presence of Mg^{2+} is replaced by GTP. Binding of GTP causes the dissociation of the α subunit (Gα.GTP) from the complex and allows it to interact with a membrane bound enzyme (eg, adenylate cyclase or phospholipase C). The βγ components remain single functional unit and reassociates with the α subunit as the intrinsic GTPase activity of the α subunit hydrolyses GTP to GDP [Kroll and Schafer, 1989]. Several G-proteins have been described in platelets including, G_P, G_S, G_I, and G_t [Negata and Nozawa, 1990].

1.5.2.3 Phosphatidylinositol hydrolysis and secondary messengers.

Platelet activation leads to the release of a phospholipid based secondary messenger system as described for other cells [Berridge and Irvine, 1984]. Agonist receptor occupancy leads to activation of G_p [Cockcroft, 1987; Kroll and

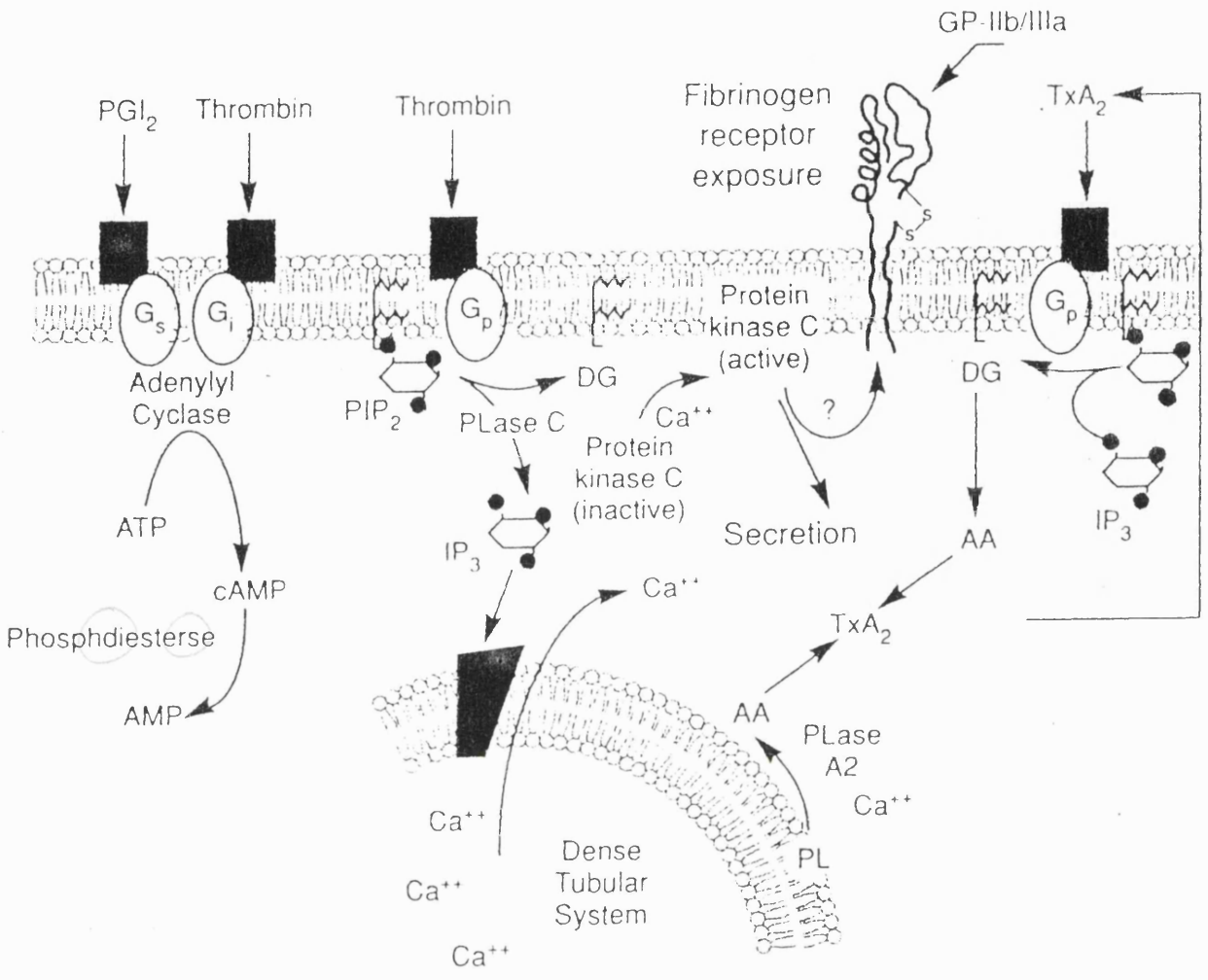


Figure 1.5: Diagrammatic representation of the pathways involved in the biochemical activation of human platelets

Schafer, 1989] and subsequent activation of phosphatidylinositol specific phospholipase C (PLC). PLC hydrolyses membrane phosphatidylinositol 4,5 bisphosphate (PIP_2) to yield two secondary messengers, inositol 1,4,5 trisphosphate ($I(1,4,5)P_3$) and diacylglycerol (DAG).

$I(1,4,5)P_3$ regulates agonist-induced increases in platelet cytosolic calcium [Berridge and Irvine, 1984]. Increases in $[Ca^{2+}]_i$ is fundamental to platelet activation (*section 1.5.2.4*). Intracellular $I(1,4,5)P_3$ is thought to bind to specific receptors on the surface of the DTS and releases Ca^{2+} from internal stores, presumably through a Ca^{2+} channel [Daniel, 1989]. $I(1,4,5)P_3$ is converted to $I(1,3,4,5)P_4$ by the action of a $I(1,4,5)P_3$ 3-kinase [Irvine *et al.*, 1986]. $I(1,3,4,5)P_4$ has been proposed to cause the influx of extracellular Ca^{2+} across the plasma membrane associated with platelet activation [Irvine and Moor, 1987]. $I(1,3,4,5)P_4$ is subsequently converted to $I(1,3,4)P_3$ by the action of a 5'-phosphomonoesterase, which is thought to be activated by protein kinase C (PKC) [Kroll and Schafer, 1989]; no function for $I(1,3,4)P_3$ has been elucidated. The same 5'-phosphomonoesterase converts $I(1,3,4)P_3$ to IP_2 ending the Ca^{2+} elevating actions of $I(1,4,5)P_3$.

The other secondary messenger, DAG, remains in the membrane matrix where it triggers the translocation of inactive PKC from the cytoplasm to the membrane. In the presence of Ca^{2+} and phosphatidylserine, PKC becomes activated; DAG is thought to increase the affinity of PKC for Ca^{2+} [Kroll and Schafer, 1989]. DAG is phosphorylated to phosphatidic acid and recycled back into phosphatidylinositol.

1.5.2.4 Calcium and platelet activation.

Ca^{2+} plays a central role in platelet activation by triggering several important pathways. Resting platelets have a $[Ca^{2+}]_i$ of approximately 100nmol/l, which is almost three orders of magnitude lower than the plasma concentration. The platelets are able to maintain the low $[Ca^{2+}]_i$ by the use of three distinct compartments for Ca^{2+} storage:-

a) Dense granules: Ca^{2+} is stored and released into the microenviroment during platelet secretion. This Ca^{2+} pool does not undergo exchange with the cytoplasmic pool.

b) DTS: This is the major storage site for Ca^{2+} in the platelet, in which Ca^{2+} is sequestered from the cytoplasm by a Ca^{2+}/Mg^{2+} ATPase pump. Ca^{2+} is released into the cytoplasm after binding of $I(1,4,5)P_3$ to its receptor on the DTS [Daniel, 1990].

c) Cytoplasm: The cytoplasmic pool (half life 17.5min) [Kroll and Schafer, 1989], which is regulated by a Na^+/Ca^{2+} antiport and possibly by a Ca^{2+}/Mg^{2+} ATPase pump in the plasma membrane.

All platelet activatory agonists with the exception of adrenaline have the capacity to induce increases in cytosolic $[Ca^{2+}]$ [Rink and Sage, 1990]. The magnitude of these increases is dependent upon the individual agonist used [Ware *et al.*, 1986]. Ca^{2+} increases are regulated by the action of $1(1,4,5)P_3$ on the DTS and by $I(1,3,4,5)P_4$ possibly acting on a Ca^{2+} channel in the plasma membrane. The activation of a 5'-phosphomonoesterase to convert IP_3 to IP_2 by PKC, has also been proposed to regulate $[Ca^{2+}]_i$ by feedback inhibition [Kroll and Schafer, 1989]. The increase in $[Ca^{2+}]_i$ is important to platelet activation because several

enzymes involved are regulated by cytoplasmic Ca^{2+} ion concentrations. These enzymes include phospholipase A_2 (PLA_2), myosin light chain kinase (MLCK) and PKC. In addition Ca^{2+} has been shown to trigger adhesion and aggregation of platelets independent of other agonists [Rink *et al.*, 1982], although this probably involves the activation of the previously named enzymes.

1.5.2.5 Tyrosine kinases and platelet activation.

Stimulation of platelets with thrombin or collagen leads to increases in tyrosine phosphorylation of a common set of proteins [Nakamura and Yamamura, 1989]. With thrombin-activated platelets the phosphorylation of these proteins occurred in three distinct phases which were thought to correspond to activation, shape change and aggregation [Ferrel and Martin, 1988]. Two studies have also shown a relationship between increases in $[Ca^{2+}]_i$ and tyrosine phosphorylation [Takayama *et al.*, 1991; Vostal *et al.*, 1991]. The implication of tyrosine phosphorylation in platelet activation signalling was further advanced by experiments in which tyrosine kinase inhibitors (eg, tyrphostin), which inhibited thrombin induced aggregation and secretion [Rendu *et al.*, 1992]. The full characterisation of tyrosine kinase activity in platelet signal transduction is yet to be elucidated.

1.5.2.6 Metabolism of arachidonic acid.

AA is a PUFA (20:4, ω-6) which is formed in the liver by the elongation and desaturation of the essential fatty acid, linoleic acid (18:4 ω-4). AA is esterified to membrane phospholipids, preferentially in the sn-2 position of the glycerol backbone. The metabolism of AA is important to vascular biology as it produces several vasoactive compounds. AA metabolism by the enzyme cyclo-oxygenase

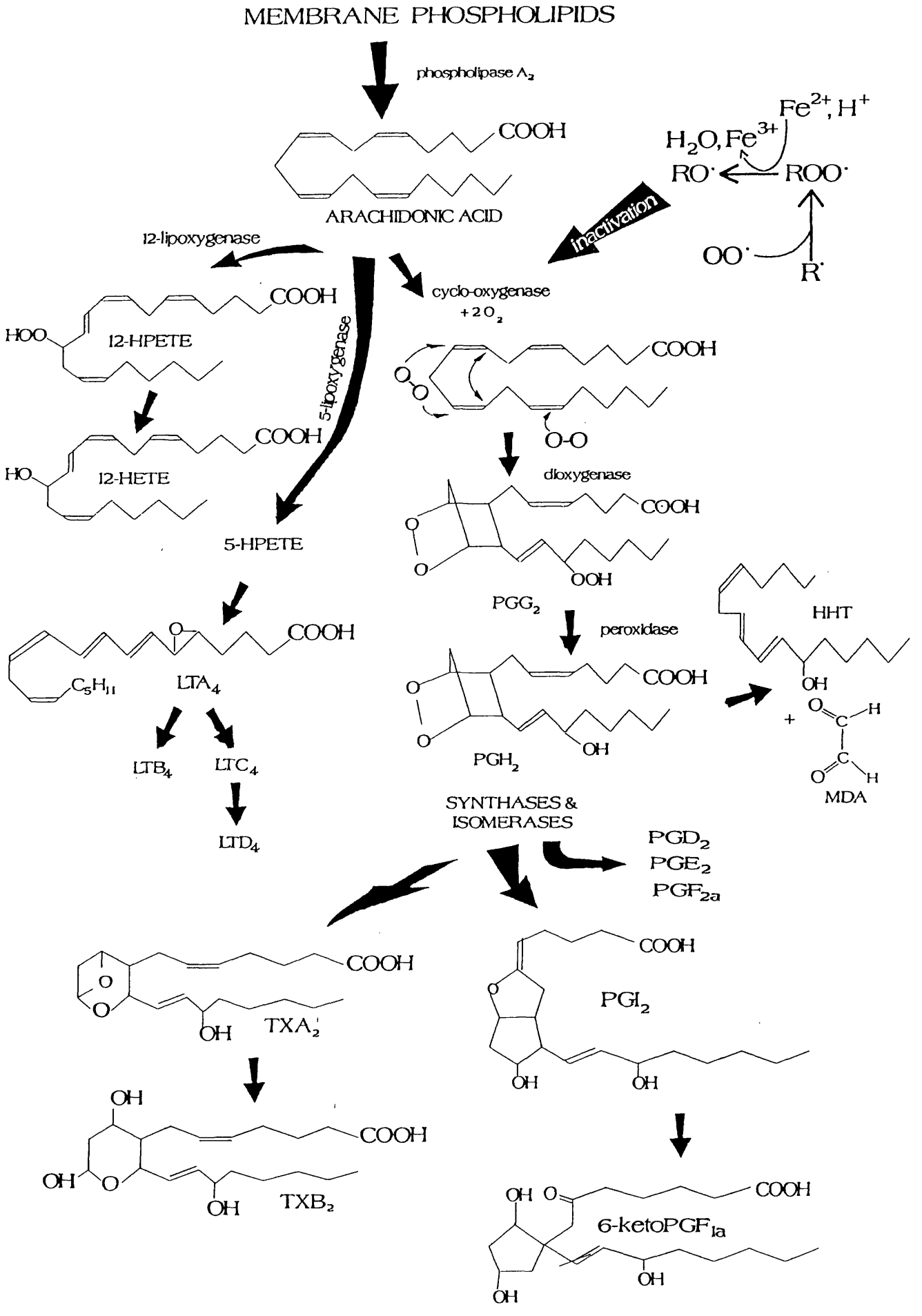
(prostaglandin H_2 synthase) in platelets results in the production of TXA_2 [Hamberg *et al.*, 1975], while metabolism via the enzyme lipoxygenase leads to formation of leukotrienes [Samuelsson *et al.*, 1979].

Increases in $[Ca^{2+}]_i$ leads to the activation of PLA_2 [Rittenhouse and Horne, 1984]. PLA_2 is the term given to a group of phospholipase enzymes, the platelet is thought to possess several isoforms of PLA_2 [Loeb and Gross, 1986]. PLA_2 hydrolyse phospholipids to release AA from both the plasma membrane and the membrane of the DTS [Brass, 1991]. Cyclo-oxygenase catalyses the cyclization of carbons 8 to 10 by concomitant insertion of four oxygen atoms to produce prostaglandin G_2 (PGG_2) [Smith and Marnett, 1991]. PGG_2 is the precursor of all AA derivatives mediated by cyclo-oxygenase, these include prostaglandins D_2, E_2, $F_{2\alpha}$, H_2, I_2 and TXA_2 *[Figure 1.6]*. TXA_2 is a potent platelet agonist, SMC mitogen and vasoconstrictor [Kroll and Schafer, 1989]. Once formed TXA_2 diffuses out of activated platelets and acts to propagate aggregation by binding to a specific receptor on the platelet surface [Hanasaki and Arita, 1991]. The production of TXA_2 and other eicosanoids can be blocked by the action of non-steroidal anti-inflammatory drugs (NSAIDS) such as aspirin and indomethacin [Vane, 1971]. This has led to the use of aspirin for anti-platelet therapy in the prevention of thrombosis.

1.5.3 Platelet shape change.

The first morphological change observed during platelet activation is a shape change from a resting discoid to a spherical shape with extending pseudopodia or spiny spheres [Zucker and Borelli, 1954]. Shape change promotes aggregation by providing a greater surface area for platelet-platelet contact.

Figure 1.6: The proposed pathways for arachidonic acid in vascular cells.


MEMBRANE PHOSPHOLIPIDS
phospholipase A2
COOH
ARACHIDONIC ACID
Fe2+, H+
H2O, Fe3+
RO·
ROO·
OO·
R·
inactivation
12-lipoxygenase
5-lipoxygenase
cyclo-oxygenase
+2O2
HOO
12-HPETE
HO
12-HETE
5-HPETE
O-O
dioxygenase
PGG2
peroxidase
PGH2
HHT
OH
MDA
LTA4
C5H11
LTB4
LTC4
LTD4
SYNTHASES &
ISOMERASES
PGD2
PGE2
PGF2a
TXA2
PGI2
TXB2
6-ketoPGF1a

During shape change the cytoplasmic granules are centralised by the contraction of the circumferential microtubule [White, 1986]. The contractile force for this process is the assembly of actin molecules into filaments and their interaction with the microtubule, probably through micotubule associated proteins [White, 1986]. Actin filaments also form parallel bundles at the periphery of the cell and their interactions may provide the cohesive strength to distort the membrane [White, 1986]. Inside the pseudopods are parallel associations of actin microfilaments, suggesting a role for actin in the support of pseudopodia structure [White, 1986]. Platelet shape change can be assessed by electron microscopy or nephelometry.

1.5.4 Platelet adhesion.

Platelets do not adhere to the endothelium under normal conditions, due to the physicochemical properties of endothelial cells, which provide an inert surface and secrete various substances to prevent this process [Vane *et al.*, 1990]. Platelets will rapidly adhere to exposed connective tissue (collagen) in injured vessels . The event is mediated by the interaction of subendothelial collagen and the glycoprotein (GP) Ia-IIa complex at the platelet surface [Pischel *et al.*, 1987]. In addition, a vascular component, von Willebrandfactor (vWF), also mediates adhesion of platelets to the subendothelium [de Groot and Sixma, 1987]. It has been suggested that the initial reversible adhesion is mediated by vWF, while irreversible adhesion results from an interaction with collagen [Siess, 1989]. vWF in synthesized by endothelial cells and is secreted partly into the circulation and partly into the subendothelial matrix, where it can exist as large multimers. It is present with other adhesive proteins, fibronectin and thrombospondin, which can also be involved with the adhesion event, but to a

lesser extent. The binding of vWF to platelets is mediated by the GPIbα,β - GPIX complex at the platelet surface [Roth, 1990]. vWF has also been shown to bind to GPIIb-IIIa complex in support of platelet aggregation. However, this is probably not physiologically relevant as FGN competes with vWF for binding and the plasma concentration of FGN exceeds that of vWF [Pietu *et al.*, 1984]. Upon adhesion, platelets spread over the damaged surface. *In vitro* studies have shown that the body of the adhered platelets infiltrate the surface and the cytoplasm to spread filling the gaps between the pseudopods, eventually forming a thin film [White, 1986].

1.5.5 Platelet aggregation.

Platelet aggregation is a process whereby platelets adhere to each other to form a haemostatic plug, a process which requires both a platelet membrane component and a plasma component. During the early phase of activation a binding domain for fibrinogen (FGN) is exposed on the platelet GPIIb-IIIa complex, this allows FGN to bind and act as an interplatelet bridge, allowing platelet-platelet interaction (*see section 1.5.8.1*). The level of exposure of platelets to agonists is proportional to the exposure of fibrinogen binding sites and thus aggregation [Peerschke *et al.*, 1980].

The introduction of the platelet aggregometer by Born, [1962] revolutionised the study of platelet function. Platelet aggregation *in vitro* is believed to mimic the formation of a haemostatic plug of platelet thrombus *in vivo*. Stirring in this system is essential to mimic the turbulent effects of blood circulation. The aggregometer allowed the characterisation of various agonists and antagonists on platelet function and providing valuable clues to the functioning of platelets

in vivo (*see section 2.6.5*) The relationship between aggregation, TXA_2 release and secretion induced by different agonists is discussed in *section 1.5.7.*

1.5.6 Platelet secretion.

Adhesion and aggregation of platelets leads to the release of a plethora of vasoactive, pro-aggregatory and thrombotic factors. These are principally from the α and dense granules, but lysosomal granule release also occurs *[Table 1.3].* The mechanism of platelet secretion remains to be fully elucidated, although a working model has been proposed. The initial event is the centralization of secretory granules by the microtubule ring, which is thought to be induced by loose polymerisation of actin [White, 1986]. The second event is the increase in $[Ca^{2+}]_i$, which activates two important enzymes, protein kinase C (PKC) and myosin light chain kinase (MLCK). Activated PKC phosphorylates a protein substrate, p47, this phosphoprotein is thought to allow the elongation of actin filaments [Kroll and Schafer, 1989]. In addition activation MLCK by Ca^{2+}-calmodulin phosphorylates myosin light chain allowing activation of the myosin hexamer by actin. These two processes are thought to promote microtubule contraction, forcing the centralized secretory granule membranes to fuse with the OCS and thus release their contents into the surrounding microenviroment [White, 1986].

Platelet secretion can be quantified by measurement of ATP release using lumi-aggregometry [Feinman *et al.*, 1977] or by RIA of a particular granule component. However, the development of monoclonal antibody (MAB)

CONTENTS	FUNCTION
α-granules	
platelet factor 4	mitogen
platelet derived growth factor (PDGF)	mitogen
transforming growth factor (TGF)	mitogen
fibrinogen (FGN)	physical support of aggregation
von Willebrand factor (vWF)	physical support of adhesion
thrombospondin	reinforcement of FGN binding
fibronectin	involved in platelet adhesion
β-thromboglobulin (β-TG)	platelet specific anti-heparin protein
factors V, VII and XI	coagulation cascade proteins
protein S	coagulation pathway protein
plasminogen activator inhibitor 1 (PAI-1)	inhibition of fibrin degradation
Dense granules	
serotonin (5-HT)	vasoconstrictor and platelet agonist
ADP, ATP	vasodilators and platelet agonists
epinephrine	vasoconstrictor and platelet agonist
pyrophosphate	function unknown
Ca^{2+}	reinforcement of platelet activation
Glycogen granules	
glycogen	provision of metabolic energy
Lysosomal granules	
acid hydrolases	enzymatic digestion

Table 1.3: The various factors released from platelets and their intraplatelet sources. The granules are released during platelet aggregation and the contents help the cell fulfil its haemostatic function.

Source: The molecular basis of platelet function [Plow and Ginsberg, 1990]

technology has led to the detection of specific protein markers which are derived from different secretory granules. These include the α granule membrane protein, P-selectin (GMP140, CD62P) [Stenberg *et al.*, 1985] and CD63 (GP53) a lysosomal granule membrane protein [Nieuwenhuis *et al.*, 1987]. Quantification of these markers can be used as an index for platelet secretion.

1.5.7 The relationship between aggregation, thromboxane A_2 release and secretion.

Although platelet aggregation is induced by a wide variety of agonists, it has become increasingly apparent that many of the agonists work via different pathways. Niewiarowski and Thomas, [1966], showed a synergistic effect on aggregation between thrombin and ADP which was greater than an additive effect and suggested the two agonists were working via different mechanisms. Later, cyclo-oxygenase inhibitors were shown to attenuate synergism between collagen and thrombin or ADP, but not between thrombin and ADP [Kinlough-Rathborne *et al.*, 1977]. These observations indicate that collagen-induced aggregation, but not thrombin- or ADP-induced aggregation is dependent on AA metabolism.

The work of Best and Holland, [1981] gave a much clearer indication of how different agonists work. Results from their study demonstrated:

a) Thrombin-induced aggregation and secretion was independent of TXA_2 biosynthesis.

b) Collagen-induced aggregation had a dual effect. At high concentrations (4μg/ml), aggregation was independent of TXA_2 production, but secretion had

a partial dependency on TXA_2 synthesis. At lower doses of collagen (1µg/ml) both aggregation and secretion were wholly dependent on TXA_2.

c) ADP-induced aggregation was independent of TXA_2, but granule secretion was TXA_2 dependent.

Balduini *et al.*, [1988] presented evidence to suggest that TXA_2 release in response to ADP was aggregation, but not FGN binding dependent. Inhibition of aggregation, but not FGN binding led to a decrease in TXA_2 release. This could have important implications for platelet analysis in non-stirred systems.

1.5.8 Platelet surface glycoproteins.

Platelets possess a family of cell surface adhesion molecule receptors, which mediate cell-cell interactions and involves use specific plasma proteins. These receptors are termed integrins, and all consist of non-covalently associated α and β subunits, with the β subunit common within the family [Phillips *et al.*, 1988]. The integrins often exist as complexes of two or more glycoproteins subunits. Important integrin complexes which are involved in the aggregation and adhesion of platelets are the vWF receptor GPIα,β-GPIX, the collagen receptor GPIa-GPIIa and GPIIb-IIIa which binds FGN, vWF and fibronectin.

1.5.8.1 GPIIb-IIIa and fibrinogen binding.

GPIIb-IIIa is the most abundant integrin present on platelets with a density of approximately 50,000 per platelet [Phillips *et al.*, 1988]. The GPIIb-IIIa complex is a heterodimeric structure which requires Ca^{2+} to maintain integrity of the complex. GPIIb consists of two polypeptide chains, one of 125kDa and a small cytoplasmic chain of 25kDa. The two chains are linked by one or more

disulphide bridges [Phillips *et al.*, 1988] *[Figure 1.7].* GPIIIa is a single polypeptide chain of mol.wt., 105KDa [Charo *et al.*, 1986]. The transmembrane regions of GPIIb-IIIa are connected to the cytoskeleton, possibly by the protein, talin [Painter *et al.*, 1985]. Physiologically, the major function of GPIIb-IIIa is the binding of fibrinogen in support of platelet aggregation. In the resting platelet GPIIb-IIIa is thought to be relatively inert. Upon activation however, the complex becomes a receptor for several adhesive proteins, of which fibrinogen is the preferred substrate. The binding of fibrinogen acts to physically support platelet-platelet contact [Peerschke *et al.*, 1980].

FGN is a trimeric plasma glycoprotein (two Aα, two Bβ and two γ chains) with a mol. wt. of 130kDa, the different chains being linked in antiparallel fashion. FGN is synthesised in the liver and secreted into the circulation and has plasma concentration of approximately 3g/l [Cook and Ubben, 1990]. Fibrinogen is also present in the α granules of platelets, this probably originated in the parent megakaryocyte [James *et al.*, 1977]. The binding of adhesive plasma proteins to GPIIb-IIIa is mediated by a specific amino acid, Arg-Gly-Asp (RGD), recognition sequence present on the particular plasma protein [Ruoslahti and Pierschbacher, 1987]. FGN possesses two RGD sequences both on the α chain and in addition contains a dodecapeptide sequence on the γ chain which also binds to GPIIb-IIIa [Phillips *et al.*, 1991].

The mechanism by which GPIIb-IIIa become activated still remains to be resolved, although two plausible mechanisms have been proposed. Shattil and Brass, [1987] suggested GPIIb-IIIa may be activated by direct interaction with

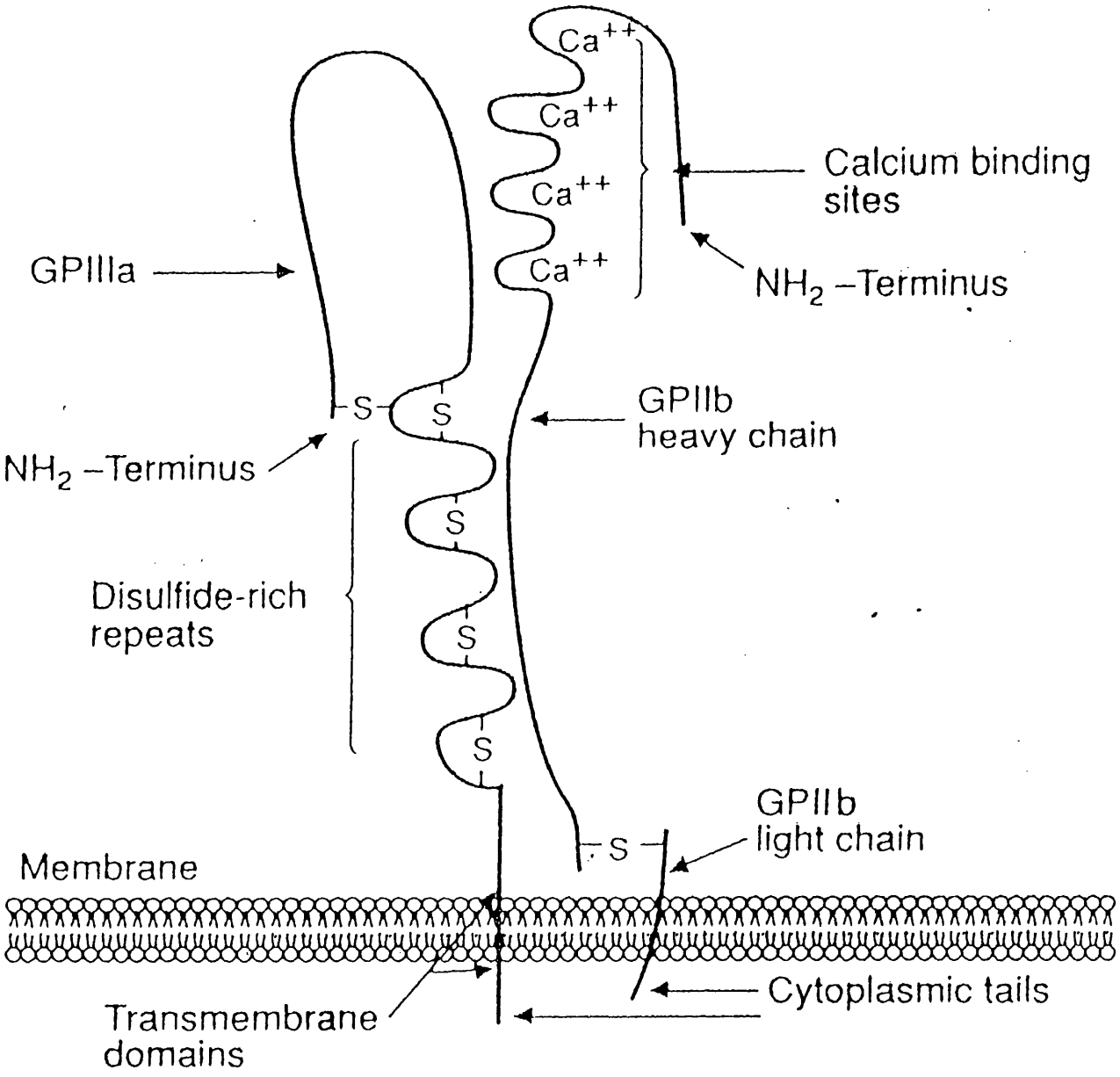


Figure 1.7: Diagrammatic representation of the structure of the platelet GPIIb-IIIa complex.

Source: The molecular basis of platelet function [Plow and Ginsberg, 1991]

a G-protein, similar to α_2-adrenergic receptors. Secondly, increases in intracellular cAMP levels and activation of PKC have both been shown to regulate exposure of FGN binding sites of GPIIb-IIIa [van Willigen and Akkerman, 1991]. The use of RGD containing peptides have provided evidence showing ligand binding to GPIIb-IIIa prior to platelet activation may trigger a conformational change in the receptor structure [Du *et al.*, 1991].

1.5.9 Platelets and the coagulation cascade.

Activated platelets, in addition to forming primary haemostatic plugs, also participate in the coagulation cascade. Platelet activation leads to a loss of membrane phospholipid asymmetry, increasing the exposure of aminophospholipids, phosphatidylserine and phosphatidylethanolamine [Schroit and Zwaal, 1991]. Phosphatidylserine provides a catalytic surface for the interaction of coagulation factors [Schroit and Zwaal, 1991]. The most important reaction is the conversion of the prothrombin zymogen to the protease, thrombin. Factor Va, which is activated from factor V or secreted from platelet α granules, binds to the phosphatidylserine at the platelet surface. Factor Xa binds to factor Va to form a 1:1 stoichiometrical, Ca^{2+} dependent complex [Tracy, 1988]. The membrane bound Va-Xa complex then interacts with prothrombin, which binds to phosphatidylserine. This assembled prothrombinase complex subsequently leads to the generation of thrombin. Thrombin in turn acts to aggregate platelets, cleave fibrinogen forming cross-linked fibrin meshworks and elicits the release various mitogens and vasoactive factors from the endothelium [Tracy, 1988].

1.6 VASCULAR ENDOTHELIUM

1.6.1 Anatomy of the vessel wall.

The vessel wall consists of three layers of cells or 'tunica':

a) Tunica intima in man is a thin layer of endothelial cells which constitute the luminal surface of the vessel. The endothelial cells are supported physically by connective tissue, consisting mainly of collagen and basal elastic lamina. The intima is separated from the tunica media by this basal membrane.

b) Tunica media is a contractile layer found predominately in large arteries such as the aorta. It is composed of a large mesh of elastin molecules, with irregular shaped SMC and small amounts of collagen fibres. The SMC present control vascular tone in response to both neuronal and humoural stimuli.

c) Tunica adventia is the outermost layer of the artery and consists principally of skin fibroblasts and some SMC surrounded by a thin layer of connective tissue, predominantly collagen.

1.6.2 Endothelial cells

1.6.2.1 Structure and function.

The endothelium is composed of a continuous layer of cells, held together by junctional complexes [Simionescu and Simionescu, 1991]. The principal function of the endothelium is the provision of a non-thrombogenic permeable barrier between the circulation and underlying tissues. However, the endothelium is also a metabolically active tissue that subserves a wide range of functions relating to vascular haemostasis [Pearson, 1991]. The endothelium synthesises glycosoaminoglycans such as heparan sulphate, which when secreted remain

on the luminal surface of the vessel wall as part of proteoglycans. Heparan sulphate is involved in the regulation of thrombin stimulation of the endothelium by catalysing the activation of anti-thrombin III [Rodgers, 1988] to reduce thrombin activity. Thrombin action is also controlled by the protein C pathway. The pathway is activated by the binding of thrombin to a endothelial surface receptor, thrombomodulin, causing the activation of protein C. Activated protein C inactivates factors Va and VIIIa which are intimately involved in thrombin generation at platelet surfaces [Rodgers, 1988].

More relevant to this study is the release of vasoactive factors from the endothelium which can influence platelet function. The endothelium synthesizes and releases two principal factors which have dual effects. Prostacyclin (PGI_2) and nitric oxide (NO) are both vasodilators and platelet inhibitors and thus may be potentially important anti-thrombotic factors [Pearson, 1991]. The release of these substances is controlled by various blood borne mediators such as thrombin, acetylcholine (ACh), histamine, adenosine, ADP, ATP, and bradykinin (BK), all of which bind to specific receptors on the endothelial cells surface. In addition, metabolic activity within endothelial cells leads to the release of $O_2^{\bullet -}$, which is converted to H_2O_2: these reactive oxygen species (ROS) can also influence platelet reactivity.

1.6.3 Prostacyclin.

PGI_2 is an AA metabolite which is synthesised via COX and prostacyclin synthase *[Figure 1.6]*. PGI_2 production is stimulated by either mechanical or chemical perturbation of the endothelial cell membrane. The binding of factors such as thrombin to cell surface receptors leads to phosphatidylinositol

hydrolysis and a subsequent increase in $[Ca^{2+}]_i$ (*see section 1.5.2.3*). Activated PLA_2 then liberates AA, which is metabolised to PGI_2. Mechanical stimuli which induce PGI_2 production include pulsatile pressure [van Grondelle *et al.*, 1984] and increased shear stress [Vane *et al.*, 1990]. In addition, moderate damage to the endothelium by experimental methods have shown an acute burst of PGI_2 release [Weksler, 1984]. PGI_2 synthesis can be inhibited by glucocorticoids, which stimulate the formation of lipocortin, an endogenous inhibitor of PLA_2 [Vane *et al.*, 1990] or by non steroidal anti-inflammatory drugs, such as aspirin or indomethacin, which inhibit COX [Vane, 1971]. In addition, high levels of lipid peroxides also inhibit PGI_2 production, by inhibiting PGI_2 synthase [Gryglewski, 1976]. PGI_2 is unstable with a half life of approximately 3minutes before catabolism to 6-keto-$PGF_{1\alpha}$ [Moncada and Vane, 1978]. The measurement of 6-keto-$PGF_{1\alpha}$ in urine has been used to quantify PGI_2 production *in vivo* and *in vitro*.

1.6.3.1 The vascular actions of prostacyclin.

PGI_2 is a potent inhibitor of platelet aggregation and vasodilator [Moncada *et al.*, 1976]. The biological effects of PGI_2 are induced by causing a transient increase in cytosolic cAMP concentrations by activation of adenylate cyclase [Tateson *et al.*, 1977]. Due to its short biological half life PGI_2, is a locally (paracrine) acting hormone rather than systemic hormone. When released into the vessel wall it acts to relax the underlying SMC, while its action luminally is to inhibit platelet activation. PGI_2 binds to specific receptor on the platelet surface which is thought to be linked to adenylate cyclase by the G-protein, G_s [Dutta-Roy *et al.*, 1987]. The subsequent rise in cAMP attenuates the phospholipid based secondary messenger system. cAMP inhibits the production of IP_3 and DAG

[Rittenhouse-Simmons, 1979], possibly by decreasing the activity of PLC. PGI_2 also increases the sequestration of cytosolic Ca^{2+} into the DTS, preventing the activation of Ca^{2+} regulated enzymes [Tao *et al.*, 1992] (*section 1.4.2.4*). The longevity of the cAMP signal is controlled by the action of specific phosphodiesterases. Additionally it has been proposed that the thrombin receptor is linked to G_i, which down regulates adenylate cyclase and favours platelet aggregation [Cooper and Rodbell, 1979].

1.6.3.2 Prostacyclin action in atherosclerosis.

The biological properties of PGI_2 may protect against atherosclerosis by suppressing the accumulation of cholesteryl esters by macrophages [Willis *et al.*, 1987] and increasing cholesteryl ester hydrolysis in vascular SMC [Hajjar *et al.*, 1982, Willis *et al.*, 1987]. A reduction of PGI_2 activity, as seen in the area of atherosclerotic plaques [Sinzinger *et al.*, 1977; De' Angelo, 1978], would favour thrombus formation. This is initiated by plaque rupture leading to activation of platelets and the coagulation cascade. Hence a reduction in PGI_2 synthesis may be detrimental to normal vascular homeostasis.

Platelets from patients with FH have been shown to have a diminished sensitivity to PGI_2 [Colli *et al.*, 1983]. Inhibition of isolated platelet aggregation by PGI_2 was attenuated by the presence of LDL, this was associated in a reduced production of cAMP [Bruckdorfer *et al.*, 1984]. In contrast to the effects on platelets, LDL increased PGI_2 release from cultured endothelial cells [Spector *et al.*, 1985], with oxLDL having an even more potent effect [Triau *et al.*, 1988]. These observations suggested a paradoxical role for LDL, increasing endothelial PGI_2 production while decreasing the sensitivity of its effects on platelets. In

general it is accepted that there is diminished PGI_2 activity in plaque affected areas [Sinzinger *et al* .,1977; De' Angelo, 1978].

1.6.4 Nitric oxide.

NO or a closely related species, has been implicated in a wide variety of biological events including regulation of vascular tone, inhibition of platelet adhesion and activation, neuronal signalling and macrophage cytotoxicity.

1.6.4.1 Discovery of endothelium derived relaxing factor (EDRF).

The endothelium plays an obligatory role in the relaxation of arterial SMC by acetylcholine (ACh) [Furchgott and Zawadzki, 1980]. In these now classic experiments aortic rings, precontracted with noradrenaline, were relaxed by the addition of ACh. The ACh induced relaxation was lost if the endothelium were removed. Relaxation in response to glyceryl trinitrate was unchanged. Furthermore, the magnitude of SMC relaxation in response to ACh was directly proportional to the amount of endothelium present. From these observations it was postulated that ACh acted on muscarinic receptors of the endothelium to release a factor (EDRF) which was not PGI_2, but induced SMC relaxation [Furchgott and Zawadzki, 1980; Griffith *et al.*, 1984]. ACh activated PLC-induced hydrolysis of AA, and it was postulated that EDRF was an AA metabolite, possibly a lipoxygenase product. Pulsatile flow through segments of canine femoral artery induced the release of a EDRF which was not PGI_2, as evidenced by bioassay Rubanyi *et al.*, [1986].

It was proposed that EDRF was NO, since no significant differences in their actions were found on arterial relaxation [Ignarro *et al.*, 1987, Palmer *et al.*,

1987]. Authentic NO solutions were compared directly to EDRF released from stimulated aortic endothelial cells cultured on microcarrier beads. No difference could be detected in biological half-life, the ability to relax contracted aortic strips, while the effects of both NO and EDRF were inhibited by the presence of Hb. Palmer *et al.*, [1987] also showed that the $O_2^{\bullet-}$ produced by aortic strips was a major contributor to the decay of both NO and EDRF.

1.6.4.2 The biosynthesis of nitric oxide.

NO is synthesised *in vivo* from a biological substrate. Palmer *et al.*, [1988] demonstrated that NO derived from cultured endothelial cells was synthesised from the amino acid, L-arginine. Incubation of endothelial cells with L-arginine (L-arg), but not D-arg, significantly enhanced the production of NO in response to BK. Studies with ^{14}C L-arg demonstrated that L-citrulline was the other product produced in NO synthesis [Iyenger *et al.*, 1987]. Inhibition of NO synthesis by N^G-monomethyl L-arg (NMMA), strongly suggested that L-arg was the substrate and the guanidino N atoms were utilised in NO formation [Palmer *et al.*, 1988]. NO production in response to calcium ionophore A23187 indicated that the pathway may be initiated by an increase in intracellular Ca^{2+} [Palmer *et al.*, 1988].

Marletta *et al.*, [1988] proposed the existence of an enzyme, NO synthase, which catalyses the conversion of L-arg to NO in the presence of Ca^{2+}. The proposed synthesis of NO is outlined in *Figure 1.8* and can be broken down into five steps:
a) Hydroxylation of a guanidino nitrogen atom to form N^G-hydroxylarginine, with NADPH acting as a co-factor.

b) Two electron oxidation of the hydroxylamine moiety; an electron donor was not proposed.

c) Loss of a proton and subsequent production of an amino acid radical.

d) Fragmentation of the unstable radical, which produces NO and an amino acid derivative, carbodiimide.

e) Reaction of carbodiimide with H_2O [Marletta, 1988], then produces L-citrulline.

It is of interest that NO is also released from macrophages, independently of Ca^{2+}, when stimulated with lipopolysaccharide [Marletta, 1988]. This could be inhibited if cells were pre-incubated with N^GMMA [Hibbs *et al.*, 1987], suggesting the presence of a NO/L-arginine pathway similar to that of the endothelium. SMC have also been shown to release an NO-like factor which inhibited platelet aggregation and was abrogated by the presence of oxyHb and N^GMMA, but potentiated by SOD [Mollace *et al.*, 1991]. Importantly, the effects of LPS on macrophages could be abolished by pre-incubation with cycloheximide. This indicated that NO production by the endothelium and macrophages occurred by slightly different mechanisms, since the effects of cycloheximide suggest protein (enzyme) synthesis was required [Mollace *et al.*, 1991]. In addition the NOS of the endothelium was Ca^{2+} dependent, while the macrophage and SMC NOS was Ca^{2+} independent and enzyme synthesis was required for NO production.

Several different NOS isoenzymes have now been characterised:

[1] Neuronal NOS (nNOS; type I) is the constitutive Ca^{2+} dependent enzyme found in neuronal tissue.

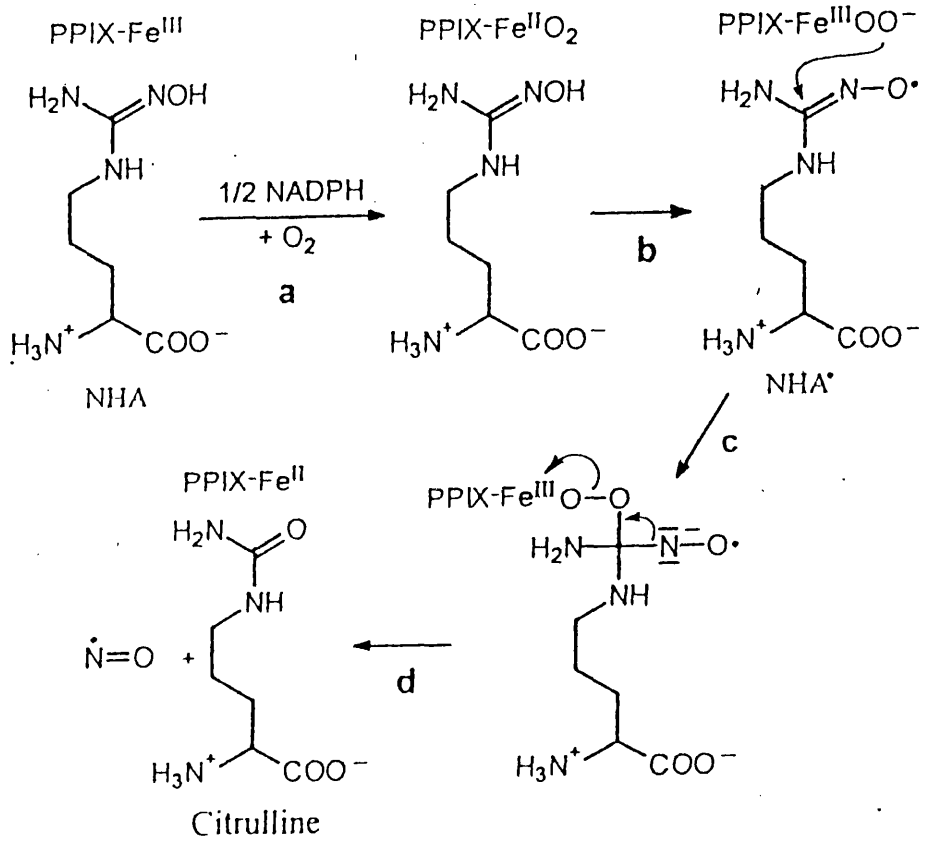


Figure 1.8: The proposed mechanism for the formation of nitric oxide from L-arginine.

[2] Inducible NOS (iNOS; type II) is the inducible Ca^{2+} independent enzyme found in macrophages.

[3] Endothelial NOS (eNOS; type III) is theconstitutiveCa^{2+} dependent enzyme found in endothelial cells.

1.6.4.3 Nitric oxide: Biological effects and mode of action.

Vascular relaxation in response to ACh and BK is mediated by the release of NO [Furchgott and Zawadzki, 1980; Griffith *et al.*, 1984]. The half-life of NO seems to be very short, ranging from 6 to 30s [Griffith *et al.*, 1984; Palmer *et al.*, 1987, Ignarro *et al.*, 1987, Myers *et al.*, 1990]. NO, although an inorganic molecule, it is extremely lipophilic and once formed diffuses out of the cell into adjacent cells, which include endothelial cells, platelets or SMC, where it exerts its biological effect. In this respect NO is unique as it can be considered a primary intercellular messenger.

More relevant to this thesis, NO has been shown to be a potent inhibitor of platelet aggregation. Azuma *et al.*, [1986] first demonstrated that the effluent from a perfused rabbit aorta stimulated with ACh, both relaxed aortic strips and inhibited AA-induced platelet aggregation. Furlong *et al.*, [1987] showed endothelial cells produced EDRF inhibited both collagen- and TXA_2-induced aggregation, this inhibition could be abolished by Hb and potentiated by SOD. NO also inhibited adhesion of radiolabelled thrombin-stimulated platelets to aortic strips stimulated with BK [Radomski *et al.*, 1987b]. In a more detailed study [Radomski *et al.*, 1987a], both endogenous and exogenous NO inhibited platelet adhesion, the inhibition was potentiated by SOD and inhibited by Hb.

Furthermore PGI_2 and cAMP phosphodiesterase inhibitors had very little affect on adhesion, while specific cGMP phosphodiesterase inhibitors potentiated the inhibition of adhesion. These observations strongly implicated NO as the active agent in respect to platelet inhibition.

Nitrates, NO and $^{\bullet}OH$ can stimulate guanylate cyclase to increase intracellular cGMP [Murad *et al.*, 1978]. It was suggested that increases in cGMP induced by exogenous NO and NO donors inhibited platelet aggregation [Mellion *et al.*, 1981]. Subsequently increases in platelet cGMP were shown to inhibit PI hydrolysis and p47 phosphorylation induced by thrombin [Takai *et al.*, 1981]. Furthermore endothelium dependent arterial relaxation was also associated with increased cytosolic cGMP [Rapoport and Murad, 1983]. The addition of exogenous NO to platelets led to a rapid increase in cGMP which occurred in the first 15s after addition [Salvemeni *et al.*, 1990, Nguyen *et al.*, 1991]. NO binds to the haem group of guanylate cyclase to form nitrosylhaem. This interaction pulls the Fe^{2+} out of the plane of the planar porphyrin ring leading to enzyme activation [Ignarro, 1990]. Incubation of NO with gel filtered platelets prior to addition of thrombin, inhibited aggregation, serotonin secretion, p47 phosphorylation and phosphatidic acid production, but enhanced $[Ca^{2+}]_i$. The inhibition of NaF induced aggregation, which activates GTP binding proteins directly, and the decrease in phosphatidic acid production, indicated that NO inhibited G-protein activation of PLC and subsequent secondary messenger production rather than affecting receptor/G protein coupling [Nguyen *et al.*, 1991]. Experimental evidence indicates that basal release of NO by endothelial cells may control platelet function and vascular tone. Adhesion of thrombin stimulated platelets to an unstimulated endothelial monolayer was inhibited by

the presence of a specific cGMP phosphodiesterase inhibitor [Radomski *et al.*, 1987b]. The passage of platelets, once through rabbit coronary vascular bed, significantly increased platelet cytosolic cGMP without ACh stimulation of endothelial cells [Pohl and Busse, 1989]. However, addition of ACh to the perfusate did increase platelet cGMP compared to the effects of unstimulated endothelial cells. Platelets also possess an L-arginine/NO pathway which is activated during platelet aggregation and may represent a self-regulatory mechanism for platelet function [Radomski *et al.*, 1990a, b]. There appears to be basal release of NO from the endothelium which enters the platelet to increase cGMP [Radomski *et al.*, 1987; Pohl and Busse, 1989]. These findings strongly suggest the biological actions of NO are mediated by increasing intracellular cGMP. The short half life of NO suggests it is a local, rather than a systemically acting messenger.

1.6.4.4. Reactions of nitric oxide.

(I) Nitric oxide and superoxide anion: formation of peroxynitrite.

Many studies involving NO observed that SOD increased the longevity of its biological effects [Rubanyi *et al.*, 1986; Palmer *et al.*, 1987; Radomski *et al.*, 1987; Furlong *et al.*, 1987]. This suggested that $O_2^{\bullet-}$ or a related species reacts with NO, resulting in the loss of its biological activity. Gryglewski *et al.*, [1986] implicated $O_2^{\bullet-}$ as the free radical species which nullifies NO, since SOD, but not catalase, protected arterial relaxations induced by BK stimulation of endothelial cells.

Beckman *et al.*, [1990] postulated that inhibition of NO activity by $O_2^{\bullet-}$ was due to a chemical interaction between the two free radicals. The product of the

reaction is the peroxynitrite anion ($ONOO^{\bullet -}$), which when protonated rapidly decomposed (half life approx. 1.9s) to produce $NO^{\bullet}_2$ and an oxidant with a similar reactivity to $^{\bullet}OH$ (reaction 1).

$$O_2^{\bullet -} + NO \rightarrow ONOO- + H^+ \rightleftarrows ONOOH \rightarrow {}^{\bullet}OH + NO^{\bullet}_2 \rightarrow NO_3^- + H^+ \quad (1)$$

If $ONOO^-$ is produced *in vivo*, it would be in close proximity to the luminal surface of arteries and as a result may elicit endothelial damage, which in turn contribute to the development of atherosclerosis. The provision of a SOD on the luminal surface of the endothelium may protect against this process.

(ii) Nitric oxide and oxygen.

NO readily reacts with O_2 to produce NO_2. NO_2 has two possible fates; firstly in the gaseous phase NO_2 could dimerise to form N_2O_4, while in solution NO_2 would yield nitrite and nitrate ions (reactions 1, 2 and 3).

$$2NO + O_2 \rightarrow 2NO_2 \quad (1)$$

$$2NO_2 \rightleftarrows N_2O_4 \quad (2)$$

$$2NO_2 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+ \quad (3)$$

Reactions 1 and 3 are the most biologically relevant and probably represent the actual inactivation pathway for NO during bioassay experimentation *in vitro*. In addition, reaction 1 indicates that the stability of NO is directly proportional to its initial concentration.

(iii) *Nitric oxide and Hemoglobin.*

Hb possesses a very high binding affinity for NO; which binds to the Fe^{2+} ion of the haem group. The product of the reaction is nitrosyl-haem, a complex which is stable for several minutes [Keilin and Hartree, 1937]. Micromolar concentrations of Hb are present in the circulation and as such may influence the half-life of NO.

1.6.4.5. Does nitric oxide exist in vivo ?

It is accepted that the properties of EDRF can be mimicked by NO. However little evidence has been presented showing the existence of NO *in vivo*. Hence, it has been postulated that released NO may be complexed and stabilised by a carrier, in the plasma. Thiol groups (R–SH) can be nitrosylated in the presence of NO [Stamler *et al.*, 1992], and it has been suggested that peptides and proteins containing free thiol groups may act as carriers for NO. Several S-nitrosothiols have been shown to inhibit platelet aggregation and increase cGMP [Mellion *et al.*, 1983]. More recently Myers *et al.*, [1990] showed S-nitrosocysteine to induce vasorelaxation of porcine aortic rings, which was 80 times more effective than authentic NO solutions. The effects of S-nitrosocysteine were abolished by the presence of Hb, suggesting a mechanism involving the release of NO. In the same study, S-nitrosoglutathione and S-nitrosomercaptoethanol were found to induce relaxation, although to lesser extent than S-nitrosocysteine. S-nitrosocysteine also inhibited platelet activation and secretion, which was associated with an elevation in cGMP [Lieberman *et al.*, 1991]. A study by Stamler *et al.*, [1992], showed that several plasma proteins could be nitrosylated and were found to be biologically active, as assessed by their abilities to relax aortic strips and inhibit platelet aggregation.

Although the above studies do not provide evidence of the biological existence of S-nitrosothiols, they do demonstrate that they possess biological activity which may mediate the release of NO. It is reasonable to postulate that S-nitrosothiols constitute a mechanism by which NO can exert a systemic effect. The biological relevance of S-nitrosothiols requires more detailed investigation.

1.6.4.6. Nitric oxide and atherosclerosis.

The development of atherosclerosis is thought to associated with a loss of vascular tone in the affected areas. Strips taken from atherosclerotic human coronary arteries showed a decreased potential to relax when challenged with BK, compared with responses in normal arteries [Fostermann *et al.*, 1987; Bossaller *et al.*, 1987]. It was proposed that LDL played a key role in the mechanism of these effects. nLDL and oxLDL both inhibited relaxations induced by ACh; the effects of nLDL were reversible and dependent on the contracting agent [Andrews *et al.*, 1987b]. In contrast, Tanner *et al.*, [1991] showed oxLDL, but not nLDL, inhibited vascular relaxation. OxLDL only inhibited endothelium-dependent relaxation of blood vessels, since relaxation in response to Ca^{2+} ionophore, A12387, and SIN-1(an NO donor) were unaffected. This indicated that the effects of oxLDL were at receptor level.

Activation of endothelial G-proteins by NaF induced the release of NO, this was only partially inhibited by pertussis toxin [Flavahan and Vanhoutte, 1990], suggesting the involvement of two distinct G-proteins, one of which was more sensitive to pertussis toxin (G_i). OxLDL at low concentrations (50mg/l) exerted its most potent effects against endothelial stimulators (thrombin and serotonin) of NO release which were G_i dependent [Kugiyama *et al.*, 1990]. At higher

concentrations of oxLDL (100 - 300mg/l) the effects were more non-specific, inhibiting the actions of A12387 and BK (G_i independent) [Kugiyama *et al.*, 1990, Tanner *et al.*, 1991]. In the above studies, different incubation times were used which may account for some of the different results obtained, but in addition they may also give an indication to the mechanisms underlying the progression of atherosclerosis. The SMC of atherosclerotic coronary arteries respond normally to NO mimetics such as SIN-1, which would indicate that the effects of oxLDL are on the endothelial cells [Tanner *et al.*, 1991; Shimokawa *et al.*, 1991]. Endothelial dysfunction in the affected area is an early event in atherosclerosis [Hansson *et al.*, 1987]. The endothelial dysfunction in atherosclerosis and that produced experimentally by oxLDL appear to be similar [Shimokawa *et al.*, 1989]. Evidence would suggest in early atherosclerosis or at low oxLDL concentrations, endothelial dysfunction is mediated by impaired G-protein function, in particular pertussis sensitive G-proteins [Shimokawa *et al.*, 1989; Shimokawa *et al.*, 1991]. In support of this concept G_i endothelial-dependent relaxations in hypercholesterolaemic animals have been shown to be reduced compared to G_i independent pathways [Shimokawa *et al.*, 1991]. As the disease progresses, equivalent to higher experimental concentrations of oxLDL and increased incubation times, the impairment of relaxation becomes less specific. Tanner *et al.*, [1991] suggested longer term exposure to oxLDL allowed the uptake of oxLDL by the endothelial cells scavenger receptor. Endocytosed oxLDL was proposed to interfere with the L-arginine/NO pathway, since pretreatment of aortic rings with L-arginine produced normal relaxation responses in the presence of oxLDL. The actions of oxLDL may be mediated by oxidation products, an example of which is lysolecithin.

1.6.5. Reactive oxygen species and platelet function.

The endothelium continually produces superoxide anions ($O_2^{\bullet -}$), which most probably originate from incomplete reduction of oxygen by the electron transfer chain in mitochondria [Ramasarma, 1982]. The respiratory burst of neutrophils is also a major source of $O_2^{\bullet -}$. Superoxide is generated by the addition of an unpaired electron to molecular oxygen (reaction 1):-

$$O_2 + e^- \rightarrow O_2^{\bullet -} \qquad (1)$$

Superoxide radicals are normally removed *in vivo* by the action of (SOD), which catalyses the dismutation of superoxide anions to hydrogen peroxide (H_2O_2) [Babior and Peters, 1981] (reaction 2):-

$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (2)$$

Hydrogen peroxide is also thought to be cytotoxic and is subsequently converted to water and molecular oxygen *in vivo* by the action of the enzyme catalase (reaction 3):-

$$2H_2O_2 \rightarrow 2H_2O + O_2 \qquad (3)$$

Although little has been published on the effects of superoxide on platelet function, H_2O_2 has been shown to influence the reactivity of platelets.

Endogenous H_2O_2 potentiated aggregation of platelets pre-exposed to ADP [Canoso *et al.*, 1974]. This effect was shown to involve enhanced release of platelet granules [Rodvien *et al.*, 1976]. Exposure of washed platelets to H_2O_2 (1μM) significantly increased $[Ca^{2+}]_i$ [Del Principe *et al.*, 1991]. These studies indicate that H_2O_2 can influence aggregation. However, a recent study has demonstrated the endogenous production of H_2O_2 by platelets [Maresca *et al.*, 1992]. Del Principe *et al.*, [1985] showed that the addition of catalase inhibited collagen-induced platelet aggregation, consolidating that H_2O_2 is involved in platelet aggregation. Subsequently, H_2O_2 was shown to be produced by collagen-, but not thrombin-stimulated platelets [Del Principe *et al.*, 1991], the authors suggested that H_2O_2 was involved in the activation of cyclo-oxygenase.

1.7 LOW DENSITY LIPOPROTEINS AND PLATELET FUNCTION.

1.7.1. Platelets and hypercholesterolaemia.

The original observations suggesting that lipoproteins may influence platelet activity were made by Farbiszewski *et al.*, [1969], who demonstrated PRP had an increased susceptibility to aggregate when challenged with ADP if isolated LDL were added. LDL was also proposed to sensitise platelets *in vivo*, since platelets from type IIa and IIb patients were more sensitive to agonist such as ADP and adrenaline. [Carvalho *et al.*, 1974]. These observations were confirmed by Joist *et al.*, [1979] and Zahavi *et al.*, [1981], who also showed increased release of platelet granules. These effects may have been due to increased plasma concentrations of catecholamines [Smith *et al.*, 1991], which are known to synergise with other platelet agonists. Hassell *et al.*, [1983a] showed a strong relationship between plasma LDL concentrations and platelet sensitivity to ADP and adrenaline. However, major differences were only observed between

individuals of low and average LDL concentrations, no increase in sensitivity occurred with individuals of high LDL concentrations. Although these studies indicate lipoproteins do influence platelet function, the criteria for selection of controls must be addressed carefully as this would have a great bearing on the interpretation of the results.

The mechanism proposed for the effects observed in hyperlipidaemic subjects was that elevated plasma LDL concentrations led directly to an increased cholesterol content in platelet membranes and a subsequent reduction in membrane fluidity [Shattil and Cooper, 1977]. Incubation of platelets with cholesterol-rich liposomes, led to platelets which were more active than controls. Conversely, incubation with cholesterol-poor liposomes produced platelets of low reactivity. An increased membrane cholesterol content may alter the activity of membrane bound enzymes, such as adenylate cyclase and phospholipases. Other studies have failed to show a link between changes in membrane phospholipid/cholesterol ratio and platelet activity in, one, atherosclerosis [Moscat *et al.*, 1986] and two, in patients with severe liver disease [Owen *et al.*, 1981]. This indicates that increased membrane cholesterol may only be important in specific circumstances.

1.7.2. Direct effects of low density lipoproteins on platelets.

Since changes in P/C ratio did not fully explain the observations of increased platelet activity in hyperlipidaemics states, the concept that lipoproteins, particularly LDL, may have direct effects on platelets was proposed. Fujitani *et al.*, [1979] first showed aggregation of gel filtered platelets *in vitro* was increased by the presence of exogenously added LDL. These observations were

subsequently confirmed by others [Aviram and Brook 1983; Hassall *et al.*, 1983b] in both PRP and washed platelet preparations. If LDL was added at pathophysiological concentrations (> 2.5g/l), secondary aggregation of isolated platelets was induced independently of other platelet agonists [Hassell *et al.*, 1983b].

Other lipoproteins such as VLDL and HDL have also been shown to influence platelet function *in vitro.* Aviram and Brook, [1983] showed VLDL to enhance platelet activation while HDL was inhibitory. Hassell *et al.*, [1983b] found VLDL to have no effect with HDL again being inhibitory. Subsequently it was recognised that HDL_2 (apoE containing) invoked inhibitory effects while HDL_3 had little effect [Desai *et al.*, 1989; Higashihara *et al.*, 1991].

Since platelets are anucleate their cholesterol requirements must be fulfilled by the megakaryocyte or provided by LDL. If platelet cholesterol is derived from LDL then this would require a receptor for LDL binding and subsequent endocytosis. Mazurov *et al.*, [1982] first showed the binding of fluorescently labelled LDL to gel filtered platelets. Labelled LDL was displaced by a twofold excess of unlabelled LDL, but required a twenty-fold excess of HDL to have the same effect. A subsequent study showed that LDL binding to washed platelets, was temperature dependent, saturable and displaced by excess LDL and HDL, divalent cation independent and independent of platelet activation state [Curtiss and Plow, 1984]. Homozygous FH subjects were also found to bind LDL, suggesting that the apoB/E receptor was not involved [Shmulewitz *et al.*, 1984]. Koller, [1986] found LDL, HDL_2 and HDL_3 all bound to isolated platelet membranes, binding was Ca^{2+} independent and not mediated by arg or lys

residues of apoB. In a more detailed study, two major lipoprotein binding membrane glycoproteins were isolated and identified as the GPIIb-IIIa complex. LDL and HDL bound to the isolated complex and to the individual subunits. Competition for binding sites on GPIIIa was observed between FGN, LDL and HDL, while FGN also inhibited binding of LDL and HDL to GPIIb without binding itself [Koller *et al.*, 1989]. The proposed binding site for lipoproteins on GPIIb-IIIa was further reinforced by Kowalski *et al.*, [1990], who inhibited binding of VLDL and LDL to the platelet surface by the presence of RGD-containing peptides and Mabs to the GPIIb-IIIa complex. Hassell *et al.*, [1990], demonstrated that LDL binding to platelets was biphasic and saturable binding was to a receptor with a different molecular weight to that of the apoB/E receptor. In all these studies, saturable binding was achieved over time periods ranging from 15min to 3h, methodological differences probably account for these and other differences in results, but the evidence strongly suggested that LDL-receptor coupling occurs at the platelet surface.

1.7.3. Influence of low density lipoproteins on post-receptor events.

LDL induced activation of platelets has been shown to influence post receptor events. Several groups have shown LDL to increase platelet $[Ca^{2+}]_i$ [Andrews *et al.*, 1987a, Dunn *et al.*, 1988, Knorr *et al.*, 1988, Block *et al.*, 1988, Katzman *et al.*, 1991], although to varying degrees. The mobilization of Ca^{2+} is crucial to platelet activation, activating several important enzymes (*see section 1.5.2.4*). The increase in Ca^{2+} was associated with phosphorylation of p47 and PKC activity [Andrews *et al.*, 1987a], increased PI turnover [Block *et al.*, 1988] and increased TXA_2 formation [Knorr *et al.*, 1988]. It is likely that all the latter effects are associated with the effects on Ca^{2+}. In addition, LDL was shown to inhibit the

activity of adenylate cyclase and the resultant increase in cAMP [Bruckdorfer *et al.*, 1984].

1.7.4. Oxidative modification of low density lipoproteins and platelet function.

LDL is thought to become oxidatively modified during the progression of atherosclerosis (*see section 1.3*). Aviram, [1989] showed oxLDL to inhibit collagen-induced platelet aggregation, while acetylated LDL, PLC treated LDL and hepatic lipase treated LDL had no effect. In another study by Ardlie *et al.*, [1989] nLDL enhanced ADP stimulated aggregation, while oxLDL induced aggregation, although preparations of oxLDL had TBARs concentrations much lower than those quoted in other studies. Similarly Meraji *et al.*, [1991] showed that oxLDL, with relatively low concentrations of oxidation products, potentiated and induce aggregation of isolated platelets. Two products of lipid oxidation, lysophosphatidylcholine and 4-HNE are known to inhibit platelet aggregation [Besterman and Gillette, 1971; Selley *et al.*, 1988]. Other oxidised lipids have also been shown to influence platelet function; 13-hydroxydienoic acid inhibited platelet activity [Simon *et al.*, 1990], while 15-HETE potentiated thrombin-induced platelet activation [Setty *et al.*, 1992].

1.8 AIMS.

There were four major aims of the present study:

[1] To investigate how the extent of oxidative modification of LDL influenced the effects of the lipoproteins on platelet function.

[2] To identify which components of LDL or modified LDL were responsible for the activation of platelets.

[3] To investigate whether LDL/modified LDL affected the sensitivity of platelets to NO.

[4] To study the influence of peroxides, including hydrogen peroxide, on platelets as target cells for NO.

2.0 MATERIALS AND METHODS.

2.1 REAGENTS LIST.

Acetylsalicylic acid

Adenosine diphosphate

1, 1, 3, 3-ethoxypropane

Human thrombin

Hydrogen peroxide

Isomethylbutyl xanthine (IBMX)

Manganese oxide

Prostaglandin I_2

The above chemicals were all purchased from Sigma Chemicals Co., U.K.

carboxy-phenyl-4,4,5,5-tetramethylimidazoloine-1-oxyl-3-oxide,(Calbiochem,U.K.)

Collagen (equine) (Hormon Chemie, France)

CHOD-iodide (Merck, U.K.)

Cyclic guanosine monophosphate RIA kit (Life Sciences Ltd, U.K.)

15 (S)-Hydroperoxyeicosatetraenoic acid (Cookson chemicals, U.K.)

Nitric oxide gas (Lyndi gas, U.K.)

Nitrogen gas (British Oxygen Company, U.K.)

S-nitrosoglutathione (Tocris Cookson, U.K.)

Rabbit anti-human anti-fibrinogen antibody (Dako Ltd, Denmark)

IOP62-FITC (The Binding Site, U.K.)

RFAC-4 (Dr. A.H. Goodall, Royal Free Hospital School of Medicine, U.K.)

All other chemicals were obtained from BDH unless otherwise stated in the text, and were of Analar grade or above.

2.2 EQUIPMENT LIST.

Platelet aggregometer (Centratronic Sales Ltd, U.K)

Platelet aggregation cuvettes (Pollution and Process Monitoring., U.K.)

Micro magnetic stir bars (Pollution and Process Monitoring., U.K.)

Flow cytometer (EPICS PROFILE II; Coulter, U.K.)

Ultracentrifuge (XL70; Beckman Ltd, U.K.)

Ultracentrifuge rotor (70Ti; Beckman Ltd, U.K.)

Polycarbonate ultracentrifuge tubes (Beckman Ltd, U.K.)

Centrifuge (Centra-7R refrigerated centrifuge; I.E.C., U.K.)

Thrombocounter (Coulter Elelectronics Ltd, U.K.)

Spectrophotometer (DU70: Beckman Ltd, U.K.)

Scintillation counter (Beckman 5000LS)

Gas sampling tubes (University College London glassblower, U.K.)

Polypropylene sterile syringes (Becton Dickinson Ltd, U.K.)

Butterfly needles (sterile, 19 or 21gauge; Venesytems, U.K.)

Needles (sterile, 21gauge; Venesytems, U.K.)

Rubber septa (Phase separators, U.K.)

Monovette tubes (Starstedt Ltd., U.K.)

Centrifuge tubes (Corning Ltd, U.K.)

Plastic universal collection tubes (B.D.H.)

Gas-tight syringes (Hamilton, U.S.A.)

Agarose gel electrophoresis kit (Universal Gel/8; Ciba Corning Ltd, U.K.)

2.3 BUFFERS AND SOLUTIONS.

2.3.1 Low density lipoprotein preparation.

[1] Acid-citrate-dextrose (pH 6.4): 72.6mM NaCl, 113.8mM glucose, 29.9mM tri-sodium citrate and 2.8mM citric acid.

[2] Density solution (1.063g/ml) (pH 7.4): 68mM NaBr, 0.3mM EDTA, 5mM NaOH and 1μM DTPA.

[3] Density solution (1.151g/ml) (pH 7.4): 260mM NaBr, 0.3mM EDTA , 5mM NaOH and 1μM DTPA.

[4] Tris buffer (for dialysis; pH 7.4): 140mM NaCl, 12mMTris, 1μM DTPA

Both density solutions were prepared with in deoxygenated water

2.3.2 Platelet preparation.

[4] Modified Tyrode's HEPES buffer (pH 7.4): 150mM NaCl, 0.5mM HEPES Na, 0.55mM $NaH_2PO_4.2H_2O$, 7mM $NaHCO_3$, 2.7mM KCl, 0.5mM $MgCl_2$ and 5.6mM glucose.

[5] HEPES buffered saline (pH 7.4): 150mM NaCl, 5mM KCl, 1mM $MgSO_4.7H_2O$ and 10mM HEPES Na.

[6] Formyl saline (0.2%v/v): 5ml formaldehyde (stock solution 40%) in 995ml saline (0.9%w/v).

[7] EDTA-wash buffer (pH 6.5): 36mM citric acid, 10mM EDTA, 5mM KCl, 90mM NaCl and 5mM glucose.

[8] Tyrode's phosphate buffer (pH 7.4): 136.9mM NaCl, 11.9mM $NaHCO_3$, 4.2mM $NaHPO_4.2H_2O$, 2.7mM KCl.

2.3.3 Electrophoresis.

[9] Barbitone buffer (pH 8.4): 100mM NaCl, 2.3mM barbitone, 14.2mM sodium barbitone, 1μM EDTA,

2.4 COLLECTION OF BLOOD SAMPLES.

All blood samples were obtained from healthy volunteers, who denied taking any medication in the previous 14 days. Blood was taken by antecubital venepuncture, with minimal stasis, using a 19 or 21gauge butterfly needle into sterile plastic syringes. The blood was transferred to sterile universal pots containing acid-citrate-dextrose (ACD, ratio 1:4) as an anticoagulant, and gently inverted. All blood samples obtained were handled in this way and centrifuged within 5min of collection, unless otherwise stated.

2.5 LOW-DENSITY LIPOPROTEIN RELATED METHODS.

2.5.1 Isolation of low density lipoproteins.

LDL were isolated from human plasma by discontinuous density ultracentrifugation using a fixed angle rotor, based on the method of Chung *et al.*, [1980]. Freshly citrated whole blood was centrifuged at 2000g for 20min at 4°C to yield plasma. The plasma density was adjusted to 1.3g/ml by addition of solid sodium bromide (NaBr; 31g per 70ml plasma), which was essentially

transition metal ion free; NaBr was dissolved in the plasma by slow stirring. 10ml of density adjusted plasma was underlayed beneath 0.9% sodium chloride (NaCl) solution in 36.5ml non-disposable polycarbonate centrifuge tubes. These were centrifuged at 200000g for 2.5h at 4°C, to yield a bright yellow band of LDL in the middle of the tube *[Figure 2.1]*. The LDL were removed using a syringe and a 19G needle, disregarding the chylomicrons and VLDL at the top of the tube, and subjected to a second centrifugation to remove possible impurities such as HDL and albumin. Here 10ml of LDL was overlayed on 6.5ml of 1.151g/ml density solution (*see section 2.3.1*) and the remainder of the tube topped up with 1.063g/ml density solution (*see section 2.3.1*). The tubes were spun at 200000g for 16h at 4° C, after which LDL floated at the top of the tube.

LDL were concentrated in disposable concentrators (Amicom Centriprep 100), which removed 95% of the salts present. This was followed by dialysis against 5L. of de-oxygenated Tris buffer (pH 7.4;*see section 2.3.2*) for 8h at 4°C, with four complete changes of buffer. Dialysis was performed in an air-tight container to prevent re-oxygenation of the buffer and in the dark to eliminate any possibility of photo-oxidation. Dialysis tubing was boiled in EDTA solution prior to use, to remove any transition metal ions which may have been present. Once prepared, all LDL samples, except aliquots to be oxidised, were supplemented with diethylenetriaminepentaacetic acid (DTPA, 1μM) to prevent any oxidation. In addition all solutions used in the isolation and preparation of LDL were supplemented with DTPA (1μM).

2.5.2 Estimation of low density lipoprotein concentration.

LDL concentration was expressed as protein concentration and determined by

a modification of the Lowry method [Markwell et al., 1978]. Bovine serum albumin (stock 200µg/ml) was used to produce a standard curve and the concentration of the stock solution checked spectrophotometrically immediately prior to each assay. Absorbance was read at 660nm in a DU-70 spectrophotometer and the concentration of LDL protein calculated from the standard curve using straight line regression constants.

2.5.3 Oxidation of isolated low density lipoproteins.

In this study two different methods of oxidation were used to modify LDL.

[1] Fully oxidised LDL (oxLDL).

LDL were exposed $Cu_2SO_4.5H_2O$ (6.4µM/mg protein/ml) under sterile conditions at 37°C for 24h. Oxidation was halted by the addition of excess DTPA (13µM/ mg protein), to chelate the Cu^{2+} ions. OxLDL was then dialysed in 5l deoxygenated Tris buffer for 4h at 4°C, with two complete changes in buffer, to remove the chelated Cu^{2+} ions. After dialysis the oxLDL was supplemented with DTPA (1µM) to prevent further oxidation.

[2] Minimally modified LDL (mmLDL).

LDL were exposed to air under sterile conditions at 37°C for 18 - 24h. The sample was placed in a sterile glass vial and sealed with a rubber septum: this was then pierced with a 19gauge needle attached to a 0.22µm disposable filter to allow gaseous exchange. On completion, the mmLDL was removed and supplemented with DTPA (1µM) to prevent further oxidation.

All LDL preparations were characterised by determination of their content of lipid

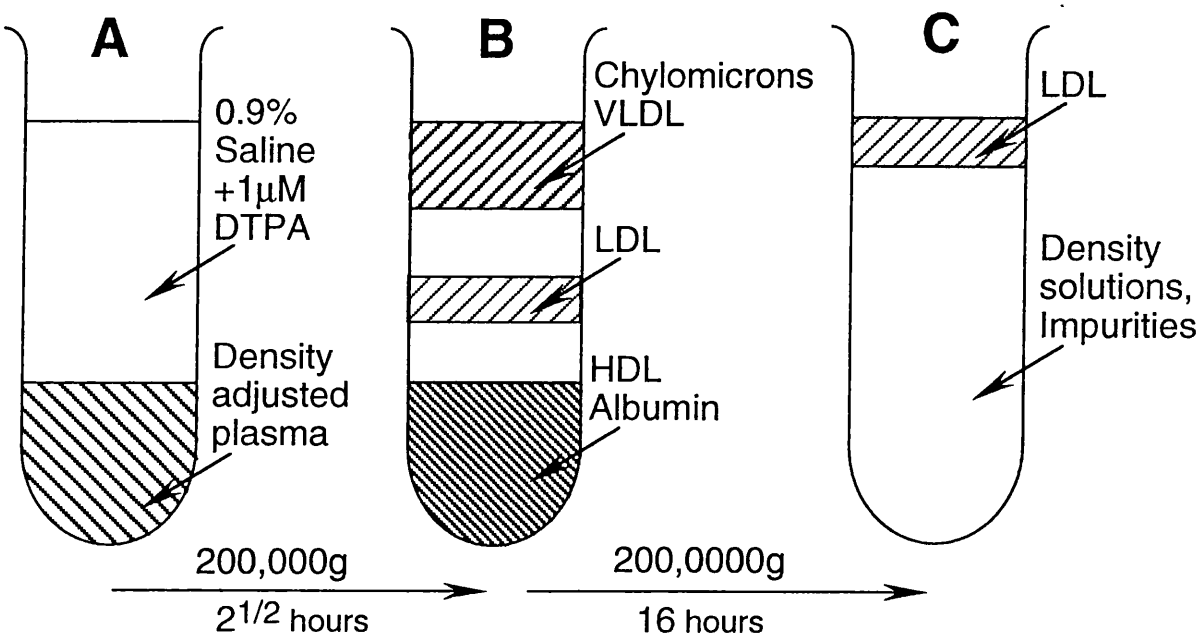


Figure 2.1: Isolation of low density lipoproteins from density adjusted plasma by ultracentrifugation.

LDL were isolated from human plasma by discontinuous density ultracentrifugation of plasma, based on the method of Chung *et al.*, [1980]. Briefly, density adjusted plasma was underlayed beneath NaCl (0.9%) centrifuged to leave LDL in the middle of the tube, as in (1). The LDL is removed and overlayed on 1.151g/ml density solution, while the remainder of the tube is topped up with 1.063g/ml density solution (2). After centrifugation the LDL flots at the top of the tube, as shown in (3).

hydroperoxides (LPO) (*see section 2.5.6*) and the formation of thiobarbituric acid reactive substances (TBARS) (*see* section 2.5.7). Samples were stored in air-tight vials at 4°C and used within one week of preparation. At no point during this study were individual LDL preparations pooled.

2.5.4 Preparation of 15(S) Hydroperoxyeicosatetraenoic acid (HpETE) and 15(S) Hydroperoxyeicosatetraenoic acid (HpETE)-LDL.

15(S)-HpETE was supplied in ethanol, therefore its effect on platelets could not be tested directly, because of the inhibitory effect of alcohols on platelet activity. Consequently, the lipid hydroperoxide was dissolved in physiological buffer before addition to platelets, as follows. The ethanolic 15 (S)-HpETE was placed in a darkened glass vial and the ethanol evaporated under nitrogen. The subsequent residue was dissolved in 0.01M NaOH and then diluted to the appropriate concentration in deoxygenated phosphate buffered saline. The whole procedure was performed in a chamber continually flushed with nitrogen. Once prepared all 15 (S)-HpETE solutions were kept on ice until added to the platelets. Within each experiment testing the effects of 15(S)-HpETE on platelet aggregation an internal control (blank) was used to account for the effects of any residual ethanol. An equal volume of ethanol to that which the 15 (S)-HpETE was resuspended from, was subjected to the same procedure as that of authentic 15 (S)- HpETE. This 15 (S)-HpETE blank was added directly to the platelets, and any effects on aggregation noted.

In some experiments nLDL were used as carrier particles for the fatty acid hydroperoxide. Here, ethanolic 15(S)-HpETE was placed in a 1ml plastic microfuge tube (Eppendorf) and the ethanol was evaporated to dryness under

nitrogen. 0.5ml of nLDL (5 mg protein/ml) was added to the tube and incubated for 60min at 4°C to allow for uptake of the fatty acid hydroperoxide by LDL. After incubation, the 15(S)-HpETE-LDL was dialysed against deoxygenated Tris buffer for 30min at 4°C to remove any residual ethanol. The total amount of LPO was measured spectrophotometrically (*see section 2.5.6*).

2.5.5 Identification of oxidised low density lipoproteins by agarose gel electrophoresis.

During the oxidation of LDL, the relative negative charge of the particles increase due to modification of $apoB_{100}$ [Morel *et al.*,1984]. Agarose gel electrophoresis, based on the method of Blix, [1941], was used to identify different forms of $apoB_{100}$ according to their relative net charge.

Agarose gels were peeled away from backing plate and the wells filled with approximately 1µl of sample or control. The gel was then loaded into the electrophoresis cassette and placed in a tank containing barbitone buffer (pH 8.6). The electrophoresis was run for 35min at 100V and 4mA. Under these conditions proteins migrated towards the anode. On completion the gel was oven dried at 55°C for 15 to 20min, cooled to room temperature and stained for 1min with Fat Red 7B. The gel was destained in methanol/water solution (ratio 2:1) and oven dried for 15 to 20min.

2.5.6 Assay for Lipid hydroperoxides (LPO) content of low density lipoproteins.

The method used for measurement of total lipid hydroperoxides in LDL was based on an assay developed by El-Saadani *et al.*, [1989]. The assay utilises

the colour reagent of a commercially available test kit for cholesterol measurement (CHOD-iodide). The test proceeds by the oxidation of iodide (I^-), in the form of potassium iodide, to iodine (I_2) by lipid hydroperoxides. This can be measured spectrophotometrically at 365nm. The colour reagent contained detergents, these provided suitable conditions for its interaction with lipid hydroperoxides, which remain associated with the lipid phase of the LDL. Values were calculated from the absorbance values at 365nm by the Beer-Lambert law (see below), using the molar extinction coefficient ($\epsilon = 2.46 \times 10^4\ M^{-1}\ cm^{-1}$) and expressed as nmol/mg $apoB_{100}$.

$$A = \frac{\epsilon \times L}{C}$$

Where A is absorbance, ϵ is the extinction coefficient for peroxides, L is the path length and C is the concentration. Results were expressed as nmol LPO /mg protein .

2.5.7 Assay for Thiobarbituric acid reactive substances (TBARs) following LDL oxidation.

The TBARs assay provided an index of reactive aldehydes produced from the breakdown of lipid hydroperoxides. The assay is widely cited, but limited to measurement of malondialdehyde (MDA). MDA is a breakdown product of arachidonic acid and is produced in relatively constant proportions to the peroxidation process [Esterbauer *et al.*,1990]. It is highly hydrophilic, which results in its release from the lipid phase of the LDL particle. In contrast, the other aldehydes produced are highly lipophilic and thus remain associated with

the LDL [Esterbauer *et al.*, 1990]. As MDA is formed only from arachidonic acid and therefore the molar concentrations found in various forms of LDL are less than those for LPO.

Trichloroacetic acid was used to precipitate the LDL leaving the soluble MDA in the aqueous phase to react with the thiobarbituric acid, producing a pink colouration measured at 532nm. Values were calculated from a standard curve of MDA, freshly prepared by the acid hydrolysis of 1, 1, 3, 3 - tetraethoxypropane [Beuge and Aust, 1978]. Results were expressed as nmol MDA equivalents/mg $apoB_{100}$.

2.6 METHODS FOR THE STUDY OF PLATELETS.

2.6.1 Preparation of platelet rich plasma.

Whole blood, taken into ACD, was centrifuged (Centra-7R refrigerated centrifuge, IEC) at 120g for 18min at 20°C to produce platelet rich plasma (PRP). PRP was removed by sterile Pasteur pipettes, leaving at least 0.5cm of PRP near to the buffy coat to limit any erythrocyte and neutrophil contamination, before being transferred to sterile plastic centrifuge tubes. Autologous platelet poor plasma was prepared by recentrifuging the PRP 800g for 10 min.

The pH of the PRP was adjusted with Tyrodes HEPES buffer prior to experimentation. All experiments were completed within 2h of isolation.

2.6.2 Preparation of washed platelets.

Platelets isolated from freshly taken whole citrated blood, was based on the method of Vargas *et al.*, [1983]. For preparation of washed platelets (WP), PGI_2 (final concentration 50nM; 1µl of 1mg/ml stock per 5ml PRP) was added directly to the PRP and the tube immediately inverted. The PRP was recentrifuged at

800g for 10min at 25°C, to leave a platelet pellet. The supernatant, platelet poor plasma (PPP), was discarded and the inside of the tubes wiped clean with tissue to remove any traces of plasma. Platelets were then carefully resuspended in Tyrodes HEPES buffer (*see section 2.3.2*). WP were counted using a thrombocounter, and diluted with Tyrodes HEPES buffer (*see section 2.3.2*) to give a final concentration of 3 x 10^8 platelets/ml (approximate plasma density). Upon resuspension of the WP they were checked for the characteristic 'swirling', which indicated the platelets had been resuspended successfully. Platelets were left for 1h to recover their sensitivity to agonists and used within 3h of venepuncture. The WP preparations were supplemented with $CaCl_2$ (1mM) and fibrinogen (0.3mg/ml) prior to experimentation.

PGI_2 transiently increased the concentration of cAMP and was used to inhibit platelet activation during the isolation procedure. Isolated platelets were left for 1h to recover their responsiveness, as the cAMP is broken down, by endogenous phosphodiesterases. This method of platelet preparation has been shown to maintain platelet functional viability to a greater extent than methods using changes in platelet pH [Brackwell *et al.*, 1982].

2.6.3 Alternative methods for platelet preparations.

For some experiments alternative platelet preparations were used:-

[1] The Tyrode's HEPES buffer was substituted by a modified Tyrode's phosphate buffer (*see section 2.3.2*).

[2] After resuspension the platelets were incubated with H_2O_2 (10μM) for 5min before being subjected to a second centrifugation at 800g for 5min at 25°C. The

platelets were then resuspended in Tyrode's HEPES buffer (*see section 2.3.2*).

[3] A second washed platelet preparation was used, based on the method of Lagarde *et al.*, [1980]. Whole blood was taken into universal tubes containing 3.8% tri-sodium citrate (ratio 1:9), inverted and centrifuged at 130g for 15min at 20°C to produce PRP. The pH of PRP was then adjusted to 6.4 by the addition of 0.3M citric acid and the platelets recentrifuged at 750g for 10min at 20°C. The supernatant was discarded and the platelet pellet resuspended in EDTA-wash buffer (*see section 2.3.2*). The platelets were then centrifuged again under the same conditions and the new supernatant was discarded, the platelet pellet resuspended in Tyrode's HEPES buffer (*see section 2.3.2*). For aggregation studies, WP were supplemented with $CaCl_2$ (1mM) and used immediately.

[4] For flow cytometric studies, blood was taken via a 21gauge butterfly needle into Monovette tubes containing 3.8% tri-sodium citrate and centrifuged at 120g for 18min at 20°C to produce PRP.

2.6.4 Acetylsalicylic acid treated platelets.

In some experiments thromboxane A_2 (TXA_2) formation was inhibited by acetylsalicylic acid (ASA), which acetylated and therefore inhibited the active site of cyclo-oxygenase enzyme. WP were incubated with ASA solution (100µM final concentration) for 30min at 37°C before use.

2.6.5 Platelet aggregation in vitro.

Platelet aggregation was assessed turbidometrically using a dual channel aggregation module, fitted with a dual pen chart recorder, based on the method

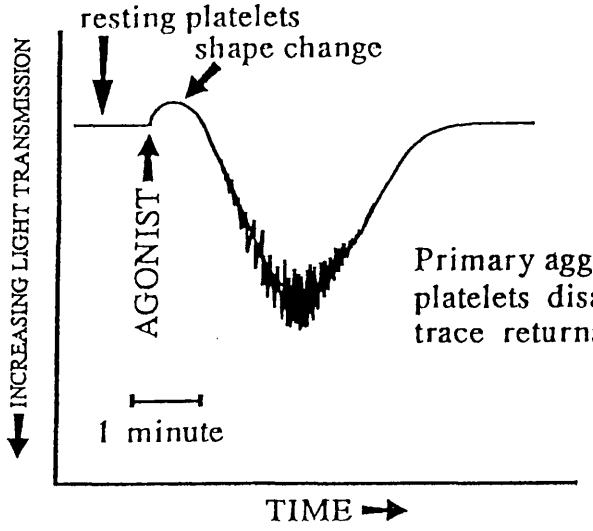
of Born, [1969]. The technique measures increases in light transmission of a stirred platelet suspension. Suspension of platelets have high light scattering properties. As platelet aggregates of increasing size are formed (induced by the addition of physiological agonists), the amount of light transmitted by the suspension increases and is detected by a photocell. Upon stimulation by agonists a slight decrease in light transmission occurs, resulting from a reversible shape change (*see section1.5.3*). This may or may not be followed by aggregation depending on the strength of the stimulus. Aggregation may be one of two types: `primary aggregation', which is reversible and does not involve release of granule contents *[Figure 2.2a]*, and `secondary aggregation' which is irreversible and is accompanied by granular secretion *[Figure 2.2c]*. A transitory phase between primary and secondary aggregation also exists. This is usually the minimum dose of agonist required to induced secondary aggregation and is termed the threshold dose*[Figure 2.2b]*.

Aggregation was measured as percentage increase in light transmission through a stimulated platelet preparation, relative to the light transmission through a resting platelet preparation. The platelet aggregometer was calibrated with an WP suspension or PRP to give the baseline reading or 0% light transmission (aggregation). While Tyrode's-HEPES buffer or autologous PPP were used to calibrate for the maximum reading or 100% light transmittance (aggregation), as these would represent an aggregated sample. Aggregation tests were performed in duplicate, using 100µl of WP or PRP in flat bottomed glass cuvettes kept at 37°C with continual stirring (800rpm) by micro magnets. The cuvettes and micro magnets had previously been soaked in dimethyldichlorosilane (BDH) and dried to create an inert surface. Platelets were stirred for 1min prior to addition of the

Figure 2.2: Diagrmmatic representation of the different types of aggregation traces.

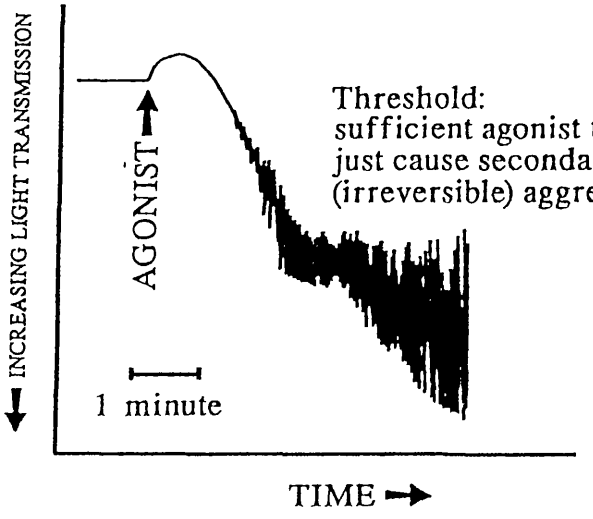
(a) Primary of reversible aggregation.
(b) Threshold response.
(c) Secondary of irreversible aggregation.

(A)



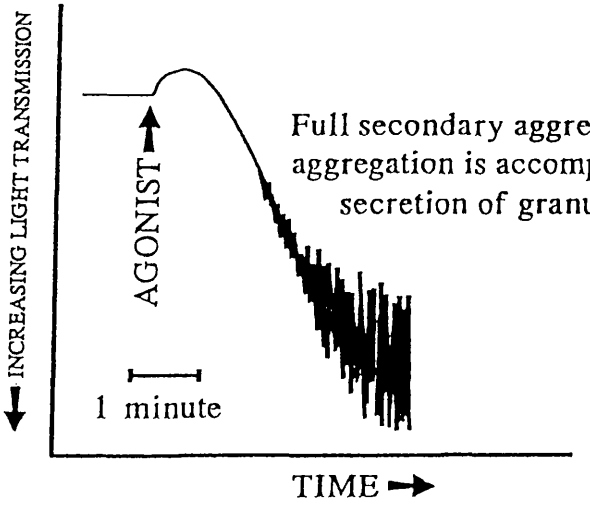
Primary aggregation:
platelets disaggregate &
trace returns to baseline

(B)



Threshold:
sufficient agonist to
just cause secondary
(irreversible) aggregation

(C)



Full secondary aggregation:
aggregation is accompanied by
secretion of granules

agonist to allow for temperature equilibration and to observe any spontaneous aggregation.

The agonists used in this study were (final concentrations):-

[1] ADP 0.1 - 10μM

[2] thrombin 0.005 - 0.1U/ml

[3] collagen 0.1 - 1.0μg/ml

Agonists were added from stock solutions in volumes no greater than 5μl and the aggregation observed for 3min after the last addition.

2.6.6 Flow cytometry.

2.6.6.1 Principles.

Flow cytometry is a technique which allows analysis of single cells. In combination with immunolabelling of antigenic epitopes by monoclonal antibodies (mabs) the method provides a specific, highly sensitive diagnostic tool [Tschope *et al.*, 1992].

Target cells, once introduced to the system, are hydrodynamically focused into single cell flow by a surrounding sheath fluid. Cells challenged by the laser beam produce a forward light scatter, the extent of which is determined by cell size and a right-angle light scatter which is determined by cell granularity. These two parameters may be used to characterise different cell populations, by means of a two-dimensional plot (bitmap). If fluorescently labelled Mabs to a particular epitope are added to the system, bound Mabs after excitation by the laser beam will emit light of a defined wavelength. This is recorded in a logarithmic intensity

scale to give both intensity and distribution of fluorescence and thus to quantify the expression of the epitope.

2.6.6.2 Application and method.

Flow cytometry combined with immunolabelling of antigenic epitopes by mabs allows the elucidation of changes in platelet surface characteristics [Abrams and Shattil, 1991]. The exposure of binding domains of GPIIb-IIIa on the platelet surface is known to be a post-activatory event. Consequently, the extent of fibrinogen binding to the platelet surface can be used as a relative index of platelet activation. The addition of fluorescein isothiocyanate (FITC) conjugated rabbit anti-human fibrinogen antibody (Rαfgn-FITC) to PRP allows the binding of fibrinogen to be quantified [Warkentin *et al.*, 1990]. In the current study this allowed comparison of the effect of LDL on early platelet activation (fibrinogen binding) and the full functional response of aggregation. The same principle can be applied to platelet degranulation. This process results in the expression of extrusion markers of post-degranulated platelet surface. Fluorescently labelled Mabs to P-selectin, IOP62-FITC, (The Binding Site) and CD63, RFAC-4, [Cox and Goodall, 1991] bind to granule specific glycoproteins. This allowed a specific and detailed quantification of platelet granule release. The technique provides a finite value for the level of degranulation within set platelet population, in this case 5000 cells.

PRP (5µl) was incubated with Rαfgn-FITC (5µl: diluted 1:1 with HEPES buffer), HEPES buffer (45µl), ADP(5µl) and in the presence or absence of LDL (5µl) for 20min. ADP was always added last as the starting point of the experiment. Each tube was mixed gently by hand and incubated for 20min before being fixed with

10-fold excess of 0.2% formyl saline (500µl) [Warkentin *et al.*, 1990]. The flow cytometer (Coulter EPICS PROFILE II) analysed 5000 platelets from each tube and produced two quantitative results, % fluorescence bound to each platelet and mean fluorescence intensity.

2.6.6.3 Methodological considerations of flow cytometric analysis.

Incubation of WP with Rαfgn-FITC in the absence of agonists indicated the platelets were activated slightly during preparation. The specificity of the technique showed that approximately 20% of WP had fibrinogen bound to their surface. This increase in fibrinogen binding occurred during the second centrifugation of the isolation procedure and subsequent manipulation, since incubation of PRP with Rαfgn-FITC produced only 4% of platelets with bound fibrinogen. Consequently, it was decided that the use of WP would not give a true representation of any effects of LDL on platelet fibrinogen binding and experiments using WP were not pursued.

In addition, the flow cytometric experiments are performed in a non stirred system, which is an important difference between this technique and nephelometry. The lack of turbulence allows the study of individual epitopes on the platelet surface without inducing the full functional response.

2.7 PREPARATION, USE AND MEASUREMENT OF NITRIC OXIDE SOLUTIONS.

2.7.1 Nitric Oxide (NO) preparation.

NO was prepared as a solution from NO gas in double distilled water (ddw) according to the method of Palmer *et al.*, [1987]. Ddw was boiled for 10min and

allowed to cool to 60°C, before being pulled under vacuum into a specially adapted 100ml glass sampling tube (prepared by a glassblower at University College, London). One end of the tube was sealed with a teflon-rubber septum and ddw was subsequently de-oxygenated by bubbling with N_2 gas for 45min. The open end of the tube was then carefully sealed with a septum to prevent reoxygenation. The appropriate amount of NO gas was then injected through the septum using a gas-tight syringe. At high concentrations of NO, if the water had not been fully de-oxygenated, the solution had a slight brownish tinge due to formation of NO_2. However, this was undiscernible with low concentrations of NO solutions, the bio-activity of which could not be assessed until tested with platelets. All glassware used in the preparation of NO solutions had been washed in nitric acid, thoroughly rinsed in ddw and dried prior to their use. NO solutions were kept on ice throughout the experiments. Concentrations of NO solutions were calculated on the basis of Avogadro's law: one mole of any gas at S.T.P. occupies approximately 22400cm^3. In some cases the concentrations of NO solutions were tested using and NO electrode or the Griess reaction (*see section 2.7.2 and appendix 1*).

2.7.2 Griess reaction: measurement of nitrite concentrations.

The Griess reaction allows the estimation of the nitrite content of a given solution, unfortunately the resolution of the assay is only 1μM. The reaction mixture consists of the sample, HCl and sulphanilic acid, to which a nitrite specific dye was added (1-(naphthyl) ethylenediamine), producing a purple colouration measured at 548nM. Values were calculated from a standard curve prepared from $NaNO_2$.

2.7.3 Preparation of s-nitosoglutathione solutions.

S-NOG solutions were prepared in deoxygenated water, with fresh stock solutions used in each individual experiment.

2.7.4 Hydrogen peroxide solutions.

H_2O_2 (stock solution 8.82M) solutions were diluted in deoxygenated water, to prolong the half life, and stored in air tight vials for the duration of the experiments. Inactive H_2O_2 solutions were prepared by leaving the solutions stirring overnight while exposed to air, or by heat treatment at 100°C for 30min.

2.7.5 Premixing of Nitric Oxide and Hydrogen peroxide.

NO or NO and H_2O_2 added simultaneously (10µl respectively), were delivered as a bolus into a platelet aggregation cuvette containing stirred Tyrode's HEPES buffer (37°C, 800rpm). The cuvettes were left stirring for the required period of time before an aliquot was removed, no more than 5µl, and delivered into a separate cuvette containing stirred WP (100µl). The concentrations of the pre-mixed NO and H_2O_2 stock solutions were 20-fold greater to allow for the extra dilution.

2.8 PREPARATION OF PEROXYNITRITE.

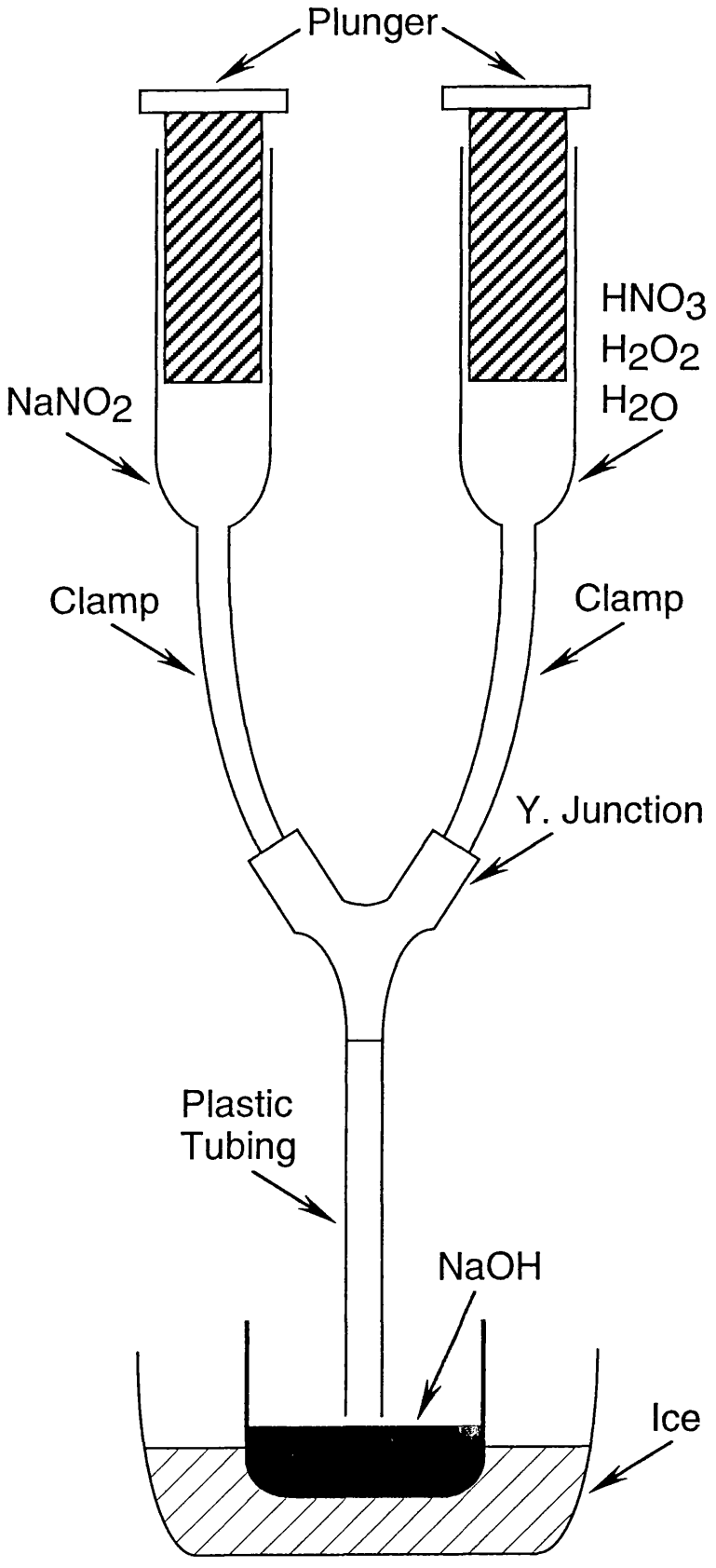
Peroxynitrite is a potent oxidant highly unstable at physiological pH, formed *in vivo* through a reaction between NO and $O_2^{\cdot-}$ [Beckman *et al.*, 1990]. Experimentally this can be achieved by a reaction involving acidified nitrite and hydrogen peroxide (reaction 1 and 2).

$$3HNO_2 \rightleftharpoons HNO_3 + 2NO + H_20 \qquad (1)$$

$$HNO_2 + H_2O_2 \rightarrow HOONO + H_2O \qquad (2)$$

$NaNO_2$ (10ml) was placed in a glass syringe, while another was filled with HNO_3 (1M, 6.3ml), H_2O_2 (8.8M, 0.85ml) and H_2O (3.15ml). The syringes were connected via sterile plastic tubing and a Y-junction. From the bottom of the Y-junction more tubing was positioned directly over a beaker of NaOH (1.2M, 10ml). The tubing in close proximity to each syringe was clamped to prevent any premature mixing of the reactants *[Figure 2.3]*. At the appropriate time both clamps were released and even pressure applied to each syringe plunger. The reactants then mixed in the tubing and were passed directly into the NaOH (1.2M). By passing the mixture into immediately into a highly alkaline environment the peroxynitrite could be preserved in its anionic form ($ONOO^-$). Excess H_2O_2 was removed by passing the peroxynitrite through a small column of manganese oxide, until all effervescence had ceased. The concentration of peroxynitrite was calculated from its absorbance value at 302nm [*Figure 2.4]* and molar extinction coefficient ($\epsilon = 1.67 \times 10^3$ M-1 cm-1) according to the Beer-Lambert law (*see section 2.5.6*). All reactants and the glass syringes were kept on ice for 30min prior to use.

Peroxynitrite controls were prepared by following the identical procedure except the mixture was passed into H_2O rather than NaOH, leading to spontaneous decomposition (reaction 1). NaOH was added to the solution later to give the correct pH.


Plunger
HNO_3
H_2O_2
H_2O
$NaNO_2$
Clamp
Clamp
Y. Junction
Plastic
Tubing
NaOH
Ice

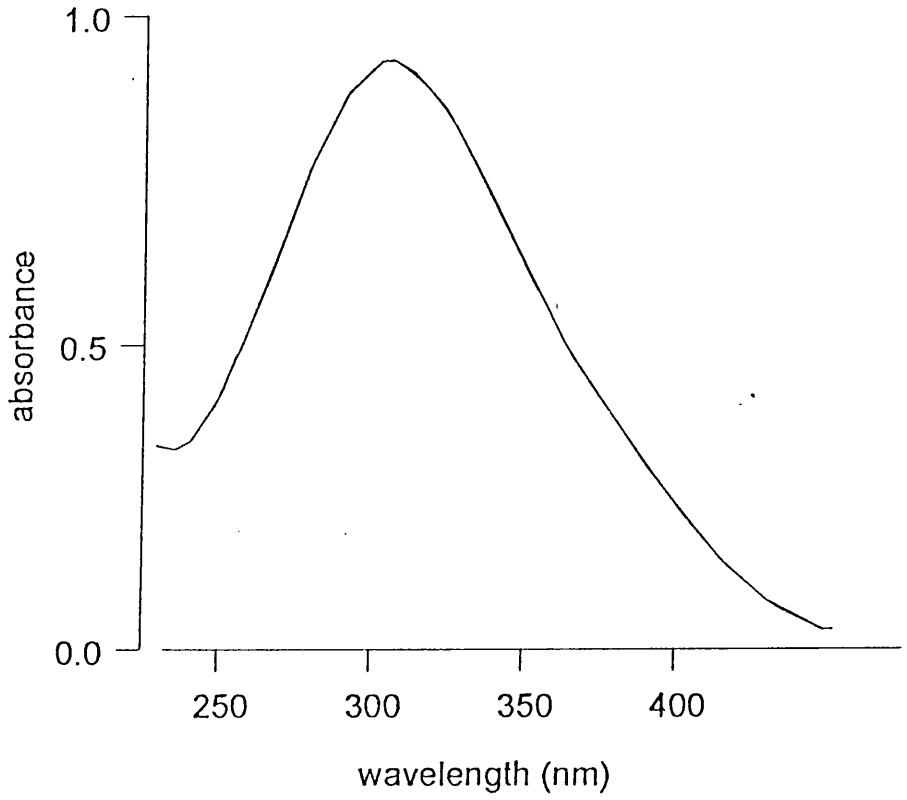


Figure 2.4: Scan of freshly prepared peroxynitrite solution.

Authentic peroxynitrite was prepared from $NaNO_2$, HNO_3, H_2O_2 and H_2O as described in section 2.8. A known volume of the solution was added to 1ml of NaOH and the mixture scanned from 250 - 400nm. The spectrophotometer was blanked on decomposed peroxynitrite.

2.8.1 Effects of peroxynitrite on the buffering capacity of Tyrode's HEPES buffer.

After dilution of peroxynitrite in water the solution was still highly alkaline in nature, therefore, the buffering capacity of Tyrodes HEPES buffer (*see section 2.3.2*) against peroxynitrite was tested. The peroxynitrite was diluted immediately before use and aliquots of known concentrations added to the stirred buffer while the pH was monitored.

2.9 RADIOIMMUNOASSAY OF 3', 5' CYCLIC GUANOSINE MONOPHOSPHATE.

cGMP levels of WP were measured by a commercially available radioimmunoassay kit (Amersham, UK). The assay was based on competition between unlabelled cGMP and a fixed quantity of ^{125}I cGMP for a limited number of binding sites on a cGMP specific antibody. This is reacted with a second antibody, which is attached to magnetisable particles, and the samples centrifuged. Supernatants were discarded to remove any unbound cGMP and the radioactivity of the pellets read in a scintillation counter. Using fixed quantities of antibody and ^{125}IcGMP, the amount of ^{125}I cGMP bound to the antibody was inversely proportional to the concentration of unlabelled cGMP.

IBMX (100µM final conc.), a phosphodiesterase inhibitor, was added to the platelets 30min before the experiment. NO, H_2O_2 or NO/H_2O_2 were added to the WP in the platelet aggregometer and left for 1min at 37°C with continual stirring. This was halted by the addition of cold perchloric acid (100µl, 0.5M), after which the WP were vortexed and stored at -70°C. On the day of the assay samples were thawed and the acid neutralised by addition of K_2HPO_4 (200µl; 1M).

Samples were vortexed and a slight precipitation of potassium perchlorate was allowed to settle before 20µL aliquots were removed and used in the assay. The results indicate the rate of formation of cGMP in the platelets.

Statistical analysis.

Data were expressed as mean ± S.E.M. and analysed by Student's t test, while the number of each experiment performed is shown on each figure legend..

CHAPTER THREE: THE INFLUENCE OF LOW DENSITY LIPOPROTEIN OXIDATION ON PLATELET FUNCTION.

3.1 INTRODUCTION.

The influence of LDL on platelet responsiveness is now well documented, although the mechanism by which this influence is exerted remains unresolved. Farbiszewski *et al.*, [1969] first showed PRP to have an increased potential to aggregate when stimulated with ADP in the presence of isolated LDL. This finding was complemented by Carvalho *et al.*, [1974], who demonstrated that PRP from type IIa hyperlipidaemic patients (increased plasma LDL and VLDL concentrations) possessed an increased susceptibility to aggregate when challenged with ADP or adrenalin, in comparison to PRP from non-hyperlipidaemic patients. These observations were confirmed Joist *et al.*, [1979] and Zahavi *et al.*, [1981], but not by Corash *et al.*, [1981]. Indirect evidence for increased platelet activity was also provided by Tremoli *et al.*, [1984] who found that patients with type IIa hyperlipidaemia possessed increased circulating concentrations of MDA and TXB_2, markers for lipid peroxidation and platelet activation respectively.

It is possible that in earlier studies the LDL used may have been partially oxidised, since the potential importance of oxidised LDL were not fully appreciated. In the work described by Ardlie *et al.*, [1989], where oxidised LDL activated platelets, the LDL were only moderately oxidised, which indicated that the level of oxidation may be an important factor in how LDL influence platelet activity. Therefore it was important to define the oxidation state of LDL which activated platelet most effectively. A detailed study of the extent to which oxidative modification of LDL influenced their effects on platelet responsiveness

was undertaken. LDL were prepared with great care to prevent any oxidation throughout the isolation procedure. The effects of nLDL on platelet function were then compared directly to those of mmLDL and oxLDL. mmLDL probably most closely represents a physiologically relevant modified LDL in plasma and for the most part in atherosclerotic plaques.

Two different platelet models were used in this study, WP allowed the assessment of direct interactions between LDL and platelets, while PRP, represented a model closer to the whole blood milieu where LDL-platelet interactions could be studied in the presence of other plasma factors normally present *in vivo*, and which both LDL and platelets would encounter under physiological conditions. Platelet function was assessed by three parameters.

[1] Aggregation, as assessed by nephelometry.

[2] Early activation, assessed by the binding of fluorescently labelled fibrinogen.

[3] Late activation (degranulation), as measured by the expression of P-selectin and CD63.

These three parameters allowed a more detailed and complete investigation of platelet function compared to previous studies. Nephelometry allowed assessment of the influence of LDL on platelets normal haemostatic function, while FGN binding was used to assess the activation process. The measurement of platelet granule extrusion markers, rather than measurement of granule contents, provided a more accurate method for quantifying

degranulation.

3.1.1 Summary of aims.

[1] To investigate how the extent of LDL modification affected their influence on platelet function.

[2] To identify the form of LDL which was most effective when activating platelets.

[3] If possible to identify the component(s) of the particles which may be responsible for their activatory effects.

3.2 RESULTS.

3.2.1 The identification of the extent of LDL oxidation by measurement of oxidation products.

Isolated nLDL possessed low concentrations of both LPO and TBARs [*Table 3.1*], despite the presence of metal ion chelators throughout the isolation procedure. When LDL were subjected to the oxidation conditions, a range of concentration of oxidation products were produced, despite identical oxidation times. This highlighted the heterogenous composition of the particles and demonstrated a variation in the rates of oxidation of individual samples. mmLDL possesses a two-fold increase in LPO, a more modest increase in TBARs relative to nLDL *[Table 3.1]* and in addition retained their yellow coloration. A small number mmLDL samples were prepared by short exposure to Cu^{2+} with similar concentrations of LPO and TBARs obtained. However, the use of Cu^{2+} was not ideal. In the presence of Cu^{2+}, the differences in rates of oxidation became more pronounced, and many samples became over oxidised. Longer term exposure of LDL to Cu^{2+} produced oxLDL, these lipoproteins possessed much greater concentrations of LPO and TBARs *[Table 3.1]*. The range of LPO concentrations with oxLDL was very large, emphasizing variations in oxidation kinetics of the individual samples. The formation of LPO during the oxidation of LDL is biphasic, increasing to a maximum before declining rapidly [Esterbauer *et al.*,1990]. Higher LPO values represented LDL with a slower oxidation rate and vice versa. Since higher LPO concentrations are indicative of oxidation halted near the peak of LPO production, while the lower values are found when LPO are converted to TBARs. OxLDL lost the yellow coloration of β-carotene, becoming white in colour and appeared to be extensively aggregated. The concentrations of oxidation products found in the different LDL preparations are

LDL preparations	n	lipid peroxides nmol/mg apoB	TBARs nmol/mg apoB
nLDL	11	10.2 - 25.0 (13.7 ± 1.3)	0.2 - 0.9 (0.5 ± 0.1)
mmLDL	23	18.9 - 56.7 (39.2 ± 2.1)	0.7 - 2.7 (1.3 ± 0.1)
oxLDL	7	100 - 369 (243 ± 33)	3.7 - 16.3 (11.4 ± 1.7)

Table 3.1: Measurement of lipoprotein oxidation in the different preparation of low density lipoproteins.

Native low density lipoproteins were oxidised by either exposure to air (mmLDL) or Cu^{2+} (oxLDL). The extent of oxidation in all the lipoprotein preparations was characterised by the concentrations of lipid peroxides (LPO) and thiobarbituric acid substances (TBARs). The results are expressed as nmol/mg LDL protein and represent the range of values obtained. Figures in brackets are the mean ± SEM and n = the number of independent experiments on individual LDL preparations.

summarised in *Table 3.1*.

3.2.2. Agarose gel electrophoresis (AGE).

A clear distinction between oxLDL and nLDL could be observed using AGE *[Figure 3.1].* OxLDL showed an increased migration towards the anode, by comparison mmLDL showed an identical electrophoretic mobility to nLDL. This apparent increased negativity of the oxLDL particles suggested modification of the positive amino acid residues of $apoB_{100}$, and also indicated that with mmLDL lipid peroxidation had occurred without significant modification of $apoB_{100}$ *[Figure 3.1]*.

3.2.3. Concentrations of endogenous LDL and HDL present in platelet rich plasma.

Experiments performed with PRP contained endogenous lipoproteins, and therefore it was important to measure their initial concentrations. The LDL and HDL cholesterol levels of all donors were within the normal ranges *[Table 3.2]*. Only in experiments where nLDL was added to PRP was the total concentration of LDL calculated, as mmLDL and oxLDL must be considered as separate species.

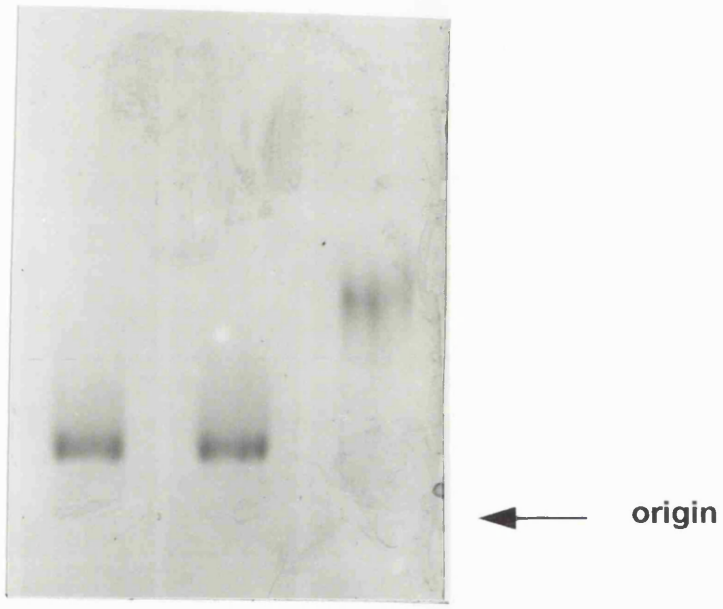


Figure 3.1: Agarose gel electrophoresis of low density lipoproteins following oxidation.

Native low density lipoproteins were isolated from fresh human plasma (see section 2.5.1) and were oxidised by exposure to air under sterile conditions for 18 - 24h (mmLDL) or by exposure to Cu^{2+} for 24h (oxLDL). The samples (1µl) were then applied to an agarose gel and electrophoresed at pH 8.6 in barbitone buffer for 35 min (100V and 4mA) and the gels stained with Fat Red B and destained in methanol/water solution (ratio2:1) and oven dried for 15 - 20min. The gel is typical of those in 4 independent experiments

Experiment	n	LDL	HDL
		(mmol cholesterol/l.)	
nLDL - aggregation	3	2.0 ± 0.7	1.6 ± 0.6
mmLDL - aggregation	6	2.1 ± 0.7	1.7 ± 0.2
mmLDL - flow cytometry	6	2.5 ± 0.5	1.7 ± 0.2
oxLDL - aggregation	3	2.4 ± 0.7	1.7 ± 0.1
oxLDL - flow cytometry	3	2.8 ± 0.1	1.4 ± 0.1

Table 3.2: Concentrations of LDL and HDL cholesterol in the donors used to prepare platelet rich plasma.

Samples of platelet poor plasma were taken for analysis of LDL and HDL by convential methods using the Friedewald formula to determine the concentration of LDL indirectly

3.3. NATIVE LOW DENSITY LIPOPROTEINS AND PLATELET FUNCTION.

3.3.1 The effects of native low density lipoproteins on the aggregation of washed platelets.

The action of nLDL on platelet aggregation was assessed by investigating four possible outcomes:

[1] Aggregation in the absence of known platelet agonists.

[2] Potentiation of aggregation stimulated by sub-maximal concentrations of platelet agonists.

[3] Inhibition of agonist-induced platelet aggregation.

[4] No activation or inhibition.

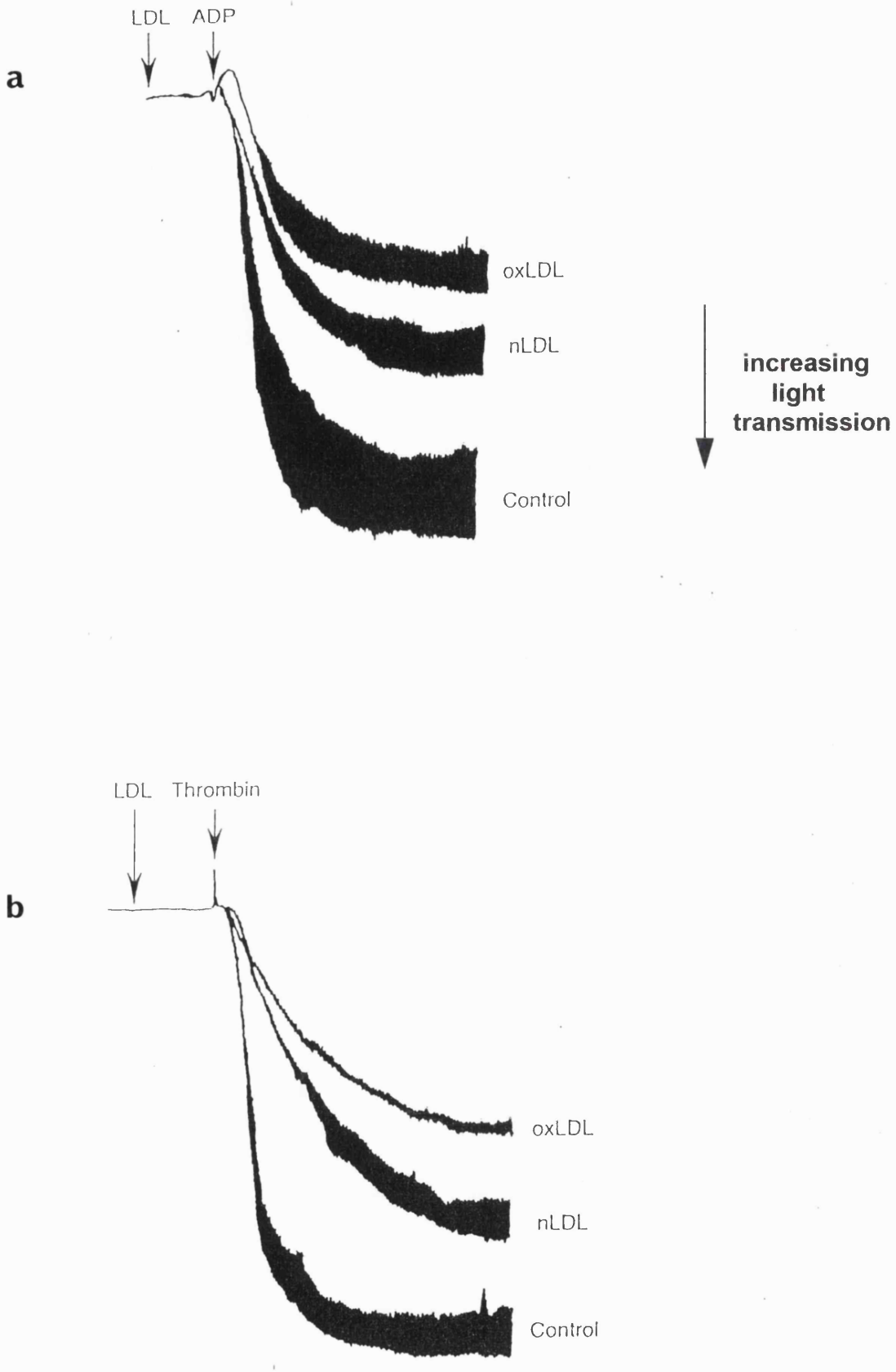
nLDL (0 - 1mg protein/ml) added directly to WP and incubated for 3min did not induce platelet aggregation. nLDL incubated with WP for 1min prior to the addition of sub-maximal doses of ADP (1 - 2μM) did not enhance aggregation, but caused a slight reduction of the normal response. Similar results were obtained when sub-maximal thrombin (0.005U/ml) was used to stimulate WP.

Stimulation of WP with ADP (10μM) or thrombin (0.1U/ml) induced normal secondary aggregation, indicating that the platelets had retained their functional viability after isolation *[Figure 3.2a, b]*. If WP were incubated with nLDL prior to addition of ADP (10μM), a significant reduction in the normal ADP-induced aggregation response was observed *[Figure 3.2a]*. The effect was concentration-

Figure 3.2: Typical aggregation traces demonstrating the effects native and oxidised low density lipoproteins on platelet aggregation.

(a) WP were activated by the addition of ADP (10μM) in the presence or absence of nLDL or oxLDL (1mg/ml). The lipoproteins were incubated with WP for 1min prior to the addition of ADP, and aggregation measured 3min later.

(b) as for a, except thrombin was used to stimulate the WP.


a
LDL
ADP
oxLDL
nLDL
Control
increasing
light
transmission
b
LDL
Thrombin
oxLDL
nLDL
Control

dependent for nLDL, with all concentrations of nLDL causing inhibition. Maximal inhibition of platelet activity, 43.6 ± 5.7% ($p \leq 0.01$), was induced by 1mg protein/ml, but the effect was also significant at 0.75mg protein/ml (41.0 ± 9.2%, $p \leq 0.05$) *[Figure 3.3]*. Repetition of the experiments using thrombin (0.1U/ml) to stimulate the platelets, also resulted in a concentration-dependent inhibition of the normal agonist-induced response in the presence of nLDL *[Figure 3.2b]*. The magnitude of the maximal effect induced by 1mg protein/ml (36.6 ± 10.7%) was less than that observed with ADP-stimulated aggregation *[Figure 3.3]*. However, all concentrations of the native lipoproteins produced significant inhibition of platelet aggregation ($p \leq 0.05$). Inhibition by nLDL showed a reduction in both total aggregation and aggregate size, the aggregation traces took the appearance of reduced secondary responses *[Figure 3.2a, b]*.

3.3.2. The influence of native low density lipoproteins on platelet aggregation in platelet rich plasma.

nLDL (0-1mg/ml) was incubated with PRP for 3min, without any change in light transmittance detected. When incubated for 1min prior to the addition of ADP (10μM), nLDL failed to inhibit aggregation compared to control responses of ADP only.

To investigate whether the interaction was time dependent, PRP was incubated with nLDL for 30min at 37°C without stirring, prior to addition of ADP. When challenged with ADP (0.1 - 10μM) there was a substantial inhibition of aggregation, as compared to PRP incubated with Tyrodes HEPES buffer (*see section 2.3.2*) *[Figure 3.4]*. nLDL induced an inhibition of platelet aggregation at all concentrations of ADP used. With submaximal concentrations of ADP (0.1

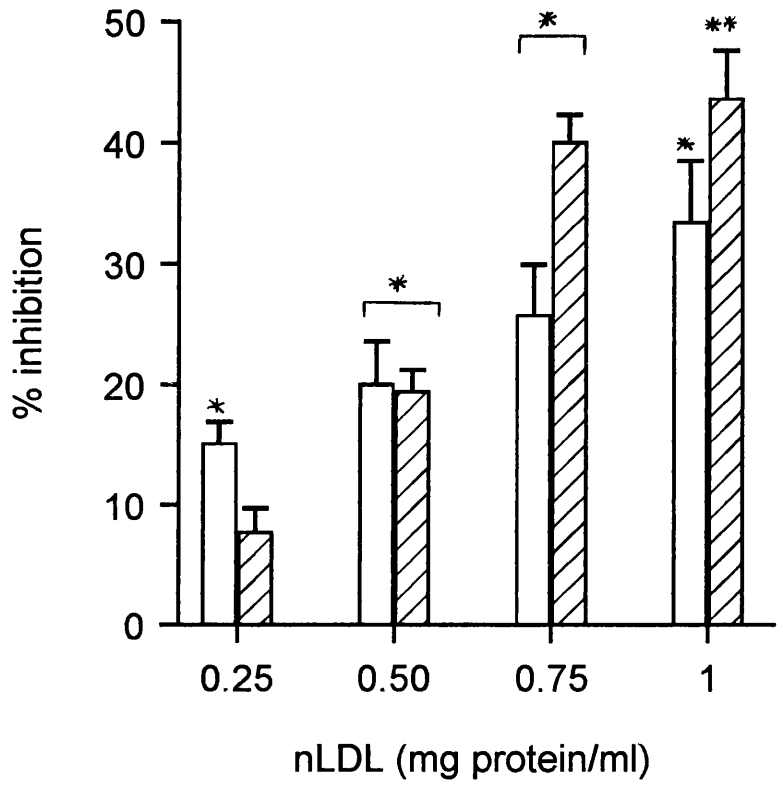


Figure 3.3: The effects of nLDL on agonist induced-aggregation of washed platelets.

Native low density lipoproteins were prepared by ultracentrifugation of fresh human plasma and incubated with WP for 1min prior to the addition of ADP (10μM; shaded bars) or thrombin (0.1U/ml; open bars). The results are presented as % inhibition of aggregation and are expressed as mean ± SEM (n=4) compared to aggregation induced by the agonist alone. * $p \leq 0.05$, ** $p \leq 0.01$ (Student's t test of paired samples were calculated from the % aggregation).

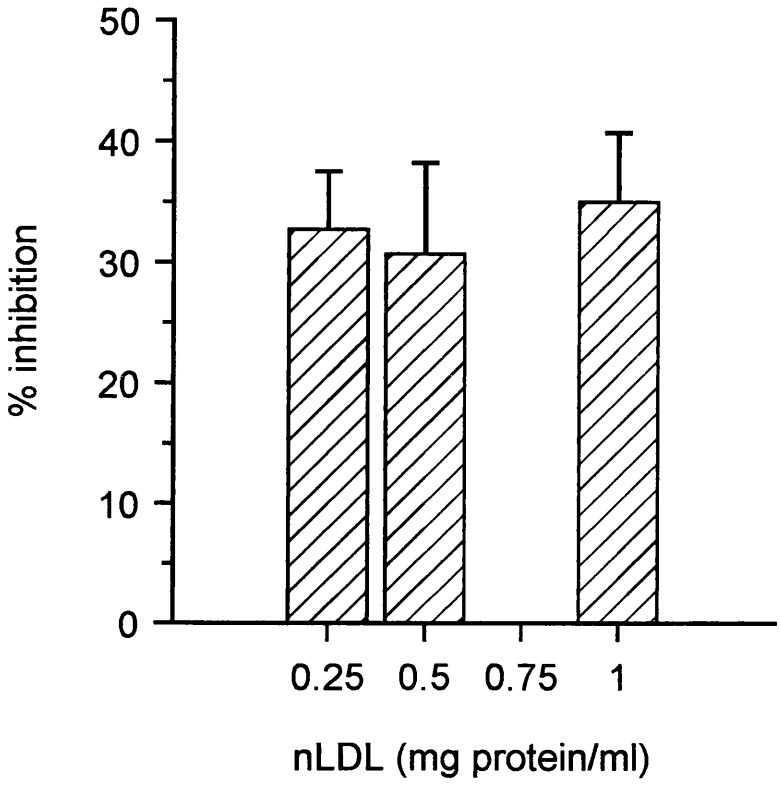


Figure 3.4: The effects of nLDL on agonist-induced aggregation of platelet rich plasma.

Native low density lipoproteins were incubated with PRP for 30min at room temperature without stirring. The lipoproteins and platelets were then transferred to an aggregation cuvette and left to temperature equilibrate for 1min before being stimulated with ADP (10μM). The results are presented as % inhibition of aggregation after 3min and are expressed as mean ± SEM (n=3) compared to aggregation induced by the agonist alone.

and 1μM) nLDL (1mg protein/ml) caused almost complete inhibition of aggregation ($p \leq 0.05$ and $p \leq 0.001$, for the two concentrations of ADP respectively). The effects were less pronounced at maximal ADP (10μM) with nLDL (1mg protein/ml) inducing 28.3 ± 5.1% ($p \leq 0.01$) inhibition *[Figure 3.4]*. The contrasting effects found between WP and PRP suggested that a plasma factor may influence any rapid interactions between the native lipoproteins and platelets.

As indicated earlier the total concentration of LDL present in the PRP experiments was greater than with WP. In the present set of experiments the mean concentration of LDL was 2mmol cholesterol which equates to approximately 0.4mg protein /ml. Therefore, the maximum augmentation of LDL concentrations above those found physiologically was approximately 3-fold at 1.4mg protein /ml. Interestingly, the maximum inhibition induced was 28.3 ± 5.1%, which was lower than the effects observed with 1mg protein/ml with WP. This demonstrates that increasing the concentration of LDL above physiological levels do not increase their anti-aggregatory effects.

3.3.3. Native low density lipoproteins and platelet activation (fibrinogen binding).

Platelets in PRP were identified by their size and side scatter characterististics, which are digitized to produce a bitmap *[Figure 3.5a]*. This was confirmed by the fluorescent detection of bound anti-GPIB Mabs (RFGP37-FITC) on the platelet surface *[Figure 3.5b]*. The influence of nLDL on the binding of fluorescently labelled fibrinogen to the platelet surface was tested using flow cytometric analysis. Stimulation of platelets in PRP with ADP (0.1 - 10μM) produced a

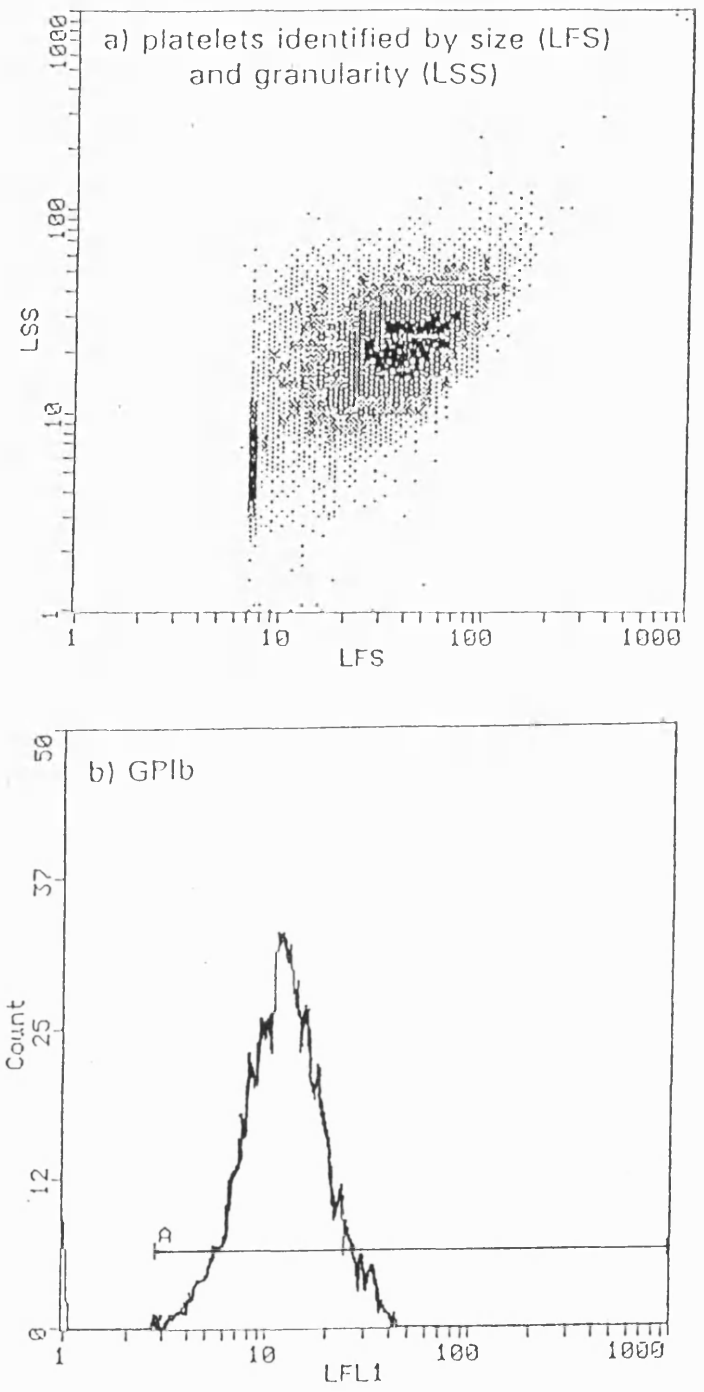


Figure 3.5: Flow cytometric analysis of platelets.

a) Platelet in PRP were identified by their size (LFS) and side scatter (LSS) charateristics.

b) Fluorescence profile of platelet incubated with FITC-conjugated anti-GPIb Mab which binds to 100% of platelets.

concentration-dependent increase in FGN binding *[Figure 3.6a].* The presence of nLDL (0 - 1mg protein/ml) inhibited ADP (1 and 10μM)-induced fibrinogen binding in a concentration-dependent manner (data not shown). Using highest ADP concentration (10μM), nLDL (0.5 and 1mg protein/ml) significantly inhibited FGN binding by 29.1 ± 5.7% and 37.7 ± 8.4% ($p \leq 0.05$) respectively, compared to controls of ADP only *[Figure 3.6b].* The magnitude of the lipoprotein induced effects increased with increasing concentration of ADP. This is not unexpected, as greater stimulation of platelets would expose more binding sites for FGN and thus allow a greater opportunity for nLDL to exert their effect on the interaction of FGN and GPIIb/IIIa.

The maximum inhibitory effect was induced by 1mg protein /ml which when added to the endogenous concentrations LDL translated to a 3-fold increase over physiological concentrations.

Two internal controls were used in the experiment:

[1] nLDL were incubated with PRP in the absence of ADP, and no increase in fluorescence was observed in comparison to PRP only. This suggested that nLDL had no activatory effects.

[2] nLDL was incubated with RFAC-4 alone to observe for non specific binding of the antibody; no fluorescence was detected.

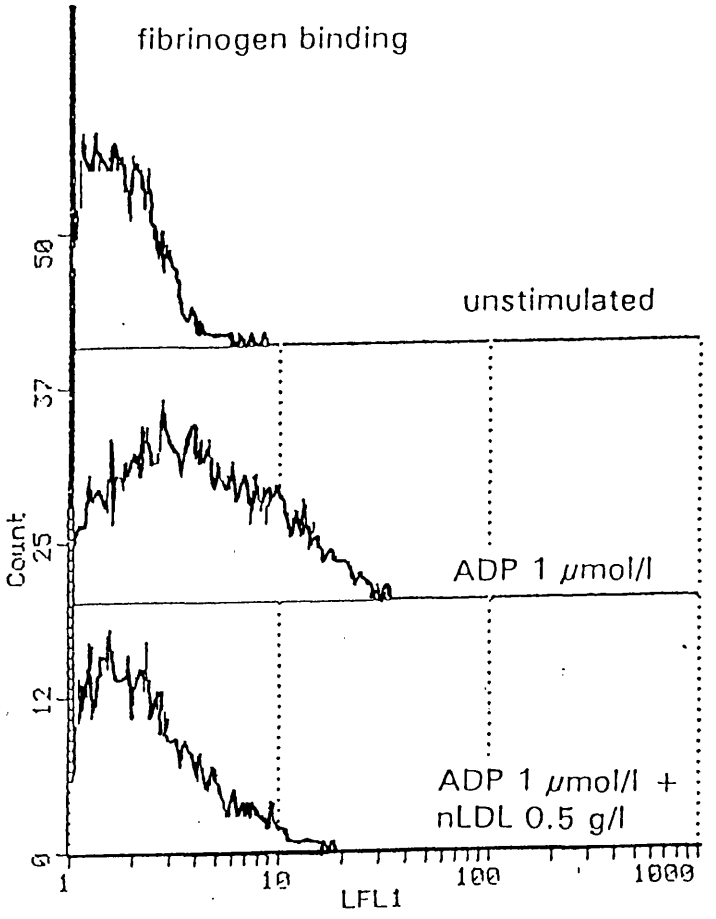


Figure 3.6a: The influence of native low density lipoproteins on binding of fluorescently labelled fibrinogen to the platelet surface.

Flourescence profiles of platelets incubated with RαFgn-FITC in the absence of exogenous agonists (resting sample; top panel), with ADP (1μM: middle panel) and with ADP (1μM) and nLDL (0.5mg protein/ml; bottom panel).

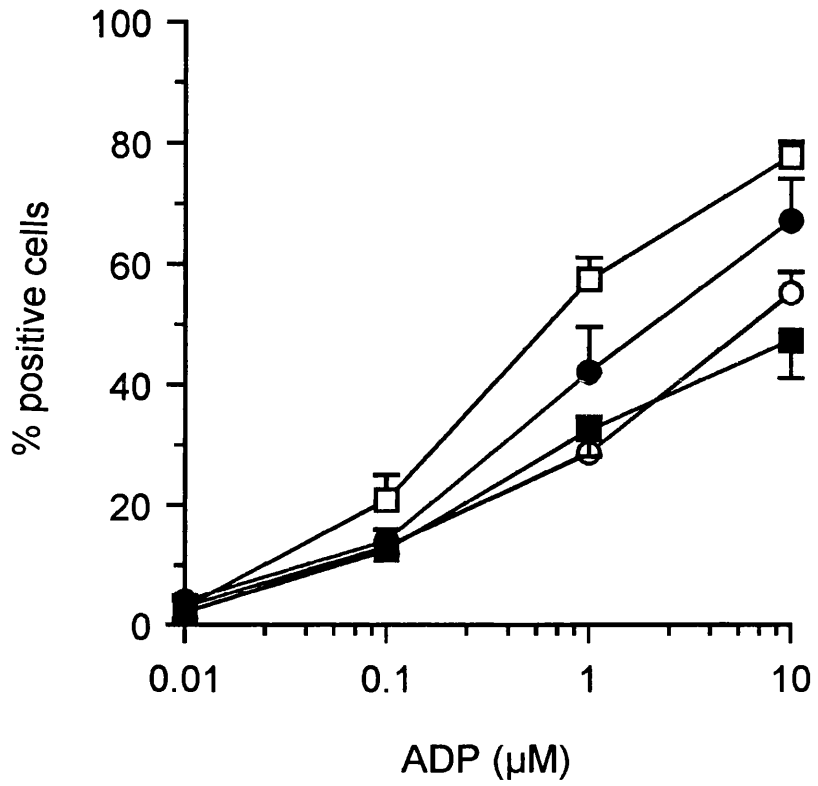


Figure 3.6b: The influence of native low density lipoproteins on binding of fluorescently labelled fibrinogen to the platelet surface.

PRP (5μl) were added to HEPES buffer (50μL) containing native low density lipoproteins (0 -1mg protein/ml) and RαFgn-FITC. The experiment was started by the addition of ADP (0.1, 1, 10μM) and then incubated at room temperature for 20min. Three concentrations of nLDL were tested 0.25mg/ml (●), 0.5mg/ml (■) and 1mg/ml (○)and the effects were compared to FGN binding induced by ADP alone (□). The platelets were then fixed with formyl saline (500μl) and analysed by flow cytometry. Platelets were identified by their size and side scatter charateristics and the results expressed as the percentage of cells positive for bound fluorescence. The data represents mean ± SEM of 3 independent experiments. For statistical significance refer to text (*section 3.3.3*).

3.3.4. The influence of native low density lipoproteins on platelet degranulation.

During platelet degranulation protein markers, specific to the different type of granule, are exposed at the platelet surface. These can be measured by the use of Mabs and thus quantify the extent of the degranulation process. In the present study P-selectin and CD63, markers for the α- and lysosomal granules respectively, were measured. P-selectin also acts as a platelet receptor for adhering leukocytes.

nLDL (0 - 1mg protein/ml) incubated with PRP in the absence of ADP did not induce expression of P-selectin or CD63. Stimulation of PRP with ADP(10μM) induced an increase in expression of P-selectin and CD63 *[Table 3.3]*. ADP-stimulated expression of P-selectin and CD63 was not affected by the presence of nLDL.

	P-selectin	CD63 antigen
unstimulated PRP	2.5 ± 0.9	6.5 ± 3.6
ADP (10μM)	16.8 ± 2.1	14.2 ± 3.6
ADP (10μM) +		
nLDL	17.0 ± 2.5	13.9 ± 0.8
mmLDL	23.2 ± 3.5***	25.4 ± 3.8*
oxLDL	15.1 ± 3.2	12.6 ± 0.9

Table 3.3: The influence of LDL on ADP-induced expression of platelet granule markers.

Platelet rich plasma (5μl) were added to HEPES buffer (50μL) containing low density lipoproteins (1.0mg protein/ml) and FITC-conjugated CD62P and CD63 Mabs. The experiment was started by the addition of ADP (10μM) and then incubated at room temperature for 20min. The platelets were then fixed with formyl saline (500μl) and analysed by flow cytometry. Platelets were identified by their size and side scatter charateristics and the results expressed as the percentage of cells positive for bound fluorescence at one concentration of ADP. The data represents mean ± SEM of 4 independent experiments. * $p \leq 0.05$, *** $p \leq 0.005$ (Student's t test of paired samples).

3.4. THE INFLUENCE OF MINIMALLY MODIFIED LOW DENSITY LIPOPROTEINS ON PLATELET FUNCTION.

nLDL did not show any pro-activatory properties, but the data suggest they may play an inhibitory role. Modified forms of LDL have been shown to exert different effects from nLDL on various cell types. The following section investigated how mmLDL, LDL which has undergone lipid oxidation only, affected platelet function.

3.4.1. Influence of minimally modified low density lipoprotein on platelet aggregation.

The work by Ardlie *et al.*, [1989] suggested modified LDL could enhance aggregation of platelets exposed to low concentrations of agonists more effectively than native lipoproteins. In the present study, WP were stimulated with sub-maximal ADP (1 -2μM) to induce primary aggregation. Incubation of mmLDL (0 - 1mg protein/ml) with WP for 1min prior to the addition of ADP, caused significant enhancement of aggregation *[Table 3.4]*. The effect was not concentration-dependent for mmLDL within the physiological range, the greatest change was observed with 0.25mg protein/ml (22 ± 2 % total aggregation; $p \leq 0.001$), compared to ADP alone. However, all concentrations of LDL, except 1mg /ml did enhance total aggregation ($p \leq 0.05$) *[Table 3.4]*. In these experiments ADP induced primary aggregation responses, which were enhanced to secondary responses by the presence of mmLDL, demonstrating the ability of these lipoproteins to sensitize platelets to ADP. However, if ADP induced a secondary response the effects of mmLDL were not detected.

The short incubation of mmLDL with WP alone in the previous experiments

caused a slight aggregatory effect (increase in light transmission), which was not observed in control experiments using Tyrode's HEPES buffer (*see section 2.3.2*). Consequently, mmLDL (0 - 1 mg protein/ml) were left to incubate with WP for 3min and the aggregation measured. mmLDL induced primary aggregation of WP, which peaked at approximately 2min after addition of mmLDL, before the aggregate disassociated *[Figure 3.7]*. The effect was concentration-dependent, with 0.5 and 1mg protein/ml mmLDL inducing 4.7 ± 2.7% and 16.5 ± 3.9% ($p \leq 0.05$) aggregation respectively *[Table 3.4]*, compared to controls (addition of equal volume of Tyrodes HEPES buffer). No additional increase in aggregation were observed with concentrations of mmLDL above 1mg protein/ml.

The data indicated that mmLDL acted as a weak platelet agonist, an effect which was concentration-dependent and saturable.

3.4.2. Platelet aggregation in PRP: influence of minimally modified low density lipoproteins.

mmLDL incubated with PRP for 1min prior to addition of ADP (0.2 - 1.0μM), enhanced total aggregation compared to ADP alone *[Table 3.4]*. The potentiation was less effective than with WP and no concentration-dependent effect was evident. Maximal potentiation of platelet aggregation was induced by 0.25mg/ml mmLDL, 18.6 ± 2.9 ($p \leq 0.05$) *[Table 3.4]*. In contrast to experiments with WP, mmLDL in the absence of ADP failed to induce aggregation, demonstrating that the plasma enviroment inhibited the direct effects of mmLDL on platelets.

mmLDL (mg protein/ml)	percentage aggregation			
	no agonist		ADP (1μM)	
	WP (n=6)	PRP (n=3)	WP (n=5)	PRP (n=6)
0	0	0	9.3 ± 1	9.6 ± 0.8
0.25	0	0	22 ± 2***	18.6 ± 2.9*
0.50	4.7 ± 2.7	0	21.6 ± 3.1***	18.3 ± 2.4**
0.75	nd	nd	14.6 ± 2*	16 ± 2.6*
1	16.4 ± 3.9	0	11.8 ± 3.7	11.7 ± 2.4

Table 3.4: The influence of minimally modified low density lipoproteins on platelet aggregation.

The effects of mmLDL were tested in WP and PRP either alone or 1min before the addition of ADP (1μM) and aggregation measured 3min after the final addition. The results represent the percent maximum aggregation and are expressed as mean ± SEM, where n= the number of individual experiments.
*< p0.05; **p< 0.01; *** p< 0.005. nd= not done.

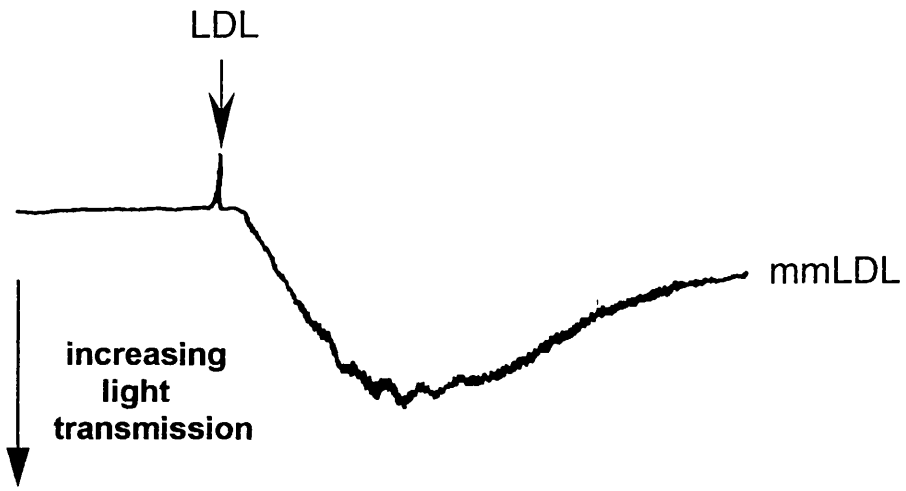


Figure 3.7: Typical trace of platelet aggregation induced by mmLDL (1mg/ml)

3.4.3. The influence of minimally modified low density lipoproteins on platelet fibrinogen binding.

In the previous sections (*3.4.1 and 3.4.2.*) mmLDL were shown to both induce and potentiate aggregation of platelets. These effects can only occur by an increase in platelet-FGN interactions and in this present section the influence of mmLDL on FGN binding was assessed. MmLDL preparations were incubated with PRP, and the platelets activated with ADP (0.1 - 10μM). The level of fibrinogen binding induced by ADP was significantly ($p \leq 0.05$) enhanced in the presence of mmLDL (0 - 1mg protein/ml) *[Figure 3.8a].* Potentiation, although modest, occurred with all concentrations of mmLDL tested. In addition, potentiation of the agonist responses also occurred at the highest concentration of ADP (10μM), a phenomenon not detected using nephelometry *[Figure 3.8b].* The specificity of the present technique probably allowed these responses to be recorded.

In subsequent experiments, incubation of mmLDL with PRP in the absence of agonists, showed increase in FGN binding, compared to PRP alone. The effects were significant at all concentrations of mmLDL tested, but no concentration-dependent pattern emerged. The largest increase in FGN binding was invoked by 0.5mg protein/ml, 11.4 ± 2.0 ($p \leq 0.05$), although the effects were more significant at 0.75 and 1.0mg protein/ml mmLDL ($p \leq 0.01$) *[Figure 3.9].* Maximal effects were reached at 0.5mg protein/ml, no significant increases in activation were observed with higher concentrations of mmLDL. Incubation of mmLDL with the antibody alone (RFAC-4) produced no fluorescence, indicating there was little or no non-specific binding of the antibody.

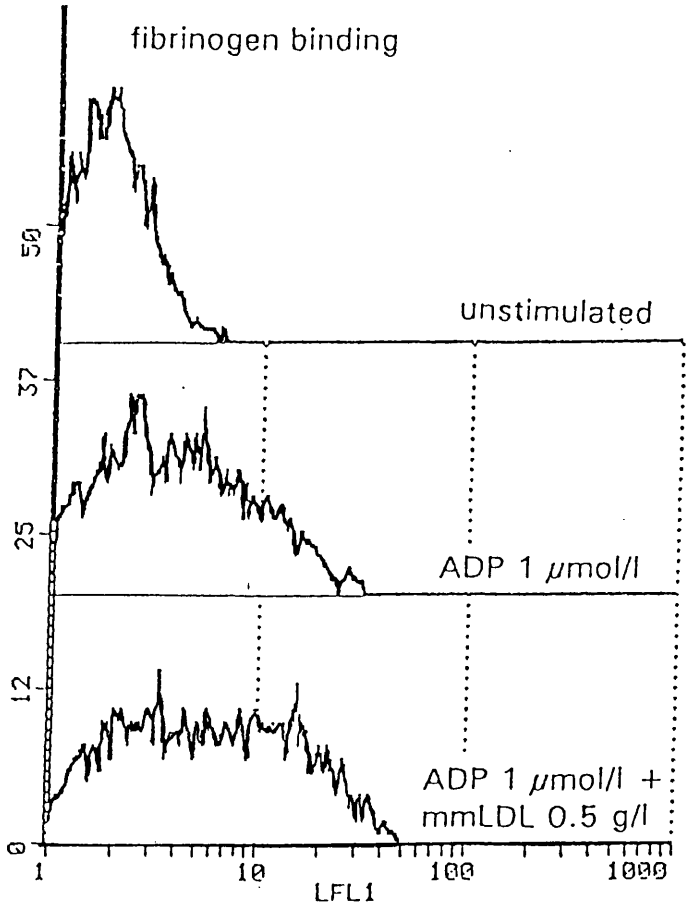


Figure 3.8a: The enhancement of ADP-induced fibrinogen binding by minimally modified low density lipoproteins.

Flourescence profiles of platelets incubated with RαFgn-FITC in the absence of exogenous agonists (resting sample; top panel), with ADP (1μM: middle panel) and with ADP (1μM) and mmLDL (0.5mg protein/ml; bottom panel).

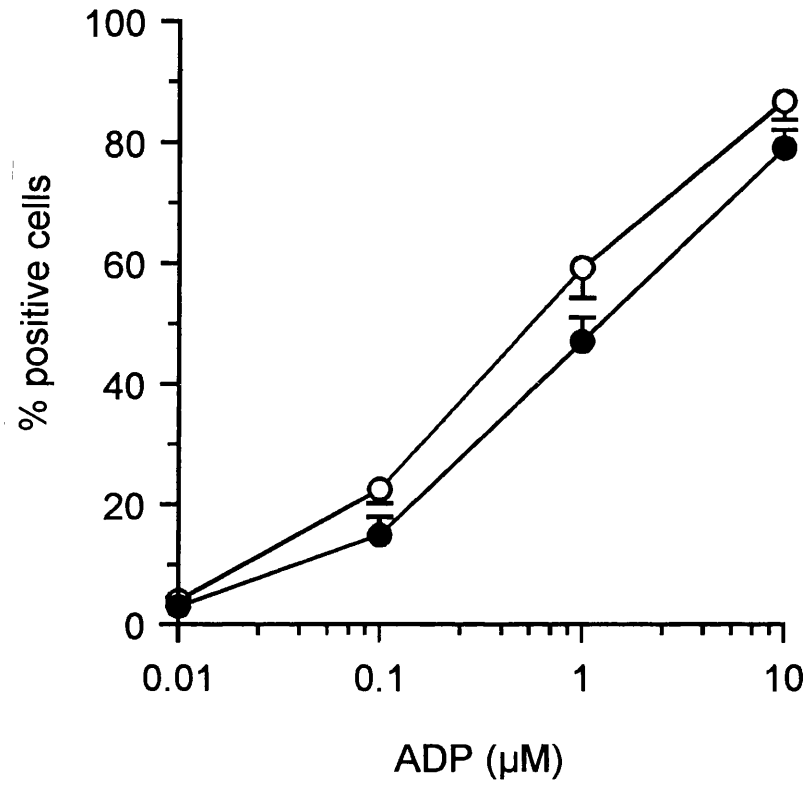


Figure 3.8b: The enhancement of ADP-induced fibrinogen binding by minimally modified low density lipoproteins.

PRP (5μl) were added to HEPES buffer (50μL) containing minimally modified low density lipoproteins (0.5 mg protein/ml) (○) and RαFgn-FITC. The experiment was started by the addition of ADP (0.1, 1 or 10μM) and then incubated at room temperature for 20min. The platelets were then fixed with formyl saline (500μl) and analysed by flow cytometry. The effects of mmLDL were compared to to ADP alone (●) .Platelets were identified by their size and side scatter charateristics and the results expressed as the percentage of cells positive for bound fluorescence. The data represents mean ± SEM of 4 independent experiments. For statistical significance refer to text (*section 3.4.3*).

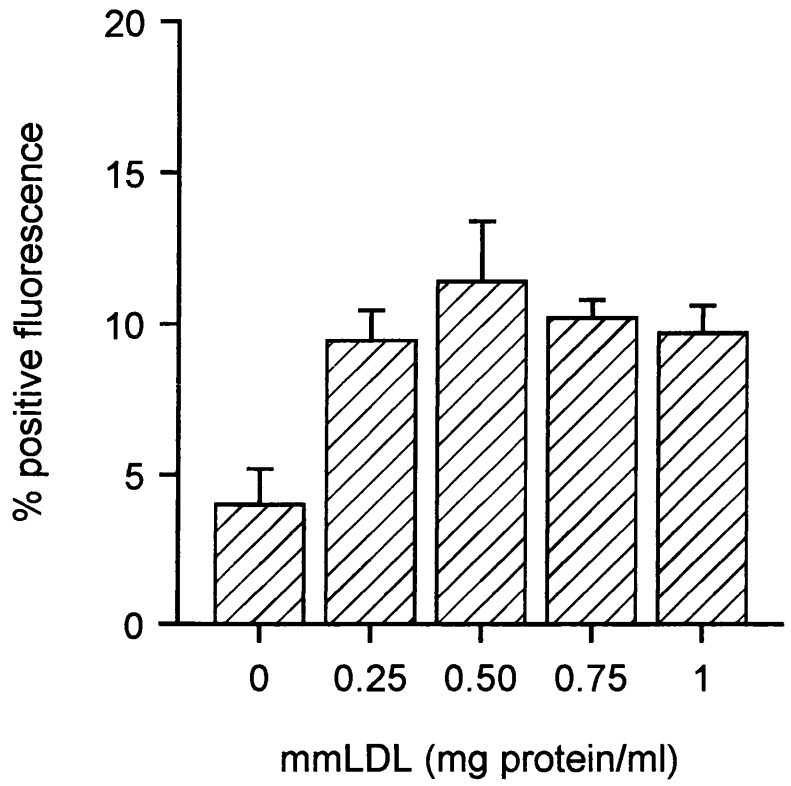


Figure 3.9: Flow cytometric analysis of fibrinogen binding induced by minimally modified low density lipoproteins in the absence of physiological agonists.

PRP (5µl) were added to HEPES buffer (50µL) containing minimally modified low density lipoproteins (0 -1.0mg protein/ml) and RαFgn-FITC and incubated, without the addition of agonist, for 20min at room temperature. The platelets were then fixed with formyl saline (500µl) and analysed by flow cytometry. Platelets were identified by their size and side scatter charateristics and the results expressed as the percentage of cells positive for bound fluorescence. The data represents mean ± SEM of 6 independent experiments. For statistical significance refer to text (*section 3.4.3*).

The results suggest that mmLDL, in contrast to nLDL, induced and potentiated the binding of FGN to the platelet surface, which indicates the increased activation of platelets in the presence of ADP and possibly other agonists.

3.4.4. Is platelet degranulation influenced by minimally modified low density lipoproteins ?

The actions of mmLDL on platelet degranulation were tested, as the release of these vasoactive factors has an important propagating effect on platelet aggregation.

3.4.4.1. The effect of minimally modifed low density lipoprotein on P-selectin expression.

mmLDL (1mg protein/ml) incubated with PRP alone did not induce expression of P-selectin. The presence of mmLDL enhanced ADP-induced expression of P-selectin ($p \leq 0.005$) *[Table 3.3].*

3.4.4.2. The effect of minimally modified lipoproteins on CD63 expression.

mmLDL (1mg/ml) alone did not induce expression of CD63. However, expression of CD63 stimulated by ADP (10μM) was significantly enhanced in the presence of mmLDL. CD63 expression increasing from 14.2 ± 3.6% to 25.4 ± 3.8% ($p \leq 0.05$) *[Table 3.3].*

3.5. THE INFLUENCE OF OXIDISED LOW DENSITY LIPOPROTEINS ON PLATELET FUNCTION.

Fully oxLDL are mostly found in the foam cells of the atherosclerotic plaque and is therefore unlikely to interact with platelets directly even under pathological conditions, unless there is fissure of the plaque. In this respect the relevance of experimentation on the actions of oxLDL may not be as important as those for nLDL and mmLDL. Nevertheless, the effects of oxLDL were investigated for comparison.

3.5.1. The effects of oxidised low density lipoproteins on aggregation of washed platelets.

OxLDL did not induce aggregation of WP after 3min incubation and inhibited sub-maximal ADP (1 - 2μM) (not shown). Incubation of oxLDL (1mg protein/ ml) with WP for 1min prior to addition of ADP (10μM), induced a concentration-dependent inhibition of agonist-stimulated aggregation *[Figure 3.2a]*. Maximal inhibition was induced by 1mg/ml oxLDL, 54.3 ± 4.4% ($p \leq 0.01$), but the effects were also significant at 0.5mg/ml (35.0 ± 4.5%, $p \leq 0.05$) *[Figure 3.10]*. Using 1mg protein/ml the inhibitory effects oxLDL on ADP-induced aggregation were greater than those of nLDL. In one experiment oxLDL was tested against thrombin-induced aggregation. OxLDL inhibited the aggregation response by 32% *[Figure 3.2b]*.

3.5.2. Platelet aggregation in PRP: influence of oxidised low density lipoproteins.

The previous experiments were repeated using PRP with similar results obtained. oxLDL did not induce aggregation and was inhibitory to both sub-

maximal and maximal ADP. The experiments with oxLDL alone and with sub-maximal ADP (0.2 - 1.0μM) were not pursued. Inhibition of ADP (10μM)-induced aggregation by oxLDL was concentration-dependent and maximal inhibition was induced by 1mg protein/ml, 50.6 ± 8.2% ($p \leq 0.05$). These results are in contrast to those found previously with nLDL (*section 3.3.2*), where inhibition of ADP-induced aggregation of PRP was only observed after a 30min incubation. This indicated that inhibition by nLDL and oxLDL may proceed via different mechanisms.

3.5.3. The effects of oxidised low density lipoproteins on platelet fibrinogen binding.

OxLDL incubated with PRP alone, did not to induce fibrinogen binding. PRP stimulated with ADP (1μM) showed a slight inhibition in the presence of oxLDL. However, stimulation of FGN bnding in PRP with ADP (10μM), was inhibited by oxLDL in concentration-dependent manner *[Figure 3.11].* The inhibition was to a greater extent than that observed with nLDL and was significant at all concentrations of oxLDL used. Maximal inhibition, 64.1 ± 5.2%, was induced by 1mg protein/ml ($p \leq 0.01$). Incubation of oxLDL with RFAC-4 alone, showed no increase in fluorescence.

3.5.4. Do oxidised low density lipoproteins influence platelet degranulation?

oxLDL had no significant effect on P-selectin or CD63 expression, either alone or in combination with ADP *[Table 3.3].*

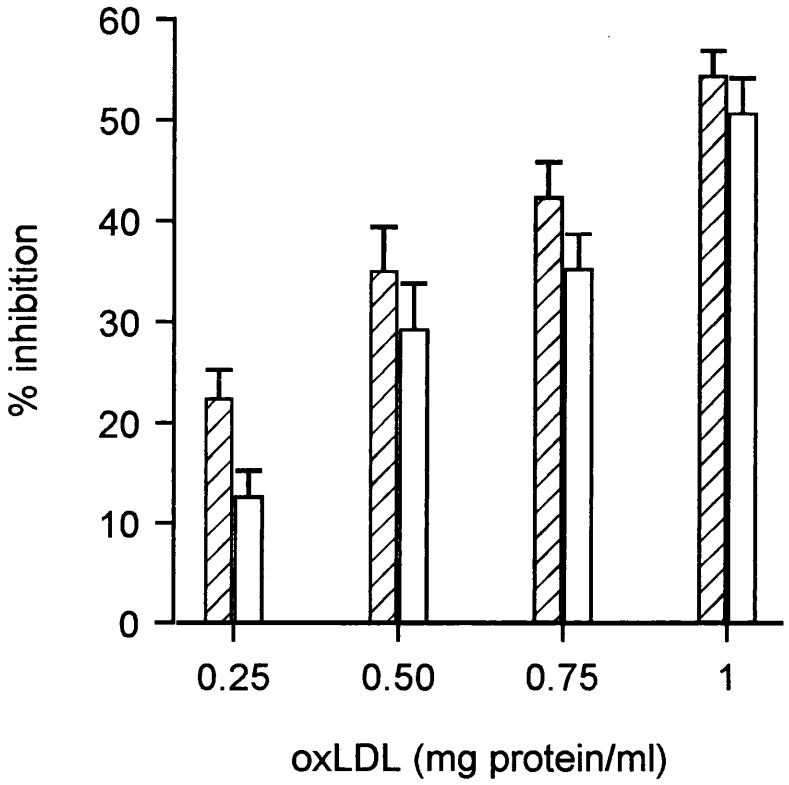


Figure 3.10: The effects of oxidised low density lipoproteins on agonist induced platelet aggregation.

Oxidised low density lipoproteins were prepared by exposure to Cu^{2+} for 24h at 37°C (*see section 2.5.3*) and incubated with WP (hatched) or PRP for 1min prior to the addition of ADP (10μM). The results are represent as % inhibition of aggregation and are expressed as mean ± SEM (n=3) compared to aggregation induced by the agonist alone. For statistical significance refer to text (*sections 3.5.1 and 3.5.2*).

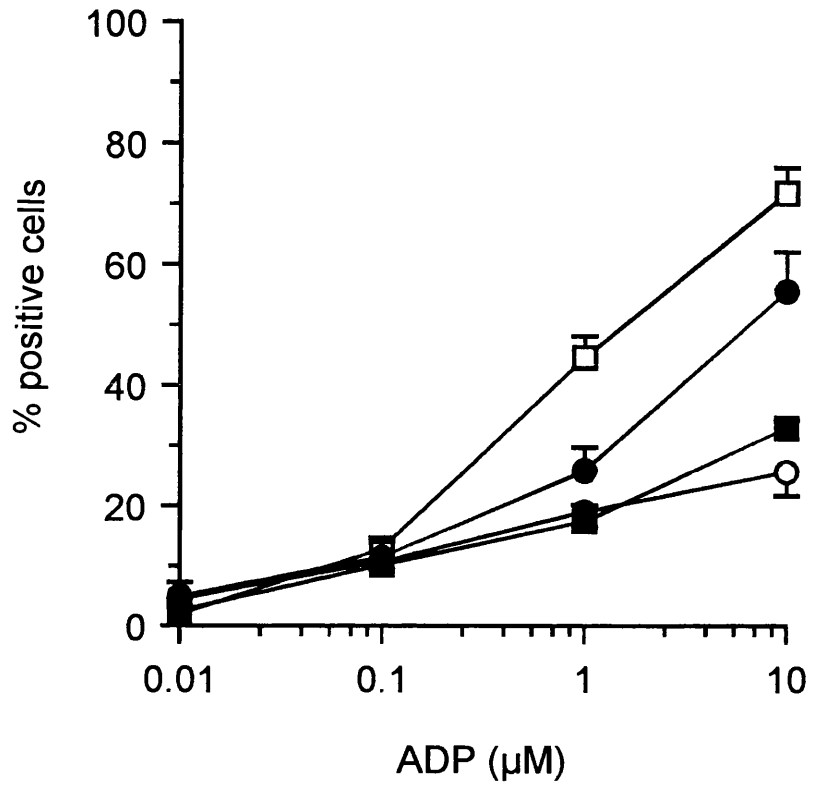


Figure 3.11: The influence of oxidised low density lipoproteins on ADP-induced fibrinogen binding.

PRP (5μl) were added to HEPES buffer (50μL) containing oxidised low density lipoproteins (0 -1.0mg protein/ml) and RαFgn-FITC. The experiment was started by the addition of ADP (0.1, 1, 10μM) and then incubated at room temperature for 20min. Three concentrations of oxLDL were tested 0.25mg/ml (●), 0.5mg/ml (■) and 1mg/ml (○)and the effects were compared to FGN binding induced by ADP alone (□). The platelets were then fixed with formyl saline (500μl) and analysed by flow cytometry. Platelets were identified by their size and side scatter charateristics and the results expressed as the percentage of cells positive for bound fluorescence. The data represents mean ± SEM of 3 independent experiments.For statistical significance refer to text (section 3.5.3).

3.6. THE INFLUENCE OF COPPER IONS AND DTPA ON PLATELET AGGREGATION.

Copper ions (Cu^{2+}) and DTPA were used in the isolation and oxidation of LDL. Theoretically neither of these substances should be present after dialysis of LDL, however their direct effects on platelet aggregation were assessed, to allow for any possible trace levels left in the LDL preparations. Cu^{2+} (0 - 5μM) added directly to both WP and PRP, did not induce aggregation.

The presence of Cu^{2+} with platelets stimulated with ADP (10μM) or thrombin (0.1U/ml) did not inhibit agonist induced aggregation. DTPA (0 - 1μM) failed to induce aggregation when added directly to platelets and also failed to alter ADP or thrombin induced responses. Cu^{2+} (1μM) and DTPA (1μM) were then added in combination and again failed to aggregate or inhibit agonist induced aggregation of platelets.

3.7 DISCUSSION.

Current evidence suggests important roles for both oxidised LDL and platelets in the pathogenesis of atherosclerosis and thrombosis. LDL have previously been shown to sensitise platelets to other agonists (*see section 1.7.2*) and hence may be important pro-thrombotic factors. Here, the effect of oxidation of LDL on platelet function was studied in detail.

3.7.1 Low density lipoprotein oxidation.

Isolated nLDL possessed a range of concentrations of both LPO and TBARs, which highlighted the heterogeneous nature of the particles and also suggested that slight oxidation of LDL may occur *in vivo*. All solutions used in the isolation procedure were supplemented with DTPA, which has been shown to completely inhibit the oxidation of LDL [Heinecke *et al.*, 1984], indicating that the products may have been preformed. There are several possibilities as to where these lipid peroxides may have originated. It is feasible that LDL may become partially oxidised in the circulation, despite the presence of antioxidants. Alternatively, the LPO originate from the oxidation of HDL and subsequently transferred through simple lipid exchange. Bowry *et al.*, [1992] demonstrated that HDL may be the major source of lipid peroxides *in vivo*: this concept is supported by the fact that HDL has less antioxidant protection than LDL. A third possibility is that the peroxides originate from a dietary source. In terms of disease development, these preformed lipid peroxides found in native LDL may an important factor in their subsequent development into plaque associated oxLDL. LDL with preformed lipid peroxides may be primed and more susceptible to oxidation by cellular free radical attack, if they become trapped in the sub-endothelium. However, it must be also accepted that the peroxides may have been formed

during the isolation procedure.

One of the aims of this study was to produce LDL at different stages of oxidation, corresponding to LDL with purely lipid oxidation (mmLDL), formed by slight oxidation of the fatty acid side chains present on the phospholipid monolayer of the particles, and also a more extensively oxidised LDL with both lipid and protein modification (oxLDL). In the present study oxidation of LDL was achieved by one of two methods, exposure to Cu^{2+} or air. These methods were used because they were logistically easier to perform, and produced more consistent oxidation kinetics than cell-induced oxidation. Oxidation induced by copper has been extensively studied and after correction for the different concentrations of ions used, direct comparisons can be made.

LDL can be modified by several methods *in vitro* to produce oxLDL (*see section 1.3*). The oxidative modification, regardless of the method, probably proceeds by a free radical chain reaction leading to the peroxidation of the PUFA associated with the phospholipid surface monolayer of LDL (*see section 1.3.1*). Oxidation of LDL can be followed and quantified by the measurement of lipid peroxidation products. The most consistently used method is the TBA assay [Beuge and Aust, 1978]. This measures MDA as a lipid oxidation product which is produced in fairly constant proportion to the peroxidation process [Esterbauer *et al.*, 1990]. When LDL are prepared at different stages of modification, the use of the assay as a sole index of oxidation is flawed because it is limited to the measurement of MDA. LDL has been shown to contain two major PUFA, linoleate and arachidonate, the relative proportions of these PUFA in LDL are 9:1 [Esterbauer *et al.*, 1990]. The problem may be circumvented by

measurement of the total amount of fatty acid oxidation products. In the present study, the measurement of total PUFA peroxidation was performed using a spectrophotometric assay for total LPO measurement (*see section 2.5.6*). Thus, oxidatively modified LDL could be quantified by both total PUFA and arachidonate oxidation products, giving a clearer indication of the extent of modification.

Oxidation of LDL is a dynamic process, whereby the concentrations of LPO and TBARs are constantly changing. Profiles of Cu^{2+}-induced oxidation show an initial lag phase, associated with the loss of vitamin E [Esterbauer *et al.*, 1989], followed by a sharp increase in the concentrations of LPO resulting from the loss of linoleate and arachidonate. LDL oxidation by Cu^{2+} is totally reliant on the presence of preformed LPO [Thomas and Jackson, 1990], since the removal of peroxides before exposure to Cu^{2+} prevented increases in the REM. This indicates that Cu^{2+} does not initiate lipid peroxidation. The extent of preformed LPO in LDL is positively correlated to an increased susceptibility to Cu^{2+} induced oxidation [Frei and Gaziano, 1993]. Since nLDL were shown to possess low levels of LPO Cu^{2+} was used to initiate the oxidation. All LDL preparations were exposed to Cu^{2+} for 24h, yet the concentrations of oxidation products varied greatly. This highlighted the heterogeneous nature of LDL particles with respect to individual oxidation kinetics. The levels of LPO and TBARs corresponds well with previous studies using similar concentrations of Cu^{2+} [Esterbauer *et al.*, 1987; Esterbauer *et al.*, 1989; Thomas and Jackson, 1990; Lodge *et al.*, 1995]. OxLDL produced by the present method has been shown to undergo extensive macrophage uptake *in vitro* [D. Mayer. PhD thesis, London University 1994]. The oxidation was halted by the addition of excess DTPA (*see section 2.5.3*).

During the early stages of LDL oxidation lipid peroxidation occurs without protein modification [Zhang *et al.*,1989]. This group demonstrated that slight oxidation of LDL, associated with small increases in LPO, did not increase the REM compared to non-oxidised LDL. In the present study, nLDL were oxidised by exposure to air to produce mmLDL. This was for two reasons, one, the method was less severe than Cu^{2+}-induced oxidation which helped to avoid protein modification, and two, under normal physiological conditions LDL is unlikely to encounter high levels of transition metal ions [Halliwell and Chirico, 1993]. Oxidation by air proceeded at a slower rate compared to Cu^{2+}-induced oxidation. After 18 - 24hrs incubation, a 2-fold in LPO and TBARs were found in these LDL preparations, compared to the native lipoproteins. In addition, the mmLDL were distinctly yellow in appearance and were almost indistinguishable from autologous nLDL. mmLDL prepared by long-term storage of nLDL at 4°C or by exposure to Fe^{3+} [Berliner *et al.*, 1989; Rajavashisth *et al.*, 1990; Watson *et al.*, 1995], possessed approximately 11nmols TBARs/mg LDL, compared to approximately 7.5nmols TBARs/mg LDL in this study. This indicated that the method used presently, reliably produced mmLDL in a relatively short period of time without exposing the lipoproteins to unphysiological levels of transition metal ions. These levels of peroxides are close to those which have been observed within the pathophysiological range in human plasma [Stringer *et al.*, 1989].

When the different forms of LDL were analysed using agarose gel electrophoresis, oxLDL showed an increased REM in comparison to nLDL, while mmLDL was indistinguishable from nLDL. These results validate the methods chosen to modify LDL, as they confirm mmLDL preparations were devoid of

significant protein modification, while the physicochemical changes of oxLDL were much more extensive.

3.7.2 Influence of native low density lipoproteins on platelet function.

In previous studies, LDL had been shown to sensitise platelets to physiological agonists and in some cases activated platelets independently of agonists [Fujitani *et al.*, 1979; Hassell *et al.*, 1983; Aviram *et al.*, 1983]. In the present study stringent isolation procedures were used for the preparation of LDL, and using preparations, nLDL induced a concentration-dependent inhibition of agonist-induced aggregation. nLDL inhibited aggregation of WP after an incubation of only 1min, which suggested a rapid surface-mediated event. In contrast, experiments with PRP required a 30min incubation for nLDL to induce a comparable effect, indicating that the presence plasma proteins or another plasma factor prevented the rapid effects seen with WP. In relation to these experiments, Surya *et al.*, [1992] demonstrated that a 30-60min incubation of platelets with LDL led to the transfer of arachidonate from platelets to LDL, thus decreasing potential platelet aggregation. It is possible that a similar phenomenon occurred here. Interestingly, the presence of endogenous nLDL in PRP did not seem to increase the inhibitory effect of LDL, suggesting that any inhibitory effect may already have been maximal.

nLDL inhibited the binding of FGN to the GPIIb-IIIa complex at the platelet surface. These observations, taken with the aggregation data, would suggest that nLDL probably inhibit platelet aggregation by attenuating the early stages of activation (FGN binding) and thus blocking interplatelet bridging. Several studies have shown LDL to be capable of binding to the platelet surface

[Mazurov *et al.*, 1982; Curtiss and Plow, 1984; Koller, 1986; Hassell *et al.*, 1990; Katzman *et al.*, 1991; Pedreno *et al.*, 1994]. The phenomenon also occurs with platelets from FH patients, indicating that the apoB/E receptor is probably not involved. Koller *et al.*, [1989] demonstrated that both LDL and HDL bound to isolated GPIIb and IIIa, the binding was saturable and in the case of GPIIIa was competitively inhibited by excess FGN. FGN did not affect lipoprotein interactions with GPIIb. However, this is not surprising as no binding site on GPIIb for FGN has been reported and the observation may represent non-specific binding. The data suggests that lipoproteins may compete with FGN for a single binding site on GPIIIa. This may also explain the apparent inhibitory effects of HDL_3 on platelet function [Desai *et al.*, 1989; Higashihara *et al.*, 1991]. However, caution must be exercised in the interpretation of these results, as the isolation of the GPIIb-IIIa complex could alter its normal binding characteristics. In support of the supposition, Kowalski *et al.*, [1990] inhibited lipoprotein binding to platelets by the inclusion of GPIIb-IIIa Mabs or RGD containing peptides, the latter suggesting lipoproteins may interact via the RGD recognition sites or regions in close proximity. Recently, an amino acid sequence on GPIIIa has been discovered which possesses a high similarity to the LDL binding regions on the apoB/E receptor [C. Ettelaie, personal communication], with 23% identity and 78% similarity *[Figure 3.12]*. This may represent a binding site for lipoproteins on platelets, but requires further investigation for the full implications to be established. Competition for binding between FGN and LDL may explain the differences in results between the WP and PRP aggregations experiments. PRP would contain much higher concentrations of FGN and hence may prevent nLDL having a rapid effect.

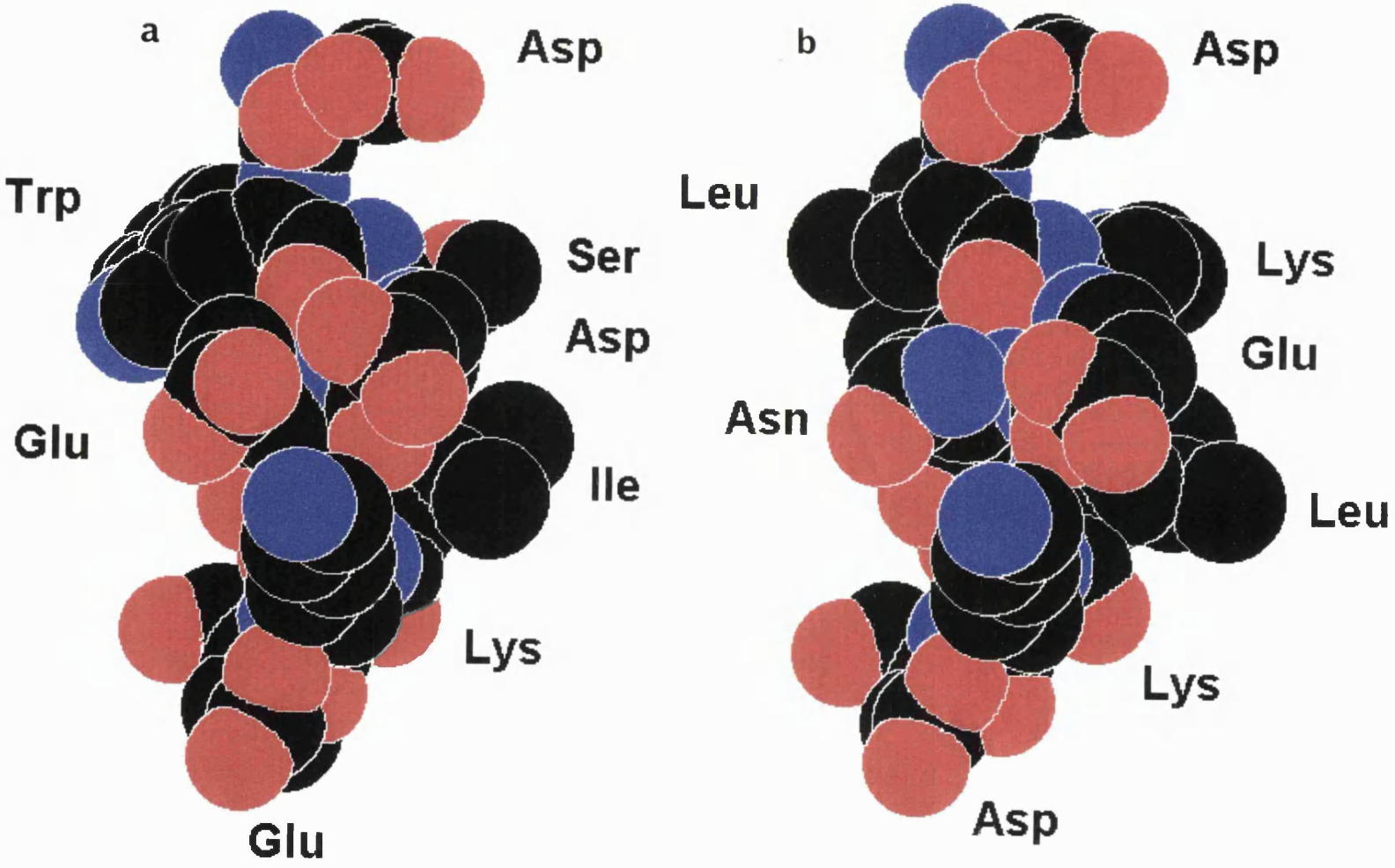
Figure 3.12. Space filling models of amino acid sequences from both apoB/E receptor and glycoprotein IIIa.

The diagram shows a sequence on GPIIIa which has a high similaritywith a sequenc found in the binding region of the apoB/E receptor, and may represent a possib binding site for LDL on platelets.

a) ApoB/E receptor residues 283 - 291.

b) Platelet GPIIIa, residues 39 - 47.

Space filling models of the two sequences were constructed using the Alchemy I program (Tripos Associates Inc, St. Louis, USA), and was a gift from Dr. C. Ettelaie Royal Free Hospital School of Medicine.


a
Asp
Trp
Ser
Asp
Glu
Ile
Lys
Glu
b
Asp
Leu
Lys
Glu
Asn
Leu
Lys
Asp

The influence of TXA_2 on platelet activity may be important to the interpretation of the results from both the aggregation and flow cytometric experiments. Platelet aggregation *in vitro* (Born aggregometry; *see section 2.6.5*) is thought to mimic thrombus formation as the stirring recreates the turbulent effects of blood flow. With ADP-stimulated platelets, TXA_2 formation is dependent on aggregation, not FGN binding [Balduini *et al.*, 1988], and thus in the aggregation system platelet derived TXA_2 would enhance aggregation. This means in the aggregation experiments nLDL is opposing the actions of both ADP and TXA_2. In a non-stirred system, TXA_2 release would be limited, hence the actions of nLDL would be an effect purely on ADP-induced FGN binding and could not be compared directly to aggregation. Thus flow cytometric analysis allows the study of the early stages of platelet activation (FGN binding), and not the full functional response, which involves granule secretion and TXA_2 formation. Therefore, caution must be exercised when comparing observations in stirred and non-stirred systems.

The results showing nLDL to inhibit platelet activity are in contrast to other studies, where LDL were reported to activate platelets. A recent study by Van Willigen *et al.*, [1994] demonstrated nLDL significantly increased the FGN binding to platelet stimulated with ADP. However, the nLDL samples were prepared in the presence of thimerosal, an antibacterial agent, which has been shown to activate platelets and remain associated with LDL [Schacter *et al.*, 1990]. nLDL has also been shown to enhance aggregation of whole blood by a new method of filtragometry, although the level of LDL oxidation was not reported [Brõijersén *et al.*, 1993].

The significance of the inhibitory effects of nLDL on platelet activity is unclear. However most importantly, nLDL prepared by the present methods does not activate platelets. This is not entirely surprising, as in evolutionary terms it would be unfavourable for a normal plasma component to activate cells in which they are in intimate contact throughout the circulation. If nLDL did increase platelet aggregation, this would be detrimental to normal haemostasis possibly leading to increased thrombotic complications. nLDL may fulfil a mild inhibitory role with respect to platelet function, although their full contribution to regulation of platelet function is yet to be elucidated. If LDL does reduce aggregation by binding to GPIIb-IIIa, the effects on FGN binding may proceed by two mechanisms. Firstly, the size of LDL, once bound, could sterically block access of the receptor to FGN, reducing interplatelet bridging. Alternatively, binding of the lipoproteins to GPIIb-IIIa may alter the binding characteristics of the receptor and thus down regulate FGN-receptor coupling. A reduction in FGN binding to GPIIb-IIIa may lead to a down regulation of FGN binding associated tyrosine phosphorylation [Ferrell and Martin, 1989], again leading to a reduction in aggregation. Therefore, the inhibitory effects of nLDL may be two-fold, a physical attenuation of FGN-binding, leading to a possible reduction in tyrosine phosphorylation, secondary messenger levels and ultimately aggregation. It is interesting to note that increasing the experimental concentrations of LDL above the physiological concentration (0.5mg/ml), did not increase their inhibitory actions, indicating that if plasma nLDL concentrations reach pathophysiological levels their possible anti-aggregatory effects are not increased. Furthermore, their beneficial effects maybe masked by the pro-aggregatory actions when oxidised (discussed later).

3.7.3 Modified low density lipoproteins and platelet function.

In contrast to the effects of nLDL, mmLDL activated platelets. They induced FGN binding and aggregation of platelets independently of agonists and also enhanced ADP-induced activation and aggregation. Previous studies have shown LDL to induce secondary aggregation of WP using pathophysiological concentrations of LDL (> 2mg protein/ml) [Hassell *et al.*, 1983]. In the present study, mmLDL only induced primary aggregation. With concentrations in excess of 1mg protein/ml, a significant increase in aggregation was not observed. However, mmLDL did potentiate sub-threshold concentrations of ADP to induce secondary aggregation. One possible explanation for these results is the effects of mmLDL on platelet secretion: mmLDL alone failed to induce expression of P-selectin and CD63 which is consistent with the inability to induce secondary aggregation. However, in the presence of ADP, mmLDL did enhance platelet secretion. The release of vasoactive factors from platelet granules may help to sustain and increase the aggregation response. Partial degranulation measured by P-selectin and CD63 expression is associated with granule content release, as evidenced by the levels of β-thromboglobulin [Janes *et al.*, 1994]. The data complement previous work showing LDL to increase dense granule secretion. Surya *et al.*, [1992] has demonstrated that short term exposure of washed platelets to LDL leads to an increase in dense granule secretion by TXA_2-dependent and independent mechanisms, after initial stimulation by α-thrombin. However, the degree of LDL oxidation was not measured in this study. LDL were also shown to enhance platelet activation stimulated by sub-maximal serotonin in PRP [Fetkovska, 1992]. In a morphological study, Zhao *et al.*, [1993] showed LDL, prepared without protection from potential oxidants, to induce platelet shape change.

The mmLDL preparations in this study were very mildly oxidised, and it is conceivable that with earlier studies, where the full implications of LDL oxidation had not been appreciated, that slight oxidation of the lipoproteins may have occurred. Resulting in a form of LDL very similar to that described here as mmLDL. The only recognisable physicochemical difference between nLDL and mmLDL was a 1.5/2-fold increase in LPO/TBARs. However, their effects on platelets were very different. Agarose gel electrophoresis demonstrated that the apoB of both nLDL and mmLDL were unmodified, indicating that LPO are of fundamental importance to the actions of mmLDL. It is plausible that mmLDL could bind to platelets in a similar fashion to that proposed for nLDL, but the presence of LPO could create an oxidative microenviroment leading to a change in the cell surface characteristics, for example, increased permeability to Ca^{2+} or an altered functioning of membrane bound enzymes. LDL have been shown in numerous studies to increase $[Ca^{2+}]_i$ concentrations in both platelets [Andrews *et al.*, 1987a, Dunn *et al.*, 1988, Knorr *et al.*, 1988, Block *et al.*, 1988, Katzman *et al.*, 1991, Bochkov *et al.*, 1991, Katzman *et al.*, 1994] and other cell types [Galle *et al.*, 1991]. An increase in $[Ca^{2+}]_i$ may explain how LDL increased other secondary messenger markers of platelet activation, since this increased $[Ca^{2+}]_i$ may activate PLA_2 (*see section 1.6.3*). Zhao *et al.*, [1993], suggest that the increased $[Ca^{2+}]_i$ requires Ca^{2+} from the extracellular compartment, as the presence of EDTA inhibited the LDL-induced increase in $[Ca^{2+}]_i$. Wiedtmann *et al.*, [1995], have recently presented work which is very similar to the present study. This group found mildly oxidised LDL to activate platelets, an effect which could not be reproduced with either nLDL or oxLDL. In contrast to work presented here, mildly oxidised LDL induced full secondary aggregation. The actions of mildly oxidised LDL were attributed to the activation of PLA_2 and COX,

since inhibition of these enzymes abolished the effects of the lipoproteins. Another recent study by Watson *et al.*, [1995] suggests that the cellular effects of mmLDL are due to oxidised phospholipids. Treatment of mmLDL with PAF acetylhydrolase (PAF-AH) eliminated its cellular effects. The data suggest a possible role for PAF in the actions of mmLDL. However, Wiedtmann *et al.*, [1995], showed that the aggregatory effects of mildly oxidised LDL, which were mediated by COX and PLA_2, were unaffected by the presence of PAF receptor antagonists. PAF-AH has been proposed to be a general scavenger of oxidised phospholipids [Strafforini *et al.*, 1993], and treatment of LDL with PAF-AH inhibited the production of a whole range of oxidised phospholipid products. These data compare favourably with the present study, as it is postulated here that the activatory effects of mmLDL, reside in the oxidised lipid moiety, in particular the LPO.

Atherosclerosis is associated with increased levels of plasma lipid peroxide levels [Stringer *et al.*, 1989]. In relation to these observations, the antioxidant status of the individual preparations may also be of importance, Salonen *et al.*, [1991] demonstrated a weak relationship between subjects with low antioxidant levels and hyper- responsiveness of platelets. The increased susceptibility of the platelet to aggregate was reversed by antioxidant supplementation. The effects of mmLDL on platelet aggregation are complemented by two other studies [Ardlie *et al.*, 1989, Meraji *et al.*, 1991], both of which showed that oxLDL potentiated and induce aggregation. The oxLDL preparations in both of the studies possessed relatively low concentrations of oxidation products in comparison to other work. The physiological relevance of the actions of mmLDL are unclear, but the enhanced aggregation in the presence of plasma suggests

that mmLDL may potentially increase the formation of thrombi.

The properties of apoB in mmLDL are not significantly different from those associated with nLDL. Therefore it is interesting to speculate that mmLDL may not be fully recognised by the reticulo-endothelial system and therefore escape from the subendothelial compartment and exist in the circulation. Two independent studies have shown the presence of a modified LDL in human circulation. Avogaro *et al.*, [1987], isolated an LDL subfraction from non-lipidaemic subjects, which had an increased electronegativity. The modified LDL had a reduced ability to bind to human skin fibroblasts compared to nLDL, but was more efficient than acLDL. These modified LDL represented approximately 5-20% of the total LDL content. These experiments were further characterised by a second study [Cazzolato *et al.*, 1991]. Here the modified LDL was found to have significantly greater levels of TBARs than nLDL, while the cholesteryl ester and phospholipid contents were reduced. In a separate study, Shimano *et al.*, [1991] isolated a similar LDL subfraction which comprised approximately 1% of the total. This subfraction bound to human fibroblasts with the same affinity as nLDL, although they had a greater susceptibility to oxidation, possibly because of the presence of preformed peroxides. In all three studies an LDL subfraction may have been isolated which may be a closely related species to the mmLDL of the present study.

OxLDL underwent both lipid and protein modification, as indicated by the concentrations of LPO/TBARs and the increased REM. Fully oxLDL, in contrast to mmLDL, did not activate platelets, but inhibited aggregation in both WP and PRP. This indicated that further oxidation of LDL again altered the properties of

the particles. In both platelet models, the inhibition occurred after a 1min incubation, suggesting a different mechanism of inhibition to that of nLDL. Oxidation of LDL yields high concentrations of lysolecithin [Besterman and Gillette 1971] and 4-HNE [Selley *et al.*, 1988], both of which have been shown to inhibit platelet function. The relative increase in concentration of these products in oxLDL may account for the results obtained. These findings are supported by Aviram, [1989], who reported significant inhibition of collagen-induced aggregation by oxLDL, but not acLDL, where lysine residues were blocked, but no oxidation products were present. This strongly suggests that lipid oxidation products, rather than the apoB modification are responsible for inhibition of platelet function by oxLDL. Direct interactions between oxLDL and platelets are unlikely as oxLDL would be held in the foam cells of the plaque, unless the plaque fissures and oxLDL is released. However, one recent study has shown oxLDL to bind to platelets with the same affinity as nLDL, although the apparent binding site was not identified [Pedreno *et al.*, 1994].

In summary, LDL have previously been shown to activate platelet to varying degrees. Many of these studies failed to report the oxidation state of LDL. Here, the effects of oxidative modification of LDL on platelet function has been studied in a more systematic manner. The extent of oxidation of LDL seems to be critical in determining the nature of their effects on platelet function. It is proposed that mmLDL are the most potent form of LDL with regard to platelet activation, a supposition which is supported by a recent publication by Wiedtmann *et al.*, [1995].

CHAPTER FOUR: THE INFLUENCE OF EXOGENOUS HYDROGEN PEROXIDE ON PLATELET AGGREGATION.

4.1 INTRODUCTION.

Lipid peroxides can influence platelet function by the activation of cyclo-oxygenase (COX) [Warso and Lands, 1983]. Plasma lipid peroxide concentrations have been shown to be raised in atherosclerosis [Stringer *et al.*, 1989], which in turn may relate to the pathophysiology of atherosclerosis and thrombosis. Evidence suggets that these LPO are associated, at least in part, with plasma lipoproteins [Nishigaki *et al.*, 1981; Bowry *et al.*, 1992]. In the previous chapter, mmLDL was shown to induce activation and aggregation of platelets independently of platelet agonists. It was proposed that the pro-aggregatory effects of mmLDL are mediated by the low levels of hydroperoxy fatty acid oxidation products which are associated with these particles.

Platelets from patients with Type IIa hyperlipidaemia are hyper-aggregable [Carvalho *et al.*, 1974] and also require more PGI_2 to inhibit aggregation compared to platelets from normal patients [Colli *et al.*, 1983]. However, there were no differences in PGI_2 stimulation of isolated membrane adenylate cyclase between the two groups [Colli *et al.*, 1983]. These later data indicate that there are differences in plasma composition rather than alterations of PGI_2 receptor function and its linked signal transduction systems. LDL were subsequently shown to reduce the sensitivity of platelet adenylate cyclase to PGI_2 [Bruckdorfer *et al.*, 1985, Colli *et al.*, 1985]. These studies were performed before the full implications of LDL oxidation had been appreciated. Thus, following the argument laid out in the previous chapter, it is reasonable to

postulate that the effects of LDL on platelet sensitivity to PGI_2 were due in part, to partially oxidised forms of LDL.

Although interactions between LDL and PGI_2, with respect to platelet function have been studied by several workers, very little research has addressed the effects of LDL on the sensitivity of platelets to NO. The influence of LDL, in particular mmLDL, on platelet sensitivity to NO may be relevant to platelet function in hyperlipidaemia and vascular disease. Hence, in the following chapter the effects of peroxides (viz. mmLDL) on platelet function were examined. Initially the simplest model peroxide available, hydrogen peroxide, was used. Several studies have demonstrated that H_2O_2 influences platelet function: H_2O_2 enhances aggregation of platelet pre-exposed to agonists [Canoso *et al.*, 1974; Rodvein *et* al., 1976; Del Principe *et* al., 1985], but has also been shown to inhibit their aggregation [Canoso *et al.*, 1974; Holmsen and Robkin, 1977].

The effects of H_2O_2 on platelet aggregation and platelet sensitivity to NO *in vitro* were investigated. As in the previous chapter, two platelet models were used in this study. The plasma environment is rich in antioxidants and free radical scavengers, such as ascorbic acid, SOD, catalase and glutathione peroxidase (GSH Px), which may have important effects on experiments with exogenous NO and H_2O_2 solutions. Using WP, the effects of NO and H_2O_2 could also be studied in the absence of these and other plasma constituents. Platelet aggregation was induced by either thrombin, collagen or ADP and the inhibitory effects of NO and an NO donor, S-nitrosoglutathione (S-NOG), were assessed. The influence of H_2O_2 on these platelet inhibitors was investigated in detail.

4.1.1 Summary of aims.

[1] To assess the effects of H_2O_2 on platelet aggregation in the presence and absence of physiological agonists.

[2] To investigate how the aggregatory effects of H_2O_2 influence platelet sensitivity to NO and the NO donor s-nitrosoglutathione.

[3] To identify the mechanisms by which H_2O_2 exerts its actions on platelets.

4.2 THE INFLUENCE OF HYDROGEN PEROXIDE AND NITRIC OXIDE ON PLATELET FUNCTION.

4.2.1 The direct effects of hydrogen peroxide on platelets.

H_2O_2 (1 - 25μM) was added as a bolus to WP and aggregation assessed after 3min. H_2O_2 (10 - 25μM) induced spontaneous aggregation, but only to a very limited extent. The effects were concentration dependent for H_2O_2, with maximal aggregation 9.3 ± 5.4% induced at 25μM *[Table 4.1]*. Substantial variations in the magnitude of responses were observed between the individual platelet preparations. In a few preparations H_2O_2 had no effect at all. The results were similar to those observed with mmLDL, although the aggregation traces produced by H_2O_2 did not appear as classical primary responses (*see section 2.6.5*).

4.2.2 The effects of hydrogen peroxide on thrombin-stimulated platelets.

The effects of H_2O_2 (1 - 25μM) were tested in combination with sub-threshold concentrations of platelet agonists. WP were 'primed' with thrombin (0.005 - 0.01U/ml) which induced an aggregation response of 4 ± 0.7%. Incubation of H_2O_2 for 1min prior to the addition of thrombin had no effect on aggregation. However when added 1min after thrombin, H_2O_2 enhanced total aggregation significantly *[Table 4.1]*. This potentiation of thrombin activity by H_2O_2 was concentration-dependent and was statistically significant at concentrations between 10 - 25μM *[Table 4.1]*. Maximal effects were obtained with 25μM H_2O_2 (27.6 ± 6.5%) aggregation ($p \leq 0.05$) compared to controls of thrombin alone (4 ± 0.7%).

4.2.3 The effects of hydrogen peroxide on collagen- and ADP- stimulated platelet aggregation.

The effects of H_2O_2 on collagen-stimulated aggregation were studied using the same protocol as for thrombin. Collagen (0.1 - 0.2µg/ml) was used to stimulate the WP and at these concentrations the aggregation responses were small (3.5 ± 2%). H_2O_2 (1 - 25µM) was added to platelets 1min after the addition of collagen and it potentiated aggregation in the concentration range of 5 - 25 µM *[Table 4.1].* The magnitude of the enhancement was concentration-dependent for H_2O_2: 25µM H_2O_2 induced 16.8 ± 2.2% aggregation ($p \leq 0.05$). The effects were less variable at lower concentrations of H_2O_2, with 5µM inducing 12 ± 1.8% aggregation *[Table 4.1].*

H_2O_2 had no effects on aggregation induced by sub-threshold ADP (1 - 2µM).

4.2.4 Investigations into the mechanism by which hydrogen peroxide mediated the potentiation of platelet aggregation.

The mechanism by which H_2O_2 potentiated platelet aggregation was investigated using aspirin-treated platelets (*see section 2.6.4*). WP were stimulated with thrombin (0.005 - 0.01U/ml), which induced very low levels of aggregation, 5 ± 2% (not shown). The addition of H_2O_2 (25µM) 1min later enhanced the aggregation to 31.3 ± 8.2% ($p \leq 0.05$) (not shown) *[Table 4.1].* Aspirin treatment of WP did not change significantly the aggregation induced by thrombin alone (6.3 ± 8%). H_2O_2 added to aspirin-treated platelets, which had been stimulated with thrombin, failed to significantly enhance aggregation. Total aggregation induced by thrombin and supplementary H_2O_2, in aspirin treated platelets, was 8 ± 2.7%, significantly lower than in the absence of aspirin ($p \leq 0.05$) [*Table 4.1*]. These data suggest H_2O_2 potentiates platelet aggregation via a cyclo-oxygenase-dependent mechanism.

H_2O_2 (μM)	no agonist	thrombin (0.005U/ml)	thrombin (0.005U/ml) + aspirin	collagen (0.1 μg/ml)
	% total aggregation			
0		4 ± 0.7	6.3 ± 1.8	3 ± 2
1	3.5 ± 1.5	4 ± 0.9	nd	4 ± 1.2
5	4 ± 0.7	7.2 ± 1.2	nd	12 ± 0.8*
10	4.5 ± 0.8	13.6 ± 2.6*	nd	16.2 ± 2.8**
15	5.8 ± 1.1	22.6 ± 6.2*	nd	15 ± 2.4**
25	9.3 ± 5.4	27.6 ± 6.5**	8 ± 2.7	16.8 ± 2.6**

Table 4.1: The effects of hydrogen peroxide on platelet aggregation induced by collagen and thrombin.

H_2O_2 was added directly to WP, from a diluted stock solution, either the the absence or presence of platelet agonists. In the experiment using platelet agonists, WP were stimulated with either thrombin (0.005U/ml) or collagen (0.1μg/ml) and left for 1min before the addition of H_2O_2, aggregation was measured 3min later. The results are presented as % total aggregation compared to thrombin or collagen alone and are expressed as mean ± SEM of 4 individual experiments. * $p< 0.05$, ** $p< 0.01$.

4.3 NITRIC OXIDE AS AN INHIBITOR OF PLATELET AGGREGATION.

NO solutions have been shown to inhibit platelet function *in vitro* [Radomski *et al.*, 1987c]. In the following sections the inhibitory effects of NO were tested against both thrombin- and collagen-induced aggregation. WP were stimulated with sub-maximal concentrations of agonists in the presence of NO solutions. When assessing the actions of NO, two parameters were considered: the concentration of NO required to induce 50% inhibition (IC_{50}) and the minimal concentration required to elicit complete inhibition. The resultant inhibition curves were used as a reference guide for the effects, if any, of H_2O_2.

4.3.1 The measurement of nitric oxide concentrations in solution.

The concentrations of NO solutions were checked by the Griess reaction (*see section 2.7.2*). This measured the nitrite (NO_2^-) ion concentration in any given solution. However, the lower limit of detection of the assay is only 1μM and therefore only NO solutions of higher concentrations could be tested using this method.

Solutions of 10 and 20μM NO were prepared, which were diluted ten-fold in the volume of the assay. The results demonstrated that in 4 individual solutions of each concentration, the expected and actual concentrations were very similar. In the case of solutions proposed to be 1μM the actual concentrations were 0.9 ± 0.05, while the range for the 2μM solutions was 1.9 ± 0.1μM. The results demonstrate that the technique used to prepare NO solutions was reliably accurate.

4.3.2 The effect of nitric oxide solutions on thrombin and collagen-induced platelet aggregation.

NO (0 - 5μM) was added as a bolus to WP and incubated for 1min prior to the addition of thrombin (0.02U/ml). NO (1nM) failed to influence thrombin-induced aggregation. However above this concentration, NO elicited a concentration-dependent inhibition of aggregation. The IC_{50} for NO against thrombin was 95 ± 40nM and complete inhibition achieved at 1.8 ± 0.5μM *[Figure 4.1].* NO also induced a concentration-dependent inhibition of collagen (0.5μg/ml)-stimulated aggregation. The IC_{50} for NO against collagen was 16.8 ± 3.3nM and complete inhibition achieved at 125 ± 50nM *[Figure 4.1].* These data indicate that inhibition mediated by NO was more effective against platelet activity induced by collagen than thrombin, this difference is possibly due to the production of endogenous NO by collagen-stimulated platelets.

4.4 THE INFLUENCE OF HYDROGEN PEROXIDE ON PLATELET INHIBITION BY NITRIC OXIDE.

4.4.1 Nitric oxide mediated inhibition of thrombin- and collagen-induced aggregation: the influence of supplementary hydrogen peroxide.

The influence of H_2O_2 on the inhibition of platelet aggregation by NO was investigated. NO (0 - 10μM) was incubated for 1min with WP before addition of thrombin (0.02U/ml), H_2O_2 was added 1min after the agonist and aggregation measured 3min later. The presence of H_2O_2 (0-25μM) antagonised the inhibitory actions of NO: the IC_{50} for NO was elevated and the NO concentration required to induce complete inhibition of aggregation was increased *[Figure 4.2].* These effects of H_2O_2 were concentration-dependent, but only significant at the highest concentration of H_2O_2 tested. H_2O_2 (25μM) increased the IC_{50} to 3.6 ± 0.8μM NO

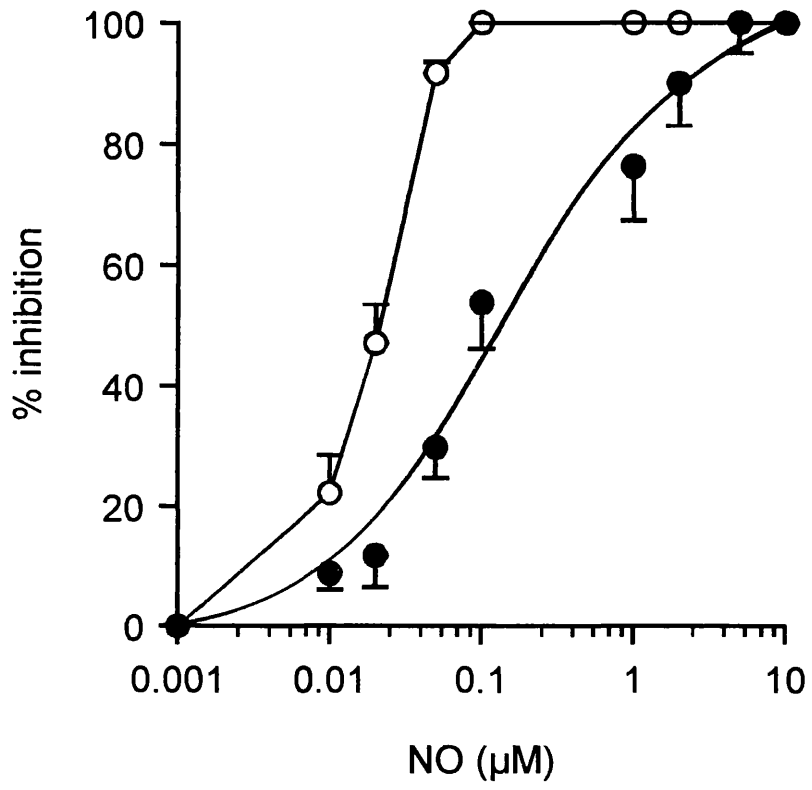


Figure 4.1: Concentration response curves for nitric oxide against both thrombin- and collagen-induced aggregation.

WP were incubated with NO solutions (0.001 - 10μM), added as a bolus, 1min prior to the addition of thrombin (0.02U/ml) (●) or collagen (0.5μg/ml) (○), and total aggregation measured 3min after the addition of the agonist. The results represent the percent inhibition of aggregation, compared to controls of inactive NO solutions. The data are expressed as mean ± SEM of 4 individual experiments.

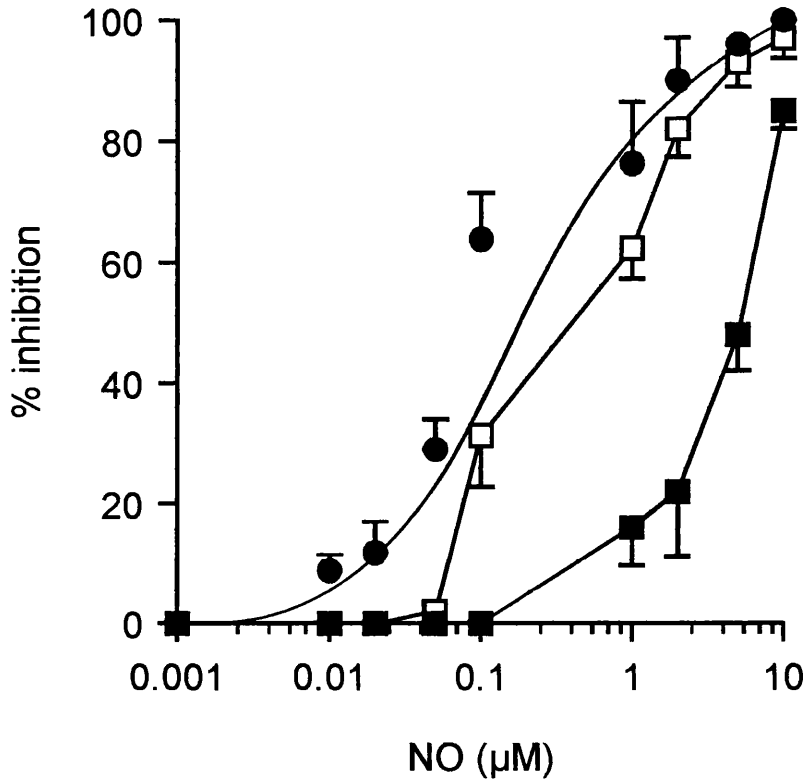


Figure 4.2: Concentration response curves for nitric oxide against thrombin-induced aggregation: effects of supplementary hydrogen peroxide.

WP were incubated with NO solutions (0.001 - 10μM), added as a bolus, for 1min prior to the addition of thrombin (0.02U/ml) (●), after a further 1min H_2O_2 (supplementary) was added at a concentration of either 25μM (■) or 5μM (□) and the total aggregation measured after a further 3min. The results represent the percent inhibition of aggregation, and are expressed as mean ± SEM of 4 individual experiments. For statistical analysis refer to text (*section 4.4.1*).

($p \leq 0.05$) compared to 110.0 ± 30nM NO against thrombin alone [*Figure 4.2*]. This represented a 30-fold increase in the requirement of NO. Complete inhibition was achieved at 3.25 ± 1.1μM NO, significantly greater than the NO concentration required in the absence of H_2O_2 ($p \leq 0.05$). H_2O_2 was most effective against low concentrations of NO, in some cases completely abolishing any inhibitory effects.

The experiments were repeated with collagen (0.5μg/ml)-stimulated WP. H_2O_2 again decreased the inhibitory actions of NO *[Figure 4.3]*. However, NO was much more potent against collagen-induced aggregation than was the case with thrombin. H_2O_2 (5μM) increased the IC_{50} for NO to 55 ± 10nM NO from 19 ± 5nM ($p \leq 0.05$), a 2.5-fold decease in the effectiveness of NO. Higher concentrations of the peroxide (15 and 25μM) did not significantly change the IC_{50} (data not shown) for NO and complete inhibition was not significantly altered at 175 ± 50nM NO (compared to 125 ± 20nM) *[Figure 4.3]*. The reduced actions of H_2O_2 against NO-mediated inhibition of collagen-induced aggregation, may reflect an influence of endogenously produced NO.

4.5 POSSIBLE DIRECT INTERACTIONS OF NITRIC OXIDE AND HYDROGEN PEROXIDE.

In the above sections, it was demonstrated that H_2O_2 antagonised the inhibitory actions of NO, when added post-agonist. In these experiments, the intracellular concentration of cGMP would be expected to be elevated before the addition of the peroxide, since NO and NO-donors increase platelet cGMP within 15sec of exposure of the cells to the antagonist [Salvemini *et al.*, 1990; Nguyen *et al.*, 1991]. The question arises as to whether the antagonism of NO by H_2O_2 was

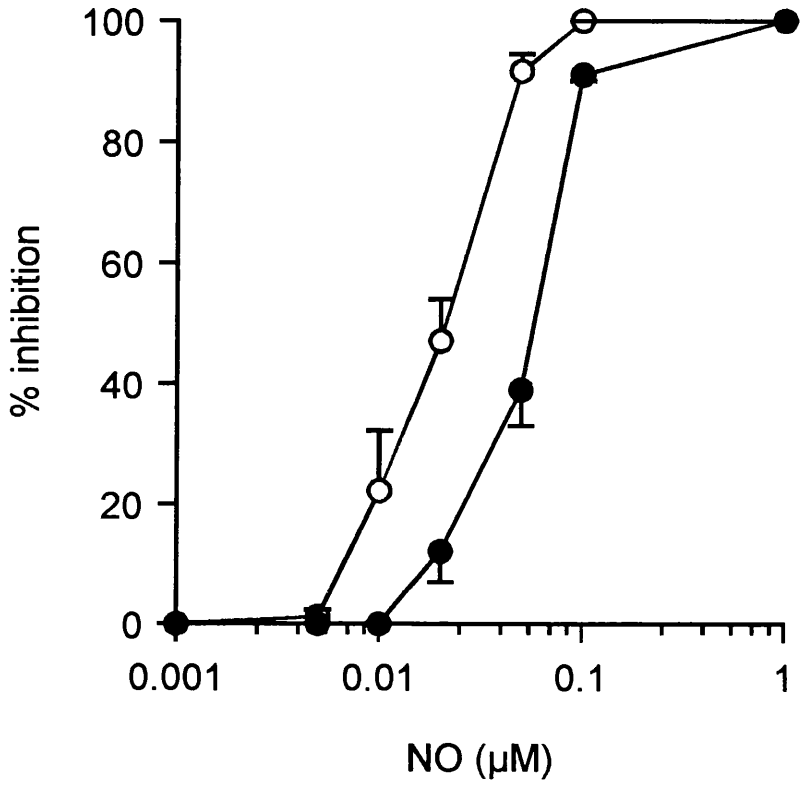


Figure 4.3: Concentration response curves for nitric oxide against collagen induced aggregation: effects of supplementary hydrogen peroxide.

WP were incubated with NO solutions (0.001 - 10μM), added as a bolus, for 1min prior to the addition of collagen (0.5 μg/ml) (○), after a further 1min H_2O_2 was added at a concentration of 5μM (●) and the total aggregation measured after a further 3min. The results represent the percent inhibition of aggregation, and are expressed as mean ± SEM of 4 individual experiments. For statistical analysis refer to text (*section 4.4.2*).

due to an affect of the peroxide on secondary messenger pathways, for example, alteration of phosphodiesterase activity, or was a consequence of a direct interaction between NO and H_2O_2. NO and H_2O_2 have been shown to react directly to form singlet oxygen [Noronha-Dutra *et al.*, 1992]. Thus, in the following experiments NO and H_2O_2 were added simultaneously to isolated platelets to investigate their effects on platelet aggregation.

4.5.1 The influence of nitric oxide and hydrogen peroxide added simultaneously on agonist-induced platelet aggregation.

H_2O_2 (5 - 25μM) was added simultaneously with NO (0 - 1μM) to WP in the absence of agonists and left for 3min after which no aggregation response was observed. If NO/H_2O_2 were incubated for 1min with WP prior to the addition of thrombin (0.02U/ml), the inhibition of aggregation was to a greater extent than with NO alone *[Figure 4.4b,c,d]*. The effects were concentration-dependent for H_2O_2, with 25μM generating the largest effect. In the presence of H_2O_2 (5μM) the IC_{50} for NO against thrombin-stimulated platelet activity was reduced from 110 ± 35nM to 38.5 ± 6.5nM ($p \leq 0.05$) and with H_2O_2 (15μM) the IC_{50} for NO was further reduced to 6.5 ± 2.5 ($p \leq 0.01$). When H_2O_2 (25μM) was added with NO the peroxide reduced the IC_{50} for NO to approximately 2 ± 1nM *[Figure 4.5]*. This is an approximate value as in some cases the inhibitory effects induced by NO/H_2O_2 began at greater than 50% inhibition. In these instances, the IC_{50} was taken as 1nM NO. The inhibitory actions of NO with all concentrations of H_2O_2 were so potent that NO solutions could not be accurately prepared at sufficiently low concentrations to abolish the inhibition (lowest final concentration 1nM NO).

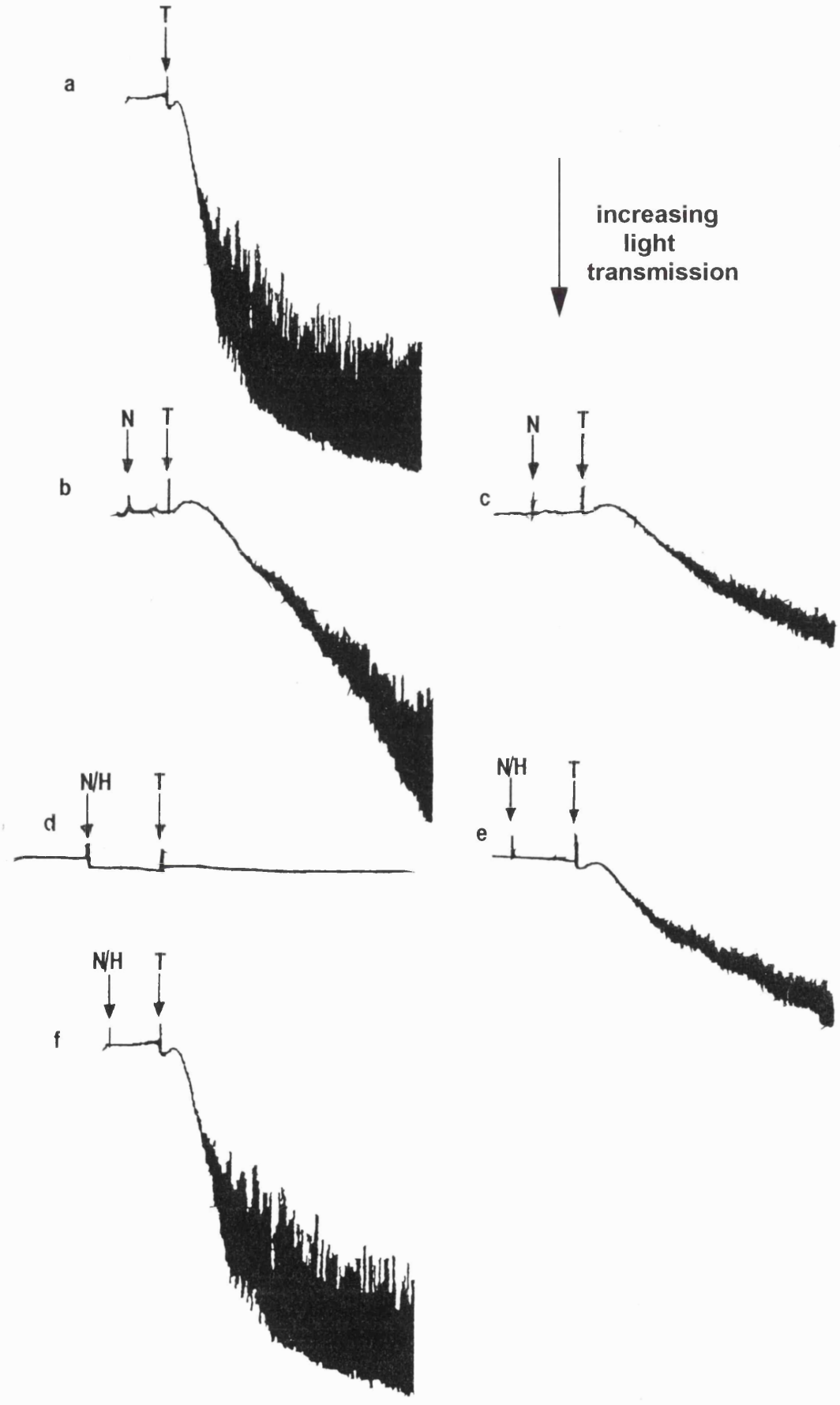
The experiments were repeated using fixed concentrations of NO (1 and 10nM)

Figure 4.4: Typical aggregation traces demonstrating the effects of nitric oxide and hydrogen peroxide on platelet aggregation.

(a) WP were incubated with thrombin (0.02U/ml) for 3min.
(b) NO (10nM) was added to WP 1min before the addition of thrombin.
© NO (100nM) was added to WP 1min before the addition of thrombin.
(d) NO (10nM) + H_2O_2 (25μM) were added to WP 1min before the addition of thrombin.
(e) NO (1nM) + H_2O_2 (25μM) were added to WP 1min before the addition of thrombin.
(f) NO (10nM) + H_2O_2 (25μM) were added to WP 30min before the addition of thrombin.

T: thrombin, N:, nitric oxide, H: hydrogen peroxide

The results are typical of at least 4 independent experiments.


T
a
increasing
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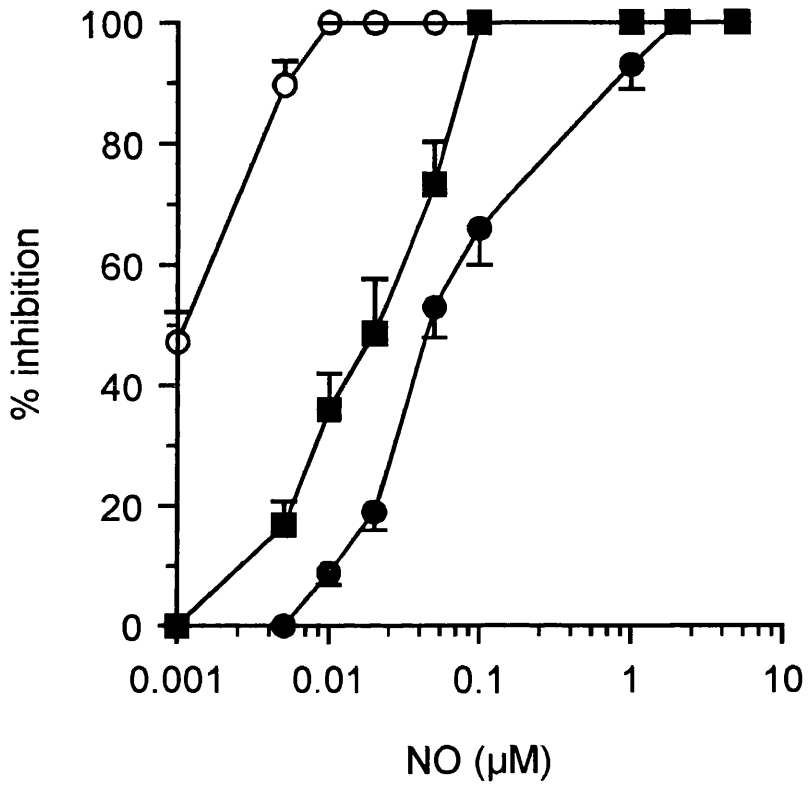


Figure 4.5: Hydrogen peroxide enhances the inhibition of thrombin-induced platelet aggregation by nitric oxide.

A range of concentrations of NO were added simultaneously with H_2O_2, from separate syringes, to WP before the addition of thrombin (0.02U/ml). The data are presented as concentration response curves for NO only (●), and NO in combination with H_2O_2 at either 25μM (○) or 5μM (■). The results represent the percent inhibition of aggregation and are expressed as mean ± SEM of 6 individual experiments. For statistical analysis refer to text (*section 4.5.1*).

and variable concentrations of H_2O_2 (0 -25μM). This was to confirm previous findings and, in addition, to explore the concentration range for H_2O_2 at which the effects were detectable. NO and H_2O_2 were added simultaneously, and in all cases inhibited platelet aggregation to a greater extent than NO alone *[Figure 4.6]*. With NO (1nM) the EC_{50} for H_2O_2 was 4.9 ± 2.3μM and in combination with H_2O_2 (1μM) induced 7.0 ± 3.5% inhibition. These results showed H_2O_2 to influence the action of NO at very low concentrations, within the physiological range [Test and Weiss, 1984], and to enhance a normally inactive concentration of NO (1nM) *[Figure 4.4e]*. The inhibitory actions were even greater with NO (10nM), with an EC_{50} for H_2O_2 of 1.6 ± 0.5μM *[Figure 4.6]*. EC_{50} (effective concentration) was used to describe the effects of H_2O_2 in this instance, as the peroxide seemed to play a positive role in the actions of NO.

These results show a potent inhibition of aggregation by NO and H_2O_2, the actions of which are in complete contrast to the observations when H_2O_2 was added after NO and thrombin (*see section 4.4.1*).

4.5.2 The effects of inactive nitric oxide and hydrogen peroxide on platelet aggregation.

To establish whether contaminants of the two solutions could have influenced the previous observations, experiments were performed with inactive solutions. NO solutions left exposed to air for 15min prior to addition to WP, failed to induce inhibition of thrombin (0.02U/ml)-stimulated aggregation either alone, or in combination with H_2O_2. Similarly H_2O_2 which had been stirred and exposed to air overnight or boiled for 30min, did not potentiate sub-maximal thrombin-induced aggregation or enhance the inhibitory actions of NO. This indicates that

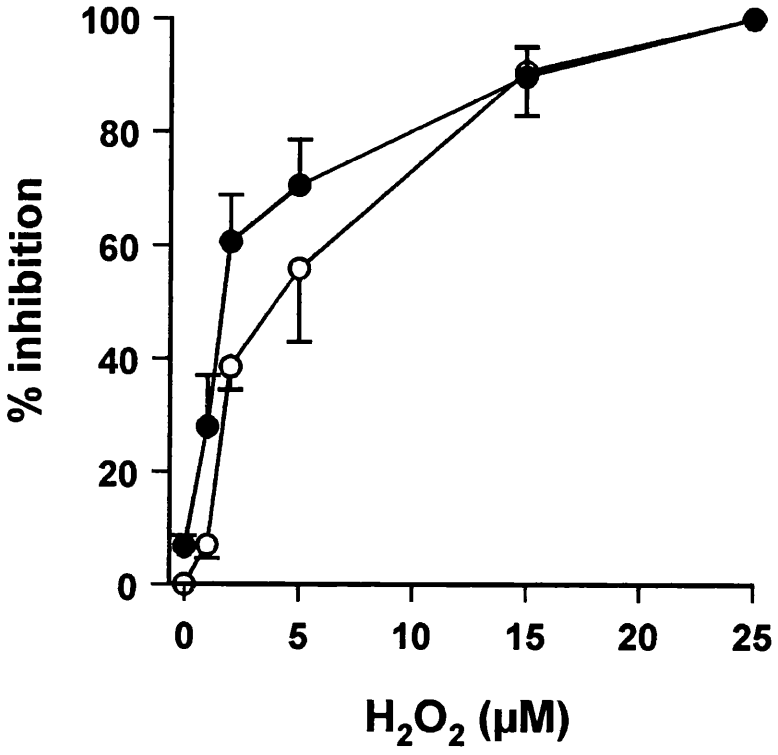


Figure 4.6: Concentration response relationship between nitric oxide and hydrogen peroxide on inhibition of thrombin induced platelet aggregation, when added simultaneously.

NO and H_2O_2 were added simultaneously, from separate syringes, to WP 1min before the addition of thrombin (0.02U/ml). Two fixed concentrations of NO, 1nM (○) and 10nM (●), were used in combination with a range of concentrations of H_2O_2. The results represent the percent inhibition of aggregation and are expressed as mean ± SEM of 4 individual experiments. For statistical analysis refer to text (*section 4.5.1*).

both active NO and H_2O_2 were required for the powerful inhibitory actions observed in previous experiments. In addition, the breakdown products of NO, NO_2^- and NO_3^- (*see section1.6.4.4*), or other possible contaminants were not responsible for the protracted effects.

4.5.3 Recovery of platelets from the effects of nitric oxide or nitric oxide with hydrogen peroxide.

To ascertain whether the enhanced inhibition of aggregation by NO in the presence of H_2O_2 was not simply a cytotoxic effect, the inhibitory effects NO/H_2O_2 were studied as a function of time.

NO (10nM, 100nM, 1μM) or NO (10nM)/H_2O_2 (5, 15, 25μM) were added to WP and left stirring for 1min, and incubated at 37°C for up to 1h before stimulation with thrombin (0.02U/ml). The magnitude and longevity of the NO mediated inhibition was concentration dependent. NO (10nM) after 1min incubation induced 8.5 ± 3.3% inhibition, which was lost after 10min. NO (1μM) induced the greatest inhibition, 83.3 ± 6.3%, which persisted until 30min (19.8 ± 13.3%), but was not detected at 60min.

Inhibition induced by simultaneous addition of NO/H_2O_2 was more effective and persisted longer than 10nM NO alone [*Figure 4.7*]. After 30min incubation NO (10nM) had no effect on platelet activity. However, in the presence of H_2O_2 the inhibition still persisted. At this time point, NO, in combination with H_2O_2 (25μM) induced 59 ± 7.3% inhibition ($p \leq 0.01$). The effects with H_2O_2 (15 and 25μM) in combination with NO (10nM) were significantly greater than inhibition of aggregation by NO (100nM) alone ($p \leq 0.05$). When H_2O_2 was tested in the

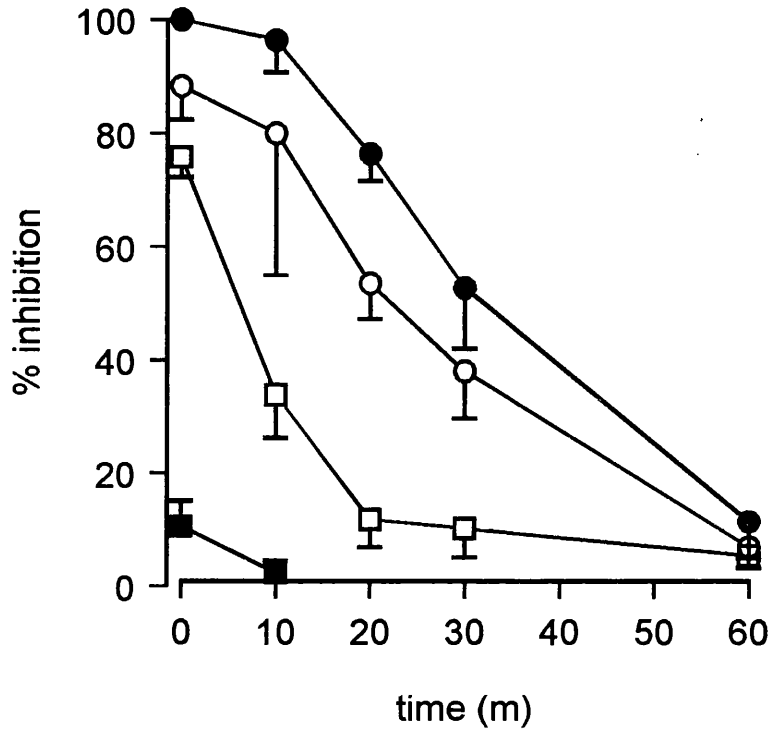


Figure 4.7: The recovery of platelets from inhibition induced by nitric oxide in combination with hydrogen peroxide.

NO only or, NO in combination with H_2O_2 were added to WP and left for up to 1h before stimulation with thrombin (0.02U/ml). A fixed concentration of NO (10nM) was added to WP alone (■), or simultaneously with H_2O_2 at 5μM (□), 15μM (○) and 25μM (●). Total aggregation was measured 3min after the addition of the agonist. The results represent the percent inhibition of aggregation and are expressed as the mean ± SEM of 4 individual experiments. For statistical analysis refer to text (*section 4.5.3*).

absence of NO, it failed to affect aggregation compared to the agonist alone. The results are summarised in *Figure 4.7.*

All WP treated with mixtures of NO/H_2O_2 fully recovered functional viability after 60min incubation. No differences in thrombin-induced aggregation responses could be detected between WP treated with Tyrode's HEPES buffer (*see section 2.3.2*) or NO/H_2O_2 *[Figure 4.4a, e].* This indicates that NO/H_2O_2 in combination are not cytotoxic, and H_2O_2 acts to both intensify and prolong the effects of NO.

4.5.4 The effects of premixing nitric oxide and hydrogen peroxide before their addition to platelets.

The protracted inhibition of aggregation induced by NO/H_2O_2 added simultaneously, raised the possibility that a new free radical species may be formed from a reaction between the two mediators. The new species may possess different characteristics to that of authentic NO. This was tested by pre-mixing NO and H_2O_2 in stirred Tyrode's HEPES buffer (*see section 2.3.2*) for up to 60sec before aliquots were transferred to WP. Mixing was performed at 37°C in a separate aggregation cuvette. WP were then left for 1min after the addition of the mixture before being challenged with thrombin (0.02U/ml). The time for transfer between cuvettes was approximately 2 - 3s which was additional to the stated pre-mixing times.

NO (10nM) induced 12.5 ± 3.7% inhibition when added directly to WP. However, after 20s premixing addition of NO (10nM) failed to affect aggregation. In contrast, in the presence of H_2O_2 there was a significant increase in both the magnitude and longevity of the inhibitory actions of NO *[Figure 4.8].* The effects

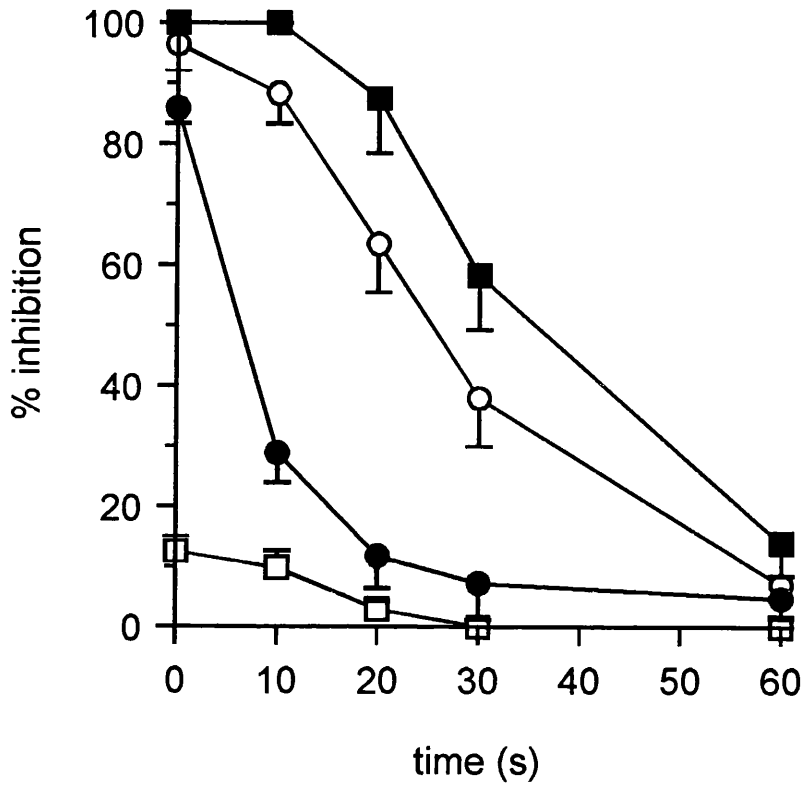


Figure 4.8: The effect of premixing of nitric oxide and hydrogen peroxide on the inhibition of platelet aggregation stimulated by thrombin.

NO alone or, NO in combination with H_2O_2 were premixed in Tyrodes HEPES buffer for up to 1min before their addition to WP. NO was used at a fixed concentration of 10nM (□), or in combination with H_2O_2 at concentrations of 5μM (●), 15μM (○) and 25μM (■). An aliquot of the mixture was added to WP and left stirring for 1min before the addition of thrombin (0.02U/ml), total aggregation was measured 3min after addition of the agonist. The time scale excludes the transfer time (2-3s) of the aliquots from the mixing cuvette to the platelets. The results represent the percent inhibition of aggregation and are expressed as the mean ± SEM of 4 individual experiments. For statistical analysis refer to text (*section 4.5.4*).

were concentration-dependent for H_2O_2 with 25μM exerting the greatest influence. NO(10nM) pre-mixed with H_2O_2 (25μM) for 20s induced 87.5 ± 9% inhibition ($p \leq 0.005$, compared to NO alone) and was still active after 60s premixing (13.9 ± 4.3%). H_2O_2 (5 and 15μM) in combination with NO also exerted significant inhibitory effects after 20s pre-mixing, 11.8 ± 3.9% ($p \leq 0.01$, compared to NO alone) and 63.5 ± 8% ($p \leq 0.05$, compared to NO alone) respectively, compared to NO alone *[Figure 4.8]*. All combinations of NO and H_2O_2 still had slight inhibitory actions after 60s pre-mixing compared to controls, where NO was added with Tyrode's HEPES buffer. This suggested that a new free radical species may have been formed, but further investigations were required to substantiate this.

4.6 THE INFLUENCE OF PEROXYNITRITE ON PLATELET AGGREGATION.

The interaction of NO and H_2O_2 in solution may produce peroxynitrite, via formation of nitrous acid, although this has not been reported to occur at neutral pH [Beckman, 1990] (*see below*). Peroxynitrite is produced *in vivo* by a reaction between NO and $O_2^{\cdot -}$ (*see section1.6.6.4*)

$$H_2O_2 + HNO_2 \rightarrow ONOO^- + H_2O + H^+$$

Peroxynitrite solutions were prepared (*see section 2.8*) and their effects on platelet aggregation were tested. Peroxynitrite (0.01 - 100μM) was added to WP and no aggregation observed after 3min incubation. Incubation of peroxynitrite for 1min prior to addition of thrombin (0.02U/ml) resulted in a concentration-dependent inhibition of platelet aggregation *[Figure 4.9]*. The IC_{50} for peroxynitrite against thrombin-stimulated platelets was 3.1 ± 1.1μM, which was

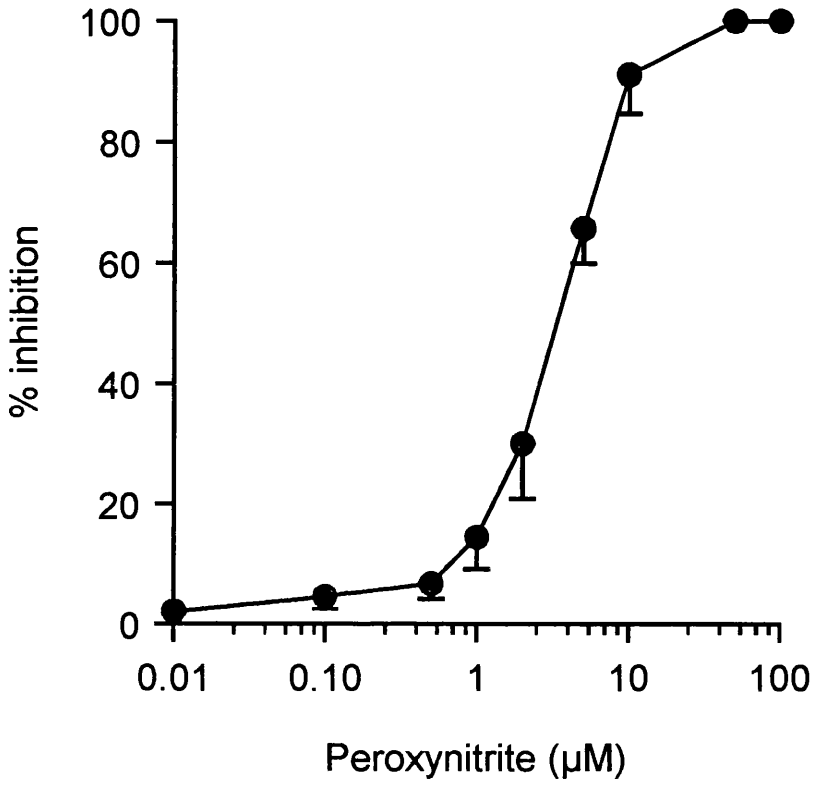


Figure 4.9: The effect of peroxynitrite solutions on platelet aggregation stimulated by thrombin.

Peroxynitrite, diluted from a stock solution immediately before use, was added to WP over a range of concentrations 1min before the addition of thrombin (0.02U/ml). Total aggregation was measured 3min after the addition of the agonist. The results represent the percent inhibition of aggregation, and are expressed as mean ± SEM of 4 individual experiments. For statistical analysis refer to text (*section 4.6*).

approximately 30 times less effective than NO (IC_{50} 110 ± 35nM) and 3000 times less effective than NO (10nM) in combination with H_2O_2 (25μM) *[Figure 4.9]*. Controls of decomposed peroxynitrite (*see section 2.8*) had no effect on platelet function. This suggested the inhibitory actions of NO/H_2O_2 were not due to formation of peroxynitrite.

Peroxynitrite, once prepared, was stored in its anionic form at alkaline pH. To check for the influence of pH on platelet function, aliquots of known concentrations of peroxynitrite were added to stirred Tyrode's HEPES buffer (*see section 2.3.2*) and the pH was monitored continuously. No significant change in pH was observed at concentrations below 100μM peroxynitrite, which suggested the inhibitory action of peroxynitrite was not mediated by an increase in pH (*not shown*).

4.7 THE INFLUENCE OF FREE RADICAL SCAVENGERS, ENZYMES, METAL CHELATORS AND NITRIC OXIDE QUENCHERS ON NITRIC OXIDE AND HYDROGEN PEROXIDE MEDIATED INHIBITION.

Results from previous sections suggested inhibition of platelet activity induced by simultaneous addition of NO and H_2O_2 may be due to a new radical species with different properties than NO or peroxynitrite. This was further investigated by addition of free radical scavengers. Specific radical scavengers were incubated with WP for 1min at 37°C before the addition of either NO(10nM), NO/H_2O_2 (10nM/25μM) or peroxynitrite (5μM). WP were stimulated with thrombin (0.02U/ml) and aggregation measured after 3min. Addition of Tyrode's HEPES buffer (*see section 2.3.2*) instead of a scavenger was used as a control.

(I) Sodium urate: a hydroxyl radical scavenger.

Sodium urate (0.5mM) when added directly to WP did not induce aggregation and had no effect on aggregation stimulated by thrombin (0.02U/ml). However, the presence of sodium urate (0.5mM) significantly decreased the inhibition of aggregation induced by both NO/H_2O_2 (89 ± 5.4%) and peroxynitrite (73.3 ± 6.2%), but had no effect on inhibition by NO alone *[Table 4.2]*. The levels of inhibition elecited by NO/H_2O_2 and peroxynitrite in the presence of urate were 65.6 ± 7.9% ($p \leq 0.01$) and 43.0 ± 5.1% ($p \leq 0.005$) respectively, significantly less than in the absence of urate *[Table 4.2]*. Sodium urate was more effective against peroxynitrite than NO/H_2O_2.

(ii) Mannitol: a hydroxyl radical scavenger

Thrombin- (0.02U/ml) induced aggregation was not affected by the presence of mannitol (50mM). Mannitol pre-incubated with WP reduced the inhibition of platelet aggregation elicited by both NO/H_2O_2 and peroxynitrite, but as in the case of sodium urate it was more effective against peroxynitrite. Inhibition by NO/H_2O_2 and peroxynitrite were 60.5 ± 5 % ($p \leq 0.01$) and 50.5 ± 4.6% ($p \leq 0.005$) respectively, a significant reduction of inhibition compared to controls *[Table 4.2]*. Mannitol had no effects on NO-mediated inhibition.

(iii) Ascorbate.

Ascorbate had no effect on inhibition of aggregation induced by either NO, NO/H_2O_2 or peroxynitrite *[Table 4.2]*.

(iv) Catalase and superoxide dismutase.

WP were incubated with catalase for 1min prior to addition of NO/H_2O_2. The

Addition	% inhibition of aggregation		
	NO (10nM) + H_2O_2 (25µM)	peroxynitrite (5µM)	NO (200nM)
buffer	89 ± 5.4	73.3 ± 6.2	42
Na urate (0.5mM)	65.6 ± 7.9	43 ± 5.1	38
mannitol (50mM)	60.5 ± 6	50.5 ± 4.6	45
ascorbate (0.2mM)	84.3 ± 7.8	50.5 ± 4.6	30
SOD (100 U/ml)	89.5 ± 4.4	74.9 ± 5.3	32
catalase (100 U/ml)	57.5		
DTPA (10µM)	86		

Table 4.2: The influence of reagents which suppress radical oxygen species on the effects of nitric oxide and hydrogen peroxide on platelet aggregation.

NO was added simultaneously with H_2O_2, from separate syringes, to WP before the addition of thrombin (0.02U/ml). WP were preincubated with the individual radical scavengers for 1min prior to testing the effects of NO and H_2O_2. The results represent the percent inhibition of aggregation and are expressed as mean ± SEM of 5 individual experiments and in some cases only two individual experiments. For statistical analysis refer to text (*see secton 4.7*).

presence of catalase (100units) reduced the inhibition of thrombin-mediated aggregation from 100% to 57.5%, but had no effect on the actions of peroxynitrite. SOD (100units) had no effect on inhibition of aggregation induced by either NO, NO/H_2O_2 or peroxynitrite *[Table 4.2]*.

(v) Diethenetriaminepentaacetic acid: a metal ion chelator.

DTPA was shown not to influence agonist-induced aggregation of platelets (*see section 3.6*). Incubation of DTPA with WP for 1min prior to addition of NO or NO/H_2O_2 did not affect inhibition of thrombin-stimulated aggregation. This indicates that the effects of NO and H_2O_2 were not catalysed by metal ions *[Table 4.2]*.

(vi) Carboxy-PTIO: an NO sequestrant.

Carboxy-PTIO reacts with NO stoichiometrically (carboxy-PTIO/NO = 1.0) in neutral solution, antagonising the biological effects of NO [Akaike *et al.*, 1993].

Carboxy-PTIO (1μM) was incubated with WP for 30s prior to the addition of NO. The presence of carboxy-PTIO significantly decreased the inhibitory actions of NO on thrombin-induced aggregation compared to controls (WP without carboxy-PTIO) *[Figure 4.10]*. NO (100nM) induced 52.5 ± 6.4% inhibition which was reduced to 2.5 ± 2.2% in the presence carboxy-PTIO. At higher concentrations of NO, the carboxy-PTIO was less effective. For example, NO (1μM) induced 93.1± 4.6% inhibition which was reduced to 35.7 ± 7.4% ($p \leq 0.01$). Although the carboxy-PTIO significantly reduced the actions of NO, it seemed in the present system that carboxy-PTIO did not work in a completely stoichiometrical manner and only induced complete abolition of NO as a ten-fold

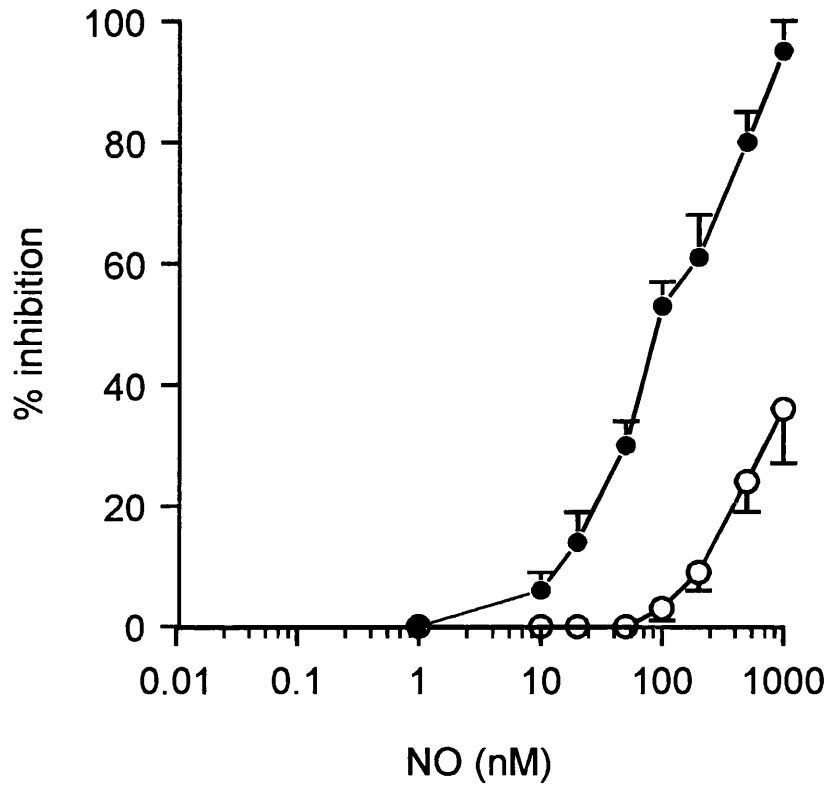


Figure 4.10: The effects of the nitric oxide quencher, carboxy-PTIO, on the inhibition of thrombin-induced platelet aggreagtion by nitric oxide.

A range of concentrations of NO were added to WP, in the presence (O) or absence (●) of carboxy-PTIO (1μM), 1min before stimulation by thrombin (0.02U/ml). Total aggregation was measured 3min after the addition of the agonist. The data are presented as the percent inhibition of aggregation and represent the mean ± SEM 3 independent experiments. For statistical analysis refer to text (*section 4.7*).

excess *[Figure 4.10]*.

Carboxy-PTIO (100nM), preincubated with platelets before the addition of NO(10nM)/H_2O_2(25μM), antagonised the inhibition of platelet activity. NO/H_2O_2 induced 89.5 ± 1.4% inhibition, which was significantly reduced to 12.0 ± 9.3% ($p \leq 0.001$) in the presence of carboxy-PTIO. In addition, when NO and H_2O_2 were premixed for 10s and carboxy-PTIO was added to the platelets simultaneously with the inhibitors, the inhibition was reduced from 94 ± 7 to 24 ± 4%. These results indicate that although other free radicals may be involved in the mechanism of inhibition by NO/H_2O_2, NO was still of major importance and suggested that H_2O_2 interacts synergistically with NO rather than reacting to form a free radical species other than NO.

4.8 THE INFLUENCE OF NITRIC OXIDE AND HYDROGEN PEROXIDE ON PLATELET GUANYLATE CYCLASE ACTIVITY.

NO exerts its cellular effects by the stimulation of soluble guanylate cyclase and a subsequent increase in cGMP [Mellion *et al.*, 1983, Radomski *et al.*,1990a, b]. Therefore, the possibility that the protracted inhibition of aggregation by NO/H_2O_2 is mediated by enhanced formation of cGMP was investigated.

4.8.1 Does inhibition of platelets by nitric oxide and hydrogen peroxide involve enhanced cGMP formation?

NO, NO/H_2O_2 and H_2O_2 were added to WP under the same conditions as the aggregation experiments, and the cGMP concentrations measured by RIA (*see section 2.9*). The platelets were pre-incubated with IBMX for 30min prior to experimentation. cGMP concentrations in platelets incubated with buffer alone

were 0.39 ± 0.07 pmol cGMP/1 x 10^8 platelets, and this was taken as the basal concentration of cGMP [*Table 4.3*]. NO (0.01 - 1μM) induced a significant concentration-dependent increase in cGMP basal levels. The concentrations of cGMP induced by NO (1μM) was 3.66 ± 0.5pmol/1 x 10^8 platelets ($p \leq 0.01$) [*Table 4.3*].

Simultaneous addition of NO(10nM) with H_2O_2 (1 - 25μM) increased the concentrations of cGMP, and significantly increased the concentration of the cyclic nucleotide levels induced by NO(10nM) alone. The effects of H_2O_2 were concentration-dependent and were significant at all concentrations except the lowest, 1μM. cGMP concentrations in platelets treated with NO/H_2O_2 (25μM) was 1.45 ± 0.21($p \leq 0.01$) compared to 0.64 ± 0.06pmol for platelets treated with NO only. H_2O_2 (15 and 25μM) elicited a 2-fold increase in intraplatelet cGMP levels The addition of H_2O_2 alone appeared to cause only a slight increase in cGMP, but the effects were not significant *[Table 4.3]*.

4.8.2 The influence of hydrogen peroxide on platelet inhibition by dibutryl-cGMP.

Dibuytryl cGMP (dB-cGMP) is a non-hydrolysable analogue of cGMP, which can enter cells. Once inside, the buytryl groups are cleaved and the cGMP becomes metabolically active. WP were incubated with dB-cGMP (0.001 - 5.0μM) for 1min prior to the addition of thrombin (0.02U/ml). Db-cGMP caused a concentration-dependent inhibition of the normal agonist induced response. The IC_{50} for dB-cGMP inhibition of thrombin-stimulated aggregation was 200 ± 50μM *[Figure 4.11]*. To replicate the conditions of the previous experiments examining the influence of H_2O_2 on the inhibitory actions of NO, H_2O_2 was added to WP

addition	NO or H_2O_2 (μM)	cGMP (pmols/ 1 x 10^8 platelets)
control (buffer)		0.39 ± 0.07
NO	0.01	0.64 ± 0.06#
	0.1	1.36 ± 0.22#
	1	3.66 ± 0.5#
H_2O_2	25	0.66 ± 0.19
NO (0.01μM) / H_2O_2 (μM)	1	0.69 ± 0.11
	5	0.79 ± 0.09
	15	1.38 ± 0.36
	25	1.45 ± 0.21**

Table 4.3: The effect of simultaneous addition of nitric oxide and hydrogen peroxide on guanylate cyclase activity in platelets.

NO alone, H_2O_2 alone or NO in combination with H_2O_2 were added to platelets, preincubated with IBMX (100μM), and left stirring for 1min before addition of cold perchloric acid (200μl, 0.5M). The samples were vortexed and stored at -70°C. On the day of the assay the samples were thawed and the acid neutralised using K_2HPO_4 (200μl, 1M). cGMP levels were measured by radioimmunoassay (*see section 2.9*). The data are representativeof 4 individual experiments and expressed as the mean ± SEM pmol cGMP/ 1 x 10^8 platelets. # $p< 0.05$, ** $p< 0.01$.

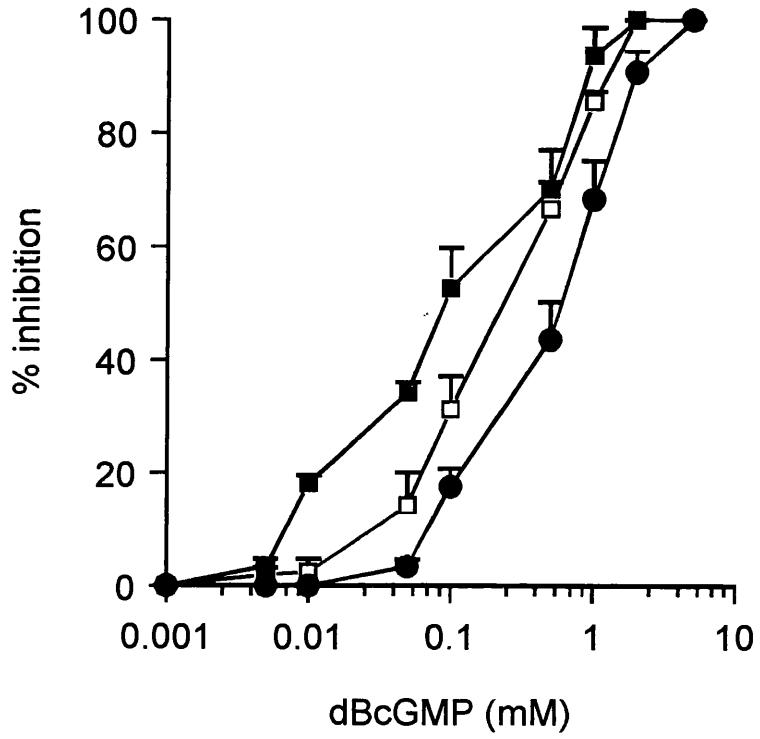


Figure 4.11: The influence of hydrogen peroxide on platelet inhibition by dibutrylcGMP.

WP were preincubated with a range of concentrations of dBcGMP for 1min before the addition of thrombin (0.02U/ml). The experiment were performed in the absence of H_2O_2 (□), or with H_2O_2 (20μM) added simultaneously with dBcGMP (■), or with H_2O_2 added 1min after the thrombin (●). Total aggregation was measured 3min after the addition of the agonist. The data are presented as the percent inhibition of aggregation and are expressed as mean ± SEM of 3 independent experiments. For statistical analysis refer to text (*section 4.8.2*).

either simultaneously with dB-cGMP or 1min after the addition of thrombin (2min after db-cGMP). Simultaneous application of dB-cGMP and H_2O_2 resulted in a small, but significant increase in the effectiveness of db-cGMP *[Figure 4.12]*. The IC_{50} for dB-cGMP against thrombin-induced aggregation was reduced to 80 ± 40μM ($p \leq 0.05$) in the presence of H_2O_2. Conversely, addition of the peroxide after db-cGMP resulted in a decrease in the effects of the platelet inibitor. These latter actions of H_2O_2 were much more powerful than when it acted synergistically with db-cGMP, increasing the IC_{50} to 830 ± 130μM dB-cGMP ($p \leq 0.001$) *[Figure 4.11]*.

The enhancement of dB-cGMP mediated inhibition by H_2O_2 was relatively small compared to the effects of the peroxide on the actions of NO. This suggests that the effects of H_2O_2 are not exerted downstream of the formation of cGMP. In contrast, addition of H_2O_2 after the platelet inhibitor, resulted in the antagonism of cGMP-mediated effects. These data corroborate the previous results which showed that H_2O_2 reduced the effectiveness of NO when added after the main agonist (*see section 4.4.1*).

4.9 INVESTIGATION OF POSSIBLE ARTIFACTS WHICH MAY AFFECT THE SYNERGISM BETWEEN OF NITRIC OXIDE AND HYDROGEN PEROXIDE.

In this section platelets were prepared by different methods than in previous sections to assess whether artifacts inherent in the methods were responsible for the novel effects observed with H_2O_2.

(I) Comparison between platelets isolated using prostacyclin and low pH.

Platelets isolated by the method of Vargas *et al.*, [1981], as used in this study, relied on a transient increase in intracellular cAMP, evoked by PGI_2, to facilitate the isolation procedure. It is therefore possible that the effects of NO/H_2O_2 reported in this chapter may have involved residual levels of PGI_2-induced cAMP. This was investigated by direct comparison of the inhibitory actions of both NO and NO/H_2O_2 on autologous PGI_2 isolated platelets (WP-PGI_2) and pH isolated platelets (WP-pH) (*see section 2.6.3*). In both preparations NO (0.001 - 10μM) induced concentration dependent inhibition of thrombin (0.02U/ml)-induced aggregation with little difference in the IC_{50}. For WP-PGI_2 and WP-pH the IC_{50}s were 80nM and 90nM NO respectively. Similarly inhibition of platelet activity induced by NO(10nM)/H_2O_2(25μM) were almost identical with both preparations, 80 and 84% inhibition for WP-PGI_2 and WP-pH respectively. This indicated that residual cAMP did not significantly influence the inhibition by NO/H_2O_2.

(ii) Comparison of different platelet buffers.

WP used in this study were normally resuspended in a HEPES based buffer (*see section 2.6.3*). To investigate whether HEPES influenced inhibition of aggregation by NO or NO/H_2O_2, autologous platelets were resuspended in either Tyrode's HEPES based buffer or Tyrode's phosphate buffer (*see section 2.3.2*) and the inhibition by NO and NO/H_2O_2 were compared. NO induced a concentration-dependent inhibition of thrombin-stimulated aggregation in both WP preparations with little difference in their IC_{50}. The IC_{50} for NO were 120 and 130nM respectively. In addition, little difference was observed with inhibition of platelet aggregation induced by NO(10nM)/H_2O_2(25μM), 93 and 100%

respectively.

(i) Oxyhaemoglobin as a possible contaminant of the platelet preparations.

NO is known to bind to OxyHb which leads to the inactivation of the inhibitor (*see section 1.6.4.4*). OxyHb is found in plasma and was thus a possible important contaminant of the WP preparations which could influence the inhibitory actions of NO and NO/H_2O_2. If oxyHb were present in the WP preparation, H_2O_2 added with NO could oxidise OxyHb to MetHb, and thus prevent the inactivation of NO. The overall effect would suggest an apparent synergism.

In order to determine whether oxyHb influences the action of NO on platelet activity, WP were pre-washed with H_2O_2 (15μM) (*see section 2.6.3*) and the effects of NO and NO/H_2O_2 compared to WP prepared normally. No significant difference in IC_{50} for NO against thrombin (0.02U/ml)-induced aggregation was observed between WP (109 ± 16nM) and H_2O_2-washed WP (122 ± 13nM). Inhibition of platelet aggregation by NO(10nM) /H_2O_2(25μM) was also unaffected by pre-washing with H_2O_2, 89.7 ± 6.4% and 86.0 ± 5.8%, for WP and H_2O_2-washed WP respectively. This demonstrates that oxyHb had little or no effect on the observed synergy.

4.10 THE INFLUENCE OF NITRIC OXIDE AND HYDROGEN PEROXIDE ON PLATELET AGGREGATION IN THE PRESENCE OF PLASMA.

4.10.1 Nitric oxide-mediated inhibition of collagen-induced platelet aggregation in PRP.

The efficiency of NO as an inhibitor of platelet aggregation was tested in the

presence of plasma. NO solutions (0.01 - 20μM final concentrations) were added directly to PRP and incubated for 1min prior to addition of collagen (0.5μg/ml). At concentrations greater than 10nM, NO induced a concentration-dependent inhibition of platelet aggregation. The IC_{50} for NO was 826.7nM ± 291.4nM ($p< 0.005$), and complete inhibition achieved with 16.7 ± 5.8μM NO ($p< 0.01$) *[Figure 4.12]*. Both the IC_{50} for NO and the concentration of NO required to completely inhibit collagen-induced platelet aggregation were significantly greater than those required to inhibit the aggregation of WP. The data demonstrate that increased concentrations of NO are required to inhibit platelet aggregation in the presence of plasma and suggests the plasma environment enhances the breakdown or sequestration of NO.

4.10.2 The effects of nitric oxide and hydrogen peroxide added simultaneously on collagen-induced platelet aggregation in PRP.

In a previous section of this chapter (*section 4.5.1*) H_2O_2 was shown to enhance the inhibition of collagen-induced aggregation of WP. Here the effect of H_2O_2 on inhibition of collagen-induced platelet aggregation by NO in the presence of plasma was examined. Simultaneous addition of NO (0 - 1μM) with H_2O_2 (25μM) significantly increased the effectiveness of NO. The IC_{50} for NO against collagen-induced agregation in the presence of H_2O_2 was reduced to 149.3 ± 66.5nM ($p < 0.05$). This concentration of NO was still much greater than the IC_{50} found in similar experiments with WP ($p< 0.001$) *[Figure 4.12]*. These data suggest that the previously found synergy between NO and H_2O_2 can occur in the presence of plasma.

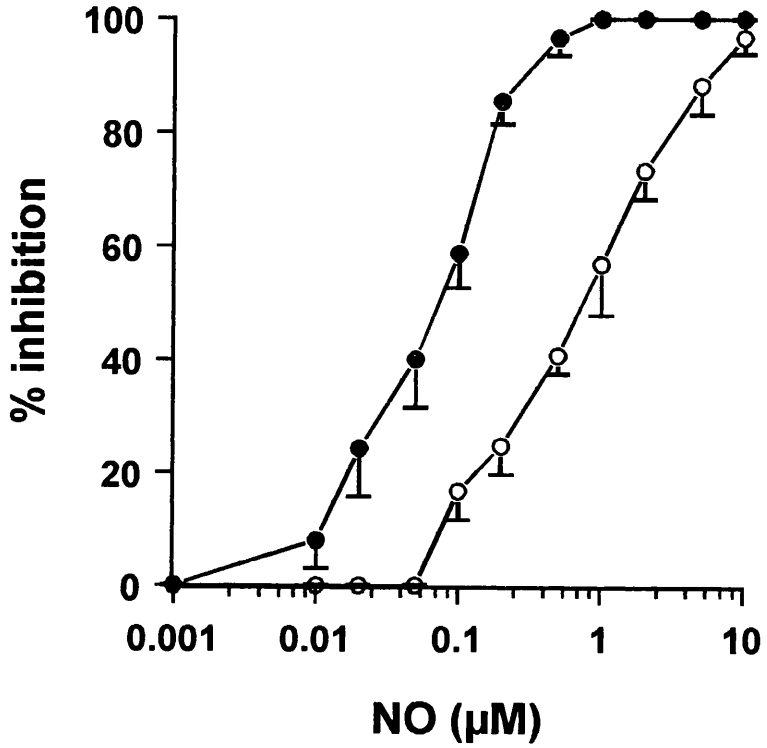


Figure 4.12: Inhibition of platelet aggregation by nitric oxide in the presence of plasma: the influence of hydrogen peroxide.

Platelet rich plasma were incubated with a range of concentrations of NO in the presence (●) or absence (○) of H_2O_2 (20μM,) for 1 minute before the addition collagen (0.5μg/ml). Total aggregation measured after 3min. In all cases the H_2O_2 was added simultaneously with NO. The results are expressed as the percent inhibition of aggregation compared to collagen alone and represent means ± SEM of 3 independent experiments.

4.11 S-NITROSOGLUTATHIONE AS AN INHIBITOR OF PLATELET AGGREGATION.

S-nitrosoglutathione (S-NOG) is a stable S-nitrosothiol which releases NO intracellularly, and inhibits agonist induced platelet aggregation by increases in intracellular cGMP concentration [Radomski *et al.*, 1992]. NO solutions may be come partially oxidised to nitrite (NO_2^-) in the aggregation cuvette during the duration of the experiment, which would reduce efficiency of its biological effects. Release of NO from dissolved S-NOG is low, but enhanced by platelet lysate suggesting the release of NO may be mediated by an enzyme present in the platelet membrane [Radomski *et al.*, 1992]. The S-nitrosothiol was employed to investigate whether the antagonistic effects of H_2O_2 were specific to the effects on exogenous NO solutions or a more general effect against cGMP-mediated inhibition. Evidence suggests that S-NOG was a more potent inhibitor of platelet aggregation *in vitro* than NO [Radomski *et al.*, 1987c], and this may relate to the enhanced release of NO by a factor associated with the platelet membrane. If NO is released from S-NOG close to the platelet membrane the possiblity of NO becoming oxidised before entry to the cell is reduced. Thus S-NOG provided a tool to investigate whether synergism between NO and H_2O_2 occurred intra or extracellularly .

4.11.1 The effects of S-nitrosoglutathione on sub-maximal thrombin and collagen-induced aggregation.

S-NOG was incubated with WP for 1min prior to addition of thrombin (0.02U/ml), which resulted in a concentration dependent inhibition of aggregation. The IC_{50} for S-NOG was 19.3 ± 2.4nM and complete inhibition aginst thrombin-stimulated aggregation was achieved at 133.3 ± 47.1nM: both values were significantly ($p<$

0.05) less than for NO solutions *[Figure 4.13a, b]*. When S-NOG was tested against collagen it was found to be more effective than against thrombin with an IC_{50} of 2.1 ± 0.8nM ($p< 0.001$). Reduced glutathione (0 - 100nM) was used as a control for S-NOG, and had no effects on platelet aggregation.

4.11.2 S-nitrosoglutathione-induced inhibition of platelet aggregation: effects of carboxy-PTIO.

In order to investigate whether S-NOG released appreciable amounts of NO extracellularly, its effects on platelet aggregation were assessed in the presence and absence of carboxy-PTIO (1μM). WP were incubated with carboxy-PTIO (1μM) or Tyrode's HEPES buffer *(see section 2.3)* for 1min prior to the addition of SNOG (0 -1μM). The presence of carboxy-PTIO had no significant effect on SNOG mediated inhibition of thrombin (0.02U/ml)-induced aggregation. The IC_{50} for WP and carboxy-PTIO incubated WP were 24 and 26nM respectively. The experiments were performed twice with similar results obtained. These results are in contrast to those obtained when carboxy-PTIO was tested against authentic NO solutions, where substantial antagonism of NO-mediated inhibition was observed. These results indicated that S-NOG released NO intracellularly or close to the membrane where carboxy-PTIO had little access to NO. These results may indicate that the synergistic phenomenon observed between NO and H_2O_2 occurs intracellularly.

4.11.3 The effects of simultaneous addition of S-nitrosoglutathione and hydrogen peroxide on platelet aggregation.

S-NOG and H_2O_2 were added simultaneously to WP in order to examine whether the synergism observed with NO/H_2O_2 could be reproduced with S-NOG.

Simultaneous application of S-NOG (0 - 10nM) and H_2O_2 (25μM) to platelets 1min prior to the addition of thrombin (0.02U/ml), resulted in a concentration-dependent inhibition of aggregation. The IC_{50} for S-NOG added with H_2O_2 was 0.71 ± 0.21nM, significantly lower than that for S-NOG alone ($p \leq 0.001$). This represented a 25-fold increase in the effectiveness of S-NOG as an inhibitor. Complete inhibition of thrombin-stimulated aggregation was achieved with S-NOG 16.7 ± 4.7nM ($p \leq 0.05$), significantly less than with S-NOG alone *[Figure 4.13a, b]*. When glutathione (0 - 100nM), used as a control for S-NOG, was added in combination with H_2O_2 the aggregation response was unaltered. The results indicated that the proposed synergistic actions may occur intracellularly.

When the experiments were repeated with a fixed concentration of S-NOG (10nM) and H_2O_2 (0 - 20μM) produced an inhibition which was concentration dependent for H_2O_2. S-NOG/H_2O_2 (20μM) induced 95.2 ± 2.1% inhibition. The IC_{50} for H_2O_2 was 2.7 ± 0.3μM, significantly ($p \leq 0.01$) greater than the EC_{50} with NO (1.6 ± 0.5μM) *[Figure 4.14]*.

4.11.4 The effects of S-nitrosoglutathione on collagen-induced aggregation in PRP.

Incubation of S-NOG (0 - 5μM) with PRP for 1min prior to addition of collagen (0.5μg/ml) produced a concentration-dependent inhibition of the normal aggregation response. The IC_{50} for S-NOG was 476 ± 162nM and complete inhibition was achieved with 2.5 ± 1.5μM S-NOG *[Figure 4.15]*.

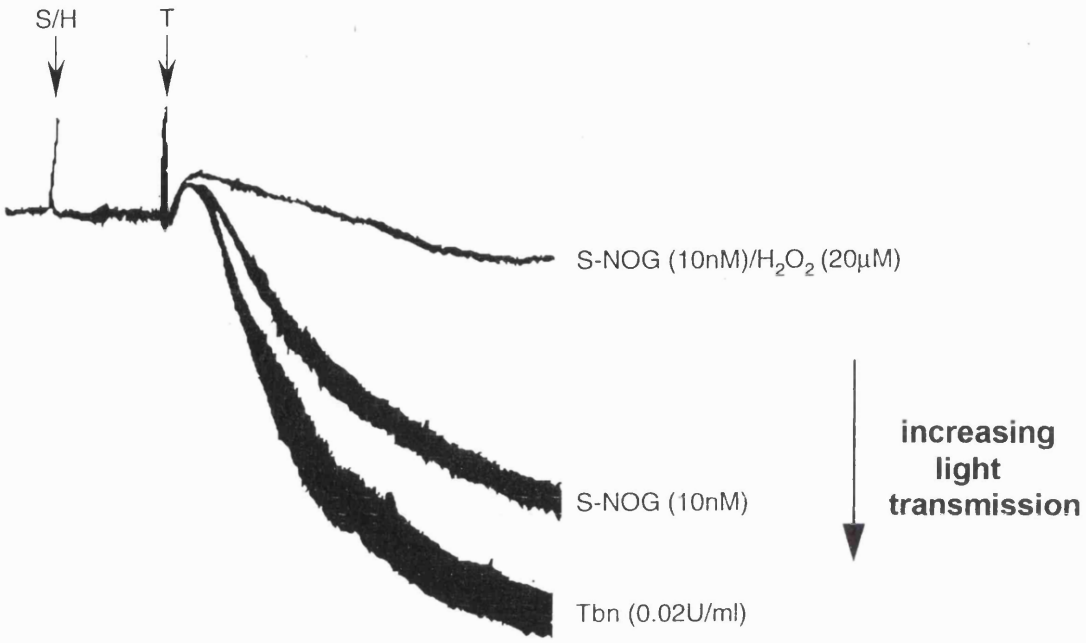


Figure 4.13a: Hydrogen peroxide enhances the inhibition of platelet aggregation by s-nitrosoglutathione.

WP were incubated with thrombin (0.02U/ml) alone or, pre-incubated with S-NOG (10nM) prior to addition of thrombin. In the third trace S-NOG as added simultaneously with H_2O_2 (20μM) and incubated for 1 minute before the addition of thrombin. T represents the addition of thrombin, while H and S represent the addition of H_2O_2 and/or S-NOG. The results are typical traces of at least 5 independent experiments.

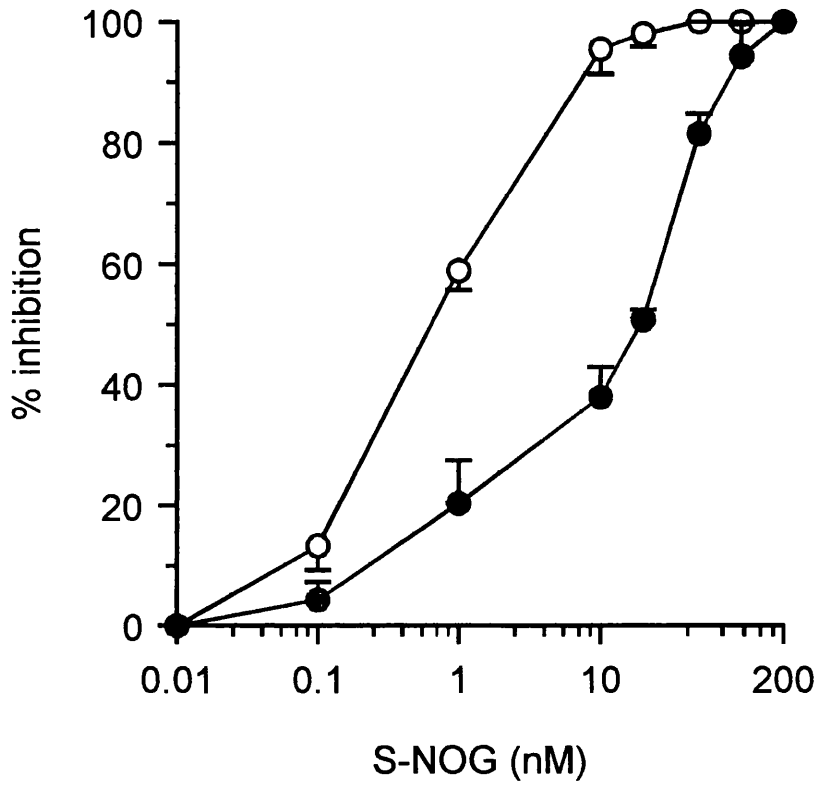


Figure 4.13b: Hydrogen peroxide enhances the inhibition of platelet aggregation by S-nitrosoglutathione.

The effect of H_2O_2 on platelet inhibition by S-NOG was invetsigated. WP were princubated with S-NOG (0 - 1μM) before the addition of thrombin (0.02U/ml), either in the presence (○) or absence (●) of H_2O_2, and aggregation measured after 3min. The data are presented as the percent inhibition of aggregation and are expressed as mean ± SEM of 5 independent experiments. For statistical analysis refer to text (*section 4.11.1*).

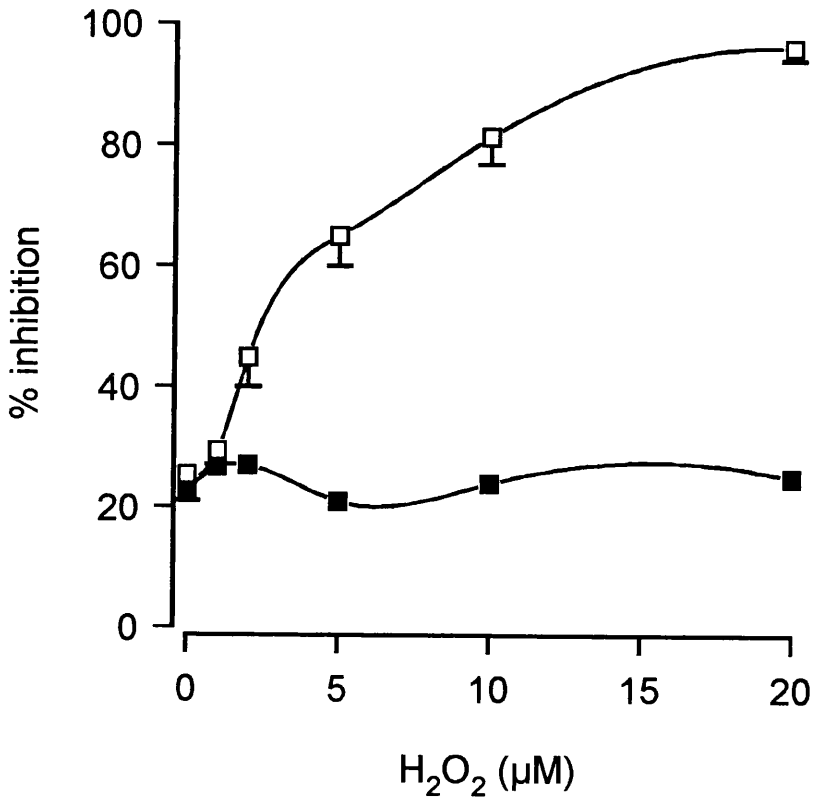


Figure 4.14: Concentration response relationship between hydrogen peroxide and S-nitrosoglutathione on platelet aggregation stimulated by thrombin.

S-NOG and H_2O_2 were added to WP 1min before the addition of thrombin (0.02U/ml) and aggregation was measured 3min later. A single concentration of S-NOG (10nM) was used in combination with a range of concentrations of H_2O_2 (□). In control experiments H_2O_2 which had previously been exposed to air for 24h were used (■). The results are expressed as the percent inhibition of aggregation and represent the mean ± SEM of 4 independent experiments, using inactive H_2O_2 the experiment was performed twice For statistical analysis refer to text (*see section 4.11.3*).

S-NOG (0 - 5μM) and H_2O_2 (25μM) were applied simultaneously to platelets to determine whether the synergism between the two inhibitors also occurred in plasma. The presence of H_2O_2 significantly enhanced the inhibitory effect of S-NOG on platelet activity. The IC_{50} for S-NOG was reduced to 32 ± 9nM from 476 ± 162nM ($p \leq 0.05$). Complete inhibition was also significantly reduced, 0.33 ± 0.18nM ($p \leq 0.05$) *[Figure 4.15]*. The synergism between S-NOG and H_2O_2 to inhibit platelet aggregation may imply that similar phenomenon could occur with other nitrosothiols. This could have wider biological implications, as nitrosothiols such as S-nitrosoalbumin are known to occur in plasma [Stamler *et al.*, 1992].

4.11.5 The influence of radical scavengers on S-nitrosoglutathione and, S-nitrosoglutathione and hydrogen peroxide induced inhibition of platelets.

The influence of sodium urate (0.5mM) and mannitol (50mM) on the actions of S-NOG and S-NOG/H_2O_2 was assessed to examine the possible influence of other radicals. Pre-incubation of the free radical scavengers with WP for 1min had no effect on the inhibition of platelet aggregation by S-NOG or S-NOG/H_2O_2. However, if urate or mannitol were incubated with platelets for 30min prior to addition S-NOG/H_2O_2 the subsequent inhibition of thrombin-induced aggregation was attenuated slightly. The level of inhibition was reduced from 94 ± 6% in the presence of the scavengers to 75.9 ± 4.0 % and 80 ± 3.3%, for urate and mannitol respectively.

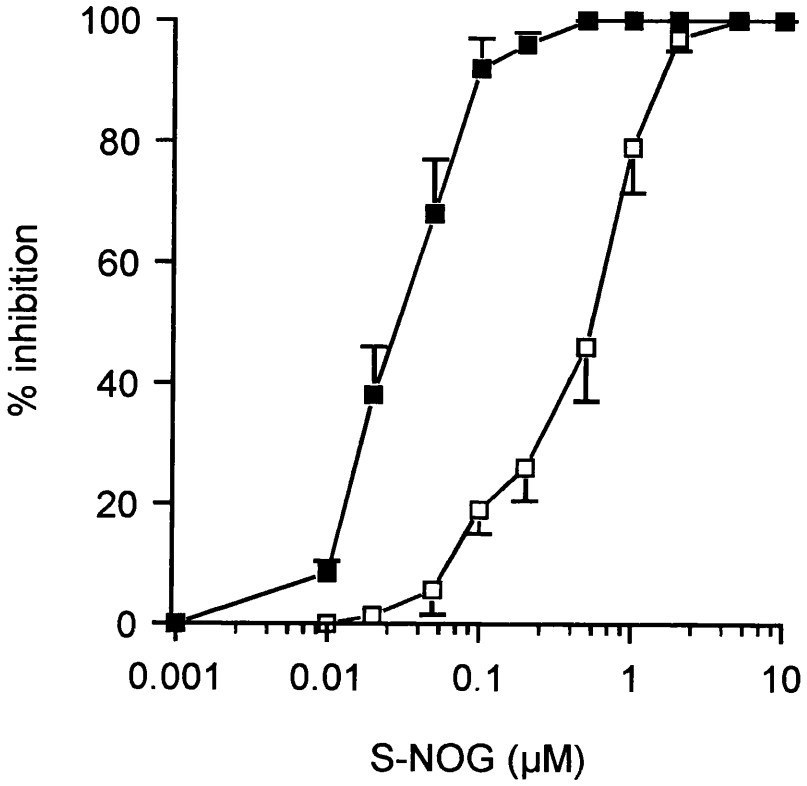


Figure 4.15: Inhibition of platelet aggregation by S-nitrosoglutathione in the presence of plasma: the influence of hydrogen peroxide.

Platelet rich plasma was incubated with a range of concentrations of S-NOG in the presence (■) or absence (□) of H_2O_2 (20μM,) for 1 minute before the addition collagen (0.5μg/ml). Total aggregation was measured after 3min. In all cases the H_2O_2 was added simultaneously with S-NOG. The results are expressed as the percent inhibition of aggregation compared to collagen alone and represent means ± SEM of 4 independent experiments. For statistical analysis refer to text (see section 4.11.4)

4.12 THE IMPORTANCE OF TIME INTERVALS SEPARATING THE ADDITION OF S-NITROSOGLUTATHIONE OR NITRIC OXIDE AND HYDROGEN PEROXIDE ON THE INHIBITION OF PLATELET AGGREGATION.

In order to establish whether simultaneous addition of NO or S-NOG and H_2O_2 was essential for the synergistic inhibition of platelet activity to occur, H_2O_2 was added upto 1min after or 1min before S-NOG/NO. In all cases the time interval between S-NOG/NO and thrombin (0.02U/ml) was 1min, so that in some experiments H_2O_2 was added immediately before the agonist. Simultaneous addition of S-NOG/H_2O_2 induced 92.2 ± 2.1% inhibition of aggregation. However, if H_2O_2 was added before S-NOG a reduction in the level of inhibition was observed *[Figure 4.16a]*. Incubation of H_2O_2 with WP for 1min prior to S-NOG induced 61.1± 6% ($p<0.01$) inhibition, while at 30s before the inhibition was 72.2 ± 1.1% ($p<0.01$), both significantly less than when added simultaneously. If H_2O_2 was added after S-NOG for up to one minute, no significant change in the level of inhibition was observed *[Figure 4.16a]*. When these experiments were repeated with NO, the effect of separation of addition of NO and H_2O_2 was greater, with a clear optimal synergism when the two were added simultaneously. However, the enhanced inhibitions were still observable at all the time intervals studied. This indicates that the key manipulation is the addition of NO and H_2O_2 before the agonist and that the effects are not totally dependent on the simultaneous addition of these agents.

Cyclic GMP concentrations were measured in platelets where NO and H_2O_2 had been added simultaneously or at intervals up to 1 minute apart.

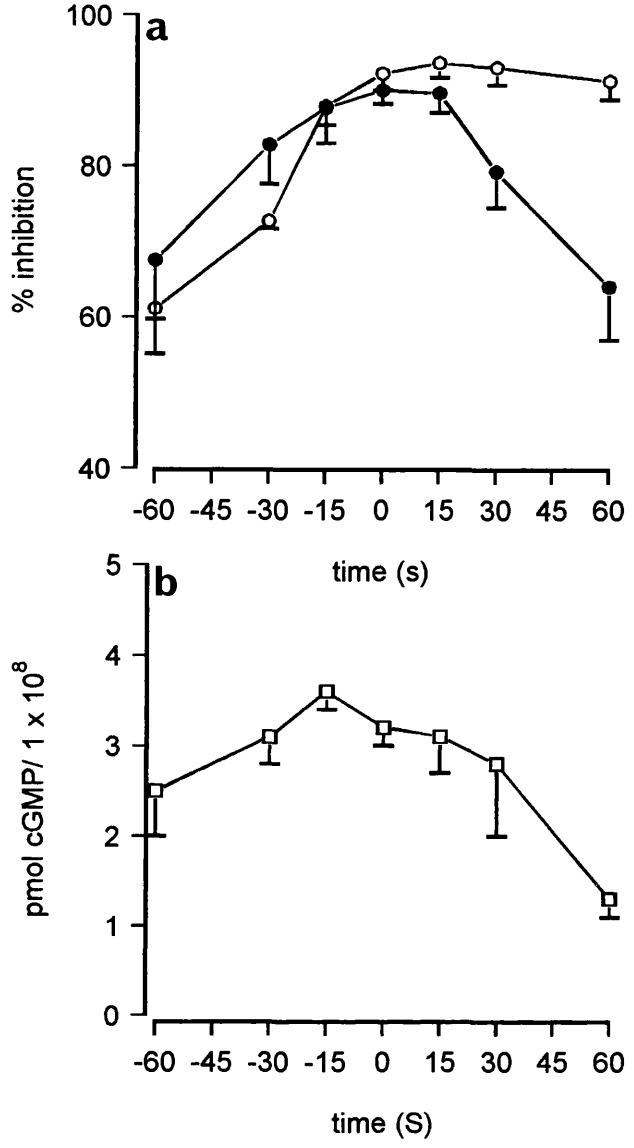


Figure 4.16: The effect of separating the addition of s-nitrosoglutathione or nitric oxide and hydrogen peroxide on the inhibition of platelet aggregation.

a) H_2O_2 (20μM) was added WP up to 1minute before or 1minute after S-NOG or NO (10nM). In all cases WP were stimulated with thrombin (0.02U/ml) 1minute after the addition of S-NOG (○) or NO (●), so in some cases H_2O_2 was added immediately before the agonist and aggregation measured after 3 minutes. The results are expressed as % inhibition of aggregation compared to thrombin alone and are representative of at least 3 individual experiments.

b) The experiments were performed as in a., except that an excess of cold HCl was added instead of thrombin and platelet cGMP measured by radioimmunoassay.

In all cases the presence of H_2O_2 significantly increased the rate of cGMP formation compared to that with NO alone. A greater variation in the inhibition of aggregation had been found with NO than if S-NOG was used (*see above*). The extent of the inhibition of aggregation was parallelled to a significant degree by variations in the concentrations of cyclic GMP *[Figure 4.16b]*, in that the relative increase in the rate of cGMP formation was greater at reduced time intervals, indicating the importance of the role of this nucleotide in the interactions of H_2O_2 and NO.

4.13 DISCUSSION.

In chapter three of this thesis mmLDL were shown to activate platelets, an action which was proposed to be mediated by the presence of low concentrations of LPO. The effects of peroxides on platelet aggregation and platelet inhibition by NO, were first investigated by assessing the influence of H_2O_2. This simple peroxide induced opposing effects on platelet function which were dependent on the order in which it was presented to the platelets relative to the main agonist.

4.13.1 The pro-aggregatory effects of hydrogen peroxide.

H_2O_2 was added directly to platelets and found to induce small amounts of primary aggregation. This contrasted with previous studies where aggregation in the absence of agonists was not observed [Canoso *et al.*, 1974; Pratico *et al.*, 1992; Iuliano *et al.*, 1992]. The aggregation traces were not conventional in appearance, and may be due to fusion of platelets in the presence of H_2O_2, as observed by Rodvein *et al.*, [1976]. When tested in combination with sub-threshold concentrations of thrombin or collagen, H_2O_2 enhanced the aggregation response to initiate secondary aggregation. However this was only observed if the peroxide was added after the agonist. This phenomenon was also reported by other workers [Canoso *et al.*, 1974; Rodvein *et* al., 1976; Pratico *et* al., 1992; Iuliano *et* al., 1992]. The potentiation of aggregation was cyclo-oxygenase mediated, since pre-incubation of platelets with aspirin, abolished the action of the peroxide. Previous work has also implicated the involvement of cyclo-oxygenase in the actions of H_2O_2 [Del Principe *et al.*, 1985, Pratico *et al.*, 1992, Iuliano *et al.*, 1992], although the effects seemed to be dependent on which agonist was used. Pratico *et al.*, [1992] found H_2O_2 to

potentiate aggregation by collagen and AA, but not thrombin, whereas in the present study enhancement of aggregation was demonstrated with both collagen and thrombin, but not ADP. One possible explanation for this apparent anomaly is the concentration of peroxide used. The optimal H_2O_2 concentrations for potentiation of collagen and thrombin-induced aggregation were 5 and 25μM respectively. In the previous study by Pratico *et al.*, [1992] the maximum concentration of peroxide used was 7.5μM, indicating that potentiation of thrombin-induced aggregation may have not occurred, because the amount of peroxide provided was not sufficient to induce the effect. Nanomolar quantities of H_2O_2 are generated by resting platelets, which is significantly increased after stimulation with either thrombin or AA [Maresca *et al.*, 1992]. H_2O_2 has been shown to increase Ca^{2+} entry in both platelets [Del Principe *et al.*, 1985, Pratico *et al.*, 1992] and endothelial cells [Kimura *et al.*, 1992]. In platelets the increase in $[Ca^{2+}]_i$ may activate PLA_2, inducing the release of AA and its subsequent metabolism by cyclo-oxygenase. The increase in $[Ca^{2+}]_i$ associated with these agonists was further enhanced by inhibition of GSH Px, but not catalase, suggesting that GSH Px is the dominant enzyme for H_2O_2 degradation in platelets [Maresca *et al.*, 1992]. The peroxide has also been proposed to be an intermediate in the platelet activation cascade. H_2O_2 is produced after stimulation with collagen, but not thrombin [Del Principe *et al.*, 1985]. However, in a separate study H_2O_2 was shown to be a poor activator of cyclo-oxygenase compared to other longer chain peroxides found in the platelet [Hemler *et al.*, 1979].

4.13.2. The influence of hydrogen peroxide on nitric oxide-mediated inhibition of platelet aggregation.

NO inhibited both thrombin and collagen-induced aggregation in a concentration dependent manner: the IC_{50} values for NO against both agonists were at concentrations found physiologically [Gross and Wolin, 1995]. NO was more potent against collagen than thrombin, but this may be an additive effect due to the production of endogenous NO. Platelet aggregation by collagen, but not thrombin, has been associated with the endogenous production of NO [Radomski *et al.*, 1990]. NO was also shown to inhibit collagen induced aggregation in PRP although greater concentrations were required, probably because of increased breakdown or complexing of NO by factors such as oxyHb.

The activatory effects of H_2O_2 on platelets were tested against the inhibitory actions of NO. H_2O_2 antagonised the effects of NO, when it was added after the agonist. This suggested that the combined effects of the peroxide and thrombin were sufficiently potent to override the effects of NO which include, sequestration of Ca^{2+}, decreased p47 phosphorylation and 5-HT secretion [Nguyen *et al.*, 1991]. This latter study also demonstrated NO was unable to inhibit aggregation induced by Ca^{2+} ionophore A23187 and in some cases even enhanced the aggregatory response. Recently Okamoto et al., [1995], showed Ca^{2+} influx induced by thrombin to have partial resistance to the inhibitory actions of NO. Thus, it is possible to speculate that an increased Ca^{2+} influx, associated with H_2O_2 [Del Principe *et al.*, 1985, Pratico *et al.*, 1992] may not only enhance thrombin-induced aggregation, but also alter the actions of NO. The results were very similar when collagen was used to stimulate platelets, although H_2O_2 was less effective, probably because of the additional formation

of endogenous NO by platelets activated by collagen. H_2O_2 is produced by the endothelium and activated PMNs [Test and Wiess, 1984], both of which are involved in the pathogenesis of atherosclerosis and are in close contact with platelets throughout the circulation. The data presented here suggest, at first sight, that the metabolic production of H_2O_2 seems to be a potentially important pro-thrombotic factor. In these particular experiments H_2O_2 was added to the platelets 2min after NO. Since the rate of formation of cGMP occurs very rapidly after addition of NO *in vitro* [Salvemini *et al.*, 1990; Nguyen *et al.*, 1991], the peroxide was added to platelets with elevated intracellular cGMP concentrations. Thus, it was unclear whether the antagonism of NO by H_2O_2 was due to an influence on cGMP breakdown or a consequence of a direct interaction between NO and H_2O_2. This required further investigation.

4.13.3 The effects of nitric oxide and hydrogen peroxide on platelet aggregation when added simultaneously.

In complete contrast to previous experiments, simultaneous addition of NO and H_2O_2, added before the agonist, led to a protracted inhibition of aggregation, which reduced the IC_{50} for NO by almost two orders of magnitude. Potent inhibitory actions for NO in the presence of H_2O_2 were found with concentrations as low as 1nM. In addition, the effect of H_2O_2 was half maximal at 1.6μM, well within the concentration ranges found physiologically [Test and Wiess, 1984]. This appears to contradict the idea that H_2O_2 as a ROS is pro-thrombotic. Three possibilities existed for these unexpected observations, a) the effects of NO/H_2O_2 were cytotoxic, b) the effects were due to formation of a new radical species possibly peroxynitrite, or c) an authentic synergism occurred between the two species.

In order to investigate the cytotoxicity, the actions of NO/H_2O_2 were assessed as a function of time. The presence of H_2O_2 significantly enhanced and prolonged the effects of NO, but importantly 1h after the addition of NO/H_2O_2 the platelets completely recovered their response to thrombin. This indicated that the reactants had no effect on the functional viability of the cells. In addition, inactive solutions of both NO and H_2O_2 failed to induce inhibition, indicating that possible contaminants of the solutions were not responsible for the observed effects.

Inhibition was not due to the formation of peroxynitrite, the IC_{50} for this oxidant was approximately 5000 times greater than NO/H_2O_2. In fact peroxynitrite was a less effective platelet inhibitor than authentic NO. Moro *et al.*, [1994] showed that peroxynitrite activated washed platelets, however the effects were modest even at 200μM, a concentration of peroxynitrite which platelets are unlikely to encounter *in vivo*. The authors also showed the oxidant to be inhibitory to platelets in the presence of plasma, but the IC_{50} was 30 times greater than observed here. Methodological differences may account for these disparities. In the present study, peroxynitrite was only tested in isolated platelets, whereas plasma would accelerate its decomposition. In addition, Moro *et al.*, [1994] used greater concentrations of agonist than the present study.

The inhibition by NO/H_2O_2 could be due to formation of a new molecular species different from that of NO. This concept was strengthened when it was found that premixing NO/H_2O_2 prior to addition to platelets, led to the mixtures being effective 3 times longer than NO alone. NO and H_2O_2 were believed to form singlet oxygen (O_2), as measured by chemiluminescence [Noronha-Dutra *et al.*,

1992] However, the half-life was less than 1sec, while the half-life of the inhibition in this study was over 30s, much greater than the half-life of most free radicals. To investigate whether the formation of a new species was important , an NO sequestrant was used. Carboxy-PTIO reacts stoichiometrically with NO (NO/C-PTIO = 1), antagonising the biological actions of NO [Akaike *et al.*, 1994]. In the present study carboxy -PTIO did not react 1:1, but had to be used in 10-fold excess, where it induced complete inhibition of the effects of NO. Importantly, when carboxy-PTIO was added simultaneously with NO/H_2O_2, which had been premixed for 10s, there was an attenuation of their inhibitory actions. In the presence of carboxy-PTIO, inhibition was reduced to 24% from a control value of 94%, indicating that NO was of key importance to the effects. However, the remaining inhibition left a possibility of other radicals still being involved. The results suggest that the protracted effects are probably due to a synergism and an increased sensitivity of platelets to residual NO in the presence of H_2O_2.

The loss of NO by auto-oxidation is represented by the following equation:-

$$2NO + O_2 \rightarrow 2NO_2$$

The reaction involves 2 molecules of NO and 1 molecule of O_2, and thus the reaction rate falls exponentially as the NO concentration falls linearly [Gross and Wolin, 1995]. This would suggest that NO at low concentrations in buffer would be fairly stable and not disappear within seconds. The rate constant for this reaction is 1000 times greater in aqueous solution than in air [Kharitonov *et al.*, 1994]. With solutions of NO (100nM) and O_2 (230μM; approximate concentration found in buffer), the half life of NO by auto-oxidation would be approximately two

hours [Kharitonov *et al.*, 1994]. From these studies, one could predict that concentration of NO would fall relatively slowly, although it may be accelerated by the continual stirring and incubation at 37°C. Hence, inhibition still occurring after 30s would probably be due to H_2O_2, which may increase the sensitivity of platelets to the residual concentrations of NO, which alone would not normally affect platelet aggregation. It is proposed that the increased levels of inhibition observed with mixtures of NO and H_2O_2 are due to an authentic synergism.

4.13.3. The possible mechanism of the proposed synergy between nitric oxide and hydrogen peroxide.

In order to establish a mechanism by which the synergy occurred, the effects of various radical scavengers, enzymes and NO quenchers were tested on the action of NO/H_2O_2. Carboxy-PTIO, an NO sequestrant, inhibited the actions of NO/H_2O_2 but not completely, which indicated that other radicals may play a role. The attenuation of the inhibition by hydroxyl radical scavengers, mannitol and urate, indicated that another radical species may be involved. However, the inhibition was not influenced by DTPA, a transitional metal chelator. This demonstrated that $^{\bullet}OH$ radicals, produced by the Fenton reaction and requiring Fe^{2+} ions, were not responsible for the protracted inhibition. This is particularly important as $^{\bullet}OH$ is a proposed activator of guanylate cyclase [Schmidt, 1992]. SOD and ascorbate, both free radical scavengers had no effect, however catalase did reduce the inhibition, presumably by decreasing the concentration of H_2O_2. None of the above affected the inhibition of platelet aggregation by NO alone and therefore the effects of the scavengers only occurred on the synergy between NO and H_2O_2.

To investigate the location of the apparent synergism, the stable S-nitrosothiol, S-NOG, was used. Nitrosothiols are becoming increasingly important not only as tools for NO research, but also as possible carriers for NO *in vivo.* S-nitrosothiols were thought to occur via a direct interaction between NO and free sulphydryl groups [Stamler *et al.*, 1992]. However, it has been suggested that peroxynitrite is a more potent nitrosating agent [Beckman *et al.*, 1992]. Platelet inhibition by peroxynitrite was proposed to occur by nitration of GSH and subsequent release of NO [Moro *et al.*, 1994]. If nitrosylation by peroxynitrite does occur physiologically, it may serve two functions. Firstly, it may protect against possible oxidative damage by peroxynitrite, and secondly, to act as a mechanism to recycle NO and thereby preventing wastage. The addition of S-NOG to platelets induced inhibition of both thrombin and collagen-induced aggregation, in all cases it was found to be a more potent platelet antagonist than NO, a phenomenon already recognised by Radomski *et al.*, [1987c]. The increased potency of S-NOG may be due to its intracellular release of NO. Radomski *et al.*, [1992] demonstrated that release of NO from S-NOG was enhanced by platelet lysate, but not whole platelets suggesting enzymatic release at membrane level. Recently Freedman *et al.*, [1995], showed that GSH Px could enhance the release of NO from S-NOG, implicating GSH Px as a possible mediator of S-NOG induced actions. In the present studies, when the inhibitory actions of S-NOG were tested in the presence of carboxy-PTIO, no attenuation of inhibition was observed. This is in complete contrast to NO, and strongly suggests that NO is released from S-NOG intracellularly or close to the platelet membrane where the quencher would have limited access, thus providing a tool to assess the location of the apparent synergism.

The intracellular release of NO, may account for the increased potency of S-NOG in comparison to NO. Authentic NO if added exogenously, may undergo partial oxidation before entering the platelet. Radomski *et al.*, [1992], found an increase in cAMP associated with platelet inhibition by S-NOG. However cAMP did not significantly increase until concentrations of at least 300nM S-NOG were used, and therefore, this would have little bearing on the results presented here where a much lower concentration was used. When S-NOG was tested in combination with H_2O_2, the inhibitory actions were significantly increased, but to a slightly lesser extent than with authentic NO. However, inhibition was observed at less than 1nM S-NOG, one order of magnitude lower than the concentration of nitrosothiols quoted to be found in plasma [Stamler *et al.*, 1992]. Mannitol and urate, as with NO/H_2O_2, were shown to attenuate inhibition by S-NOG and H_2O_2, however a 30min incubation of the scavengers with platelets was required before they had an effect. Platelets are strongly pinocytotic and over this time period they may accumulate mannitol/urate in intracellular vacuoles, where they may have partial access to S-NOG/H_2O_2. This is in contrast to NO/H_2O_2 where the effects of the scavengers occurred more rapidly. When the effects of both NO and S-NOG were tested in the presence of plasma both of them inhibited aggregation, but greater concentrations of these antagonists were required. This is probably due to their increased breakdown in the presence of plasma factors. In the case of NO one such inhibitor would be maybe oxyHb, while a study by Freedman *et al.*, [1995] suggested that the concentration of S-NOG would be affected by plasma glutathione peroxidase (GSH Px). GSH Px was reported to increase the inhibitory actions of S-NOG on platelet aggregation. The authors suggested that GSH Px increased the availability of NO from S-NOG or converted it into a from that could more readily

interact with platelets. In the presence of H_2O_2, the concentrations of NO and S-NOG required to induce inhibition were reduced. The results produced using S-NOG suggest that the synergy may occur intracellularly and importantly the occurrence of the synergy in the presence of plasma suggests the phenomena may be of physiological importance. It also indicates that synergism may occur between H_2O_2 and other nitrosothiols such and S-nitrosoalbumin and S-nitrosocysteine.

4.13.3.1 Does the order of addition of hydrogen peroxide relative to the agonist affect platelet function?

The effects of H_2O_2 on platelet function have been studied many times with contradictory results: presumably many of these discrepancies are due to methodological differences. H_2O_2 has been shown to activate platelets [Patscheke, 1977; Del Principe *et al.*, 1985; Pratico *et al.*, 1992; Iuliano *et al.*, 1992], in all these studies washed or gel filtered platelets were used. When H_2O_2 was tested in PRP it failed to activate platelets [Salvemini *et al.*, 1991; Ambrosia *et al.*, 1994], which suggested that the activatory effects of H_2O_2 may not be that important physiologically. Other investigators have shown H_2O_2 to inhibit platelet aggregation [Canoso *et al.*, 1974; Rodvein *et al.*, 1976; Holmsen and Robkin, 1977; Ambrosia *et al.*, 1994]. The study by Holmsen and Robkin, [1976], proposed that exposure of platelets to H_2O_2 leads to changes in energy metabolism. H_2O_2 induced a rapid depletion of platelet ATP levels, albeit at concentrations greater than used here, which had little effect on aggregation until the energy charge was reduced. However minor changes did occur at concentrations of H_2O_2 relevant to this work, and may be interesting to speculate that NO could enhance these action of H_2O_2. Obvious differences such as H_2O_2

concentrations and whether H_2O_2 was added as a bolus or via a generating system would account for some of these inconsistencies. However, what seems to be the most important factor is the order in which the peroxide is presented to the platelets relative to the agonist. Generally it seems that addition of H_2O_2 before the agonist leads to platelet inhibition, while addition of H_2O_2 after the agonist results in the activation of platelets. This phenomenon was recognised by Canoso *et al.*, [1974] and largely ignored, but was confirmed by the data presented here.

4.13.3.2 The effects of nitric oxide and hydrogen peroxide on cGMP.

NO exerts its biological actions by entering platelet and stimulating the formation of cGMP, this leads to a number of intracellular events the most important of which is the increased sequestration of Ca^{2+} [Haynes, 1993]. In the present study NO induced a concentration dependent increase in cGMP formation, which in the presence of H_2O_2 was significantly enhanced, even though H_2O_2 alone did not increase cGMP. This suggested the potent inhibition by NO/H_2O_2 was a cGMP mediated effect. However, an anomaly within the data became apparent. The presence of H_2O_2 increased the effects of NO (10nM) so that it produced cGMP concentrations equivalent to that produced by 100nM NO alone. This concentration of NO however, only produced approximately 50-60% inhibition, while the NO/H_2O_2 mixture almost completely inhibited the platelets. This indicated that an additional mechanism to the increased cGMP formation may be responsible for the potent inhibitory actions of the mixtures. The peroxide was tested in combination with dBcGMP, which also inhibits platelets. Here, H_2O_2 enhanced the inhibition induced by dBcGMP, but the effect was modest in comparison to the enhancement of NO/S-NOG mediated inhibition by

H_2O_2. The results suggest that H_2O_2 may have the ability to enhance the actions of the cyclic nucleotide, but its major action is prior to cGMP formation. Interestingly, when H_2O_2 was added after the agonist it antagonised the action of dBcGMP, the magnitude of this effect was far greater than the complementary effects on cGMP and fits well with the earlier data showing the diminution of NO mediated effects by H_2O_2 when added after the agonist.

When the addition of NO and H_2O_2 was separated by up to 1 min, the level of inhibition was altered. The data showed substantial, but reduced, inhibition if the additions were separated. In all cases when NO and H_2O_2 were added before the agonist the inhibitions were greater than those of NO alone. The inhibition was greatest when the two reagents were added simultaneously. The results were slightly different with S-NOG, in that inhibition was reduced if H_2O_2 was added first: but remained constant if S-NOG was added first, with NO all inhibitions were reduced if the two reagents were not added simultaneously. These inconsistencies probably represent difference in how the NO is presented to the platelets. In the case of S-NOG, not all the NO is probably released immediately, allowing longer exposure of the cell to NO. This may account for the increased longevity of the synergy. The reduced inhibition observed if H_2O_2 is added prior to NO/S-NOG possibly relates to loss of active H_2O_2 by evaporation or enzymatic decomposition.

cGMP concentrations rise very rapidly after addition of NO and are maintained for several minutes [Salvemini *et al.*, 1990; Nguyen *et al.*, 1991]. When the addition of NO and H_2O_2 were separated there were variations in the level of cGMP formation. The presence of H_2O_2 again increased the cGMP levels

induced by NO compared to NO alone, but the greater the time interval between NO and H_2O_2 the lower the concentration of cGMP formed. NO/H_2O_2 added simultaneously induced almost complete inhibition, without reaching maximal cGMP concentrations (those levels produced by concentrations of NO which induce complete inhibition alone). This again gives credence to the possibility of that there may be another mechanism operating alongside the increased formation of cGMP. In these studies it is clear that H_2O_2 has no direct effects on guanylate cyclase, since alone it did not raise cGMP concentrations. In addition the peroxide has no effect on the activity of purified soluble guanylate cyclase [*Mayer, personal communication*]. Schmidt, [1992] showed that H_2O_2 had no direct effect on soluble guanylate cyclase, which is in agreement with data presented here. However, a recent study by Ambrosio *et al.*, [1994], suggests that H_2O_2 may be an activator of guanylate cyclase. These workers presented evidence showing that H_2O_2 induced inhibition of platelet aggregation which was associated with a 10-fold increase in cGMP above basal levels. The inhibition was totally cGMP dependent, as the inhibition was prevented if methylene blue, a guanylate cyclase inhibitor, was added. The action of H_2O_2 on cGMP mediated inhibition seem surprising initially, however other studies have also recognised a similar phenomenon. H_2O_2 was shown to elicit the relaxation of isolated pulmonary arteries by a mechanism which was associated with increases in cGMP [Burke-Wolin *et al.*, 1987]. The mechanism of these effects seems very complex, but it is thought to involve catalase and higher oxidation state of Fe. Wolin and Burke, [1987] demonstrated that activation of purified guanylate cyclase by H_2O_2 was dependent on the presence of catalase, and that cGMP formation was intimately related to the production of catalase-compound I. Compound I intermediates are thought to be formed during the interaction of

peroxides with Fe (III). Here the redox reaction involves Fe(III) derivatives which are formally Fe(V). The general equation is given as:

$$Fe(III) + HOOH \rightarrow Fe(V) + H_2O$$

Therefore, the compound I intermediates store the oxidising potential of the peroxide [Jones, 1989]. It is possible that compound I intermediates act as a radical species, allowing it to interact with the haem centre of guanylate cyclase. Whether H_2O_2 can activate guanylate cyclase is open to debate, in this study H_2O_2 did cause a slight increase in cGMP, but this was not significant.

It is likely that the mechanism of the inhibitory effects of H_2O_2 observed by Ambrosio *et al.*, [1994] and other workers may be very closely linked to the results presented in this study. Neither Ambrosio *et al.*, [1994], Canoso *et al.*, [1974] nor Rodvein *et al.*, [1976], tested the effects of H_2O_2 against thrombin stimulated platelets, which could be of fundamental importance of the interpretation of their results. Thrombin-induced platelet aggregation occurs without a significant increase in the action of endogenous NOS and production of NO [Radomski *et al.*, 1990a, b]. The previously cited studies used collagen and/or ADP, both of which induce the production of NO during aggregation. It is possible that the inhibitory actions are due to synergy between endogenously produced NO and exogenously added/generated H_2O_2 in the fashion previously described. The levels of inhibition cited in previous studies are lower than described here, but separation of their presentation to the platelets may account for this. The inhibitions were in the order of 40 - 50%, which is similar to the level of inhibition found in *section 4.12,* when the addition of NO and H_2O_2 were

separated by 1min. A related mechanism has also been shown to occur in arterial relaxation. Zembowicz *et al.*, [1993], showed H_2O_2 to induce a concentration dependent increase in relaxation of rabbit aorta: the effect was 2-3 fold greater if the endothelium was still present and was inhibited by the presence of NOS inhibitors. This strongly suggested that H_2O_2 induced the activation of NOS. It is possible that H_2O_2 increases NOS activity in platelets, but it is unlikely that this accounts for the results in the present study. Malinski *et al.*, [1993], demonstrated that platelets could produce 10-20nM NO during collagen-induced aggregation. However, this concentration of NO could not account for the almost complete inhibition of aggregation: NO alone is unable to achieve this *in vitro* at concentrations less than 1μM. Although 50% inhibition was achievable at 80nM, and in the presence of H_2O_2 this concentration of NO (10-20nM) may be adequate to induce inhibition.

In conclusion, H_2O_2 has been shown both to enhance and to diminish the actions of NO. The most interesting aspect is the possible synergy leading to the potentiation of the inhibition of aggregation by NO. This may to occur intracellularly and is associated, at least in part, by increased cGMP formation. Implicitly, this would suggest that formation of H_2O_2 from $O_2^{\cdot -}$ by SOD on endothelial cells, may not only be a mechanism for preventing the formation of peroxynitrite, but could have a positive role in prolonging and intensifying the anti-aggregatory actions of NO. A recent study has shown that NO can reversibly inhibit the activity of both purified and intracellular GSH Px, leading to an increase in intracellular peroxide concentration [Asahi *et al.*, 1995]. It is interesting to speculate that NO itself may be able to regulate the intracellular levels of H_2O_2, and thus favour a potential synergism. In relation to the previous

observation, Leoncini, [1994], found that inhibition platelet GSH Px with N-ethylmaleimide, led to an increase in intracellular H_2O_2 concentration and a concomitant decrease in platelet aggregation, suggesting an inhibitory role for the peroxide.The concept of a ROS acting as a positive secondary messenger is idiosyncratic, as ROS have been generally viewed as toxic and potentially damaging to normal homeostasis. However, brain NOS has been reported to produce H_2O_2 at low L-arg concentrations [Heizel *et al.*, 1993], which may be a compensatory mechanism to enhance the effect of reduced NO formation. In addition, the endogenously produced NO and H_2O_2 may synergise to control platelet function.

CHAPTER FIVE: THE INFLUENCE OF LOW DENSITY LIPOPROTEINS AND LIPID PEROXIDES ON PLATELET SENSITIVITY TO NITRIC OXIDE

5.1 INTRODUCTION.

Lipid hydroperoxides are formed by the peroxidation of unsaturated fatty acid side chains of both phospholipids and cholesteryl esters (*see section 1.3.1*). In recent years, evidence has accumulated linking increased plasma concentrations of lipid hydroperoxides (LPO) to various disease states. Plasma or serum LPO levels have been found to be increased in diabetes [Sato *et al.*, 1979], hyperlipidaemia [Loeber *et al.*, 1983], hypertension [Uysal *et al.*, 1986] and atherosclerosis [Stringer *et al.*, 1989]. The involvement of LPO in atherosclerosis was first demonstrated by Glavind *et al.*, [1952], who showed the presence of LPO in human atherosclerotic plaques. A subsequent study by Harland *et al.*, [1973] demonstrated that the severity of aortic atherosclerosis was strongly correlated with the concentration of LPO in the arterial wall. The possible importance of LPO in the atherosclerotic process has become more topical since it was postulated that LDL undergo post-secretory oxidative modification, which contributes heavily to the pathogenesis of the disease [Steinberg *et al.*, 1989]. Nishigaki *et al.*, [1981] analysed the LPO content of serum lipoproteins and found VLDL, LDL and HDL were all vehicles for LPO, but the most prominent was LDL.

The direct effects of LPO on the development of atherosclerosis was investigated by Yagi *et al.*, [1982]. Linoleic hydroperoxide was injected into rabbit ears and the serum LPO levels measured. After injection, the serum LPO levels were raised, but more importantly scanning electron microscopy revealed that the aortic endothelium had undergone morphological changes which were

similar to those observed in the early stages of atherosclerosis. The half life of the injection was approximately 50min, indicating that only short term exposure of the vasculature to LPO may induce physiological alteration of the endothelium.

Elevated LPO levels have been proposed to be causative factors for the induction of atherosclerosis. Three possible mechanisms by which LPO exert these effects have been suggested. In the first instance, high peroxides levels, may increase platelet aggregation. Injection of a large amount of polyunsaturated fatty acid, particularly arachidonate, has been shown to cause formation of microthrombi leading to death in rabbits [Goto, 1982]. Aggregation and adhesion of platelets may initiate atherosclerosis, by release of PDGF with subsequent SMC proliferation [Ross and Glomset, 1976]. Secondly, LPO may exert a direct effect on endothelial cells leading to altered functionality. Exposure of the endothelium to LPO could lead to reactions between membrane proteins and the peroxides altering enzyme function and possibly membrane fluidity. In the third instance high lipid peroxides concentrations may cause an imbalance in the production of vasoactive mediators. High levels of LPO attenuate the activity of cyclo-oxygenase [Gryglewski *et al.*, 1979]. The increased retention of oxLDL in the sub-endothelial space may provide the local high peroxide concentration which accounts for the diminished production of PGI_2 in the area of atherosclerotic plaques [Sinzinger *et al.*, 1977; De' Angelo, 1978]. A reduction in the levels of PGI_2 would favour platelet aggregation and increase thrombotic potential.

The influence of lipid peroxides, and their breakdown products, on endothelial

cell function have been studied previously. However, less research has been devoted to how exogenous LPO affect platelet function. Lipid peroxides have been shown to be of importance in the functioning of platelet COX, where their presence is required as an activator of the enzyme [Warso and Lands, 1983]. In chapter three of this thesis, mmLDL were shown to activate platelets after only a 1min incubation. It was proposed that these effects were mediated by the low levels of LPO present, since this was the only compositional difference between mmLDL and nLDL which could account for their contrasting actions on platelet function. The short duration of these experiments would limit the amount of lipid exchange between LDL and platelets, suggesting that exogenous LPO may have a direct effect of platelet activity.

Elevated plasma lipoprotein levels are thought to cause a increase in PGI_2 production by EC [Spector *et al.*, 1988], but reduce PGI_2 sensitivity in platelets [Beitz *et al.*, 1983; Bruckdorfer *et al.*, 1985; Colli *et al.*, 1985]. Isolated LDL has also been shown to reduce the effects of the other EC-derived vasodilator, NO. Jacobs *et al.*, [1990] demonstrated that both native and oxidised LDL reduced the bioactivity of NO on aortic rings. Intense research has shown the effectiveness of NO as a biological mediator is reduced in atherosclerotic and hyperlipidaemic models [review: Flavahan, 1992]. However the question which has not been addressed is how LDL, and in particular modified forms of LDL, affect platelets as target cells for NO. In the following sections, preliminary investigations into how specific peroxides affect platelet aggregation and platelet sensitivity to NO, were performed. A water soluble peroxide, cumene hydroperoxide, and an authentic lipid hydroperoxide, 15(S)-hydroperoxyeicosatetraenoic acid (15 (S)-HpETE), were used to investigate

further the proposal that the pro-aggregatory actions of mmLDL were mediated by the presence of LPO. In doing so, the effects of lipid hydroperoxides with those of H_2O_2 were compared.

In the second part of the study, the influence of LDL at different stages of oxidation, on platelet sensitivity to NO was investigated. The activation of platelets was inhibited with a range of NO or S-NOG concentrations to determine the IC_{50} in the presence of the lipoproteins.

5.1.1 Summary of aims.

[1] To examine the effects of short term exposure of platelets to cumene hydroperoxide and 15 (S)-hydroperoxyeicosatetraenoic acid on platelet aggregation.

[2] To determine the influence of these peroxides on platelet sensitivity to NO.

[3] To investigate the influence of oxidative modification of LDL on the sensitivity of platelets to NO.

5.2 THE INFLUENCE OF PEROXIDES ON PLATELET AGGREGATION.

mmLDL were shown to induce and potentiate the aggregation of platelets (*see section 3.4.1*), whereas both nLDL and oxLDL were inhibitory. The activatory properties of mmLDL may have been mediated by the associated lipid peroxides, present in low concentrations. Higher concentrations of peroxides may be associated with inhibition of platelet activation, as was the case with oxLDL. In *sections 4.2.1 and 4.2.2*, H_2O_2 induced small amounts of aggregation alone and potentiated aggregation induced by sub-threshold concentrations of thrombin and collagen. In the following section the effects of more complex peroxides on platelet aggregation were examined. The peroxides used were cumene hydroperoxide (cum-OOH), a water soluble aromatic peroxide, and 15(S) -hydroperoxyeicosatetraenoic acid (15-HpETE), an oxygenated metabolite of AA, known to be present in oxLDL [Esterbauer, 1993]. The peroxides were presented to the platelets either 1min before or 1min after thrombin and the aggregation measured 3min later.

5.2.1 The effects of cumene hydroperoxide on thrombin-induced platelet aggregation.

The addition of cum-OOH (0 - 100μM) alone to WP did not induce aggregation. Pre-incubation of cum-OOH (0 - 50μM) with WP for 1min prior to addition of thrombin (0.02U/ml) also failed to influence aggregation: at 100μM, cum-OOH caused a significant inhibition of aggregation (11.2 ± 3.5%, $p \leq 0.05$).

Pre-incubation of cum-OOH (0 - 100μM) with WP prior to addition of a subthreshold concentration of thrombin (0.005U/ml) also failed to affect aggregation, except at 100μM when it was slightly inhibitory. However, addition

of cum-OOH to platelets 1min after the agonist, led to a significant enhancement of thrombin-induced aggregation *[Figure 5.1]*. The potentiation of platelet activity reached statistical significance at 10, 30 and 50μM cum-OOH. Thrombin alone induced 7.7 ± 1.8% aggregation this was increased to 15.7 ± 3.8 ($p \leq 0.05$), 28.7 ± 4.9 ($p \leq 0.01$) and 27.3 ± 5.1 ($p \leq 0.05$) respectively for the three concentrations of peroxide. The enhancement of aggregation was maximal at 30μM cum-OOH and at 100μM was able to exert only a slight effect (9.3 ± 1.8%) *[Figure 5.1]*. The potentiation effects are comparable to those observed with supplementary H_2O_2, as it also enhanced aggregation when added post-agonist.

5.2.2. The effects of 15 (S)-hydroperoxyeicosatetraenoic acid on thrombin-induced platelet aggregation.

The incubation of 15 (S)-HpETE (0.01 -10μM) with WP did not induce aggregation. Furthermore, 15 (S)-HpETE (0.01 - 10μM) was shown not to influence thrombin-induced aggregation, regardless of whether the peroxide was added to the platelets before or after the agonist. Stimulation of platelets with thrombin (0.005U/ml) resulted in aggregation of only 6.3 ± 1.9%. The addition of 15 (S)-HpETE either before or after thrombin had little effect (6 ± 1 and 7.7 ± 1.2% aggregation respectively).

5.3 THE INFLUENCE OF PEROXIDES ON THE SENSITIVITY OF PLATELETS TO NITRIC OXIDE.

Previously, H_2O_2 was shown to both diminish and enhance the effectiveness of NO as a platelet inhibitor (*see sections 4.4.1 and 4.6.1*). In the following section, the effects of the more complex peroxides on platelet sensitivity to NO were examined. The peroxides were added to platelets either 1min before NO, as in

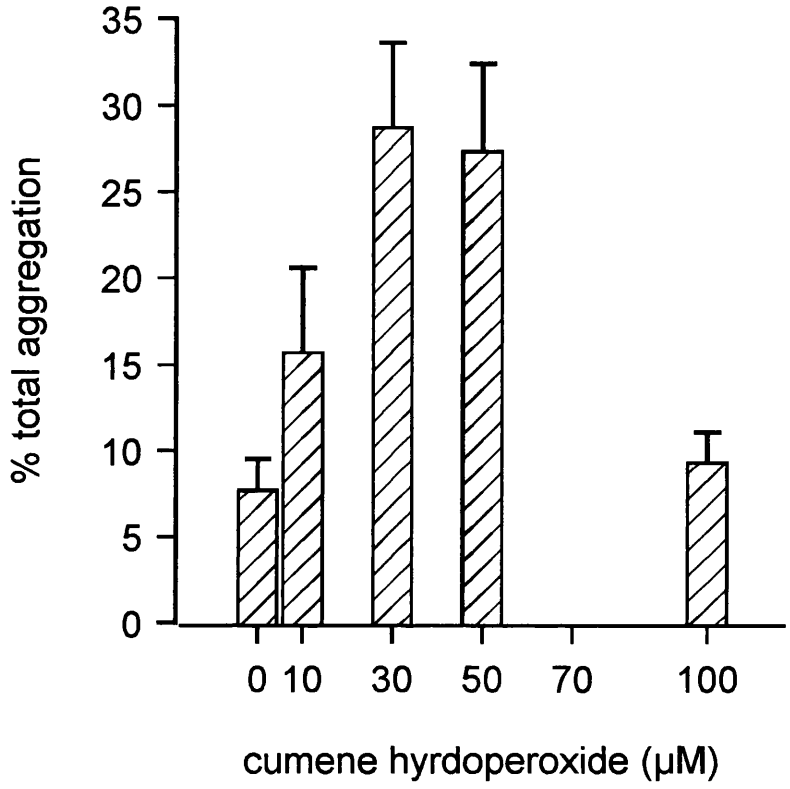


Figure 5.1: The effects of cumene hydroperoxide on sub-threshold platelet aggregation induced by thrombin.

WP were stimulated with thrombin (0.005U/ml) and left stirring for 1min before the addition of cum-OOH (0 -100μM), total aggregation was measured 3min later. The data represent the percent total aggregation and are expressed as mean ± SEM of 4 independent experiments.

[a], or 1min after thrombin, as in [b].

[a] peroxide → NO → thrombin [b] NO → thrombin → peroxide

A 1min gap between the addition of NO and thrombin was maintained throughout the experiments.

5.3.1 The influence of cumene hydroperoxide on the sensitivity of platelets to nitric oxide.

The influence of cum-OOH (30μM) on the inhibition of platelet aggregation by NO was determined. Cum-OOH added to WP, pre-incubated with NO 1min after thrombin, reduced the inhibitory actions of the platelet inhibitor ([b]). Cum-OOH (30μM) increased the IC_{50} for NO against thrombin-induced aggregation from 133 ± 30nM to 706 ± 160nM ($p< 0.05$) *[Figure 5.2].* These results are very similar to those observed when H_2O_2 was added after the agonist (*see section 4.4.1*). If cum-OOH was incubated with the platelets for 1min before the addition of NO, as in [a], no change in the IC_{50} for NO was observed. This contrasted with actions of H_2O_2 which enhanced the inhibitory actions of NO when it was added before the agonist.

5.3.2. The effect of simultaneous addition of cumene hydroperoxide and nitric oxide on platelet aggregation.

The simultaneous addition of NO and H_2O_2 resulted in a potent enhancement of the inhibitory actions of NO (*see section 4.6.1*). When cum-OOH (20μM) was added to WP simultaneously with NO (10nM), before thrombin (0.02U/ml), it did not modify the inhibitory actions of the antagonist. NO alone induced 5.8 ± 1.2% inhibition of platelet aggregation which remained unaltered at 6.7 ± 3.5%, when

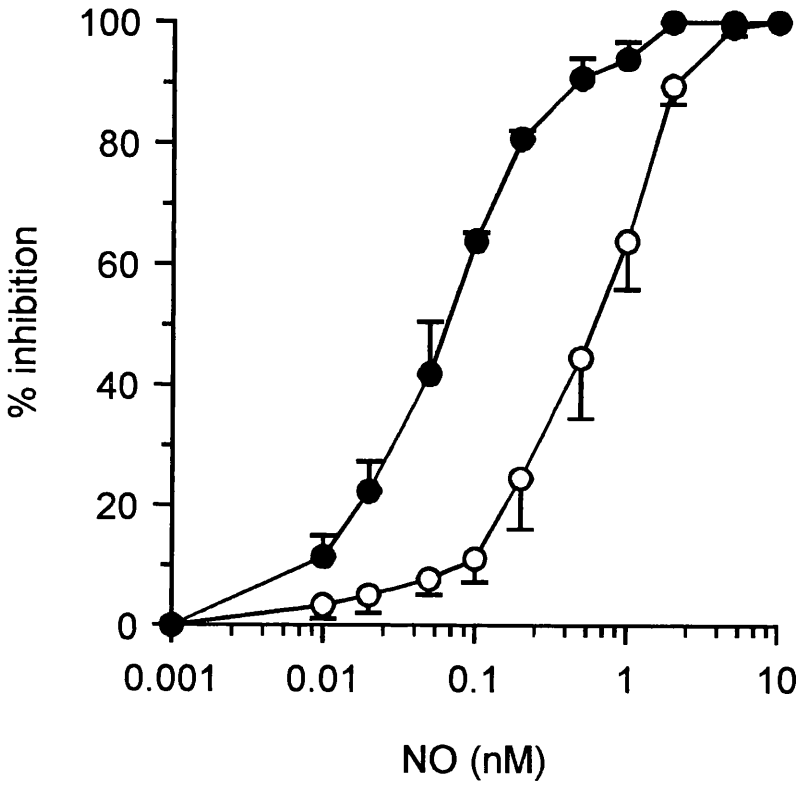


Figure 5.2: Concentration response curves for nitric oxide against thrombin-induced aggregation: effects of supplementary cumene hydroperoxide.

WP were incubated with NO solutions (0.001 - 10μM), added as a bolus, for 1min prior to the addition of thrombin (0.02U/ml) (●), after a further 1min cum-OOH was added at a concentration of 30μM (○) and the total aggregation measured after 3min. The results represent the percent inhibition of aggregation, and are expressed as mean ± SEM of 4 individual experiments.

cum-OOH was added simultaneously with NO. These data indicate that the enhancement of NO mediated inhibition was a specific effect of H_2O_2.

5.3.3 The influence of 15(S)-hydroperoxyeicosatetraenoic acid on platelet sensitivity to nitric oxide.

Pre-incubation of 15(S)-HpETE with WP for 1min before the addition of NO caused a modest reduction in the effectiveness of the platelet inhibitor. In the presence of 15 (S)-HpETE (10μM) the IC_{50} for NO against thrombin (0.02U/ml)-stimulated aggregation was increased significantly from 122 ± 14nM to 367 ± 50nM ($p < 0.01$) [*Figure 5.3*]. In contrast, the addition of the LPO 1min after thrombin (2min after NO) failed to affect platelet inhibition by NO (IC_{50} 106 ± 5.8nM).

These data show 15(S)-HpETE to differ from both H_2O_2 and cum-OOH, with regard to their effects on platelet sensitivity to NO. The presence of 15 (S)-HpETE with platelets before the addition of NO led to a reduction in the effects of the inhibitor, while H_2O_2 intensified platelet inhibition by NO and cum-OOH had no effect. Conversely, the addition of 15 (S)-HpETE to platelets after thrombin had no effect on platelet inhibition by NO, while both H_2O_2 and cum-OOH antagonised the inhibition when added at this point.

5.4 NITRIC OXIDE-MEDIATED INHIBITION OF PLATELET AGGREGATION: EFFECTS OF LOW DENSITY LIPOPROTEINS.

LDL may cause a reduction in the bioactivity of NO in atherosclerosis [Flavahan, 1993]. Hence, the influence of LDL on platelets as target cells for NO was investigated. LDL were incubated with WP for 1min either before ([a]) or after

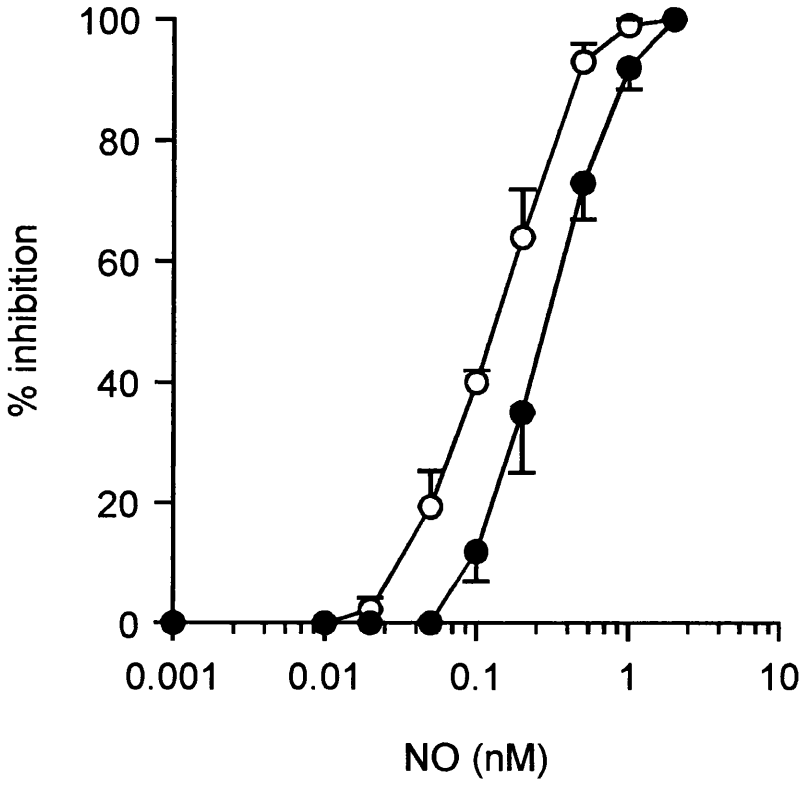


Figure 5.3: The influence of 15 (S)-hydroperoxyeicosatetraenoic acid on platelet sensitivity to inhibition by nitric oxide.

WP were incubated with NO (0 - 10μM) for 1min prior to the addition of thrombin (0.02U/ml), either in the presence (●) or absence(○) of 15 (S)-HpETE (10μM), and total aggregation measured 3min later. The results are expressed as the percent inhibition of aggregation compared to thrombin alone and represent means ± SEM of 4 independent experiments.

([b]) addition of NO, but prior to stimulation of platelets by thrombin, collagen or ADP.

[a] LDL → NO → thrombin [b] NO → LDL + thrombin

A 1min gap between the addition of NO and thrombin was maintained, and thus in experiments ([b]), the LDL were added immediately before the thrombin. The results in each section represent 3 independent experiments.

5.4.1 The influence of native low density lipoproteins on nitric oxide-mediated inhibition of platelet aggregation.

In *section 3.3.1*, nLDL were shown to inhibit thrombin-induced aggregation of WP in a concentration dependent manner. In order to assess the effects of nLDL on the inhibition of platelet activity by NO and to account for the independent effects of nLDL on aggregation, thrombin was used at a higher concentration (0.1U/ml) than in previous experiments (0.02U/ml).

Pre-incubation of WP for 1min with nLDL (1mg protein/ml) inhibited thrombin-induced platelet aggregation by 12.4 ± 6%. NO (0 - 10μM) induced a concentration-dependent inhibition of thrombin-stimulated aggregation (IC_{50} 1.3 ± 0.1μM). nLDL did not significantly affect platelet sensitivity to NO. In the presence of nLDL the IC_{50} for NO was increased slightly to 1.9 ± 0.4μM. When

the lipoproteins were added after NO the IC_{50} remained unaltered at 1.2 ± 0.3μM. Collagen (1μg/ml) activated platelets were inhibited by nLDL (1mg protein/ml) by 10.3 ± 5.2%. Again nLDL did not significantly affect inhibition of aggregation mediated by NO. The IC_{50} for NO was 0.42 ± 0.01μM, compared to 0.57 ± 0.09μM when nLDL was added before NO, and 0.28 ± 0.07μM when added after NO.

5.4.2 The influence of minimally modified low density lipoproteins on nitric oxide-mediated inhibition of platelet aggregation.

mmLDL (1mg protein/ml) incubated with WP 1min prior to addition of thrombin (0.1U/ml) failed to affect aggregation compared to that induced by thrombin alone. This is similar to results obtained in *section 3.4.1*, where mmLDL potentiation of ADP-induced aggregation was not detectable if the agonist alone induced secondary aggregation. NO (0 - 10μM) induced a concentration-dependent inhibition of thrombin stimulated aggregation of WP (IC_{50} 1.3 ± 0.1μM). The presence of mmLDL seemed to antagonise slightly the inhibitory actions of NO. Pre-incubation of mmLDL with WP prior to NO, as in [a] (*see above*), increased the IC_{50} for NO against thrombin-induced aggregation to 2.4 ± 0.3μM ($p < 0.05$) *[Figure 5.4].* If the mmLDL were added after NO, as in [b], they had little effect on platelet inhibition by NO (IC_{50} 1.7 ± 0.3μM). Similar results were obtained when platelets were stimulated with collagen. The IC_{50} for NO against collagen-stimulated aggregation was 0.57 ± 0.06μM, which increased to 0.86 ± 0.2μM ($p < 0.05$) in the presence of mmLDL. However addition of mmLDL after NO failed to affect the IC_{50} for the inhibitor (0.54 ± 0.12μM). These data suggested that the presence of mmLDL may decrease the sensitivity of platelets to NO. However, this is a cautious interpretation of the

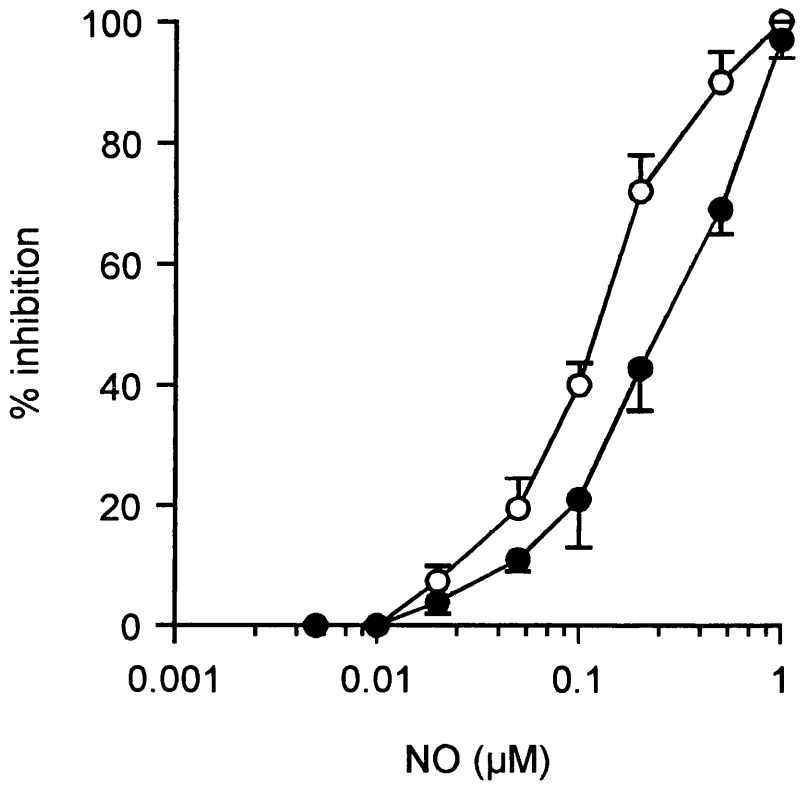


Figure 5.4: The influence of minimally modified low density lipoproteins on the inhibition of platelet aggregation by nitric oxide.

mmLDL prepared by the ultracentrifugation of human plasma followed by air oxidation (*see section 2.5.3*). Concentration response curves for NO (0 -10μM) against thrombin (0.02U/ml)-induced aggregation of WP were performed in the presence (●) and absence (○) of mmLDL (1mg protein/ml). Total aggregation measured 3min after the addition of thrombin. The results represent the percent inhibition of aggregation and are expressed as the mean ± SEM of 3 independent experiments

results and greater numbers of experiments are required to establish their true nature.

5.4.3 The influence of oxidised low density lipoproteins on nitric oxide-mediated inhibition of aggregation.

Thrombin-induced aggregation of WP was inhibited by 12.2 ± 7% in the presence of oxLDL (1mg protein/ml). oxLDL did not affect platelet sensitivity to NO, regardless of when they were presented to thrombin-stimulated platelets. However, the oxidised lipoproteins did enhance the inhibition of collagen-induced aggregation by NO. oxLDL alone inhibited the normal aggregation response to collagen by 11.4 ± 4.5%. The presence of oxLDL (1mg protein/ml) enhanced inhibition of collagen-induced aggregation by NO. OxLDL were most effective when added before NO, reducing the IC_{50} for the platelet inhibitor from 0.51 ± 0.06μM to 0.28 ± 0.05μM ($p< 0.01$), but when added after NO, the IC_{50} was also lowered to 0.32 ± 0.08μM ($p< 0.05$). The data represent three independent experiments. These results indicated that oxLDL may increase platelet sensitivity to NO, although it is likely that these are additive effects of oxLDL.

5.5 PLATELET INHIBITION BY S-NITROSOGLUTATHIONE: INFLUENCE OF LOW DENSITY LIPOPROTEINS.

Since NO is released from S-NOG intracellularly or close to the membrane (*see section 4.11.2*), it is possible to demonstrate that the effects of LDL on platelet sensitivity to NO, are independent of direct interactions between lipoproteins and NO. LDL were added to WP either 1min before or 1min after SNOG. In each case, a 1min gap was maintained between the addition of S-

NOG and collagen, and, in some cases, the lipoproteins were added immediately before the agonist (*see section 5.4*). Collagen (1µg/ml) was used to stimulate WP, because the effects of mmLDL and oxLDL on platelet sensitivity to NO were more potent against collagen-induced aggregation (*see sections 5.4.2 and 5.4.3*).

5.5.1. Native low density lipoproteins and S-nitrosoglutathione-mediated inhibition of platelet aggregation.

nLDL (1mg protein/ml) inhibited collagen-induced platelet aggregation by 18.2 ± 6.3% ($p \leq 0.05$). S-NOG added to platelets 1min before collagen inhibited aggregation in a concentration dependent manner (IC_{50} 0.072 ± 0.022µM). The incubation of nLDL with WP for 1min before addition of S-NOG, increased significantly the inhibition of platelet activity by the nitrosothiol *[Figure 5.5]*. Here, the IC_{50} for S-NOG was reduced to 0.020 ± 0.004µM ($p \leq 0.05$). This was also the case when nLDL was added 1min after S-NOG (IC_{50} 0.044 ± 0.015µM; $p \leq 0.05$) *[Figure 5.5]*.

5.5.2 Minimally modified low density lipoproteins and inhibition of platelet aggregation by S-nitrosoglutathione.

Collagen-induced platelet aggregation was not affected by the presence mmLDL (1mg protein/ml), as compared to controls. This phenomenon was also found in *section 3.4.1*, where mmLDL did not potentiate ADP-induced aggregation if the agonist alone induced a secondary response. S-NOG (0 - 10µM) produced a concentration dependent inhibition of aggregation of WP (IC_{50} of 0.091 ± 0.036µM). mmLDL, added to the platelets before or after S-NOG, failed to alter the IC_{50} for the inhibitor (0.081 ± 0.039µM and 0.084 ± 0.034µM) against

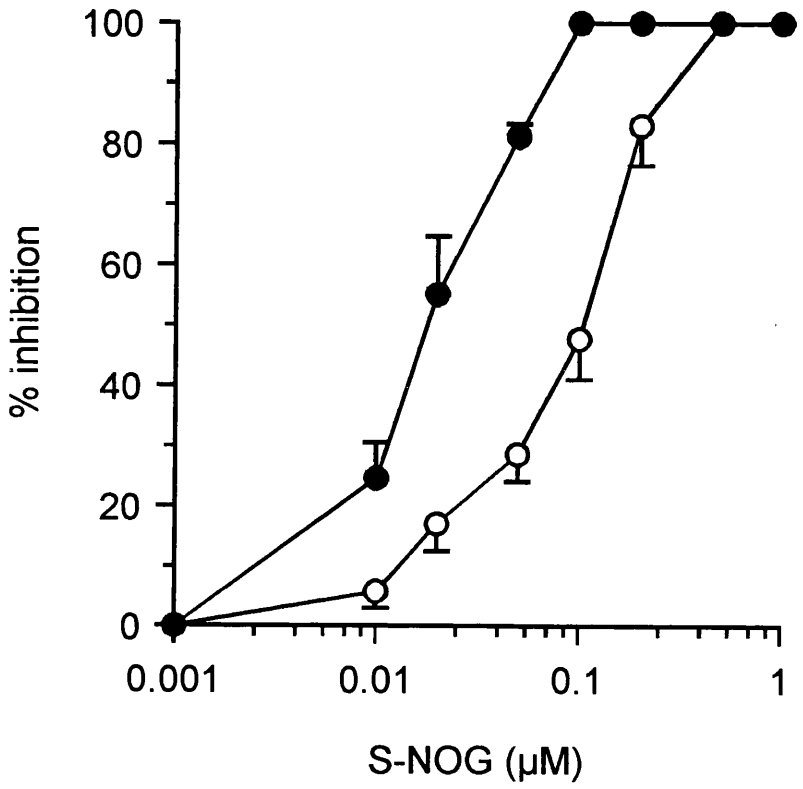


Figure 5.5: The influence of native low density lipoproteins on the inhibition of platelet aggregation by s-nitrosoglutathione.

nLDL (1mg protein/ml) prepared by the ultracentrifugation of human plasma (*see section 2.5.1*). Concentration response curves for NO (0 -10μM) against collagen (0.02U/ml)-induced aggregation of WP were performed in the presence (●) and absence (○) of nLDL (1mg protein/ml). Total aggregation measured 3min after the addition of thrombin. The results represent the percent inhibition of aggregation and are expressed as the mean ± SEM of 3 independent experiments

collagen-induced aggregation. This suggested that mmLDL has no significant effect on the sensitivity of platelets to the secondary messenger actions of NO once inside the cell.

5.5.3 The influence of oxidised low density lipoproteins on S-nitrosoglutathione-mediated inhibition of platelet aggregation.

In these experiments, oxLDL (1mg protein/ml) incubated with WP for 1min before the addition of collagen induced 21.9 ± 6.4% ($p \leq 0.05$) inhibition. S-NOG alone inhibited collagen-induced platelet aggregation in a concentration-dependent manner with an IC_{50} of 0.073 ± 0.022μM. The IC_{50} for the nitrosothiol was reduced to 0.035 ± 0.006μM in the presence of oxLDL and to 0.029 ± 0.007μM when oxLDL was added after the S-NOG. However, neither of these effects were statistically significant.

5.5.4. Résumé.

The native lipoproteins slightly enhanced the actions of cGMP-mediated inhibition of platelet aggregation by NO and S-NOG. These observations may be related to the independent inhibitory actions of the LDL preparations and suggests that a direct interaction between these lipoproteins and NO does not occur. In contrast, mmLDL seemed to attenuate slightly the inhibition of platelet activity by NO, but had a minimal effect on the actions of S-NOG. This suggested that the activatory effects of mmLDL may be potent enough to attenuate the actions of NO, but not S-NOG. The entry of NO into platelets may be impeded by mmLDL, although the effects are likely to have been small.

5.6 THE SUPPLEMENTATION OF NATIVE LOW DENSITY LIPOPROTEINS WITH 15 (S)-HYDROPEROXYEICOSATETRAENOIC ACID, AND THEIR EFFECTS ON PLATELET FUNCTION.

In chapter three of this thesis mmLDL were shown to induce the activation and aggregation of WP (*see section 3.4*). The mediator of these pro-activatory effects is proposed to be the low levels of lipid peroxides associated with the particles. Consequently, the direct effects of an authentic lipid peroxide, 15 (S)-HpETE, which is an oxygenated metabolite of arachidonic acid, on platelet aggregation was investigated (*see section 5.2.2*). 15 (S)-HpETE was of particular importance, since the phospholipid surface monolayers of LDL are known to be rich in arachidonate [Esterbauer *et al.*, 1990], and thus the peroxide represents a key LDL oxidation product. The effects of both the LPO and LDL on platelet aggregation and platelet sensitivity to NO have been examined individually. In the following experiments, both factors are combined. nLDL were utilised as a carrier particle to test the effects of 15 (S)-HpETE on WP (*see section 2.5.4*). Autologous nLDL were used as controls within each experiment. Supplementation of LDL with LPO, allowed assessment of the effects of LPO on platelet function in a more realistic model: peroxides have been demonstrated to be associated with lipoproteins *in vivo* [Nishigaki *et al.*, 1981; Bowry *et al.*, 1992].

5.6.1 The measurement of total lipid hydroperoxide of native low density lipoproteins supplemented with 15 (S)-hydroperoxyeicosatetraenoic acid.

Native LDL (5mg protein/ml) were incubated with 300nmol of 15 (S)-HpETE which equated to 60nmol 15 (S)-HpETE/mg protein. Measurements of LPO content (*see section 2.5.6*) for both nLDL and the corresponding 15 (S)-HpETE-

LDL were performed to assess the efficiency of the incubation procedure for the uptake of 15 (S)-HpETE.

nLDL incubated with buffer under the same conditions as nLDL and 15 (S)-HpETE showed no significant change in total LPO. Freshly isolated nLDL possessed 14.0 ± 3.5 nmol LPO/mg protein which only increased to 15.7 ± 3.4 nmol/mg LDL protein after further incubation and dialysis. In contrast, total LPO of nLDL was increased significantly from 14.0 ± 3.5nmol/mgLDL protein to 48.7 ± 7.3nmol/mg LDL protein ($p \leq 0.005$) after incubation with 15 (S)-HpETE.

5.6.2 The effects of 15 (S)-hydroperoxyeicosatetraenoic acid supplemented low density lipoproteins on platelet aggregation.

The influence of 15 (S)-HpETE-LDL on platelet aggregation was tested in the same manner as mmLDL (*see section 3.4.1*), assessing their actions in the presence and absence of platelet agonists. WP were activated with a sub-threshold concentration of ADP (1μM), which induced 11.3 ± 1.3% aggregation. The presence of 15 (S)-HpETE-LDL (0.25, 0.5 and 1mg protein/ml) for 1min before addition of ADP significantly enhanced platelet aggregation, at all concentrations used ($p < 0.05$) *[Figure 5.6]*. The actions of 15 (S)-HpETE-LDL were not concentration-dependent within the physiological range of concentrations for LDL, with 0.5mg protein/ml inducing the greatest effect (23.3 ± 1.5%) *[Figure 5.6]*. In control experiments, WP were incubated with autologous nLDL (1mg protein/ml) preparations. In the presence of nLDL, ADP-induced aggregation was reduced to 4.7 ± 0.9% ($p < 0.05$). When experiments were repeated with sub-threshold concentrations of thrombin (0.005U/ml) or collagen (0.1μg/ml), no potentiation of aggregation with 15 (S)-HpETE-LDL was

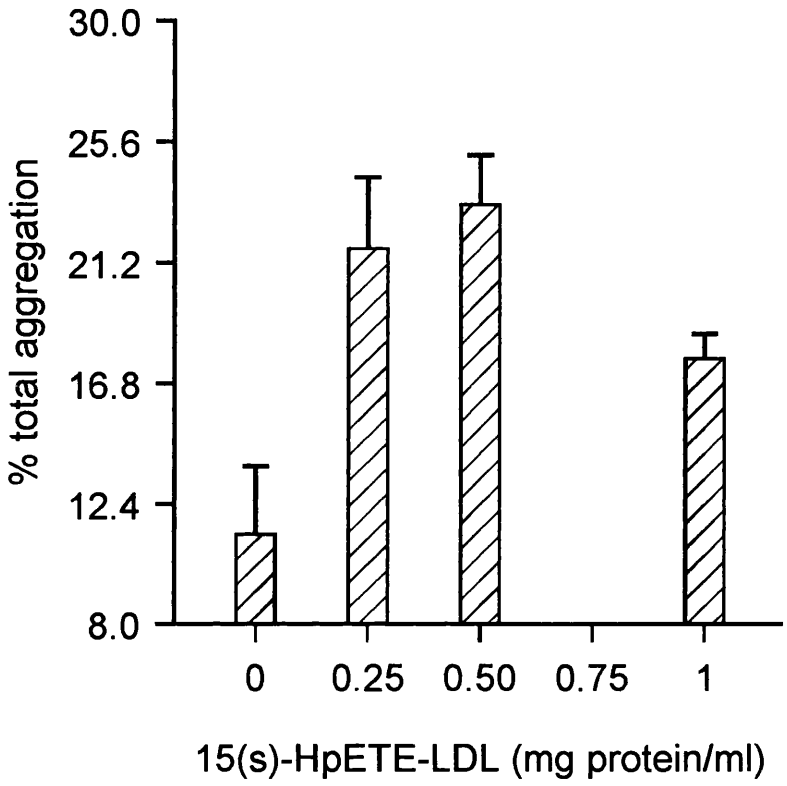


Figure 5.6: The influence of 15 (S)-hydroperoxyeicosatetraenoic acid on platelet aggregation stimulated by ADP.

15 (S)-HpETE was presented to WP as a complex with LDL to form 15 (S)-HpETE-LDL (*see section 2.5.4*). The 15 (S)-HpETE-LDL (0-1mg protein/ml) were incubated with WP for 1min prior to the addition of ADP (1μM). Total aggregation was measured 3min after the addition of ADP. The data represent the percent total aggregation and are expressed as mean ± SEM of 3 independent experiments.

observed. These experiments were not pursued. Incubation of 15 (S)-HpETE-LDL with WP in the absence of agonists failed to induce platelet aggregation. These data provide clear evidence that the presence of LPO associated with the particles alter their normal properties, allowing them to activate platelets.

5.6.3 The influence of low density lipoproteins supplemented with 15(S)-hydroperoxyeicosatetraenoic acid on nitric oxide mediated inhibition of platelet aggregation.

Following the same line of investigation as for H_2O_2, cumene-OOH and 15 (S)-HpETE alone, the effects of 15 (S)-HpETE-LDL on inhibition of aggregation by NO were investigated. WP were stimulated with ADP, but not thrombin or collagen, as the aggregatory actions of 15 (S)-HpETE-LDL were limited to ADP. NO (0 - 10μM) pre-incubated with WP (1min) induced a concentration dependent inhibition of ADP (10μM)-induced aggregation (IC_{50} 63.7 ± 8.4nM). Incubation of 15 (S)-HpETE-LDL (1mg protein/ml) with WP for 1min before the addition of NO, caused a very small, but statistically significant reduction in the actions of the antagonist. Here, the IC_{50} for NO was increased to 76.0 ± 7.7nM, ($p< 0.01$) *[Figure 5.7]*. This phenomenon was also observed with 15 (S)-HpETE and mmLDL. 15 (S)-HpETE-LDL added 1min after NO, and immediately before ADP failed to affect inhibition by NO.

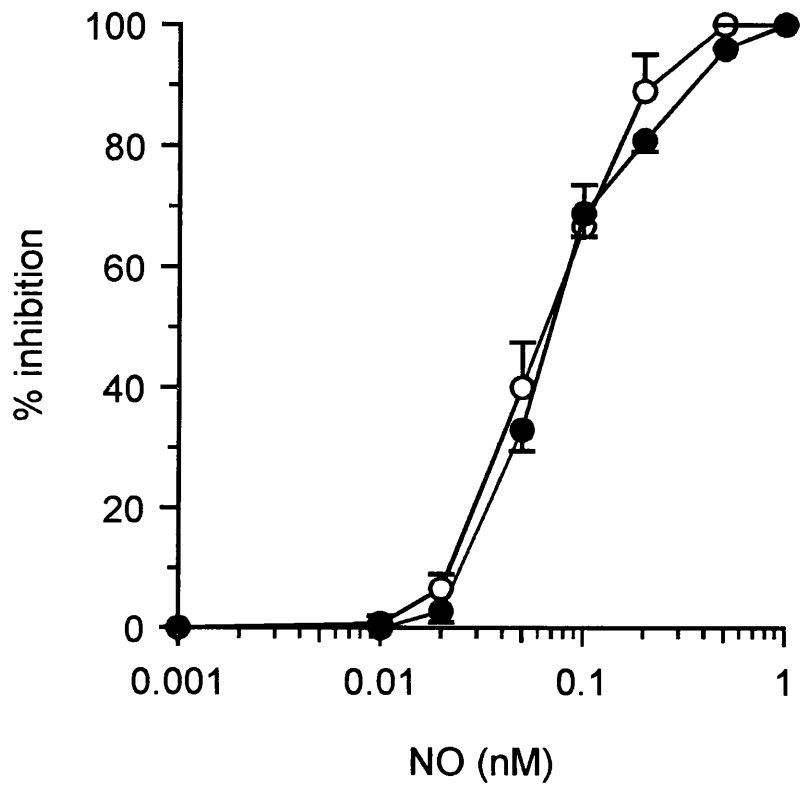


Figure 5.7: Concentration response curves for nitric oxide against ADP-induced aggregation: the influence of 15(S) Hydroperoxyeicosatetraenoic acid.

15(S)HpETE was presented to WP as a complex with LDL to form 15(S)HpETE-LDL (*see section 2.5.4*). IP were incubated with a range of concentrations of NO in the presence (●) or absence (○) of 15(S)HpETE-LDL (1 mg protein/ml) for 1min before stimulation with ADP (10μM), and total aggregation measured after 3min. In all cases the 15(S)HpETE-LDL was incubated with IP for 1min before the addition of NO. The results are expressed as the percent inhibition of aggregation compared to ADP alone and represent means ± SEM of 3 independent experiments.

5.7. DISCUSSION

5.7.1 Organic peroxides and platelet function.

The possible actions of peroxides on platelet function and platelet sensitivity to NO were investigated further using cum-OOH and 15(S)-HpETE. Cum-OOH was selected as it was water soluble and to complement previous work suggesting that NO reacts with organic peroxides leading to its inactivation [Padmaja and Huie, 1993]. Cum-OOH potentiated aggregation induced by sub-threshold concentrations of thrombin, but failed to induce aggregation. This peroxide only enhanced platelet aggregation when added to the platelets after the main agonist, as was the case with H_2O_2. Pre-incubation of cum-OOH with platelets prior to the agonist failed to influence aggregation, except at 100μM where a slight inhibition was observed. 15(S)-HpETE, in contrast to cum-OOH, had no significant effects on platelet aggregation at any of the concentrations tested. The effects of the different peroxides on platelet aggregation are summarised in *Table 5.1*.

The results suggest that peroxides such as cum-OOH and H_2O_2 behave differently from a lipid soluble peroxide, such as 15(S)-HpETE, with respect to platelet function *[Table 5.1]*. Both cum-OOH and H_2O_2 were able to potentiate aggregation, while 15(S)-HpETE had little effect. These differences may be due, at least in part, to differences in cellular permeabilities between the individual peroxides. H_2O_2 has been shown to increase platelet [Ca^{2+}] when added after the agonist [Del Principe *et al.*, 1985, Pratico *et al.*, 1992] leading to an increase in PLA_2 activation [Pratico *et al.*, 1992]. However, H_2O_2 has also been proposed to be an activator of cyclo-oxygenase [Del Principe *et al.*, 1985]. In *section 4.2.4* evidence was presented demonstrating the pro-aggregatory effects of H_2O_2 were

peroxide	no agonist	before agonist	after agonist
H_2O_2	primary aggregation	no effect	enhanced aggregation
cum-OOH	no effect	no effect	enhanced aggregation
15 (S)-HpETE	no effect	no effect	no effect
15 (S)-HpETE/LDL	no effect	enhanced aggregation	no effect

Table 5.1: Summary of the effects of different peroxides on platelet aggregation

totally reliant on COX. No evidence is presented here to indicate that cum-OOH enhances aggregation by the same mechanism. However, water soluble properties of cum-OOH may allow it to enter the cell. If this is the case, it may be speculated that cum-OOH also enhances aggregation by activation of COX. The more complex structure of 15(S)-HpETE would negate rapid entry of the peroxide into the platelet, and thus any effects would probably be mediated extracellularly.

A mechanism by which the peroxides may enhance aggregation could involve both COX and PLA_2. The addition of peroxides after an agonist may increase the $[Ca^{2+}]_i$ to a greater extent than the agonist alone, as observed by Del Principe *et al.*, [1991]. The elevated $[Ca^{2+}]_i$ may activate PLA_2, which is known to be calcium-dependent [Rittenhouse, 1984] and release AA from the membrane, which would then be metabolised by COX. These processes may enhance TXA_2 production and subsequent aggregation of the platelets [*summarised in Figure 5.8*]. This concept fits well with the present observations that peroxides only enhance activation of platelets when added after the agonist. The peroxides alone are unable to mobilize AA from the membrane to induce functioning of COX. However, if the agonist is added first, the additive effect of peroxides on platelet $[Ca^{2+}]_i$, may be sufficient to induce PLA_2 activation: H_2O_2 alone does not increase $[Ca^{2+}]_i$ in platelets [Del Principe *et al.*, 1991]. A study by Hashizume *et al.*, [1991], demonstrated that tert-butyl hydroperoxide enhanced collagen-induced aggregation and AA release. These effects were inhibited by the presence of mepacrine and indomethacin, PLA_2 and COX inhibitors respectively. Alternatively, it is possible that early addition of peroxides to the platelet leads to their enzymatic removal before the agonist is added. The

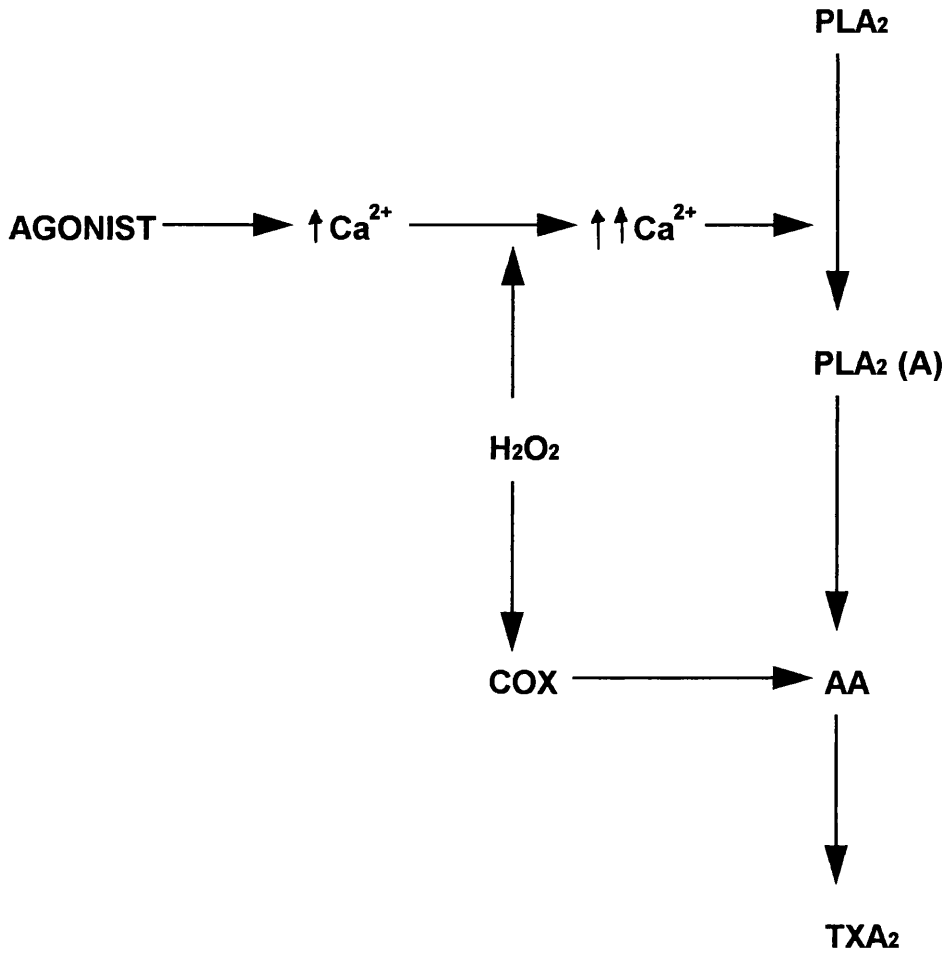


Figure 5.8: The proposed mechanism by which hydrogen peroxide and cumene hydroperoxide may influence platelet aggregation induced by submaximal concentrations of agonists.

In this scheme the peroxide increases $[Ca^{2+}]_i$ induced by the agonist, which causes activation of PLA_2 and release of AA. The peroxide also enhances activation COX, leading to the conversion of AA to TXA_2 and a subsequent increase in platelet aggregation.

Ca^{2+}: calcium ions, COX: cyclooxygenase, PLA_2: phospholipase A_2, TXA_2 ; thromboxane A_2.

reduced peroxide concentration may not be sufficient to exert a pro-aggregatory effect. Platelets possess two enzymes, GSH Px and catalase, which reduce peroxides to their hydroxy derivatives. H_2O_2 and cum-OOH induced different levels of aggregation which to some extent were dependent on the agonist, they were all optimally effective at similar concentrations (20 - 30μM).

The actions of peroxides may be related to the structure of the individual peroxide being investigated. Hemler *et al.*, [1979] demonstrated that the efficiency of lipid hydroperoxide activation of cyclo-oxygenase was dependent upon chain length, position of double bonds and location of the peroxide group. It is possible that the activation of platelets by LDL associated peroxides may be governed by such structural determinants. Indeed, in the present study 15(S)-HpETE had no significant effect on platelet activation, while Aharony *et al.*, [1982] demonstrated that 12(S)-HpETE inhibited platelet aggregation (IC_{50} 2-3μM).

5.7.2 The effects of peroxides on the sensitivity of platelets to nitric oxide

The pattern observed when cum-OOH enhanced aggregation, with respect to the order of addition to the platelets, also emerged when the effects of cum-OOH were tested against NO. Addition of the peroxide to WP after the thrombin, decreased the inhibition by NO. In contrast, addition of cum-OOH prior to NO, had no effect on the actions of the antagonist. This suggested that cum-OOH did not influence the entry of NO into the platelets, and that the reduced inhibition by NO, when the peroxide was added after thrombin, was due exclusively to its pro-aggregatory influence on platelet function. In this respect, cum-OOH behaved similarly to H_2O_2: both peroxides increased platelet activation and

decreased platelet sensitivity to NO when added after the main agonist and were maximally effective at similar concentration (20 - 30μM). In contrast to H_2O_2, when cum-OOH was added simultaneously with NO before the agonist, no change in the level of inhibition was observed. It has been proposed that NO can react with organic peroxides, leading to the inactivation of the inhibitor. In the present study there is no direct evidence to substantiate this previous study [Padmaja and Huie, 1993], as addition of NO to platelets pre-incubated with cum-OOH did not alter the efficiency of the platelet antagonist. The mechanism of action of these peroxides seem to differ from that of 15(S)-HpETE. The lipid hydroperoxide did reduce slightly the inhibition of platelet aggregation by NO, but only if present before the NO. As 15(S)-HpETE showed no pro-aggregatory effects, this would suggest that the peroxide reduced the entry of NO into the platelet. Recently, it has been shown that NO can undergo direct reactions with $LOO^{\bullet}$ and $LO^{\bullet}$ radicals of linoleic acid [Rubbo *et al.*, 1994]. It is possible that the formation of reaction products between 15(S)-HpETE and NO may prevent its entry into the platelet. However, this requires further investigation.

In conclusion, cum-OOH and 15(S)-HpETE, unlike H_2O_2, can only reduce the inhibition of platelets by NO. Both 15(S)-HpETE and cum-OOH, increased the IC_{50} for NO, although the mechanisms by which the effects were induced seemed to be different. Since, cum-OOH was only effective against NO when added before the agonist, whilst 15(S)-HpETE was antagonistic when present before NO. The effects of the different peroxides on platelet sensitivity to NO are summarised in *Table 5.2.*

peroxide	before NO	simultaneously with NO	after NO
H_2O_2	enhanced inhibition	enhanced inhibition	reduced inhibition
cum-OOH	no effects	no effects	reduced inhibition
15 (S)-HpETE	reduced inhibition	nd	no effects
15 (S)-HpETE/LDL	no effects	no effects	no effects

Table 2: Summary of the effects of different peroxides on platelet sensitivity to nitric oxide.

5.7.3 Low density lipoprotein and platelet sensitivity to nitric oxide.

NO is an extremely lipophilic molecule, which allows it to freely enter platelets and exert its biological effects. The hydrophobic characteristics of the molecule indicate it may be possible that NO is attracted to the hydrophobic cores of lipoproteins, and this may represent a process of NO deactivation. This could be particularly relevant in small localised areas of high lipoprotein concentration such as an atherosclerotic plaque [Smith, 1974; Hoff *et al.*, 1977]. Incubation of nLDL with platelets prior to the addition of NO, decreased slightly the IC_{50} for NO. This is not unexpected as nLDL itself has been shown to inhibit agonist induced aggregation of WP and, more importantly, suggests that there is no direct interaction between the lipoproteins and NO. Oxidised LDL gave essentially similar results, although the reduction in the IC_{50} was greater than observed with nLDL. This probably reflects the fact that oxLDL is a more potent inhibitor of platelets than nLDL (*see sections 5.4 and 5.5*).

NLDL was shown to have little effect on inhibition by NO, but to synergise with S-NOG, which again contradicts the earlier concepts implicating these lipoproteins as pro-thrombotic. This effect was lost even when the lipoproteins became only partially oxidised. The presence of mmLDL, led to an increase in the IC_{50} for NO against thrombin-stimulated platelets. However the lipoproteins had little effect on platelet inhibition when added after the NO. This indicated that the pro-aggregatory effects of mmLDL were not sufficiently potent to antagonise the intracellular actions of NO. When mmLDL is added before NO, the pro-aggregatory effects of the lipoproteins may reduce the inhibition, but it is also possible that there is a direct interaction between the lipoproteins (or their constituents) and NO. This implies that the reduced effectiveness of NO in

the presence of mmLDL may be due to a direct interaction between NO and LPO present in the lipoproteins, thereby reducing the amount of NO available to enter the platelet. It has been demonstrated that NO can react with organic peroxyl radicals [Padmaja and Huie, 1993] and lipid peroxyl radicals [Rubbo *et al.*, 1994]. A reaction between NO and LPO would be detrimental to haemostasis as it could lead to the loss of NO. The presence of GSH Px and GSH which act to reduce LPO [Maddipati *et al.*, 1987], may protect against this. Conversely, a reaction between NO and peroxyl radicals, could protect cells and lipoproteins against free radical mediated damage, and in this respect NO may be viewed as an antioxidant.

In contrast to the experiments with authentic NO, mmLDL did not affect inhibition by S-NOG, regardless of when mmLDL was presented to the platelets. There are two possibilities for the different results obtained with NO and S-NOG in the presence of mmLDL. S-NOG has been shown to be a more effective platelet inhibitor than NO [Radomski *et al.*, 1987], and this increased potency may be sufficient to attenuate the activatory effects of mmLDL. Alternatively, the LPO associated with mmLDL may interact directly with free NO, reducing its entry into the platelet (as discussed above). However, stabilisation of NO by a thiol group, such as that found on GSH, could reduce the possibility of a reaction. The effects of LDL and more specifically mmLDL on both platelet activation and platelet sensitivity to NO, may tip the balance in favour of aggregation. At present the mechanism of these actions are unknown, but it indicates a possible important role for mmLDL in thrombosis.

If mmLDL causes the inactivation of NO, in addition to activating platelets, it may

have important implications for the maintenance of vascular tone, in addition to haemostasis.

5.5.5 The supplementation of low density lipoproteins with the lipid peroxide 15(S) -hydroperoxyeicosatetraenoic acid

15(S)-HpETE was used to examine how LPO affected the influence of plasma lipoproteins on platelet function. Supplementation of nLDL with 15(S)-HpETE changed the properties of the particles from being inhibitory to activatory. 15(S)-HpETE alone did not induce aggregation, but enhanced aggregation induced by ADP. This provided strong evidence that LPO are at least partly responsible for the effects of mmLDL on platelets. The full pro-aggregatory effects associated with mmLDL may be a cumulative effect of different peroxides, of which 15(S)-HpETE may be one of many. It is possible that other modified components of mmLDL are also involved in their pro-aggregatory actions. A number of reports have shown that lipoproteins bind to platelets, but this does not result in endocytosis [Mazurov *et al.*, 1982; Curtiss and Plow, 1984; Shmulewitz *et al.*, 1984]. This would suggest that the actions of 15(S)-HpETE, and possibly other peroxides associated with mmLDL, are probably exerted at or near the cell surface.

Essentially, the 15(S)-HpETE/LDL behaves similarly to mmLDL: both species enhance aggregation when pre-incubated with platelets before the addition of the agonist. This again suggests that LPO interact with platelets differently from the simpler water soluble peroxides, which only activated platelets when added after the agonist *[Table 5.1]*. However, a very recent study by Wiedtmann *et al.*, [1995] showed that mildly oxidised LDL-induced aggregation of platelets was

inhibited by mepacrine and indomethicin, inhibitors of PLA_2 and COX respectively. This is the same activation pathway proposed for the potentiation of aggregation by H_2O_2 and cum-OOH (*see section 5.5.1*). This may indicate a common pathway for the enhancement of platelet function by peroxides, although the mechanism by which the enzymes involved in signal transduction are activated may be different in the two cases.

15(S)-HpETE/LDL attenuated the inhibition of aggregation by NO, although the effects were very small. This reduction in NO sensitivity was only observed if 15(S)-HpETE/LDL was pre-incubated with platelet before the addition of NO. In this respect 15(S)-HpETE/LDL behaved like 15(S)-HpETE alone and mmLDL, both of which antagonised NO in this manner. Interestingly 15(S)-HpETE/LDL had little effect on NO-mediated inhibition when added after the agonist, which may correspond to its inability to enhance aggregation when added at this point. Since 15(S)-HpETE/LDL, 15(S)-HpETE alone and mmLDL attenuated inhibition by NO when present before the antagonist, this may suggest that the lipoproteins reduce the entry of NO into the platelets. However, this proposal needs to be investigated further before a conclusive answer can be given.

Peroxides, therefore, have a varied action on blood platelets which may have significant consequences in the modulation of their activity in the normal and diseased state.

CHAPTER SIX : GENERAL DISCUSSION.

6.1 Plaque development and platelet aggregation.

Atherosclerosis is characterised by the presence of lipid-laden plaques in the intima of the arterial wall [Hoff and Gavbatz, 1982]. The plaques are prone to rupture which expose their contents to flowing blood, increasing the risk of platelet aggregation and formation of a thrombus. It is widely accepted that plaque fissure leads to thrombosis [Constantinides, 1966; Friedman and van den Bovencamp, 1966], and these two events are the precursors to acute coronary syndromes [Davies and Thomas, 1985; Falk, 1989]. Fibromuscular plaques have an increased size compared to the lipid-laden plaques, because of the incorporation of mural thrombi. Evidence suggests that plaque size increases by recurrent minor fissures of soft plaques, with subsequent mural thrombi and fibrin meshwork formation [Fuster *et al.*, 1990]. Morphologically, plaques are composed of healed fissures with overlying mural thrombi, suggesting that most fissures of the plaque reseal and incorporate the thrombus, without producing acute clinical complications.

Platelet activation and aggregation are major events during the formation of thrombi, suggesting a close relationship between platelet aggregation and vascular disease [Rubenfire *et al.*, 1986]. Several studies have shown an alteration in platelet function after coronary complications. The Caerphilly Collaborative Heart Disease Study, involving a cohort of over 200, demonstrated a strong positive relationship between previous myocardial infarction (M.I.) and ADP-induced platelet aggregation [Elwood *et al.*, 1990]. In a separate study of patients with past M.I., the subjects with platelets which more readily underwent spontaneous aggregation had a significantly greater mortality rate than those

with unresponsive platelets [Trip *et al.*, 1990]. These studies strongly implicated platelet aggregation as an integral part in the development of atherosclerotic plaques and their clinical sequelae. However, the most convincing evidence of the role of platelets, is the reduction of coronary events by treatment with aspirin [Anti-platelet trialists collaboration, 1988].

6.2 Platelet function is modulated by plasma low density lipoproteins.

Early observations in hyperlipidaemic patients suggested a causal relationship between plasma LDL concentrations and increased susceptibility of platelets to aggregate [Carvalho *et al.*, 1974, Joist *et al.*, 1979, Zahavi *et al.*, 1981]. Subsequently, LDL was shown to have direct effects on platelet aggregation and secondary messenger levels (*see section 1.7.2*). The discovery that LDL may undergo a post-secretory oxidative modification and that this process alters the properties of lipoproteins, rendering them more atherogenic, put a question mark over the findings of previous studies. It is reasonable to speculate that slight oxidation of the isolated LDL preparations may account for some of the observations. Subsequently, oxLDL were shown to have a greater effect on platelet activation than nLDL [Ardlie *et al.*, 1989, Meraji *et al.*, 1992]. In the present study, attention was paid to the extent of which the degree of oxidative modification of LDL may affect their influence on platelet function.

Stringent oxidation procedures were used in the isolation of LDL, before their oxidation. In contrast to previous studies, nLDL did not activate platelets, but inhibited aggregation in a plasma free environment, and early activation in the presence of plasma. It is proposed that these observations are attributed to nLDL interfering with the normal binding of FGN to the GPIIb-IIIa receptor.

Several studies have shown LDL to bind to the platelet surface [Mazurov *et al.*, 1982; Curtiss and Plow, 1984; Shmulewitz *et al.*, 1984; Katzman *et al.*, 1991; Pedreno *et al.*, 1994], although no direct evidence has been presented to show this binding site is GPIIb-IIIa. Whether the binding of LDL to platelets is specific or non-specific, or to GPIIb-IIIa or an alternative site remains to be resolved. More importantly, the present study demonstrates that LDL in their native form do not activate platelets, but have a small inhibitory effect. A recent finding demonstrating that nLDL and isolated apoB inhibited thromboplastin (tissue factor) activity [Ettelaie *et al.*, 1995] raises an interesting question. As nLDL seems to inhibit both platelet activation and thromboplastin activity, does this point to a role for apoB in haemostasis ? These preliminary data may suggest that apoB at physiological concentrations, could have a functional role in the regulation of some haemostatic functions.

Modified LDL modulate platelet activity, although the nature of the effect is dependent on the extent of oxidation. OxLDL inhibited platelet activation and aggregation. The potency of these effects were greater than observed with nLDL, and seemed to occur via a different mechanism. Since oxLDL inhibited aggregation after a 1min incubation both in the presence and absence of plasma. OxLDL has been shown been to be cytotoxic to various cell types [Morel *et al.*, 1984; Cathcart *et al.*, 1985; Zhao *et al.*, 1994], and it is plausible that it exerts similar effects of platelets. In addition two oxidation products of LDL, lysolecithin [Besterman and Gillette, 1971] and 4-HNE [Selley *et al.*, 1988], both inhibit platelet aggregation independently. mmLDL induced and enhanced early activation, aggregation and increased the expression of markers for degranulation. The effects occur in the presence of plasma and implicate

mmLDL as potentially important pro-thrombotic factors, in addition to their documented pro-atherosclerotic effects (*see section 1.4.3*). The results indicated that the activatory effects of mmLDL are due to the formation of oxidised lipids, since the protein moiety of the particles was not significantly modified *(see section 3.2.3).* The actions of oxidised and minimally modified LDL taken together suggested that the products of lipid oxidation may have a biphasic effect on platelet function. MmLDL, characterised by small increases in both LPO and TBARs concentrations, activated platelets, while increased concentrations of these products seemed to induce a potent inhibition of aggregation, as was the case with oxLDL. This phenomenon has already been recognised for 4-HNE, an LDL oxidation product. Selley *et al.*, [1988] demonstrated that 4-HNE could activate platelets at low concentrations, but at concentrations in excess of 100μM it inhibited aggregation. The initial results of the study suggested both LPO and their breakdown products exert a strong influence on platelet activation. Consequently, the effects of peroxides on platelet aggregation were investigated in greater detail.

6.3 The influence of peroxides and platelet function.

Warso and Lands, [1985] found plasma LPO concentrations of approximately 0.5μM in healthy subjects. However, plasma LPO concentrations have been shown to be raised in patients with atherosclerosis [Stringer *et al.*, 1989]. These studies found an upper limit of 5 - 7μM peroxides in the plasma of patients with IHD. Platelet samples from subjects with various complications associated with atherosclerosis were shown to be hyper-responsive to physiological agonists [Cavarlho et al., 1974; Joist *et al.*, 1979, Zahavi *et al.*, 1981; Trip *et al.*, 1990; Elwood *et al.*, 1990; Buczyński *et al.*, 1993]. In the present study mmLDL were

found to activate platelets. It is possible to speculate that the actions of mmLDL may link the above observations. The increased levels of plasma LPO may be in part due to the presence of mmLDL, since it is feasible that they may exist in circulation (*see section 3.7.3*). Increased plasma concentrations of mmLDL may increase the sensitivity of platelets to physiological agonists. This may be particularly relevant to the previously cited studies (see above), since their experimental analysis of platelet function were performed in PRP.

The actions of peroxides on platelet function were tested by addition of both water soluble and LPO directly to platelets. All the peroxides tested were shown to increase platelet activation in response to agonists, although some differences in the mechanism of these effects were found (*see section 5.1*). H_2O_2 and cum-OOH activated platelets when added post-agonist, which was not the case with 15 (S)-HpETE. The LPO (15 (S)-HpETE) only activated platelets when it was complexed with LDL and only if present before the agonist. It is proposed that H_2O_2 and cum-OOH may enter the platelets where they activate PLA_2 and COX (*discussed in section 5.5.1*). The more complex structure of 15 (S)-HpETE would preclude rapid entry into the platelet. However, if the LPO is complexed with LDL, the lipoprotein may bind to the platelets and thus expose the cells to a high local concentration of the peroxide. This may facilitate platelet activation in the same manner as proposed for mmLDL (*see section 3.7.4*). The results indicated that LPO are partially responsible for the actions of mmLDL.

The prevention of LPO induced effects are mediated by the activity of anti-oxidant enzymes such as SOD, catalase and GSH Px. In a recent study by Buczyński *et al.*, [1993], it was found that increased platelet activity in patients

with CHD was associated with reduced activities of these enzymes. Of particular importance was GSH Px, the major hydroperoxide reducing enzyme in platelets [Merasca *et al.*, 1992]. The activity of platelet GSH PX was reduced by 38% in these patients. The authors did not speculate as to how these decreased activities occurred. However, it may be related to the increased circulating peroxides associated with the disease. Exposure of endothelial cells to linoleic hydroperoxide led to the peroxidation of cellular phospholipids [Pacifici *et al.*, 1994a] and produced a rapid release of fatty acids, particularly arachidonic acid, into the culture media [Pacifici *et al.*, 1994b]. If a similar effect occurred in platelets it would lead to enhanced platelet aggregation. This concept is supported by a clinical study of patients at risk from cardiovascular disease. A sub-group of people with low plasma antioxidant status was selected and various platelet parameters measured [Salonen *et al.*, 1991]. These subjects exhibited enhanced platelet activation compared to controls, which was positively correlated with higher plasma LPO. The activation markers were reduced after oral administration of antioxidants. Interestingly, LPO may also increase thrombogenicity by a mechanism different to that of platelet activation. They have been shown to increase the pro-coagulant activity of the blood by inhibiting anti-thrombin III [Grey and Barrowcliffe, 1985].

6.4 Influence of lipoproteins and peroxides on platelet sensitivity to nitric oxide.

The endothelium releases PGI_2 and NO, two factors which protect the blood vessel against pathological platelet deposition (*see section 1.6*). PGI_2 has been shown to mainly inhibit aggregation [Higgs *et al.*, 1978], while NO inhibits both aggregation and adhesion [Radomski *et al.*, 1987a, b]. PGI_2 and NO have been

shown to synergise in the inhibition of platelet aggregation [Moncada *et al.*, 1990]. Reduced formation or reduced cellular sensitivity to NO or PGI_2 would favour both activation and adhesion of platelets. Platelet sensitivity to PGI_2 has been shown to be reduced in the presence of LDL, a process associated with reduced production of cAMP [Bruckdorfer *et al.*, 1985; Beitz *et al.*, 1985].

In the present, study evidence is presented to suggest that mmLDL, water soluble and lipid peroxides, all reduce platelet sensitivity to NO. The most effective peroxide was H_2O_2, although the pathophysiological relevance of these actions is not clear. H_2O_2 may be produced locally in high concentrations by activated neutrophils [Test and Wiess, 1984], and thus could be an important mediator of platelet function in local inflammatory events. However, it is unlikely that this peroxide is raised in the circulation, and evidence presented earlier in this thesis suggests it may play a contrasting role to other peroxides with respect to platelets (*discussed in section 6.5*). The actions of LPO associated with lipoproteins seem to be slightly different to those of other organic peroxides. The reduced effectiveness of NO induced by H_2O_2 and cum-OOH seem dependent on their pro-aggregatory actions, since they were only effective when added after the agonist. Pre-incubation of mmLDL, 15(S)HpETE alone or LDL-15(S)HpETE, but not nLDL with platelets before the addition of NO, attenuated the inhibitory effects of the platelet antagonist. This suggests that LPO associated with LDL may impede the entry of NO into the platelet or operate by some other mechanism. In a recent publication it was demonstrated that NO has the ability to terminate radical induced peroxidation of linoleic acid. This involves a direct interaction between NO and $LOO^{\bullet}$ or $LO^{\bullet}$ radicals, leading to the formation of nitrogen containing lipid products [Rubbo *et al.*, 1994]. The authors

speculate that NO could prevent the oxidation of LDL by terminating lipid radical chain propagation reactions, and thus possess antioxidant properties. Conversely, these antioxidant properties may lead to a reduction the vascular actions of NO, favouring SMC contraction and luminal platelet adhesion and aggregation.

The mechanism of how peroxides, in particular those associated with LDL, could reduce the effectiveness of NO is undefined. A direct reaction between NO and peroxides would reduce NO entry to the platelets and hence decrease cGMP production. However, it is possible that the pro-aggregatory effects of the peroxides could impair or oppose the actions of cGMP, without a direct interaction. The full mechanism of these observations remains to be elucidated.

6.5 The potential anti-thrombotic actions of hydrogen peroxide.

In contrast to the proposed vascular effects of peroxides and ROS, evidence is presented here which suggests that H_2O_2 may be an important secondary messenger which increases the activity of NO. The influence of H_2O_2 on platelet function has been tested many times with contradictory results produced (*see section 4.13.3.1*). Similarly, H_2O_2 increased cGMP formation in rabbit aortas [Zembowics *et al.*, 1993], while H_2O_2 induced oxidative stress can reduce the effectiveness of NO production and cGMP formation in cocultures of calf pulmonary EC and SMC [Marczin *et al.*, 1991]. Many of these studies used unrealistically high concentrations of H_2O_2 (0.5 - 2mM). In the present study using physiologically relevant concentrations, H_2O_2 acted positively to increase the effectiveness of NO, both in the presence and absence of plasma. The mechanism of these effects is poorly understood, as increased formation of

cGMP does not completely account for the protracted inhibition of aggregation. It is possible that NO and H_2O_2 synergise to reduce energy charge in the cell. H_2O_2 has been shown to decrease platelet ATP levels [Holmsen and Robkin, 1977], while NO inhibits several enzymes involved in mitochondrial respiration in rat hepatocytes [Stradler *et al.*, 1991]. NO also inhibits the action of glyceraldehyde-3-phosphate dehydrogenase [Molina *et al.*, 1992]. In fact, inhibition of mitochondrial respiration was one of the initial processes investigated to explain the action of nitrovasodilator drugs [Gross and Wolin, 1995]. The combined inhibition of glycolysis and aerobic mitochondrial respiration would profoundly impair ATP synthesis. However it is unclear whether the two antagonists exert such an effect in the time span used at these low concentrations.

The data presented here shows that H_2O_2 may have opposing effects on platelets which are dependent upon the point when it is applied to the cells. If H_2O_2 formation by the action of SOD outstrips the GSH Px activity, it would result in oxidative stress. Buczyński *et al.*, [1993] showed that GSH PX activity in platelets with CHD was reduced. Since this is the major enzyme for H_2O_2 degradation in platelets [Maresca *et al.*, 1992], excess H_2O_2 production in this instance may well lead to cellular damage. The work in this thesis supports the concept that H_2O_2 is more likely to play an inhibitory role in platelet function. H_2O_2 formation is a continual process *in vivo* [Ramasarma, 1982], produced both by platelets and the endothelium. Local concentrations of H_2O_2 may be significantly increased in inflammatory events, as neutrophils are known to produce large quantities of H_2O_2 [Test and Wiess, 1984]. Thus H_2O_2 would be present both intra- and extracellularly, with respect to the platelet. This may

imply that the synergy between NO and H_2O_2 to inhibit platelet aggregation, could occur between NO and H_2O_2 from different cellular sources. It may be important to confirm whether NO and H_2O_2 produced endogenously by the platelet can limit their activation, or that their presence is required from sources external to the platelet.

The results from chapter four of this thesis indicate that the potent inhibitory actions of NO/H_2O_2 are not due to the formation of peroxynitrite. However, this does not exclude the possibility of other free radicals or products of free radicals being involved in the inhibition of platelet aggregation. Indeed, the hydroxyl radical scavengers, mannitol and sodium urate, antagonised the inhibition of platelet activity by both NO/H_2O_2 and peroxynitrite, implying the inhibition may have a common factor.

Peroxynitrite has been implicated in the formation of nitrosothiols, by a reaction between the oxidant and free sulphydryl groups on proteins and other peptides [Beckman *et al.*, 1992]. The presence of nitrosothiols have been demonstrated in human plasma [Stamler *et al.*, 1992]. In this thesis H_2O_2 was shown to enhance the inhibition of platelet aggregation by S-NOG, both in the presence and absence of plasma. Several workers have shown H_2O_2 alone can inhibit platelet activity in PRP. It is reasonable to speculate that these inhibitory effects of H_2O_2 in PRP, could be due to a synergism with naturally occurring nitrosothiols present in the plasma, for example S-nitrosoalbumin [Stamler *et al.*, 1992]. The full implications of the synergy between H_2O_2 and NO remain to be fully defined, but the actions of H_2O_2 may be pro- or anti-thrombotic depending on the conditions at the time.

6.6 Conclusions.

From the data presented in this thesis, it is postulated that organic peroxides are critical determinants of platelet function. Many studies have shown patients suffering from various manifestations of heart disease have increased plasma peroxide concentrations and platelet which are hyper-responsive. It is possible that the peroxides are associated with LDL, which have undergone a post-secretory modification. Thus rendering the particles with similar characteristics to the mmLDL used here. These mildly oxidised forms of LDL may be responsible for the increased potential for platelet aggregation. Recently, it has been demonstrated that platelets involved in the formation of occlusive thrombi *in vitro* produce free radicals which can oxidise LDL [Gorog and Kovacs, 1995]. Hence, platelet themselves may initiate an increase in the formation of atherogenic factors such as mmLDL and thus enhance chances of thrombosis.

NO produced by the endothelium may act to regulate platelet activity *in vivo*. Here data is presented which show that LDL in their native state, may reduce platelet activity and increase the inhibitory properties of NO. Mild oxidation of LDL leads to the loss of these actions, mmLDL increase platelet aggregation and seem to oppose the actions of NO. The actions of mmLDL were proposed to be mediated by the low levels of peroxides associated with the particles. The effects of organic peroxides were on platelet aggregation and platelet sensitivity to NO were investigated. H_2O_2, cum-OOH and 15(S)-HpETE all antagonised the inhibitory actions of NO. Conversely, H_2O_2 also enhanced the actions of NO, leading to a prolongation and intensification of NO-mediated inhibition of platelet activity. This effect seemed specific for H_2O_2, and give a new insight into cellular regulation by NO.

APPENDIX ONE

The measurement of nitric oxide solutions using a sensor probe.

In this thesis, NO solutions are quoted as concentrations. Thus, it was important to verify the actual concentration of NO in each solution. This was achieved by the use of a specific sensor probe for NO(ISO-NOP: World Precision Instruments, USA).

Principle.

NO diffuses through a selective membrane and is oxidised at the working electrode resulting in an electrical current. The redox current is proportional to the concentration of NO in solution.

Calibration of the probe.

The sensor probe is calibrated using a finite amount of nitrite (NO_2^-) which is converted to NO, according to the following equation:-

$$2KNO_2 + 2KI + 2H_2SO_4 \rightarrow 2NO + I_2 + 2H_2O + 2K_2SO_4$$

The reaction goes to completion, an therefore KNO_2 is the rate limiting reagent. The ratio between KNO_2 and NO is 1:1, and therefore the amount of NO generated in solution will be equal to the amount of KNO_2 added.

For calibration of the probe, KNO_2 solutions were added to a 10ml reservoir to give final concentrations between 5 and 100nM. The response in picoamps (pA) is then used to generate a standard curve (*see accompanying figure*).

Procedure and results.

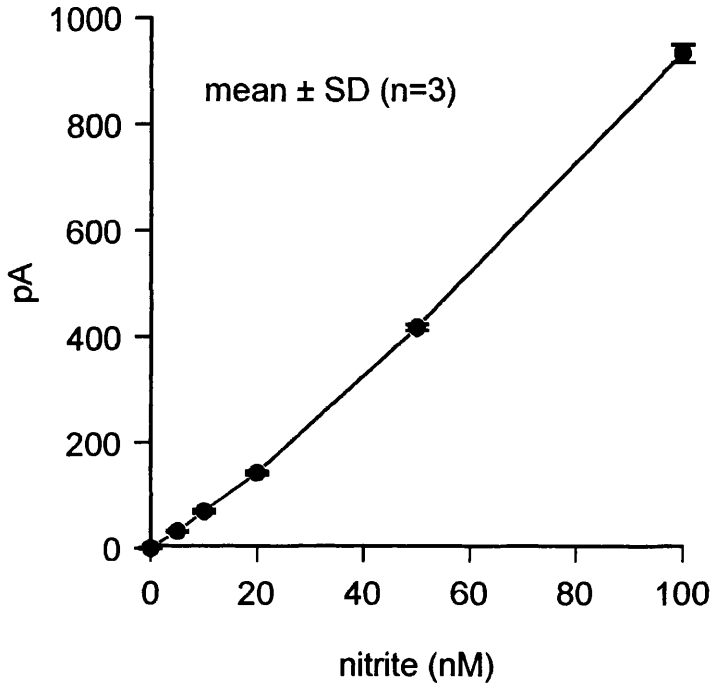
Two solutions of NO (20μM) were prepared (*see section 2.7.1*). The tip of the probe was placed in a 10ml reservoir of Tyrode's HEPES buffer (*see section 2.3.2*) and left for 30min to equibrilate. Aliquots (100μl) of the NO solutions were added the reservoir to give a final concentration of 20nM NO and the maximal change in pA measured. The pA could then be used to measure the concentration of the solutions from the standard curve. Each solution was measured three times and the results presented below.

	concentration of NO (nM)			mean
solution 1	16	19	20	18.3 ± 2.1
solution 2	17	18	19	18 ± 1

results are expressed as mean ± sd

The results suggest that the method employed to prepare solutions of NO are reliably accurate.

Standard curve used for the measurements of NO solutions.



APPENDIX TWO

Future work.

There are several possible avenues of study which could be followed up from the results presented in this thesis.

(1) Characterisation of the interactions between LDL and platelet GPIIb-IIIa.

It may be important to investigate the mechanism of this interaction under various conditions to assess whether the inhibition of platelet fibrinogen binding by nLDL could be of physiological relevance.

(2) Investigation into the mechanism by which H_2O_2 enhances the inhibitory action of NO on platelet activity.

The possible synergism between NO and H_2O_2 to inhibit platelet aggregation may have wider biological implications. It is important to investigate the contribution of endogenously produced NO and H_2O_2 on platelet aggregation. It is possible that a new radical species may be formed between NO and H_2O_2 which is more potent than NO with respect to platelet inhibition: this possibility should be examined. Finally the effects of H_2O_2 on NO-mediated functions in other cell types should be assessed.

(3) Further investigations into the effects of LPO on NO-mediated cellular function.

Preliminary evidence is presented in this thesis to suggest that LPO may antagonise the action of NO. This observation needs to be examined since LPO are known to be raised in several pathological states, and this may contribute to the disease.

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