Regulation of Human Neutrophil Apoptosis by Nitric Oxide and Peroxynitrite

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Presented for the degree of Doctor of Philosophy

The University of Edinburgh

October 2003

Declaration

I hereby declare that the data presented in this thesis is the result of my own work, carried out under the supervision of Drs. Adriano Rossi and Ian Megson at the University of Edinburgh, and that this thesis was composed entirely by myself.

EMMA LOUISE TAYLOR

Edinburgh, October 2003

To my parents, in recognition of their unstinting and continued investment in my educational and personal development

"If we knew what it was we were doing, it would not be called research, would it?" — Albert Einstein

Abstract

Apoptosis of inflammatory cells is a critical event in the resolution of inflammation, as failure to undergo this form of cell death leads to increased tissue damage and exacerbation of the inflammatory response. Prolonged survival of the neutrophilic granulocyte has been implicated in the pathogenesis of many chronic inflammatory conditions. Many factors are able to influence the rate of apoptosis in neutrophils. Among these is the signalling molecule, nitric oxide (NO), which possesses both anti-apoptotic and pro-apoptotic properties in many cell types, depending on the concentration and flux of NO, and also the source from which NO is derived.

Characterisation of the NO-related species generated by four 'NO-donating' compounds, spermine diazeniumdiolate (SPER/NO), diethylamine diazeniumdiolate (DEA/NO), 1,2,3,4-oxatriazolium, 5-amino-3- (3,4-dichlorophenyl)-chloride (GEA 3162) and 3-morpholinosydnonime (SIN-1) was carried out. It was demonstrated that the diazeniumdiolates, SPER/NO and DEA/NO both spontaneously liberated free NO in aqueous solution at physiological temperature and pH. In contrast, both GEA 3162 and SIN-1 produced NO and superoxide anion (O_2^-) concomitantly, that rapidly react to form the powerful oxidant species, peroxynitrite ($ONOO^-$). These two compounds should therefore be considered $ONOO^-$ donors rather than NO donors.

Apoptosis studies demonstrated the biphasic pro- and anti-apoptotic effects of pure NO donors, SPER/NO and DEA/NO, and the pro-apoptotic effects of $ONOO^-$ donors, GEA 3162 and SIN-1, in human neutrophils. Low concentrations of the pure NO donors delayed the rate of neutrophil apoptosis, while high concentrations of all compounds tested accelerated cell death. Time course analyses of four independent events of apoptosis revealed that morphological and biochemical parameters of neutrophil apoptosis may proceed independently of internucleosomal DNA fragmentation, which has long been considered a key hallmark of apoptosis and is frequently used as the sole criterion for assessment of this form of cell death. Treatment with high concentrations of $ONOO^-$ donors and, to a lesser extent, with

the longer-lasting pure NO donor, SPER/NO, induced morphological and cell surface (CD16 shedding and phosphatidylserine exposure) changes characteristic of neutrophil apoptosis, but paradoxically inhibited internucleosomal DNA fragmentation, as measured by propidium iodide intercalation and gel electrophoresis. In contrast, treatment with the short-lasting NO donor, DEA/NO, produced no such inhibition. An oxidation reaction was shown to be responsible for the suppression of the DNA fragmentation pathway, as the reducing agent, dithiothreitol, restored DNA fragmentation back to control levels. However, GEA 3162-mediated inhibition of DNA fragmentation did not occur upstream or at the level of degradation of inhibitor of caspase-activated DNase (ICAD/DFF45), as Western blotting showed breakdown of this protein was enhanced rather than inhibited in GEA 3162-treated neutrophils.

Therefore, NO can exert either pro- or anti-apoptotic effects on human neutrophil apoptosis, depending on its concentration and flux, whereas only pro-apoptotic effects are achieved with $ONOO^-$ donors. Cell death promoted by $ONOO^-$ or SPER/NO is independent of an increase in internucleosomal DNA fragmentation; this process is inhibited via an oxidative mechanism, but not through inhibition of ICAD/DFF45 breakdown or upstream mechanisms. Thus, NO and $ONOO^-$ are able to modulate the rate of neutrophil apoptosis, which may have implications for chronic inflammatory conditions.

Acknowledgements

I would firstly like to express my gratitude to the Wellcome Trust for their extremely generous funding of this PhD and to the University of Edinburgh Cardiovascular Research Initiative for giving me the opportunity to undertake this research, and also for the invaluable extra training and transferable skills acquired over the past 4 years, in particular during the first year. I would also like to thank the Centre for Inflammation Research for acting as the 'host' laboratory and providing me with the facilities and equipment required to complete my PhD.

On a more individual basis, I would like to acknowledge the superb supervision of this project by Drs. Adriano Rossi and Ian Megson. Needless to say, without their continued support and assistance, I would not be in a position to be writing this thesis. Additionally, I would like to express my appreciation to Dr. Ian Dransfield for his invaluable contribution to discussions about this project and the direction it should take.

On the technical side of things, so many people have been such a great help with this project, that it would be very difficult to name them all individually. However, particular thanks must go to Carol Ward, who has been incredibly helpful with regards to both technical assistance in the laboratory and scientific discussions. I would like to thank Annemieke Walker for help with preparation of neutrophil lysates, Shonna McCall for flow cytometry assistance and Mike Crane and Katie Shaw for NO electrode advice. Also, I would like to thank everybody who has been involved with the blood preps and generously donated cells for experiments.

On a personal note, I'd like to thank my family, who have been extremely supportive over the past four years, through good times and bad. I would never have got this far without them. Also, everyone in Rooms A7 and A8 has been fantastic, sharing the good times and getting me through the bad; they have done as much as anybody to keep me sane, especially my own personal agony aunt, Lorna, and special mention must also go to Sharon, Sarah, Karen and Anna. So thanks to everybody for the laughter and the banter! Last but by no means least, I'd like to thank all my various drinking buddies – far too many to mention!

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Chapter 1: Introduction

Neutrophil apoptosis is believed to be impaired in chronic inflammatory conditions, thus delaying resolution of inflammation and increasing tissue injury. Therefore, promotion of this form of cell death in neutrophils offers potential therapeutic benefit in a number of conditions. Nitric oxide (NO) and related species, such as peroxynitrite ($ONOO^-$), have been shown to exert pro-apoptotic effects in neutrophils, and many other cell types, therefore it is of interest to characterise the effects of compounds that generate these species on neutrophil apoptosis. This thesis focuses on a comparison of the effects of a number of different NO and $ONOO^-$ generators on apoptosis in neutrophils obtained from the peripheral blood of healthy volunteers.

1.1 Nitric Oxide

The free radical, nitric oxide (NO), has been identified as a factor that is a major factor involved in endothelium-dependent vasorelaxation, first observed by Furchgott and Zawadski in 1980 (Furchgott and Zawadzki, 1980). Although NO was first discovered in its capacity as an endogenous regulator of vascular tone (Palmer *et al.*, 1987), it is now known to be a key mediator in a great number of physiological and pathophysiological processes (Quinn *et al.*, 1995). NO is formed naturally in the body from the amino acid L-arginine, by the NO synthase (NOS) family of enzymes, with L-citrulline being formed in the process (Palmer and Moncada, 1989); Figure 1.1A). Tetrahydrobiopterin and NADPH are required cofactors for NO synthesis (Tayeh and Marletta, 1989). The NOS isoforms found in endothelial cells (eNOS) and in neurones (nNOS) are constitutively expressed, with their activity regulated by the calcium-calmodulin system, but they synthesise only small amounts of NO (pM – nM; (Berdeaux, 1993). A third, calcium-independent, isoform located in several cell types is termed inducible NOS (iNOS), and is stimulated to produce

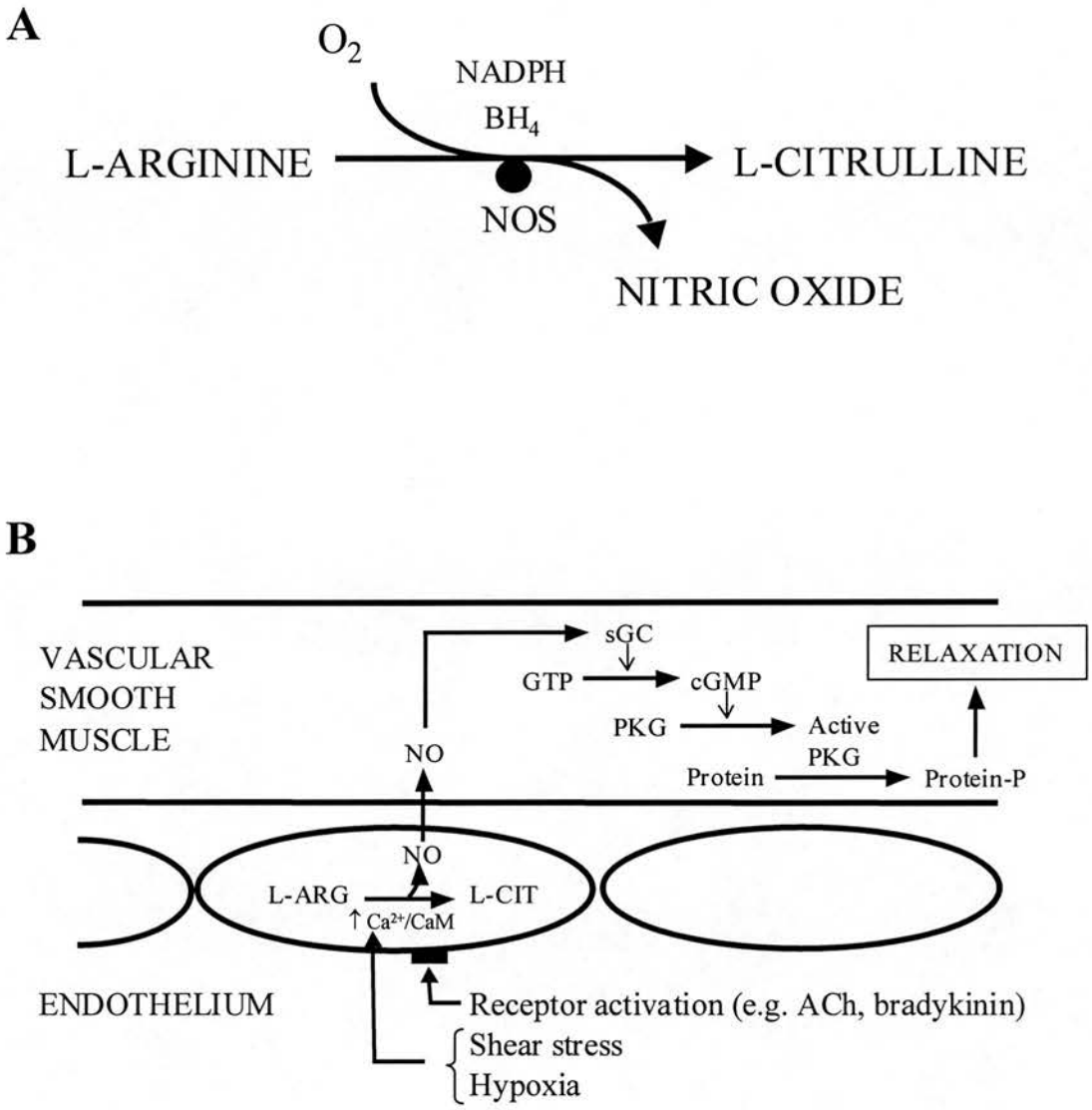


Figure 1.1 Synthesis and mechanism of vasodilatory action of nitric oxide. (A) Nitric oxide (NO) is synthesised from the conversion of the amino acid, L-arginine, to L-citrulline by NO synthase (NOS) enzymes. NADPH and tetrahydrobiopterin (BH_4) are required as cofactors. (B) Physiological stimuli such as hypoxia, shear stress or stimulation of endothelial receptors by ligands such as acetylcholine (ACh) or bradykinin causes NO synthesis from L-arginine via an increase in calcium/calmodulin (Ca^{2+}/CaM) levels. NO diffuses into vascular smooth muscle cells where it activates soluble guanylate cyclase (sGC), which catalyses the conversion of GTP to cyclic GMP (cGMP). This causes activation of the cGMP-dependent protein kinase (PKG), which phosphorylates a number of cellular targets to cause relaxation of the smooth muscle.

larger quantities of NO (nM) by a variety of cytokines and other factors (Berdeaux, 1993;Lloyd-Jones and Bloch, 1996).

1.1.1 Biology:

NO has a number of biological effects, although the possibility exists that some of its properties are mediated *in vivo* by the formation of other NO-related species such as dinitrogen trioxide (N_2O_3) or $ONOO^-$ by auto-oxidation or combination with superoxide anion (O_2^-) respectively. Such reactions limit the potential for NO itself to be responsible for some of the longer-term biological roles attributed to NO. The chemistry of NO with respect to the formation of species that may have an important role *in vivo* is discussed below.

The vasodilator effects of NO have been shown to proceed via the 'classical' NO-mediated pathway, in which NO from vascular endothelial cells diffuses to and reacts with the haem group of soluble guanylate cyclase (sGC) within vascular smooth muscle cells, thereby activating this enzyme (Figure 1.1B). Activation occurs through tertiary and quaternary structural changes in sGC on NO binding, followed by a further relaxation in protein structure (Sharma and Magde, 1999). This leads to a protein base becoming exposed, which allows the nucleophilic substitution required for the conversion of GTP to cGMP to occur (Sharma and Magde, 1999). Vasodilation then occurs via the consequent elevation of cGMP (Lincoln *et al.*, 1996); this cyclic nucleotide causes relaxation of vascular smooth muscle principally through the activation of cGMP-dependent protein kinase (PKG), which acts via several intracellular pathways to decrease the intracellular Ca^{2+} concentration and to decrease the sensitivity of the contractile system to Ca^{2+} (Carvajal *et al.*, 2000). PKG phosphorylates a number of cellular targets, including myosin light chain kinase, ion channels, ion pumps, enzymes and receptors: in particular PKG phosphorylates and decreases the activity of the receptor for inositol triphosphate (IP_3), which is an important mediator of smooth muscle contraction (Carvajal *et al.*, 2000). Levels of cGMP are regulated by the activity of

phosphodiesterases (PDEs), which act to convert cGMP to inactive 5'-GMP (Carvajal *et al.*, 2000).

Since then, interest has expanded to include the other biological effects of NO, in particular its protective effects on the cardiovascular system. NO has anti-thrombotic effects, having been shown to inhibit platelet aggregation and adhesion to vascular endothelium (Radomski *et al.*, 1987). In addition, this versatile molecule inhibits endothelial cell mediated oxidation of low-density lipoprotein (Malo-Ranta *et al.*, 1994), keeps the vascular endothelium impermeable to leukocytes (Granger and Kubes, 1996), prevents adhesion of neutrophils and monocytes to the endothelial wall (Kubes *et al.*, 1991;Kosonen *et al.*, 1999;Niebauer *et al.*, 1999;Gluckman *et al.*, 2000) and inhibits proliferation of vascular smooth muscle cells (VSMC) (Mooradian *et al.*, 1995). Moreover, NO inhibits neutrophil function, such as degranulation, shape change, chemotaxis and O_2^- release (Moilanen *et al.*, 1993;Ward *et al.*, 2000;Gluckman *et al.*, 2000).

Within the heart itself, the role of NO is somewhat controversial. However, it now appears that NO-mediated effects relating to contractility and heart rate are biphasic; low concentrations increase both contractility and heart rate, but higher concentrations have the opposing effect (Casadei and Sears, 2003). At physiological concentrations, however, it has been proposed that NO exerts predominantly positive inotropic and chronotropic effects (Casadei and Sears, 2003). Furthermore, NO has been demonstrated to play a role in the Frank-Starling response to a stretch stimulus within the heart (Casadei and Sears, 2003).

Alongside release from the endothelial isoform of NOS, NO also acts as a neurotransmitter that mediates smooth muscle relaxation in the gastrointestinal, respiratory and urogenital systems (Gibson and Lilley, 1997). Additionally, NO plays several protective roles within the central nervous system, regulating blood flow in the brain, the coupling of blood flow and metabolism and the neuroendocrine system, and is involved in the formation of memories and in behaviour (Szabo, 1996), and in the gastrointestinal system, as a mediator of mucosal defence and repair (Muscara and Wallace, 1999).

$ONOO^-$ possesses several biological characteristics in common with NO; for example, both species can cause vasorelaxation, although the effects of $ONOO^-$ may be mediated by detoxification by thiols to S-nitrosothiols, which can then elicit vasodilation through a cGMP-dependent pathway (Ronson *et al.*, 1999). As with NO, $ONOO^-$ from SIN-1 has been shown to have anti-adhesive and antithrombotic effects in an *in vivo* model of balloon angioplasty (Provost *et al.*, 1997). Similarly, inhibition of neutrophil adhesion to endothelium and various cardioprotective effects of low concentrations of $ONOO^-$ during ischaemia-reperfusion has been demonstrated by several groups. These include protection of contractility and reduction of infarct size *in vivo* (although $ONOO^-$ usually has a deleterious effect on contractility *in vitro,* unless glutathione is also present; (Ronson *et al.*, 1999). In all these cases, $ONOO^-$ may act through a mechanism remarkably similar to NO, again suggesting that conversion of $ONOO^-$ to an S-nitrosothiol or related species may be responsible for these effects, which are not due to generation of NO itself, as they could not be blocked by haemoglobin (Ronson *et al.*, 1999). It has been suggested that $ONOO^-$ from SIN-1 is able to activate sGC, but that authentic $ONOO^-$ cannot do so (Trakranrungsie and Will, 2001).

As for NO, the effects of $ONOO^-$ appear to be dependent on the concentration, with low concentrations being protective but high concentrations deleterious (Ronson *et al.*, 1999). It is tempting to speculate that protective species may be formed in the presence of low concentrations of $ONOO^-$ but that higher concentrations may overwhelm the protective and detoxifying mechanisms and be injurious to tissues. However, some similar biological actions of NO and $ONOO^-$ are believed to proceed through different mechanisms; for example, both species inhibit mitochondrial respiration, but act at different respiratory complexes and are differentially regulated (Lizasoain *et al.*, 1996). Additionally, both NO and $ONOO^-$ may induce apoptotic cell death in various cell types, with a large number of potential mechanisms being proposed.

Taken together, these studies demonstrate that NO is a highly relevant molecule in cardiovascular and inflammatory cell biology, and there is the potential for its effects to be manipulated therapeutically.

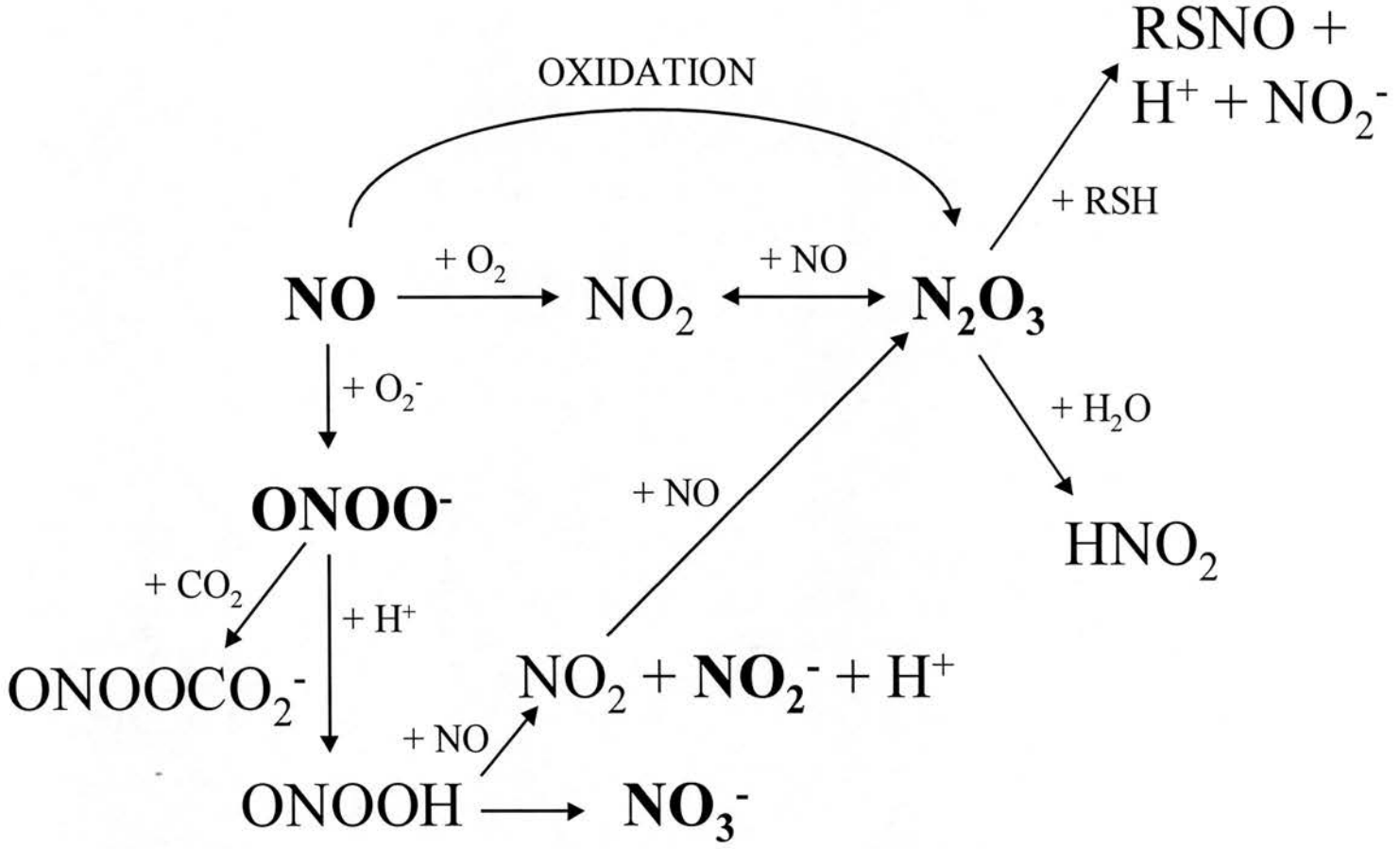


Figure 1.2 Important chemical reactions of NO relevant to biology. Nitric oxide (NO) can be oxidised to N_2O_3, which is able to donate a nitrosonium ion (NO^+) to reduced cysteine residues, to form S-nitrosothiols (RSNO). NO can also react with superoxide anion (O_2^-) to form the oxidant species, peroxynitrite ($ONOO^-$). However, $ONOO^-$ can further react with H^+ to generate peroxynitrous acid (ONOOH), then combination of ONOOH with NO can lead to the generation of N_2O_3.

1.1.2 Chemistry:

The chemistry of NO is complex, as NO is a free radical and therefore able to rapidly combine with other molecules to form reactive nitrogen oxide species (RNOS), particularly at high fluxes of NO, which may have indirect biological effects of their own. The issue is further complicated by the fact that NO and its by-products may have opposing effects, having either antioxidant or pro-oxidant properties, depending on the species; therefore NO and NO-related species may have a profound impact on the redox status of a cell (Espey *et al.*, 2000).

NO is a highly diffusible and lipophilic molecule and as such has no need for membrane receptors; rather, this molecule simply permeates cell membranes in order to stimulate intracellular biological effects (Lincoln *et al.*, 1996). NO may bind directly to haem groups of proteins (forming a nitrosyl-haem complex) to regulate their activity, for example activation of sGC proceeds through this mechanism (Craven and DeRubertis, 1978). However, *in vivo*, NO is extremely short-lived, as it may be auto-oxidised to N_2O_3 by O_2 in the gaseous or aqueous phases, via formation of NO_2 as an intermediate (Espey *et al.*, 2000); Fig 1.2). N_2O_3 is a hugely important mediator of cGMP-independent effects in NO biology, as it appears to be the central species involved in S-nitrosation reactions. It is able to donate a nitrosonium ion (NO^+) to reduced cysteines, forming S-nitrosothiols (RSNO), which may regulate protein functions and possibly act as an important storage form of NO (Stamler *et al.*, 1992). The breakdown of S-nitrosothiols or nitrosyl-metal complexes leads to the formation of nitroxyl (NO^-) which may itself have damaging oxidative effects including the formation of double strand DNA breaks (Espey *et al.*, 2000).

Another critical reaction of NO in biological systems is that with O_2^- to form $ONOO^-$ (Figure 1.2); this reaction is particularly prevalent in inflammatory cells, which produce O_2^- as part of the respiratory burst that occurs on activation. $ONOO^-$ is a powerful oxidising species that may be responsible for the cytotoxicity of NO in many cell types. $ONOO^-$ is able to cause DNA strand breaks and lipid peroxidation, nitrate tyrosine residues and oxidise reduced cysteine residues, forming either S-nitrosothiols or disulphide bridges in proteins (Kuo and Kocis, 2002;Virag *et al.*,

2003). Such modifications can alter the protein activity, resulting in profound biological effects. It is thought that some of the damaging effects of $ONOO^-$ are mediated through the hydroxyl radical ($OH^\bullet$), which is formed as an intermediate of ONOOH conversion to NO_3^-, or through interaction of O_2^- with Fe^{3+} (Espey *et al.*, 2000;Virag *et al.*, 2003). N_2O_3 can also be formed from $ONOO^-$, through interaction with H^+ to form ONOOH, followed by reaction with NO (Espey *et al.*, 2000), possibly explaining why many of the effects of NO are also obtained with $ONOO^-$ donors (Figure 1.2).

In summary, NO may act as an antioxidant by scavenging potentially harmful ROS to convert them to less damaging RNOS, or by forming relatively stable nitrosyl-haem complexes which reduces the potential for formation of strong oxidants through reaction of H_2O_2 with transition metals. It may also have oxidant properties through generation of oxidative species such as $ONOO^-$ and NO^- (Espey *et al.*, 2000). Although these species are themselves oxidants, NO can further react with many RNOS such as NO^- to convert them to less harmful molecules (Espey *et al.*, 2000). The relative abundance of NO and other molecules with which NO can interact in a given environment determines the anti- or pro-oxidant status of this molecule.

1.1.3 NO Donors:

The traditional NO donors currently in clinical use for the treatment of cardiovascular disease each have limitations that make them far from ideal, such as tolerance or toxic by-products. As a consequence, the search is ongoing for novel compounds that retain biological activity, but are free of inadequacies. This section outlines the mechanisms of NO generation by existing drugs and describes novel NO donors that may have potential therapeutic benefits.

1.1.3.1 Traditional NO donors:

The most frequently used NO donor drugs are the organic nitrates, which are esters of nitric acid and mono- or polyhydric alcohols (Feelisch, 1991). The best-known compound in this class is glyceryl trinitrate (GTN), which is still in use today - mainly in angina and acute heart failure, but occasionally in myocardial infarction or severe ischaemia (Megson, 2000). Organic nitrates exert selective vasodilatory effects on the veins through the release of NO and subsequent stimulation of soluble sGC and increased cGMP, leading to decreased venous return and reduced cardiac workload. Although the mechanism of NO release remains controversial, it is thought that both enzymatic and non-enzymatic processes may contribute to a complex multistep reaction mechanism. Initially, it was believed that NO is released by reaction of the nitrate with endogenous thiols, forming S-nitrosothiols, which decompose to release NO (Feelisch, 1991). However, it is now doubted whether thiols are sufficiently powerful reducing agents to cause decomposition in this way. Ascorbic acid and NADPH have been proposed as potential alternative reducing agents for this process (Kishnani and Fung, 1996). Considerable evidence has been accumulated for the involvement of at least three enzymes; glutathione-S-transferase, cytochrome P450 and a 200kDa microsomal enzyme in vascular smooth muscle cells in the release of NO (Kishnani and Fung, 1996), and a further enzyme, mitochondrial aldehyde dehydrogenase, has recently been demonstrated to have a role in NO generation from GTN (Chen *et al.*, 2002). Whether NO generation from the organic nitrates occurs by enzymatic or non-enzymatic means, or a combination of both, remains to be determined. The major problem encountered with long-term use of organic nitrates is that of tolerance. Depletion of tissue thiols, increased oxidative stress or activation of opposing (e.g. sympathetic nerves, endothelin, renin-angiotensin) systems have all been suggested as possible explanations for this phenomenon (Megson, 2000).

Sodium nitroprusside (SNP), used to treat acute hypertension, is also beset by difficulties in clinical use, as it can only be administered intravenously. In addition, potentially toxic cyanide can be generated as a by-product of NO release from SNP with long-term therapy (Megson, 2000). The vasodilator effects of SNP are believed

to be mediated at least partially by NO and sGC activation (Feelisch, 1991). Although initially believed to decompose spontaneously in aqueous solution (Feelisch, 1991), release of NO from SNP is now thought to occur via membrane-bound proteins (Kowaluk *et al.*, 1992) and possibly also enzymatic processes (Kishnani and Fung, 1996).

Sydnonimines are heterocyclic NO donor drugs, which are known to cause biological effects such as vasodilation and inhibition of platelet aggregation, primarily through activation of sGC (Feelisch *et al.*, 1989), although activation of K^+ channels has also been implicated (Mistry and Garland, 1998). Sydnonimines are occasionally used in stable angina, coronary vasospasm and heart failure, and their beneficial effects in cardiovascular disease are likely to include actions other than vasodilation, including inhibition of platelet activation and VSMC proliferation, and protection of LDL against oxidation. They may also help to protect against reperfusion injury following ischaemia (Megson, 2000).

1.1.3.2 Novel NO donors:

The search for improved cardiovascular drugs has resulted in the development of several groups of compounds that have the ability to generate NO by a variety of mechanisms. It is hoped that these drugs may be free of the inadequacies associated with the drugs currently in clinical use, and may even show additional beneficial actions on the cardiovascular system.

One such group of compounds is the diazeniumdiolates – nucleophile/NO adducts – also referred to as NONOates. These contain two molecules of NO attached to a nucleophilic molecule through a nitrogen atom (Maragos *et al.*, 1991). NO is released predictably and spontaneously in aqueous solution, with no enzyme or thiol requirement, to activate sGC (Keefer *et al.*, 1996). Overall, these compounds are useful research tools due to their wide range of rates of NO release, their stability as solids and their predictable generation of NO. Furthermore, a transdermal delivery system has been developed for these compounds (Smith and Simmons,

1998), thus giving them further potential for therapeutic use, as they could easily be self-administered by the patient.

Mesoionic 3-aryl substituted oxatriazole-5-imine derivatives such as GEA 3162 and GEA 3175 are able to induce neutrophil apoptosis, inhibit neutrophil function, suppress tumour cell proliferation, and have bronchodilatory, antibacterial, vasodilator, antiplatelet, fibrinolytic and LDL oxidation inhibitory activities (Ward *et al.*, 2000;Kankaanranta *et al.*, 1996).

GEA 3162 decomposes spontaneously in physiological solution, although reducing agents may contribute to this process *in vivo* (Megson, 2000). Many of the biological actions of liberated NO are mediated at least partially through sGC, although a role for opening of large conductance K^+ channels has also been proposed (Vaali *et al.*, 1998). Release of NO from GEA 3175 but not GEA 3162 is increased in the presence of human plasma, suggesting that decomposition of this compound may be accelerated by enzymatic degradation, perhaps degradation of the sulphonamide moiety, or by thiols, before it is able to release NO (Kankaanranta *et al.*, 1996).

These compounds could be particularly beneficial in the respiratory system, due to the combined effects on bronchi and inflammatory cells. They may be preferred to existing drugs as no tolerance has been observed and O_2^- is thought not to be generated alongside NO (Megson, 2000), although it has been suggested that this group of compounds has the ability to donate $ONOO^-$ (Kankaanranta *et al.*, 1996).

The mechanism of NO release by some NO donors such as nitrates, nitrites and furoxans has been suggested to involve the intermediate formation of S-nitrosothiols, chemically RS-N=O (Feelisch, 1991). Some of these have now been synthesised in order to study their effects and potential therapeutic benefits. These compounds form stable red or green solids, solutions of which decompose on exposure to heat or light, (Feelisch, 1991;Megson, 2000).

NO is generated nonenzymatically *in vitro* by homolytic cleavage of the S-N

bond, the rate of which is increased by heat, light, oxygen and alkaline pH (Feelisch, 1991). However, *in vivo* other processes such as transnitrosation of tissue thiols, interaction with ascorbate or O_2^-, or enzymatic degradation by Cu/Zn superoxide dismutase, glutathione-S-transferase, glutathione peroxidase, cell-surface protein disulphide isomerase and xanthine oxidase may contribute to NO release (Megson, 2000). Disulphide and thiyl radicals are formed as a result of the liberation of NO (Feelisch, 1991;Williams, 1996), which may have biological effects of their own, and transnitrosation of proteins may affect enzyme activity, which in turn may regulate cell function (Feelisch, 1991;Megson, 2000).

The rate of S-nitrosothiol decomposition varies from ms to h, depending on the identity and chemical nature of the bulk of the molecule (R; (Megson, 2000), and the ability to stimulate sGC increases with decreasing stability (Feelisch, 1991). However, the decomposition of S-nitrosothiols can be somewhat unpredictable, due to NO release being catalysed by trace amounts of Cu(I) (Dicks and Williams, 1996), the concentration of which can vary between laboratories, and even experiments.

Particular interest has been focused on the endogenous S-nitrosoglutathione (GSNO), as it is relatively stable in solution and its degradation produces non-toxic by-products. This compound has been shown to have great potential for therapeutic use, as its effects include arterial vasodilation and inhibition of platelet aggregation and embolisation (Megson, 2000). Endogenous NO may be stored within cells as GSNO, or in plasma as S-nitroso-albumin, therefore these are useful compounds to study, particularly S-nitrosoalbumin because the generation of NO from this thiol complex follows more predictable kinetics than that from the commonly-used synthetic compound S-nitroso-acetyl-penicillamine (SNAP;(Gluckman *et al.*, 2000).

One method of reducing sensitivity to catalytic effects of copper is to change the molecular structure so that Cu^+ is no longer able to catalyse decomposition. Such compounds include RIG200 (SNAP coupled to acetylated glucosamine) and SNVP, which is formed by lengthening the acetyl side chain of SNAP. These have prolonged vasodilator activity, suggesting that they may be retained in blood vessel walls from where they can decompose slowly to produce a sustained biological effect

(Megson *et al.*, 1997). It is hoped that S-nitrosothiols, especially RIG200, may be used to target delivery of the drug to regions of endothelial damage, as their lipophilic nature allows absorption into the endothelium-denuded vessel wall from where prolonged NO release can occur (Megson *et al.*, 1997).

A further group of NO-donating compounds, which may have therapeutic potential in inflammatory conditions, are the NO-conjugated non-steroidal anti-inflammatory drugs (NO-NSAIDs), several of which have recently been synthesised, including NO-aspirin and nitroparacetamol (Keeble and Moore, 2002). These drugs have an improved pharmacological profile versus traditional NSAIDs because they retain the anti-inflammatory and anti-pyretic properties of the parent compounds but greatly attenuate the gastro-intestinal irritation observed as a side-effect of traditional NSAIDs (Fiorucci *et al.*, 2003;Keeble and Moore, 2002). Thus, this group of compounds represents a highly exciting prospect for the clinical treatment of inflammation.

Additionally, NO moieties have been conjugated to steroids such as prednisolone. Steroids are widely-used anti-inflammatory agents, but are associated with side effects such as osteoporosis. However, it has been shown that addition of NO to prednisolone reduces such side effects by reducing the activity of osteoclast cells, which are involved in bone resorption (Perretti *et al.*, 2003). Such compounds have been shown to have increased anti-inflammatory activity (Paul-Clark *et al.*, 2000) and are effective in *in vivo* models of inflammation (Paul-Clark *et al.*, 2002;Paul-Clark *et al.*, 2000). These novel NO donors also represent a potential novel anti-inflammatory therapy.

1.2 Inflammation:

Inflammation is a physiological response to cellular injury or infection, which is characterised by redness, oedema, heat, leukocytic infiltration and pain. Its purpose is to destroy or sequester the infectious agent and injured tissue. There are

two main types of leukocytes involved in inflammatory responses in the body; granulocytes, which can be subdivided into neutrophils, eosinophils and basophils, and mononuclear cells including monocytes/macrophages and lymphocytes. These leukocytes arise from a common myeloid precursor, and exposure to different growth factors causes differentiation into the distinct classes of white blood cell.

1.2.1 The Inflammatory Response:

The neutrophilic granulocyte plays a critical role in the host defence against bacterial and fungal infection, whereas eosinophils are primarily involved in parasitic infection and allergic reactions. The neutrophil differentiates from the myeloid precursor cell under the influence of colony-stimulating factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and, in particular, granulocyte colony-stimulating factor (G-CSF(Metcalf and Nicola, 1983). It is packed with pre-formed granules, of which 4 distinct types have been identified, containing a battery of molecules designed to eliminate infectious agents (Witko-Sarsat *et al.*, 2000). The vast majority of microbicidal molecules are contained within azurophilic granules, alongside various proteases to digest extracellular matrix and intracellular proteins, therefore these granules are regarded as critical in combatting infection and injury (Witko-Sarsat *et al.*, 2000).

The neutrophil is the first leukocyte to migrate into the inflammatory site in response to chemoattractant molecules released from invading microorganisms, host tissue or dying cells (Rossi and Haslett, 1998). For example, activated cells release pro-inflammatory mediators such as IL-8 and LTB_4, whereas invading microorganisms secrete fMLP and LPS, and plasma factors such as C5a also contribute to the inflammatory process (Rossi and Haslett, 1998).

Interactions between selectins and integrins ($\beta1$ or $\beta2$ integrins) on the neutrophil and cell adhesion molecules (CAMs, e.g ICAM-1, ICAM-2 and ICAM-3) and selectins (E-selectin, P-selectin) on endothelial cells allow the neutrophil to adhere loosely to, then 'roll' along, the endothelium. If the leukocyte is activated by

chemoattractants (e.g. PAF, IL-8, fMLP, C5a), cytokines, growth factors (TNFα, GM-CSF) or bacterial products such as LPS, it adheres more firmly and spreads. The neutrophil then passes between endothelial cells through modified tight junctions (diapedesis), and moves into the site of inflammation down a number of chemoattractant gradients (Witko-Sarsat *et al.*, 2000). Once there, it exerts its antimicrobial and cytotoxic effects by various mechanisms. The neutrophil releases its stores of granules, which are packed with microbicidal proteins, collagenase, gelatinase, proteases and plasma proteins into the extracellular medium. These molecules kill the organism, but tissue damage can also occur as a result of their action. Reactive oxygen species (ROS) are produced by NADPH oxidase in the cell membrane, which kill micro-organisms, but can also damage normal tissue if produced inappropriately.

Although it is well established that macrophages are able to generate iNOS-derived NO on stimulation with inflammatory mediators such as LPS and IFN-γ (Brune *et al.*, 1997), there remains some controversy over the ability of human neutrophils to generate NO in a similar manner (Armstrong, 2001). Some studies have reported NO generation in neutrophils (Wright *et al.*, 1989;Schmidt *et al.*, 1989;Carreras *et al.*, 1996). However, others have observed no NO release from neutrophils (McBride and Brown, 1997). In other cases, NO release has been enhanced in the presence of SOD (Carreras *et al.*, 1994) and has been detectable in patients with chronic granulomatous disease (CGD) in which O_2^- production is impaired (Condino-neto *et al.*, 1993). This suggests that NO is produced alongside O_2^- in human neutrophils, thus generating $ONOO^-$ (Carreras *et al.*, 1994), and that unmasking of NO by removal of O_2^- allows detection of NO, and may explain why in some studies, NO has not been detected in human neutrophils.

The cell also generates and secretes pro-inflammatory cytokines, which recruit further inflammatory cells (neutrophils and monocytes) to the site (Witko-Sarsat *et al.*, 2000). The neutrophil also acts as a phagocyte, ingesting and destroying the target micro-organisms and cell debris through Fcγ receptors and complement receptors (Witko-Sarsat *et al.*, 2000). This process is thought to induce

the neutrophil to undergo programmed cell death or apoptosis, as described below, and it becomes unable to respond to external stimuli, causing downregulation of chemotaxis, phagocytosis and degranulation (Hart *et al.*, 2000). Specific molecules, including carbohydrates, thrombospondin-binding molecules and phosphatidylserine, on the surface of the apoptotic neutrophil allow monocyte-derived macrophages to recognise and phagocytose the apoptotic cells via a non-inflammatory mechanism, thus resolving the inflammation (Rossi and Haslett, 1998).

1.2.2 Role of NO in Inflammation:

As previously described, constitutively-generated NO from eNOS has a number of anti-inflammatory actions that protect the cardiovascular system from formation of inflammatory foci. Anti-thrombotic effects are thought to be due in part to cGMP-mediated inhibition of P-selectin expression on the surface of the platelets (Granger and Kubes, 1996). However, recent studies have demonstrated that cGMP-independent mechanisms may also play their part (Sogo *et al.*, 2000). It has been shown that the prolonged effects of NO on inhibition of platelet aggregation may be due to the formation of S-nitrosothiols, through reaction with plasma proteins such as albumin in the presence of low molecular weight thiols, allowing prolonged exposure of the platelets to NO (Crane *et al.*, 2002).

Endothelial cell-mediated oxidation of low density lipoprotein can be inhibited by GEA 3162; although the precise mechanism was not elucidated, the authors proposed elevation of cGMP, inhibition of 15-lipoxygenase, free radical scavenging or iron chelation as potential mechanisms (Malo-Ranta *et al.*, 1994). Of further interest for the prevention of atherogenesis, vascular smooth muscle cell (VSMC) proliferation may be inhibited by NO, with the most profound response obtained with NO donors that release NO at a slow rate (Mooradian *et al.*, 1995). Once again, sGC stimulation was suggested as a potential mechanism, alongside inhibition of ribonucleotide reductase (thus preventing DNA synthesis), inhibition of respiration, and effects on cytokines and growth factors influencing cell growth

(Mooradian *et al.*, 1995).

NO has been demonstrated to produce a profound inhibition of neutrophil functions. Leukocyte adhesion and emigration through vascular endothelium was increased in the presence of inhibitors of NO synthesis through the neutrophil adhesion molecule, CD11/CD18, demonstrating that endogenous NO possesses anti-neutrophil properties via effects on adhesion molecules (Kubes *et al.*, 1991). Treatment of human neutrophils with exogenous NO/$ONOO^-$ donors, SIN-1 or GEA 3162, caused an inhibition of the chemotactic response and shape change in these cells, potentially through modification of cytoskeletal components (Ward *et al.*, 2000). Additionally, neutrophil degranulation was inhibited in response to the $ONOO^-$ donor, SIN-1, however this was reported to be due to inhibition of Ca^{2+} elevation by a metabolite rather than $ONOO^-$ itself (Kankaanranta *et al.*, 1997). In further studies, a number of NO or $ONOO^-$ donors were shown to inhibit neutrophil functions such as leukotriene B_4 (LTB_4) production, degranulation, chemotaxis and O_2^- release, with elevation of cGMP proposed as a potential mechanism (Moilanen *et al.*, 1993). In monocytes and macrophages, NO caused a cGMP-independent inhibition of the oxidative burst through activation of PPAR-γ and subsequent downregulation of the p47 phagocyte oxidase (von Knethen and Brune, 2002).

However, the effects of NO are not exclusively protective and beneficial in inflammation; excessive production of NO from iNOS in activated inflammatory cells has been implicated in the pathogenesis of a number of diseases. For example, in septic shock, iNOS-derived NO is produced from both inflammatory cells and other cell types in the cardiovascular system in response to LPS from invading bacteria, which contributes to systemic hypotension and cardiac failure (Brady and Poole-Wilson, 1993). There are many conditions in which NO plays a dichotomous role, whereby endogenous low levels of NO serve to protect the tissue or organ in question, but overproduction of NO contributes to the pathogenesis of the condition. Such conditions include gastrointestinal conditions (sepsis, inflammatory bowel syndrome, ulceration; (Kubes and McCafferty, 2000;Muscara and Wallace, 1999), CNS disorders (stroke, seizures, acute and chronic inflammatory and neurodegenerative disorders;(Szabo, 1996), asthma and cystic fibrosis (Robbins *et*

al., 2000), chronic pulmonary hypertension (Hampl and Herget, 2000), interstitial lung disease (Suga *et al.*, 1998), chronic fatigue syndrome, multiple chemical sensitivity and posttraumatic stress disorder (Pall and Satterle, 2001) and surgical infection and sepsis (Johnson and Billiar, 1998). In addition, overproduction of NO is implicated in a whole range of myocardial defects, such as ischaemia-reperfusion injury, left ventricular hypertrophy, heart failure, transplant vasculopathy and rejection and myocarditis (Shah and MacCarthy, 2000), despite the protective effects of low-dose NO on cardiovascular function described above. In almost all cases, the injurious species has been proposed to be $ONOO^-$ formed from the combination of excessive NO with $O_2^{\cdot-}$.

It is therefore clear that within many systems, NO may act as a protective or injurious molecule, whereby physiological production of NO has beneficial physiological effects but excessive production may lead to the formation of cytotoxic molecules that damage tissue and may ultimately contribute to the pathogenesis of many disease states.

Levels of NO may be manipulated in several ways; a number of NOS inhibitors are available, some of which are non-selective for the NOS isoform. However, more specific inhibitors are being developed, for example, aminoguanidine, L-NIL and 1400W have all been reported to be relatively specific for the inducible form of NOS, iNOS, although they may still have inhibitory effects on the other isoforms of NOS (Alderton *et al.*, 2001). Other NOS inhibitors, such as L-NMMA and L-NAME are non-selective, affecting the activity of all isoforms of NOS (Alderton *et al.*, 2001). In contrast, NO levels may be increased through the use of NO donors (see Chapter 1.1.3), or the effects of NO may be potentiated through PDE inhibitors such as sildenafil, which inhibit breakdown of cGMP (Pauvert *et al.*, 2003). Such modifications of NO levels may have important effects on the inflammatory process *in vivo*, as discussed in Chapter 1.5.

1.3 Apoptosis

Apoptosis is a controlled, physiological form of cell death, first named in 1972 (Kerr *et al.*, 1972), although the process had been described in histological studies for some years previously (Kerr, 2002). A large number of pro- and anti-apoptotic factors exist within cells, and it is the net balance between them that determines whether or not a cell undergoes apoptosis. In contrast to necrosis, the cell is required to expend energy to undergo this form of cell death, but the process of apoptosis is ultimately beneficial to the organism as a whole, because the cell membrane integrity is maintained, thus preventing the release of cellular contents and elicitation of an inflammatory response (Wyllie, 1997). Phagocytes then recognise cells that have undergone apoptosis and clear them from the site.

1.3.1 Characteristics and Pathways of Apoptotic Cell Death:

Apoptosis is characterised by a number of morphological and biochemical changes, executed in a sequential and highly regulated manner. The most striking and definitive events of apoptotic cell death are the membrane 'blebbing' in which the cell surface appears to 'boil', division of the cell into a number of 'apoptotic bodies', and condensation and ultimate fragmentation of the nuclear material (Wyllie, 1997;Zimmermann *et al.*, 2001). Various cell surface changes, including the exposure of phosphatidylserine on the outer cell surface allow recognition and engulfment by phagocytes (Hoffmann *et al.*, 2001). The central effectors of the apoptotic pathway are the caspase family of cysteine proteases, which cleave proteins at aspartic acid residues (Thornberry, 1997;Kidd, 1998). A number of these enzymes have been discovered in humans, and they exist as inactive procaspases in non-apoptotic cells, but become activated on proteolytic cleavage following initiation of apoptosis (Wyllie, 1997). Target proteins include those involved in homeostasis and repair, such as PARP and PKC-δ, which allows DNA degradation and ensures that apoptosis is an irreversible process, downstream effectors such as protein

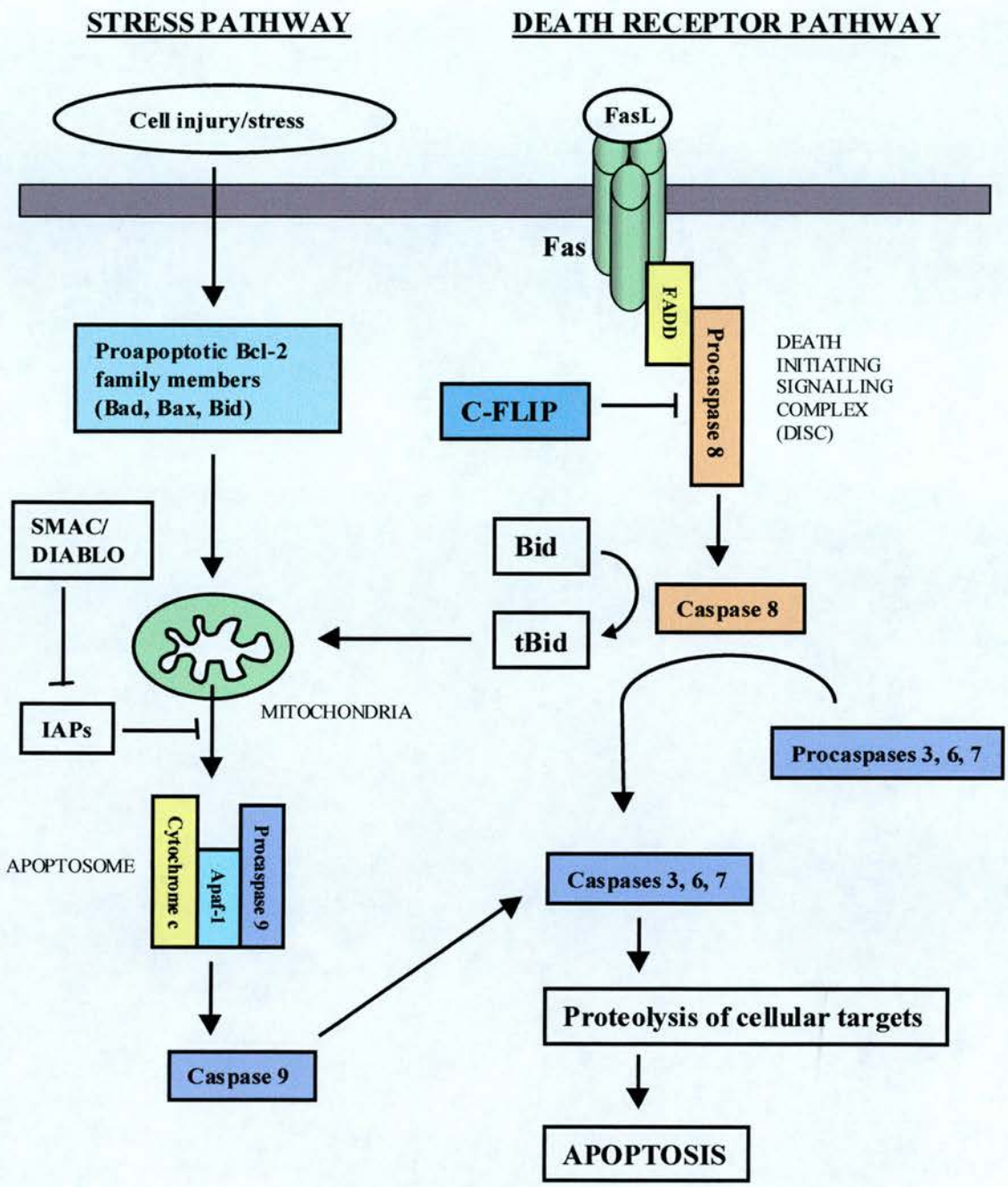


Figure 1.3 Schematic representation of the two main pathways of apoptosis, the stress or mitochondrial pathway and the death receptor pathway. The stress pathway involves translocation of pro-apoptotic Bcl-2 family proteins to the mitochondria, release of pro-apoptotic factors, apoptosome formation and activation of caspase 9 and effector caspases, 3, 6 and 7. Engagement of death receptors (e.g. Fas) causes receptor trimerisation, DISC formation and activation of caspase 8 followed by activation of effector caspases, which are responsible for proteolysis of a large number of target proteins, leading to apoptotic cell death.

kinases, and structural proteins (e.g. actin, fodrin, lamin), cleavage of which allows disassembly of the cytoskeleton and nucleus, and therefore cell shrinkage and nuclear condensation (Thornberry, 1997;Kidd, 1998).

Two principal pathways exist for the initiation of the apoptotic cascade (Figure 1.3). Firstly, engagement of death receptors such as TNF-R1 or Fas with their appropriate ligands induces receptor trimerisation and formation of the death initiating signalling complex (DISC) through interaction of the receptors with adapter proteins (e.g. TRADD, FADD) via specific 'death domain' amino acid sequences (Wyllie, 1997;Kidd, 1998). This complex can then recruit procaspase 8 and generate the active apoptosis initiator, caspase 8, through proteolytic cleavage, although c-FLIP acts as a competitive inhibitor of procaspase 8 binding to the DISC and therefore has anti-apoptotic effects (Zimmermann *et al.*, 2001).

Once active, caspase 8 can cleave procaspases 3, 6 and 7 to their respective active effector caspases, which are responsible for the vast majority of proteolytic cleavage in the apoptotic process (Zimmermann *et al.*, 2001). In addition, in some cell types (e.g. Type 2 Fas cells), caspase 8 may also initiate the mitochondrial pathway described below, by promoting translocation of the truncated form of Bid (tBid) to the mitochondria (Scaffidi *et al.*, 1998;Zimmermann *et al.*, 2001).

Inflammatory cells express Fas and TNF-R1, on the cell surface. TNF-R1 is unusual in that it mediates both pro- and anti-apoptotic signals in neutrophils (Murray *et al.*, 1997). Caspase activation and consequent induction of apoptosis may occur through adaptor proteins such as FADD. However, the TNFα signal can also recruit an alternative cell survival pathway via TRAF, to activate NF-κB (Kidd, 1998;Barkett and Gilmore, 1999). The ultimate downstream effect of receptor engagement (apoptosis or proliferation) depends on the adaptor proteins recruited to the receptor complex; those such as FADD that contain a death effector domain (DED) allow caspase recruitment and subsequent apoptotic signalling, whereas those such as TRAF that have no such domain instead signal down the proliferative pathway (Kidd, 1998).

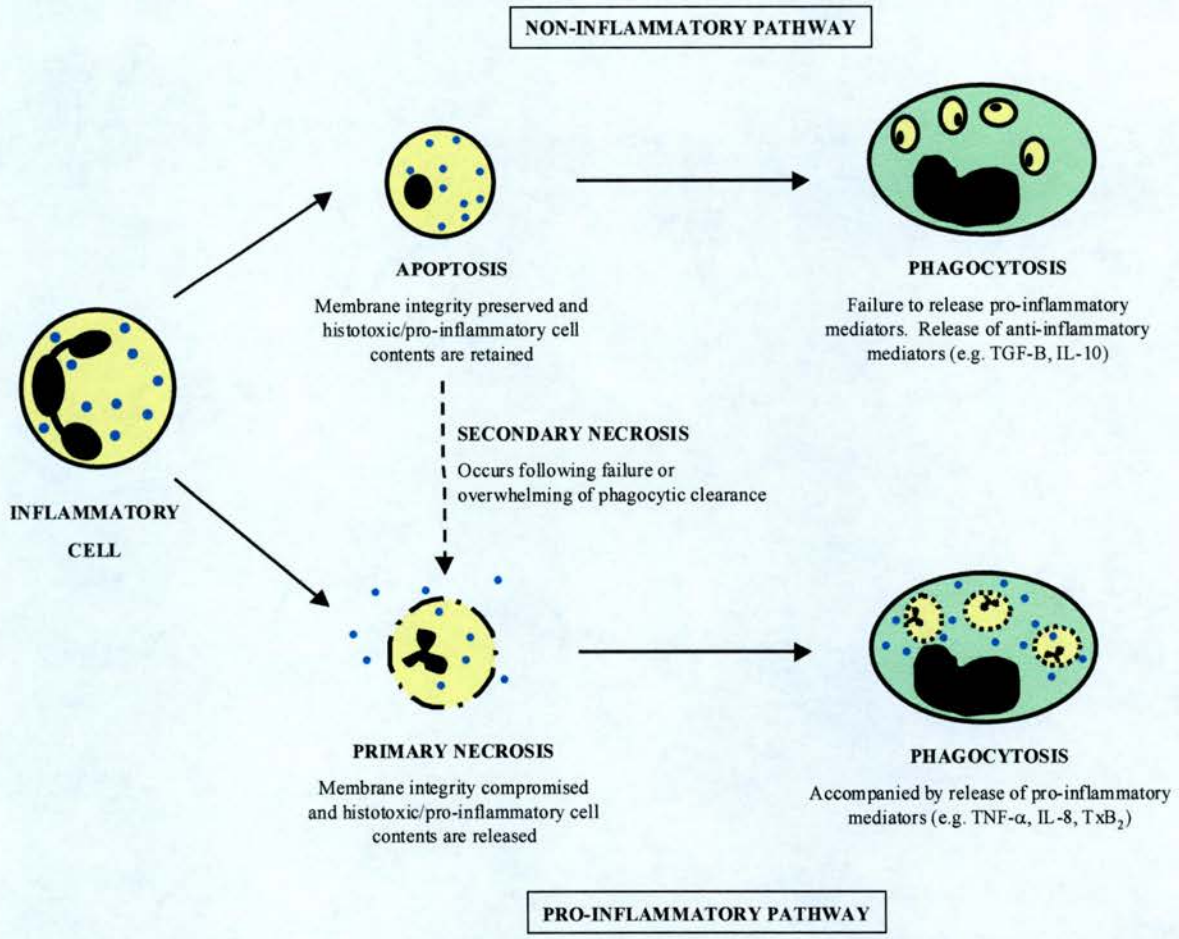


Figure 1.4 Schematic representation of neutrophil death and clearance by macrophages. For successful resolution of inflammation, neutrophils must undergo apoptotic cell death, which maintains cell membrane integrity and allows non-inflammatory phagocytosis by macrophages. Failure of cells to undergo apoptosis, or failure of clearance, leads to necrotic death of neutrophils, in which membrane integrity is lost and pro-inflammatory and histotoxic cell contents are lost, thus exacerbating inflammation. Furthermore, phagocytosis of necrotic particles by macrophages leads to the release of pro-inflammatory mediators which further enhances the inflammatory response.

The second pathway is the mitochondrial pathway, which is recruited primarily in response to cellular injury or stress. Examples of stresses that initiate the mitochondrial apoptotic pathway include DNA damage, damage to cell membranes or mitochondria, cytotoxic T lymphocyte killing and viruses (Wyllie, 1997).

A large number of pro- and anti-apoptotic Bcl-2 family proteins regulate this pathway; anti-apoptotic family members, such as Bcl-2, Bcl-$_{XL}$ and A1 tend to be located on the outer mitochondrial membrane and block the release of pro-apoptotic factors stored within mitochondria. Pro-apoptotic members (Bax, Bak, Bid) are more likely to be cytosolic (Zimmermann *et al.*, 2001). Phosphorylation of some members of the Bcl-2 family is able to regulate their activity. For example, in its active (unphosphorylated) state, Bad binds to anti-apoptotic Bcl-2 family members that usually bind to and sequester pro-apoptotic Bax. Binding of these anti-apoptotic proteins by Bad allows Bax to homodimerise, translocate to the mitochondria and induce cytochrome c release. However, phosphorylation of Bad makes it unable to bind anti-apoptotic Bcl-2 family members so they are free to heterodimerise with Bax and prevent its pro-apoptotic activity (Franklin and McCubrey, 2000).

Upon initiation of apoptosis, the pro-apoptotic proteins may undergo a conformational change and translocate to the mitochondria, where the mitochondrial transmembrane potential is lost and further pro-apoptotic proteins are released, including cytochrome c, which combines with cytosolic apoptotic protease activating factor-1 (Apaf-1) and procaspase 9 to form an 'apoptosome' (Wyllie, 1997;Zimmermann *et al.*, 2001). Cleavage of Apaf-1-bound procaspase 9 then releases active caspase 9, which subsequently cleaves procaspase 3, the principal effector protease in apoptosis (Zimmermann *et al.*, 2001).

Other pro-apoptotic proteins, SMAC/Diablo and Omi/HtrA2, are also released from the mitochondria alongside cytochrome c, and inhibit the actions of endogenous 'inhibitor of apoptosis' proteins (IAPs), thus releasing the intrinsic brake on apoptosis provided by IAPs (Zimmermann *et al.*, 2001;Los *et al.*, 2003). The IAP family of proteins includes XIAP, ML-IAP, cIAP-1 and cIAP-2, and can directly

interfere with caspase activity via binding to caspases of both mitochondrial and death receptor apoptotic pathways (Los *et al.*, 2003). SMAC/Diablo and Omi/HtrA2 are able to bind to and inhibit the function of these IAP's, thus allowing caspase activity (Los *et al.*, 2003).

1.3.2 Neutrophil Apoptosis:

Failure of inflammatory cells to undergo apoptosis, or failure of the phagocytic removal of apoptotic cells leads to exacerbation of inflammation, as neutrophils undergo primary or secondary necrosis, whereby their pro-inflammatory and histotoxic granule contents are released into the surrounding tissue (Haslett, 1992); Figure 1.4). *In vivo*, neutrophil apoptosis occurs within 8-20 h in circulating cells as part of the rapid turnover of these cells (Akgul *et al.*, 2001). In an inflammatory site, however, inflammatory mediators prolong the lifespan of neutrophils, although the neutrophil is programmed to die once it has performed its biological functions, such as killing and phagocytosis of bacteria, in response to cytokine withdrawal (Fortenberry *et al.*, 1999). Phagocytosis-induced cell death proceeds through the involvement of the β2 integrin, Mac-1, and requires the generation of reactive oxygen species by the neutrophil. Caspases 8 and 3 become activated during this process, but the activation of caspase 9 via the mitochondrial pathway is not observed (Zhang *et al.*, 2003a). However, inflammatory mediators such as GM-CSF and G-CSF promote activation of opposing survival pathways (Blaylock *et al.*, 1998), with GM-CSF acting through MAPK/ERK, which may delay neutrophil apoptosis following phagocytosis (Zhang *et al.*, 2003a) and is responsible for the accumulation of neutrophils seen in several inflammatory conditions (Ward *et al.*, 2000).

A large number of stimuli that are likely to be present at inflammatory sites, such as LPS, fMLP, PAF, LTB_4, C5a, inflammatory cytokines, hypoxia and oxidative stress, inhibit neutrophil apoptosis (Ward *et al.*, 1999b). It has been shown that some of these stimuli exert their effects by influencing NF-κB activation (Pahl,

1999). Under normal conditions, NF-κB is kept sequestered in the cytoplasm by inhibitory IκB proteins such as IκBα (Barkett and Gilmore, 1999). An activating stimulus causes phosphorylation of IκB by IκB kinase (IKK), making IκB susceptible to proteolytic degradation, leaving NF- κB free to translocate to the nucleus and bind to DNA. There, it induces transcription of target genes that promote cell survival in most cell types (Barkett and Gilmore, 1999). The cell survival effects of NFκB are largely due to upregulation of anti-apoptotic genes. These include the Bcl-2-like genes A1/Bfl1, Bcl-$_{XL}$ and Nr13, plus the adaptor proteins TRAF-1 and TRAF-2, and the inhibitors of apoptosis proteins H-IAP1 and H-IAP2 (Pahl, 1999). It has been suggested that A1 inhibits release of cytochrome c from mitochondria, and may also inhibit caspase-3 activation, while the adaptor proteins and IAPs may block activation of caspase-8 (Barkett and Gilmore, 1999). Another way in which NF-κB can promote cell survival is by affecting transcription of genes that regulate the cell cycle, thus making the cell more or less sensitive to apoptotic stimuli. Genes encoding certain transcription factors, such as c-Myb, c-Myc and c-Rel fall into this category (Pahl, 1999). *C-myc* may achieve its anti-apoptotic effects by induction of survival factors, by repression of death-inducing genes, or by a combination of these two mechanisms (Thompson, 1998). In addition, NF-κB may alter the free levels of cellular proteins, such as the transcriptional co-activator proteins CBP/p300, through direct protein-protein interactions. These proteins are able to bind to various cell signalling proteins such as nuclear receptors, p53 and Rel-A, (Barkett and Gilmore, 1999). Thus, several cell signalling pathways may be affected in ways that would alter the sensitivity of the cell to pro-apoptotic stimuli.

Neutrophils undergo constitutive apoptosis in culture, however the mechanisms by which cell death proceeds remain to be fully elucidated. Engagement of the Fas death receptor (Liles *et al.*, 1996) has been proposed as a potential mechanism for spontaneous neutrophil apoptosis, although this is controversial (Simon, 2003). Activation of p38 MAPK has also been reported to be involved (Aoshiba *et al.*, 1999). Virtually the only certainty surrounding constitutive apoptosis is the involvement of caspases, with caspases 1, 3, 8 and 9 all being

reported to become activated during this process (Khwaja and Tatton, 1999;Simon, 2003), although caspase-independent spontaneous neutrophil apoptosis has been reported (Harter *et al.*, 2001). Such results suggest that both death receptor and mitochondrial pathways are responsible for apoptosis. Downstream of caspase activation, PKC becomes activated by proteolysis (Khwaja and Tatton, 1999), which allows increased reactive oxygen species generation through NADPH oxidase which may also play a role (Kasahara *et al.*, 1997;Simon, 2003). Additionally, proteolytic activity of calpains and the proteasome contribute to constitutive apoptosis (Knepper-Nicolai *et al.*, 1998), including cleavage of the inhibitor of apoptosis protein, XIAP (Kobayashi *et al.*, 2002), and actin (Brown *et al.*, 1997) by calpains.

It is clear that pro- and anti-apoptotic members of the bcl-2 family of proteins play a role in regulating the rate of spontaneous neutrophil apoptosis. Translocation of Bax into the mitochondria during neutrophil apoptosis has been observed, thus activating the mitochondrial apoptotic pathway and causing caspase 3 activation (Maianski *et al.*, 2002). Truncation and translocation of Bid has also been observed (Simon, 2003). The role of Bak remains controversial; this molecule has been detected in neutrophils (Pryde *et al.*, 2000;Moulding *et al.*, 2001), but levels remained unchanged in the presence of factors that modulate the rate of neutrophil apoptosis (Bazzoni *et al.*, 1999). Expression of anti-apoptotic bcl-2 has been reported to occur in neutrophils and to be upregulated by LPS (Hsieh *et al.*, 1997). However other groups have found that bcl-2 is absent from mature neutrophils and that A1 represents the major anti-apoptotic member of the bcl-2 family in neutrophils, and is upregulated by GM-CSF and LPS (Chuang *et al.*, 1998). Moulding et al reported that protein levels of pro-apoptotic Bad, Bax, Bak and Bik remain constant during culture and that anti-apoptotic $Bcl\text{-}_{XL}$ protein was undetectable despite the presence of mRNA. They proposed that relatively unstable anti-apoptotic proteins, such as Mcl-1 and A1, are rapidly broken down, unmasking the effects of the more stable pro-apoptotic proteins named above (Moulding *et al.*, 2001). Thus, it is believed that Mcl-1 and A1 are likely responsible for the regulation of neutrophil apoptosis.

Neutrophil apoptosis can be modulated during *in vitro* culture, with many

agents able to either delay or enhance the rate of apoptosis in these cells; the net balance of pro- and anti-apoptotic signals determines their longevity. Corticosteroids have opposing effects in the different inflammatory cell types, as they induce apoptosis in eosinophils but inhibit neutrophil apoptosis (Haslett, 1997;Meagher *et al.*, 1996). Interestingly, the effects of glucocorticoids are mimicked by agents that increase the intracellular calcium concentration ($[Ca^{2+}]_i$), in that an increase in $[Ca^{2+}]_i$ delays neutrophil apoptosis but promotes eosinophil apoptosis (Cousin *et al.*, 1997;Ward *et al.*, 1999b). Moreover, the proinflammatory cytokine TNF-α induces neutrophil apoptosis (Ward *et al.*, 1999a), but has no such pro-apoptotic effect in eosinophils unless NF-κB activation is blocked, unmasking the pro-apoptotis effects of TNF-α (Fujihara *et al.*, 2002).

On the other hand, the bacterial product lipopolysaccharide (LPS) and agents that increase the intracellular concentration of cAMP (e.g. db-cAMP, prostaglandins) delay both neutrophil and eosinophil apoptosis (Ward *et al.*, 1999b), as do IL-3 and GM-CSF. Protein synthesis inhibitors such as actinomycin D and cycloheximide have the ability to induce apoptosis in neutrophils (Ward *et al.*, 1999b). Other potential mechanisms that may regulate apoptosis in granulocytes include oxidative damage caused by $ONOO^-$, which is generated when NO and O_2^- combine (Blaylock *et al.*, 1998;Dimmeler and Zeiher, 1997;Ward *et al.*, 2000), direct DNA damage (Dimmeler and Zeiher, 1997), S-nitrosylation of cysteines of enzymes involved in the regulation of apoptosis (Melino *et al.*, 1997) or inhibition of mitochondrial respiration at the level of cytochrome c oxidase (Melino *et al.*, 1997).

1.3.3 Nitric Oxide and Apoptosis:

NO is rapidly being recognised as an important regulatory molecule in apoptosis in many cell types, and a large number of studies have demonstrated effects of either endogenously-produced or exogenously-supplied NO on apoptosis.

1.3.3.1 General Effects of NO on Apoptosis:

This ubiquitous signalling molecule can regulate the rate of apoptosis, or programmed cell death, in many cell types, including human inflammatory cells. Whether or not cells undergo apoptosis depends on the net balance of a large number of pro- versus anti-apoptotic factors. Studies have revealed that NO has both pro- and anti-apoptotic properties, depending largely on the concentration and flux of NO, and the cell type under scrutiny (Nicotera *et al.*, 1997;Kim *et al.*, 1999). It has been proposed that low concentrations of NO, derived from constitutively active endothelial and neuronal isoforms of NO synthase (eNOS and nNOS), usually have a protective effect on cells whereas higher concentrations derived from the inducible isoform (iNOS) are more likely to drive cell death (Nicotera *et al.*, 1997). The current line of thinking is that NO has the ability to either induce or inhibit apoptosis, depending on its concentration, the cell type, the redox state of the cell and the cell's own defence mechanisms (Nicotera *et al.*, 1997).

Induction of apoptosis has been observed in VSMC (Pollman *et al.*, 1996), macrophages (Wang *et al.*, 1999), neutrophils (Ward *et al.*, 2000), cardiomyocytes (Kawaguchi *et al.*, 1997) and neurons (Nicotera *et al.*, 1997). These effects are generally believed to be cGMP-independent, and may be due to the action of $ONOO^-$ (Wang *et al.*, 1999;Ward *et al.*, 2000), S-nitrosylation of proteins (Nicotera *et al.*, 1997), the MAPK and Jun kinase (JNK) pathways (Nicotera *et al.*, 1997) or upregulation of p53, which in turn affects the apoptotic caspase enzymes (Nicotera *et al.*, 1997). In contrast, other studies have shown NO to inhibit endothelial cell, lymphocyte and hepatocyte apoptosis (Wang *et al.*, 1999;Nicotera *et al.*, 1997). This protective effect may be due to elevated cGMP or increased antioxidant levels (Nicotera *et al.*, 1997).

In stark contrast to cells undergoing necrosis, apoptotic inflammatory cells fail to release their pro-inflammatory and histotoxic contents (Haslett, 1997). Furthermore, their clearance by professional phagocytes such as macrophages occurs via a non-phlogistic mechanism, which additionally aids the resolution of the inflammatory response (Meagher *et al.*, 1992). Thus, apoptosis is generally regarded

to be non-inflammatory and is crucial for the successful resolution of inflammation.

Chronic inflammatory conditions are frequently characterised by an apparent failure of myelocytic inflammatory cells to respond to apoptotic stimuli, or of phagocytes to remove apoptotic cells. Persistence of these cells leads to damage of the surrounding tissue and exacerbation of inflammation, as cells ultimately undergo either primary or secondary (following failed clearance of apoptotic cells) forms of the extremely pro-inflammatory necrosis (Haslett, 1997;Ward *et al.*, 1999b); Figure 1.4).

Manipulation of the rate of apoptosis in critical inflammatory effector cells such as neutrophils, eosinophils, monocytes and macrophages, could therefore be of therapeutic benefit (Ward *et al.*, 1999b). NO is capable of inducing inflammatory cell apoptosis and, given its other anti-inflammatory properties, manipulation of NO concentrations is a particularly promising candidate to alter leukocyte function and rates of apoptosis in inflammatory conditions.

1.3.3.2 NO and Neutrophil Apoptosis

In recent years, it has become clear that NO also has the ability to induce apoptosis in neutrophils. Wong *et al.* demonstrated that the oxatriazole-5-imine derivative, GEA 3162, and SIN-1 increased the rate of apoptosis in human neutrophils (Wong *et al.*, 1997). Simultaneously, Brennan *et al.* found increased markers of DNA fragmentation treated with high (8-32 mM) concentrations of SNP in these cells (Brennan *et al.*, 1996). However, this effect may have been due to respiratory inhibition by cyanide derived from the liberation of NO from such high concentrations of this compound.

Since then, a number of groups have validated these findings through the use of several different sources of NO. NO gas (20 and 50 ppm) reduces cell viability and augments DNA fragmentation over 2 or 24 h of culture, an effect that is particularly pronounced in the presence of 80% O_2 (Fortenberry *et al.*, 1998).

Whether this cell death comprises apoptosis or merely necrosis, however, remains unclear. An induction of neutrophil apoptosis has also been observed using both traditional NO donors such as SNP (Singhal *et al.*, 1999) and different sources of NO such as GSNO (0.5-5 mM; (Fortenberry *et al.*, 1999).

Blaylock *et al.* (1998) reported that GEA 3162 (10 μM) increased neutrophil apoptosis as assessed by Annexin V binding to exposed phosphatidylserine (PS) at 4 and 8 h, but not at 16 h. On the other hand, the $ONOO^-$ donor, SIN-1 (1 mM), showed no significant increase in PS exposure compared to control cells, although there was a small enhancement of annexin V binding at 4 h (Blaylock *et al.*, 1998).

The effects of these two compounds were also studied by Ward *et al.*, who demonstrated that concentrations of 10-100 μM GEA 3162 and 0.3-3 mM SIN-1 enhanced caspase dependent morphological neutrophil apoptosis at 6 and 20 h, and 30-100 mM GEA 3162 increased annexin V binding at 6 h (Ward *et al.*, 2000). In contrast to Blaylock's study, however, the increase in morphological apoptosis by 10 μM GEA 3162 was not significant at 6 h, whereas Blaylock observed significant differences at just 4 h. The principal methodological difference between the two studies is in the culture conditions. It has previously been demonstrated that the micro-environment, including cell density and concentration of plasma proteins, has a critical effect on the rate of neutrophil apoptosis *in vitro* (Hannah *et al.*, 1998). It must therefore be emphasised that the choice of culture conditions may subtly alter the effects of NO on neutrophils, and experimental design should receive careful consideration.

1.4 Mechanisms of Action of NO

The mechanisms through which NO is able to both promote and delay inflammatory cell apoptosis still remain to be fully elucidated. However, it is generally believed that low concentrations tend to be anti-apoptotic, acting through a

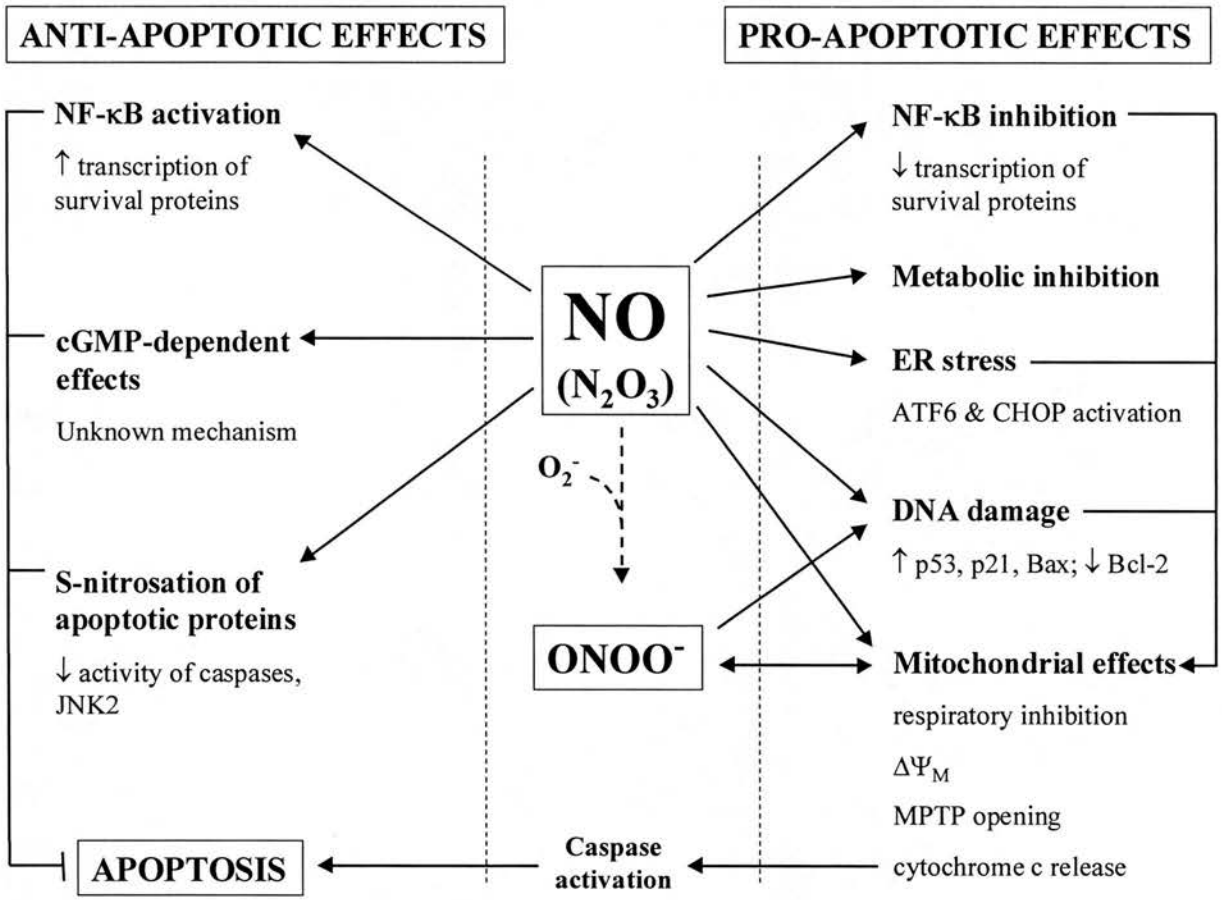


Figure 1.5 Proposed mechanisms of action for the delay or acceleration of apoptosis in leukocytes. Reduced apoptosis is largely considered to be mediated by elevation of cyclic GMP (cGMP) and/or S-nitrosation of caspases and activation of the transcription factor, NF-κB. In contrast, enhanced apoptosis is frequently attributed to cGMP-independent mechanisms, in particular activation of the stress pathway of apoptosis, prompting mitochondrial changes and ultimately cytochrome c release, apoptosome formation and caspase activation.

rise in cGMP or S-nitrosation of caspase enzymes. On the other hand, high concentrations of NO are generally more toxic, inducing either apoptosis or necrosis, with most reports suggesting that such actions occur independently of the sGC signalling pathway (see below and Figure 1.5).

Another possibility is that NO may indirectly interfere with alternative pathways that regulate inflammatory cell survival. For example, glucocorticoids (GCs) promote eosinophil apoptosis but inhibit neutrophil death (Meagher *et al.*, 1996;Ward *et al.*, 1999b). It has been shown that NO can inhibit GC receptor (GR) binding through S-nitrosation (Galigniana *et al.*, 1999), and therefore could attenuate both the pro- and anti-apoptotic effects of glucocorticoids on inflammatory cells.

1.4.1 Anti-apoptotic mechanisms:

The pathway by which NO inhibits apoptosis in neutrophils has not yet been investigated. However, it is known that NO induces a rise in cGMP in neutrophils through activation of sGC (Moilanen *et al.*, 1993;Kosonen *et al.*, 1999). Given that the cell permeable analogues of cAMP (db-cAMP) and cGMP (db-cGMP), can delay constitutive neutrophil apoptosis (Ward *et al.*, 2000), and that a rise in cGMP has been postulated to at least partially account for NO-mediated inhibition of apoptosis in other cell types (Li and Billiar, 1999;Kolb, 2000), it is possible that an increase in one or other of these cyclic nucleotides mediates the inhibition of apoptosis in neutrophils exposed to low concentrations of NO.

A role for cyclic nucleotide (cGMP or cAMP) signalling has also been proposed in NO-mediated inhibition of both constitutive and Fas-triggered eosinophil apoptosis. Beauvais et al observed that NO could only reduce eosinophil apoptosis when compounds that form nitrosyl-haem in their liberation of NO were used, which was mimicked by the permeable cGMP analogue db-cGMP and reduced by the sGC inhibitor LY-83583 (Beauvais *et al.*, 1995). Pure NO donors alone, in contrast, failed to increase eosinophil survival, although inhibition of apoptosis could be seen if hematin was added alongside a pure NO donor to artificially create a nitrosyl-heme

complex (Beauvais and Joly, 1999).

It has been suggested that such inhibition occurs through activation of sGC by the nitrosyl-haem complex (Beauvais and Joly, 1999), which has been shown to occur *in vitro* using purified sGC (Edwards *et al.*, 1981), but as yet there is no evidence to suggest that this may occur in intact cells *in vivo*. However, the mechanism is not fully understood: the issue is complicated by the fact that haem groups are known to scavenge NO, and therefore might be expected to inhibit NO-mediated effects.

Inhibition of Fas-mediated apoptosis was reproduced by both db-cGMP and db-cAMP, and potentiated by the PDE inhibitor, IBMX (Hebestreit *et al.*, 1998), again suggesting a role for cyclic nucleotides. This group localised the site of Fas receptor death pathway blockade to downstream of SMase activation and ceramide generation but upstream or around the level of JNK activation. So et al showed inhibition of the stress protein JNK2 by NO *in vitro* via S-nitrosation (So *et al.*, 1998).

Secondly, the transcription factor NF-κB is known to regulate neutrophil apoptosis as its inhibition leads to increased apoptosis (Ward *et al.*, 1999a). Agents such as LPS have been shown to delay apoptosis in this cell type through stimulation of NF-κB and subsequent caspase-1 dependent activation of IL-1β (Watson *et al.*, 1998). It has been demonstrated in several cell types that activation of NF-κB leads to the transcriptional upregulation of survival factors such as the immediate early gene, IEX-1L, inhibitor of apoptosis proteins (IAPs), and members of the anti-apoptotic Bcl-2 gene family, including Mcl-1, A1/Bfl1, Bcl-$_{XL}$ and Nr13, potentially explaining the survival effects of NO which, at low concentrations, induces NF–κB activation in macrophage cell lines (von Knethen *et al.*, 1999b;Connelly *et al.*, 2001) and in human peripheral blood mononuclear cells (Fowler, III *et al.*, 1999).

Activation of NF–κB may occur through a cGMP-dependent mechanism (Kalra *et al.*, 2000) and NO has been demonstrated to modulate the expression of several proteins affecting the activity of Bcl-2 family members through sGC

activation. A cGMP-dependent mechanism has been proposed to account for the NO-induced downregulation of BNIP3, a dominant pro-apoptotic Bcl-2 family member in hepatocytes (Zamora *et al.*, 2001). Levels of MAPK phosphatase-3 (MKP-3) mRNA, which causes degradation of Bcl-2 via ERK1/2 dephosphorylation, were seen to be decreased by NO, thus protecting Bcl-2 levels and promoting survival of endothelial cells (Rossig *et al.*, 2000), although the role of Bcl-2 in neutrophils is questionable, as described above. The pro-apoptotic adaptor protein, p66shc, is also downregulated by NO via sGC activation (Andoh *et al.*, 2000). Furthermore, survival genes such as Bcl-2 and $Bcl\text{-}_{XL}$ have been shown to be upregulated in the presence of NO in endothelial cells (Delikouras *et al.*, 2001) and human neuroblastoma cells (Andoh *et al.*, 2000), therefore it seems possible that there is a role for NF-κB-mediated transcriptional regulation in the anti-apoptotic effects of NO in inflammatory cells.

Contrasting studies in macrophage cell lines (Brune *et al.*, 1997;Vouldoukis *et al.*, 2000;Boggs *et al.*, 1998) suggest that the redox status of the cell may partially determine the effects of NO. An inhibitory effect of endogenous NO on J774 cell apoptosis can be unmasked when O_2^- is scavenged (Vouldoukis *et al.*, 2000), and overexpression of SOD also protects RAW 264.7 cells against apoptosis induced by endogenous or exogenously-supplied NO (Brockhaus and Brune, 1999). These studies suggest a role for $ONOO^-$ in mediating NO-induced apoptosis.

Conflicting evidence suggests that RAW 264.7 cells that overproduce O_2^- are resistant to NO-mediated apoptosis (Brune *et al.*, 1997), and von Knethen et al observed that O_2^- activates NF-κB, thus mediating survival in these cells (von Knethen *et al.*, 1999c). Furthermore, Brockhaus et al found that $ONOO^-$ had no role in NO-evoked apoptosis, despite the protective effect of SOD in RAW 264.7 cells (Brockhaus and Brune, 1999).

Others have found that such protection is observed when cellular thiols are depleted in RAW 264.7 cells (Boggs *et al.*, 1998). It has been reported that endogenous antioxidant levels (Rosenberg *et al.*, 1999), or the balance between oxidative and nitrosative stress (Espey *et al.*, 2000), can determine the cellular

response to NO. It has been proposed that in low thiol concentrations, NO actually protects against cell death, whereas it induces death in cells with normal thiol levels (Rosenberg *et al.*, 1999). Exogenous glutathione has also been shown to enhance neutrophil apoptosis and increase H_2O_2 levels, possibly leading to hydroxyl radical-mediated damage (Adrie *et al.*, 2000).

In the absence of large quantities of scavenger thiols such as glutathione, but in the presence of oxygen, it is possible that NO S-nitrosates critical effector molecules of apoptosis such as caspases, thus preventing their activation and having an inhibitory effect on the proteolytic cascade. It has been shown by several groups that NO can inhibit a number of apoptotic proteins (Melino *et al.*, 1997) including caspase 3 (the protease responsible for the initiation of internucleosomal DNA fragmentation) (Mohr *et al.*, 1997;Zech *et al.*, 1999;Li *et al.*, 1997;Rossig *et al.*, 1999;Kim *et al.*, 1998;Kim *et al.*, 1997), caspase 8 (Dimmeler *et al.*, 1997;Dimmeler *et al.*, 1998;Li *et al.*, 1997), caspase 9 (Torok *et al.*, 2002), caspase 1 (Dimmeler *et al.*, 1997;Dimmeler *et al.*, 1998) and caspases 2, 3, 4, 6 and 7 (Li *et al.*, 1997) activation via S-nitrosation. Inhibition of caspase 3 has been reported to involve two distinct mechanisms in hepatocytes – direct protein S-nitrosation, and another mechanism, which has not yet been elucidated, but is dependent upon cGMP (Kim *et al.*, 1997). Therefore, the cGMP-dependent anti-apoptotic effects of NO in inflammatory cells may be mediated through an inhibitory effect on caspases.

1.4.2 Pro-apoptotic mechanisms:

Studies have shown that apoptosis in neutrophils and macrophages proceeds via activation of caspase protease enzymes (Ward *et al.*, 2000;Williams *et al.*, 1997;Knepper-Nicolai *et al.*, 1998), part of the classical apoptotic effector cascade. However, the upstream mechanisms by which exposure to NO causes these enzymes to become activated has not been clarified, although several theories have been suggested.

$ONOO^-$ is considered to be one of the most likely candidates for the increased

apoptosis of inflammatory cells observed with higher concentrations of NO, particularly in the case of neutrophils (Ward *et al.*, 2000;Fortenberry *et al.*, 1998;Blaylock *et al.*, 1998), which generate large quantities of O_2^- that rapidly combines with NO to form $ONOO^-$. Compounds that generate $ONOO^-$, such as SIN-1, may promote neutrophil apoptosis similar to that evoked by NO (Ward *et al.*, 2000), and primary human monocytes also undergo apoptosis in response to $ONOO^-$ by a mechanism that involves mitochondrial membrane depolarisation, release of cytochrome c and caspase activation (Adrie *et al.*, 2000); see Figure 1.5). $ONOO^-$ has been shown to reversibly or irreversibly inhibit a number of mitochondrial respiratory complexes as well as inducing mitochondrial swelling, depolarisation, calcium release and permeability transition (Clementi *et al.*, 1998;Brown and Borutaite, 2001).

Macrophage apoptosis can also be induced by exposure to $ONOO^-$, via oxidative stress, which can be reduced by antioxidants such as ascorbic acid (Sandoval *et al.*, 1997b) or phytolens (Sandoval *et al.*, 1997a). Induction of apoptosis by NO in elicited murine macrophages or RAW 264.7 cells has also been attributed to formation of $ONOO^-$ within mitochondria, as nitrotyrosine residues were detected in cytochrome c (Hortelano *et al.*, 1999). $ONOO^-$ and metabolites of NO (e.g. N_2O_3), can cause direct DNA damage or inhibit DNA repair enzymes (Wink *et al.*, 1996), leading to an increase in the tumour suppressor protein p53, which has been shown to accumulate in NO-treated macrophages and may be the factor responsible for driving them towards apoptosis (Brune *et al.*, 1997;von Knethen *et al.*, 1999a;Messmer *et al.*, 1994;Messmer and Brune, 1996b). The p53 protein promotes apoptosis through upregulation of the apoptotic proteins Bax and cyclin-dependent kinase p21 and downregulation of the anti-apoptotic protein Bcl-2 (Kolb, 2000); Figure 2). However, Gotoh et al measured no increase of p53 in NO-mediated apoptosis in RAW 264.7 cells stimulated with LPS/IFN-γ (Gotoh *et al.*, 2002). Instead, this group proposed a role for the endoplasmic reticulum stress pathway involving the transcription factors ATF6 and CHOP leading to cytochrome c release (Figure 1.5). Also, studies in murine macrophages (Brockhaus and Brune, 1999) suggest little or no involvement for $ONOO^-$ in NO-induced apoptosis in this

cell type.

As previously described, the activation status of the survival factor, NF-κB, has been shown to play a role in regulation of the induction of inflammatory cell apoptosis (Ward *et al.*, 1999a). It has been demonstrated that high concentrations of NO can inhibit NF-κB activation in macrophage cell lines (Connelly *et al.*, 2001) and human macrophages (Raychaudhuri *et al.*, 1999), monocytes (Welters *et al.*, 2000) and neutrophils (Welters *et al.*, 2000;Fortenberry *et al.*, 2001). NO may inhibit NF-κB DNA binding through S-nitrosation of the p50 subunit of the transcription factor, as has been demonstrated in isolated NF-κB protein (Matthews *et al.*, 1996) and in human respiratory cells and murine macrophages (Marshall and Stamler, 2001). Alternatively, transcriptional induction and stabilisation of the inhibitory molecule I-κB, that keeps NF-κB sequestered in the cytoplasm, may account for the inhibition of NF-κB activity (Peng *et al.*, 1995). Furthermore, it has been reported that NO inhibits NF-κB activation in rat vascular smooth muscle cells via a cGMP-independent inhibition of the phosphorylation and proteasomal degradation of I-κB (Katsuyama *et al.*, 1998), and inhibition of the proteasome by NO has additionally been demonstrated by Glockzin et al in macrophages (Glockzin *et al.*, 1999). The result of such inhibition would be downregulation of survival factors under the control of this transcription factor, such as the anti-apoptotic Bcl-2 family members. Indeed, this has been observed by a number of studies, as exogenous NO downregulates Bcl-2 but upregulates the pro-apoptotic protein, Bax, in neurons (Tamatani *et al.*, 1998;Matsuzaki *et al.*, 1999) and upregulates Bad and Bax but downregulates Bcl-2 in human colon adenocarcinoma cells (Ho *et al.*, 1999).

In non-small cell lung cancer cells, it has been shown that NF-κB inhibition leads to apoptosis by increasing mitochondrial permeability, thus allowing release of cytochrome c and subsequent caspase activation (Jones *et al.*, 2002). This concurs with findings by Borutaite et al, who demonstrated increased mitochondrial permeability and cytochrome c release from isolated rat mitochondria, and increased caspase activation in J774 cells treated with S-nitrosothiols (SNAP, GSNO) but not NONOates (DETA/NO), which activate caspases through an as yet unidentified

alternative mechanism (Borutaite *et al.*, 2000). As S-nitrosothiols readily transnitrosate endogenous cysteine residues, this supports the concept of S-nitrosation of the NF-κB p50 subunit as the mechanism of inhibition.

In addition, the biphasic effects of NO on NF-κB activation reported by Connelly et al are mirrored by its effects on the open probability of the mitochondrial permeability transition pore (MPTP). Low concentrations of NO donors (GEA 3162, SNAP, SIN-1; 1-20 μM) delayed or had no effect on MPTP opening, while at higher concentrations (20-100 μM), opening was enhanced (Piantadosi *et al.*, 2002). In this study GEA 3162 was found to be particularly effective at inducing MPTP opening compared to the other two NO donors, and this drug induces neutrophil apoptosis at lower concentrations than NONOates or SIN-1 (Ward *et al.*, 2000), as described in Chapter 4. Enhanced MPTP opening on exposure to NO was also reported by Hortelano et al (Hortelano *et al.*, 1997). Therefore there is growing evidence to suggest that the pro-apoptotic effects of NO in inflammatory cells may be mediated, at least in part, through inhibition of NF-κB.

Albina et al proposed metabolic inhibition as a potential mechanism, as glucose starvation and inhibition of glycolysis or the TCA cycle all pushed macrophages into apoptosis (Albina *et al.*, 1993). On the other hand, inhibition of the electron transport chain of respiration had no effect (Albina *et al.*, 1993), and Messmer et al showed no reduction in NAD^+ or ATP levels in NO-induced apoptosis (Messmer and Brune, 1996a), suggesting that NO does not act through respiratory inhibition.

In contrast, others have reported that NO inhibits mitochondrial respiration through two distinct pathways (Clementi *et al.*, 1998;Brown and Borutaite, 2001). Reversible inhibition of cytochrome oxidase was seen with low concentrations of NO, whereas higher concentrations caused an inhibition of alternative respiratory chain complexes (Brown and Borutaite, 2001). Inhibition of complex IV was reversible, whereas inhibition of complex I was irreversible (Clementi *et al.*, 1998). Of course, the mechanisms described above may not be mutually exclusive. For example, respiratory inhibition by NO may enhance the production of reactive

oxygen species by mitochondria, leading to the formation of $ONOO^-$ and providing a $ONOO^-$-mediated pathway for NO-induced cytotoxicity (Brown and Borutaite, 2001).

1.5 *In Vivo* Effects of NO and its Therapeutic Potential

Delayed apoptosis of activated granulocytes has been reported to occur in a number of inflammatory conditions in humans or animal models (Haslett, 1997;Simon, 1999), including rheumatoid arthritis (Ottonello *et al.*, 2002), acute pancreatitis (O'Neill *et al.*, 2000), bacterial pneumonia (Droemann *et al.*, 2000), inflammatory bowel disease (Brannigan *et al.*, 2000), asthma (Turlej *et al.*, 2001;Woolley *et al.*, 1996;Kankaanranta *et al.*, 2000) and following surgery (Matsuda *et al.*, 2001;Chello *et al.*, 2002). Failure of these cells to undergo programmed cell death and to be cleared by phagocytes allows persistent and inappropriate inflammation to occur, as activated granulocytes release a number of pro-inflammatory mediators, which may contribute significantly to the aetiology of the disease. Therefore, the induction of apoptosis in these cells is a potential target for therapeutic intervention, by removal of the inflammatory effector cells, thereby minimising tissue damage and oedema.

Although iNOS has been implicated in the pathogenesis of certain inflammatory diseases, such as arthritis, SLE and irritable bowel syndrome (Clancy and Abramson, 1995), a number of studies have demonstrated a protective effect of NO against several conditions characterised by inflammation, such as glomerulonephritis (Heeringa *et al.*, 2000), acute hepatic necrosis (Billiar *et al.*, 1990), arthritis (Veihelmann *et al.*, 2001;McCartney-Francis *et al.*, 2001), endotoxemia (Billiar *et al.*, 1990) and acute lung injury (Liu *et al.*, 2001) *in vivo* (Clancy and Abramson, 1995). Most studies attribute these effects to the wide range of general anti-inflammatory properties of NO, as reviewed by Granger and Kubes (Granger and Kubes, 1996). Of particular interest, however, is the increasing

evidence that the protection afforded against inflammation and immunity by NO may be mediated in part through the induction of inflammatory cell apoptosis. As for apoptosis induction *in vitro*, the concentration of NO in the local environment (Ormerod *et al.*, 1999;Ross and Reske-Kunz, 2001), the timing of administration (Okuda *et al.*, 1998;Xu *et al.*, 2001) or the route of administration, and perhaps therefore the NOS isoform targeted (Paul-Clark *et al.*, 2001) appears to be critical. Studies have suggested that lower doses of NO may be detrimental but that higher doses may attenuate the inflammatory response, with some authors proposing a role for the cytotoxic effects of NO on myeloid inflammatory cells (Okuda *et al.*, 1997;Wang *et al.*, 1999;Niebauer *et al.*, 1999;Ross and Reske-Kunz, 2001).

NO appears to be particularly effective in autoimmune conditions, such as experimental allergic encephalomyelitis (EAE), which serves as a model for human multiple sclerosis (Bogdan, 1998). In this model, several studies have reported that iNOS-deficient rats or mice that were immunised directly with MBP developed exacerbated disease, although results obtained following immunisation with MBP-specific T cells often contradict these findings (Bogdan, 1998). One proposed mechanism for the protective effect of iNOS-derived NO is induction of apoptosis in macrophages or T cells (Puerta *et al.*, 2000;Xu *et al.*, 2001).

Other autoimmune disease models in which NO has been reported to be protective are the rat model of autoimmune interstitial nephritis and experimental autoimmune uveitis, as inhibition of NOS caused exacerbated injury in both models, although again other studies have produced conflicting results (Bogdan, 1998). Therefore, NO may be protective in a number of autoimmune conditions, although further research will be required to fully understand the apparent contradictory effects of NO.

A beneficial protective effect of NO has also been shown in the elicitation phase of contact hypersensitivity, as prolonged inflammatory reactions were observed in iNOS knockout mice (Ross and Reske-Kunz, 2001). Again, it has been suggested that this effect may be partly due to the induction of apoptosis in infiltrating cells. In a human model similar to sunburn and psoriasis, Ormerod et al

described a cytotoxic effect of high concentrations of topically-administered NO to immunocompetent cells, which was not seen with low concentrations (Ormerod *et al.*, 1999). Aminoguanidine, an inhibitor of iNOS, prevented the impairment of renal vascular bed responses and reduced urine nitrate levels and apoptotic mononuclear cells in a rat model of experimental nephropathy (Ozen *et al.*, 2001). However, although inflammatory apoptosis might be expected to be beneficial, the effect of this form of cell death on prognosis was not studied within the late, sclerotic phase of the disease during this study. In the same model, Rangan et al discovered that NOS inhibitors exacerbated progression of the disease (Rangan *et al.*, 2001), and tubulointerstitial injury was also found to be increased in the presence of NOS inhibitors in a model of thrombotic microangiopathy (Shao *et al.*, 2001).

Two *in vivo* studies were published in 1999, investigating the effects of NO on macrophage apoptosis in cardiovascular disease of cholesterol-fed rabbits. Niebauer et al demonstrated that provision of L-arginine (the substrate for NOS) in drinking water could reduce the formation of inflammatory lesions following balloon angioplasty (Niebauer *et al.*, 1999), while Wang et al showed that it decreased existing atherosclerotic lesions, by inducing macrophage apoptosis (Wang *et al.*, 1999).

Thus, there is a significant body of evidence to suggest that supplementation of NO may be beneficial in certain inflammatory diseases and that the induction of apoptosis in infiltrating cells may have a role in mediating this protection. However, these issues remain controversial, with much conflicting evidence. It is clear that provision of NO may not be suitable for the treatment of all inflammatory conditions, and indeed may only be appropriate at certain stages of disease progression or disease provoked by a particular mechanism. Much further work is required to clarify these issues.

1.6 Summary

NO has a biphasic effect on apoptosis in many cell types, in which low concentrations delay but higher concentrations enhance this form of cell death, a pattern that has recently been confirmed in neutrophils. This correlates with the dichotomous action of NO on the activity of caspase enzymes responsible for the execution of apoptosis *in vitro*. Inhibition of caspases by S-nitrosation is a direct consequence of exposure to low concentrations of NO or, more likely, its oxidation products (e.g. N_2O_3). On the other hand, activation of these enzymes observed during pro-apoptotic actions of higher concentrations represents a downstream event following initial effects on DNA or mitochondria, and can therefore be considered an indirect effect of NO. Although the mechanism of inhibition has not yet been investigated, it is likely that cGMP production, NF-κB activation and subsequent expression of survival proteins, or S-nitrosation of apoptotic proteins will play a role.

It has been demonstrated that exogenous NO can induce apoptosis in monocytes, monocyte-derived macrophages, neutrophils and eosinophils. In addition, endogenous NO from iNOS also promotes apoptosis in macrophages. There still remains some controversy over the mechanism by which this molecule causes this form of cell death in these cells, although it involves activation of caspase proteases, and most agree that this occurs through a cGMP-independent pathway. Moreover, mitochondria appear to play a key role in the initiation of apoptosis by NO through release of cytochrome c, resulting in caspase activation.

It is clear that $ONOO^-$ derived from SIN-1 or other agents has the ability to promote apoptosis in its own right, but its role in mediating NO-induced apoptosis remains controversial. Some groups have reported that NO-evoked cytotoxicity is likely to be effected through $ONOO^-$ formation, while studies by others have indicated little or no role for $ONOO^-$. Modulation of the activation status of the transcription factor NF-κB has also been proposed to account for NO-induced apoptosis in neutrophils and macrophages, and there is an increasing body of evidence to support this theory. On the other hand, DNA damage (by N_2O_3 or

$ONOO^-$) has also been shown, leading to an accumulation of pro-apoptotic p53.

Differences may exist in the mechanisms by which NO causes apoptosis in different cell types that could potentially be exploited to target a particular inflammatory cell type in certain conditions. Despite the uncertainties and controversies surrounding the regulation of inflammatory cell apoptosis by NO, it is clear that the class and concentration of NO-donating compound used and the cell type are critical determinants of the response. Major differences between different classes of NO donors and opposing effects with low and high concentrations of certain NO donors are observed. Thus, the amount and rate of NO release and the redox status of the target cell appear to be key factors in the cellular response to NO exposure, and certain NO donors appear to be more effective than others at promoting inflammatory cell apoptosis.

It is also important to realise that the concentration of NO donor used may not necessarily reflect the concentration of NO to which the cells are exposed. Equivalent concentrations of different NO donors may liberate NO to different extents or at different rates, or may produce different reactive nitrogen species, such as $ONOO^-$. Culture conditions may also affect NO levels; for example plasma proteins such as albumin are able to scavenge NO through the formation of S-nitrosothiols (Stamler *et al.*, 1992;Butler and Rhodes, 1997;Ramachandran *et al.*, 2001). Therefore, the concentration of free NO in the vicinity of the cells at any given time may vary from compound to compound, and the NO concentration in the system needs to be measured in order to directly compare different NO donors. Further studies are required to fully elucidate the initiation of apoptosis by NO, in order to identify potential targets for the treatment of human inflammatory conditions and to evaluate the sources of NO that provide greatest therapeutic potential.

1.7 Aims of Thesis

The aims of this thesis were to characterise the effects of several distinct compounds that release 'pure' NO or a combination of NO and O_2^- on neutrophil apoptosis *in vitro*. The intention was to study a number of independent events of apoptosis to determine the temporal relationship between them and to evaluate whether different compounds affected them differently. The aim was to ascertain whether NO or $ONOO^-$ is more effective at regulating neutrophil apoptosis, and whether a rapid burst of high concentrations of NO has different effects than prolonged release of lower concentrations. In addition, the mechanisms of action of these compounds were to be studied and compared.

Chapter 2: Materials and Methods

2.1 Isolation of Human Neutrophils

Human neutrophils were isolated from the blood of healthy volunteers as previously described (Haslett *et al.*, 1985;Ward *et al.*, 2000). Venous blood (36 ml) was removed into 50 ml polypropylene tubes containing 4 ml of sterile 3.8% sodium citrate solution to prevent coagulation, and mixed by gentle inversion. This was followed by centrifugation at 350*g* for 20 min at room temperature with the deceleration rate set to zero, which separated the blood into two fractions; a leukocyte- and erythrocyte-rich lower layer and a platelet-rich plasma upper layer. The platelet-rich plasma was aspirated from the tubes without disturbing the lower fraction, and aliquots of 10 ml transferred to sterile glass tubes in order to prepare autologous serum by addition of 220 µl 1M $CaCl_2$ (final concentration 20 µM) followed by incubation at 37°C to initiate platelet aggregation.

Erythrocytes were sedimented from the remaining cellular fraction by addition of 2.5 ml of 6% T500 dextran solution (pre-warmed to 37°C) per 10 ml haematocrit, followed by adjustment of the volume to 50 ml using pre-warmed 0.9% saline solution. Cells were allowed to sediment for no longer than 30 min at room temperature. The leukocyte-rich upper layer was then carefully removed into 50 ml polypropylene tubes, the volume was made up to 50 ml with saline, and tubes were centrifuged at 350*g* for 6 min at room temperature. Following this, the supernatant was decanted and the cell pellet resuspended in 2.5 ml of 55% isotonic Percoll (prepared as a 9:1 (v:v) ratio of Percoll:10x PBS without Ca^{2+}/Mg^{2+}) in 1x PBS without Ca^{2+}/Mg^{2+}. Discontinuous gradients were prepared by overlaying 2.5 ml of 68% isotonic Percoll onto 2.5 ml of 81% isotonic Percoll in a 15 ml Falcon tube, then layering the resuspended cells in 55% Percoll as the upper layer of the gradient. Gradients were then centrifuged at 720*g* for 20 min at room temperature with the deceleration rate set to zero in order to separate the subpopulations of leukocytes. Granulocytic leukocytes were harvested from the 81:68% interface of the gradient

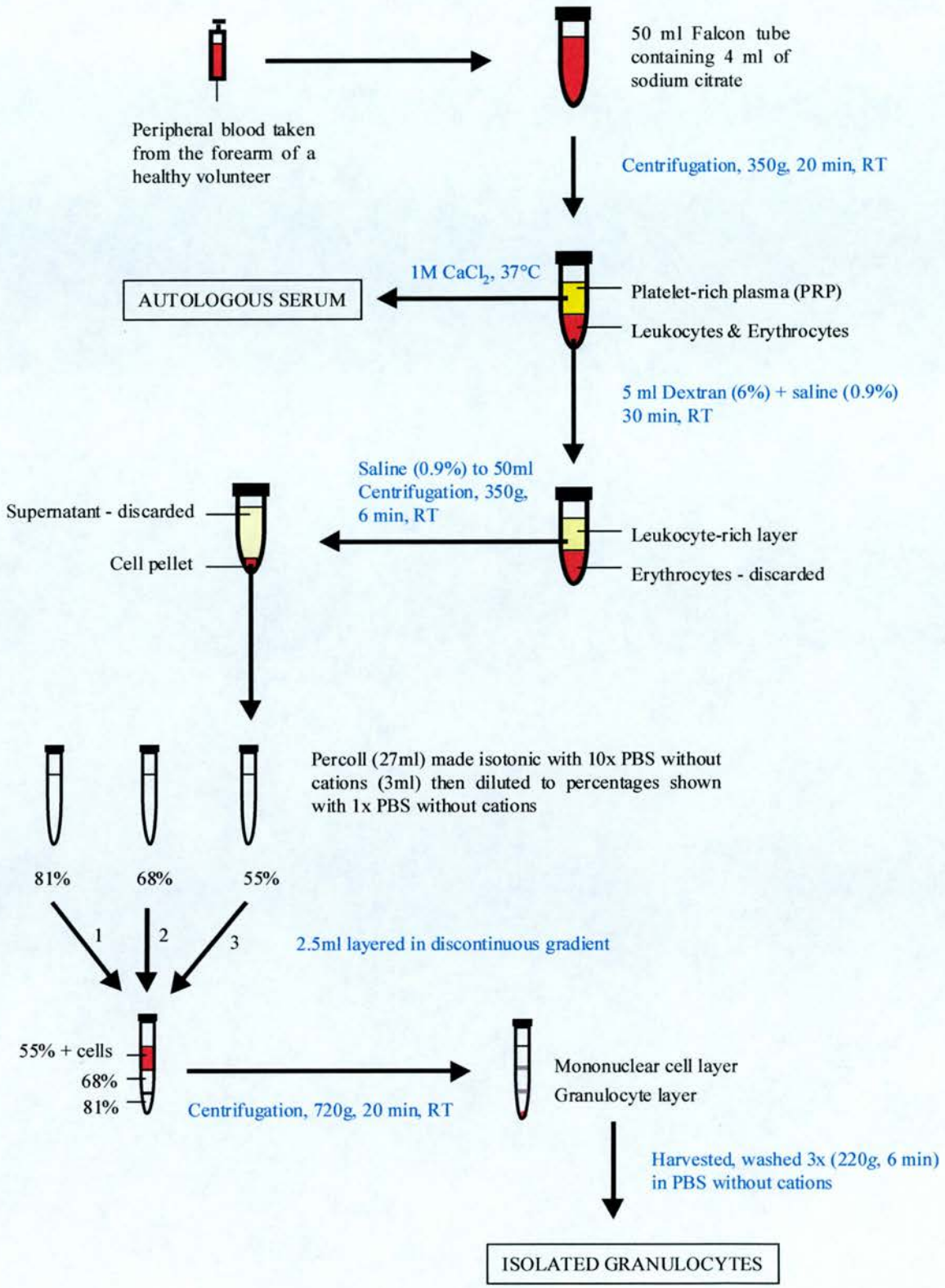


Figure 2.1 Schematic representation of the isolation of granulocytes and preparation of autologous serum from the peripheral blood of healthy volunteers.

and washed twice in 1x PBS without Ca^{2+}/Mg^{2+} (centrifuged at 220*g*, 6 min, room temperature). Granulocyte bands that were heavily contaminated with erythrocytes were discarded, as haemoglobin is able to scavenge NO and may affect the results of experiments using NO donors.

2.2 Assessment of Neutrophil Yield, Purity and Viability

Neutrophil yield was assessed using a haemocytometer. A single drop of granulocytes suspended in 50 ml PBS without Ca^{2+}/Mg^{2+} was added to the haemocytometer and the number of neutrophils in 25 squares was counted by light microscopy, and cell activation, in the form of shape change, was assessed at the same time, with activated neutrophil preparations being discarded. This number represented the neutrophil count in 0.1 µl PBS, and was therefore multiplied by 10,000 to obtain the number of neutrophils present in 1 ml. In order to obtain the total neutrophil yield from the preparation, this number was then multiplied by the number of ml PBS in which the cells were suspended (usually 50 ml). The typical neutrophil yield was 250 x 10^6 neutrophils from 160 ml whole blood, although it varied considerably between donors.

The neutrophil isolation procedure is unable to separate neutrophils from basophils and eosinophils, therefore the percentage contamination by other types of granulocyte was assessed by cytocentrifugation. A volume of 100 µl of isolated granulocytes was removed, diluted using PBS without Ca^{2+}/Mg^{2+} if required and cytocentrifuged (300 rpm, 3 min). The resulting slide was fixed in methanol (100%, 1 min) and stained using Diff-Quik physiological stain (1 min each in pink and blue dyes), then observed by oil immersion light microscopy. The percentage of contaminating basophils and eosinophils was measured by counting 500 cells at random. Basophils were rarely seen, and eosinophils usually represented only a small percentage of the total cell count. Preparations containing > 5% basophils or eosinophils were discarded. Figure 2.2 shows the different morphologies and staining of neutrophils and eosinophils from cytospun freshly-isolated leukocytes.

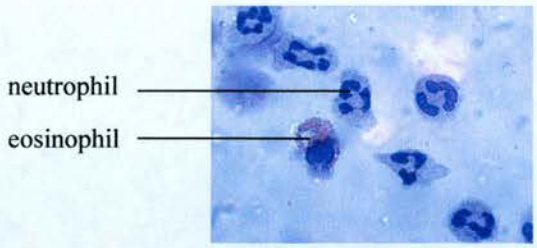


Figure 2.2 Fresh neutrophils and contaminating eosinophil from the peripheral blood of a healthy volunteer.

Mononuclear cell contamination was assessed by flow cytometry. A small aliquot of isolated granulocytes in PBS without Ca^{2+}/Mg^{2+} was removed and further diluted with PBS without Ca^{2+}/Mg^{2+}. The suspension was then run by flow cytometry and a dot plot generated on the basis of side scatter (x-axis, representing cell granularity) and forward scatter (y-axis, representing cell size). Using these parameters, cells can be distinguished on the dot plot, and the percentage of cells that falls into each subset of leukocyte can be measured; neutrophils are more granular than either monocytes or lymphocytes and therefore form a distinct population when assessed by flow cytometry (Figure 2.3). A representative flow cytometry profile showing distinct leukocyte populations and neutrophil cell purity is shown in Figure 2.1. Typically, ~98% of the isolated cells were neutrophils, and contamination levels of > 5% by mononuclear cells led to the granulocyte preparation being discarded.

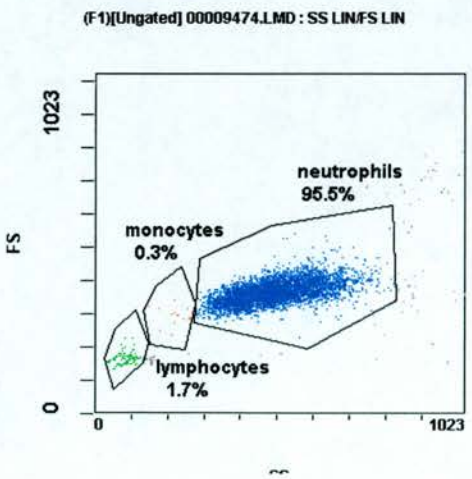


Figure 2.3 Typical flow cytometry profile showing purity of isolated human neutrophils.

Neutrophil viability was assessed by Trypan Blue exclusion. Viable cells have the ability to exclude the vital dye, Trypan Blue. Isolated granulocytes (10 µl) were placed on a haemocytometer and 40 µl Trypan Blue added. Microscopic examination then allowed the determination of the percentage of cells that excluded the dye. Only granulocyte preparations that exhibited > 99% viability were used.

2.3 Cell Culture

2.3.1 Neutrophil Culture

Harvested neutrophils were routinely resuspended at 5 x 10^6 cells/ml in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with penicillin (100 U/ml) and streptomycin (100 U/ml), plus 10% (v:v) autologous serum. Cells were cultured in flat-bottomed 96-well Falcon Flexiwell plates at 37 °C in a humidified 5% CO_2 atmosphere. Extensive studies, such as recovery experiments and shape change asays, carried out previously in the laboratory have demonstrated a lack of neutrophil adherence to these polypropylene plates and lack of cell activation.

Neutrophils (135 µl; final cell density 4.5 x 10^6 cells/ml, i.e. approximately 675,000 cells/well) were added to 15 µl of PBS (controls) or the $NO/ONOO^-$ donors, GEA 3162 (0.1 µM – 100 µM), SPER/NO (0.1 µM – 1 mM), DEA/NO (0.1 µM – 1 mM) or SIN-1 (1 mM – 3 mM), and cultured for between 1 and 20 h. In certain experiments, neutrophils were preincubated for 10 min with pharmacological agents such as the reducing agent, dithiothreitol (DTT 123; 100 – 300 µM), dihydrorhodamine 123 (DHR 123; 1 µM) or the protein kinase C inhibitor, Ro 31-8220 (1 µM). In other experiments, where neutrophils were to be incubated with two or more reagents (e.g. $NO/ONOO^-$ donors and superoxide dismutase; SOD), cells were resuspended at 10 x 10^6 cells/ml and 75 µl of cells were plated out with 15 µl of each reagent and volumes made up to 150 µl with PBS.

2.3.2 3G8 Hybridoma

The hybridoma, 3G8, raised against the leukocytic cell surface Fcγ receptor III (CD16) was grown and maintained in CL350 concentrator flasks. The cell line was initially grown up in 75 ml cell culture flasks before being seeded into CL350 flasks (8 x 10^6 cells per flask). The nutrient compartment membrane was dampened with 10 ml 'nutrient medium'; Dulbecco's Modified Eagle's Medium (DMEM) supplemented with L-glutamine, penicillin (100 U/ml), streptamycin (100 U/ml) and 1% foetal calf serum (FCS). After splitting, cells were resuspended in 5 ml of 'complete medium' (as nutrient medium except containing 10% FCS) and seeded into the cell compartment. A further 340 ml of nutrient medium was then added to the nutrient compartment, then cells were cultured for 7 days. To harvest cell supernatant containing the anti-CD16 antibody, liquid was aspirated from the nutrient compartment of the flask then the cell compartment liquid was removed using a pipette and transferred to a 15 ml Falcon tube. Cells were centrifuged (220*g*, 6 min) and supernatant transferred to a separate tube. The cell pellet was resuspended in 10 ml of complete medium and a 5 ml volume was seeded back into the flask as before. Cell supernatant was harvested every 3 days. In order to assess the binding activity of antibodies, supernatant was added to 100,000 freshly-isolated neutrophils in the CD16 assay described below. Supernatant aliquots that produced high fluorescence were pooled and used in subsequent experiments at a 1:10 dilution in PBS without Ca^{2+}/Mg^{2+}.

2.4 Assessment of Apoptosis

Apoptosis was measured according to a number of different criteria thought to represent apoptotic cell death in neutrophils: morphological changes, shedding of cell surface CD16, annexin V binding and DNA fragmentation.

2.4.1 *Morphological changes*

Viable neutrophils have a characteristic nuclear morphology, with distinctive multilobed nuclei. During apoptosis, cells become shrunken and the chromatin condenses and rounds up and stains more darkly than in viable cells; these alterations are clearly visible by oil immersion microscopy, as shown in Figure 2.4 (Ward *et al.*, 2000).

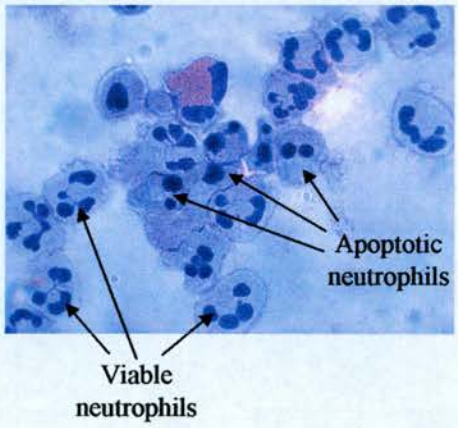


Figure 2.4 Photograph (x100 objective) demonstrating altered morphology of apoptotic vs. viable neutrophils. Viable neutrophils have multilobed nuclei whereas apoptotic cells have characteristic darkly stained rounded and condensed nuclei following staining with DiffQuik™ solution. Typical neutrophil diameter is 8-12 μm.

Following incubation, 100 μl of recovered cells were cyto-centrifuged in duplicate, fixed in 100% methanol and stained using Diff-Quik™ physiological stain, then observed by oil-immersion light microscopy (x100 objective) to determine the proportion of darkly-stained cells with condensed nuclei. At least 500 cells per slide were counted, with the observer blinded to the experimental conditions by numbering the slides rather than labelling the conditions and counting them in a random order.

In order to verify that the effects of the diazeniumdiolate compounds on neutrophil apoptosis were caused by NO generation rather than being an effect of the

parent amines that remain after decomposition of the diazeniumdiolates, a control experiment was carried out to examine the effects of spermine tetrahydrochloride and diethylamine hydrochloride on morphological apoptosis compared to control (spontaneous) apoptosis over 20 hours. Concentrations of 0.1 μM and 1 mM of these compounds were tested, as these concentrations correlated with the lowest and highest concentrations of the diazeniumdiolates that were to be used in the concentration-response studies. Figure 2.5 shows the lack of effect of spermine or diethylamine at either concentration on neutrophil apoptosis over a 20-hour incubation period.

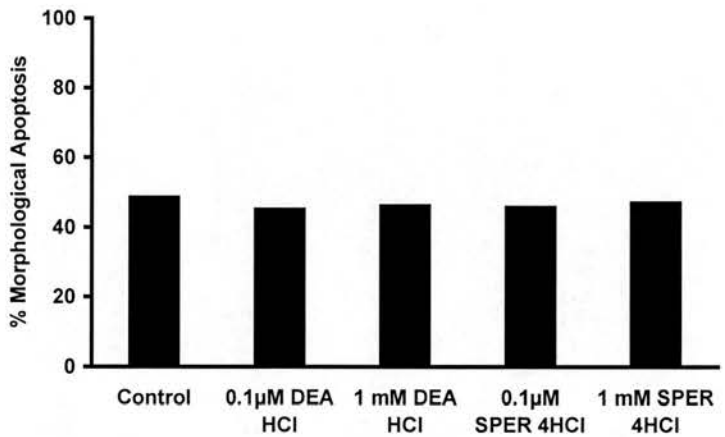


Figure 2.5 Control experiment to determine the effects of spermine and diethylamine on neutrophil apoptosis. Neutrophils (4.5 x 10^6/ml) were incubated for 20 hours in the presence of PBS (control), spermine tetrahydrochloride (0.1 μM or 1 mM) or diethylamine hydrochloride (0.1 μM or 1 mM), then apoptosis was assessed by morphology.

2.4.2 Shedding of cell surface CD16

CD16 is the Fc-γ receptor III glycoprotein antigen present on the surface of granulocytes. During the apoptotic process in neutrophils, CD16 is shed from the cell surface (Dransfield *et al.*, 1994;Homburg *et al.*, 1995). This change can be exploited using flow cytometry to determine the percentage of cells undergoing apoptosis within a given population (Dransfield *et al.*, 1994). Antibody binding in

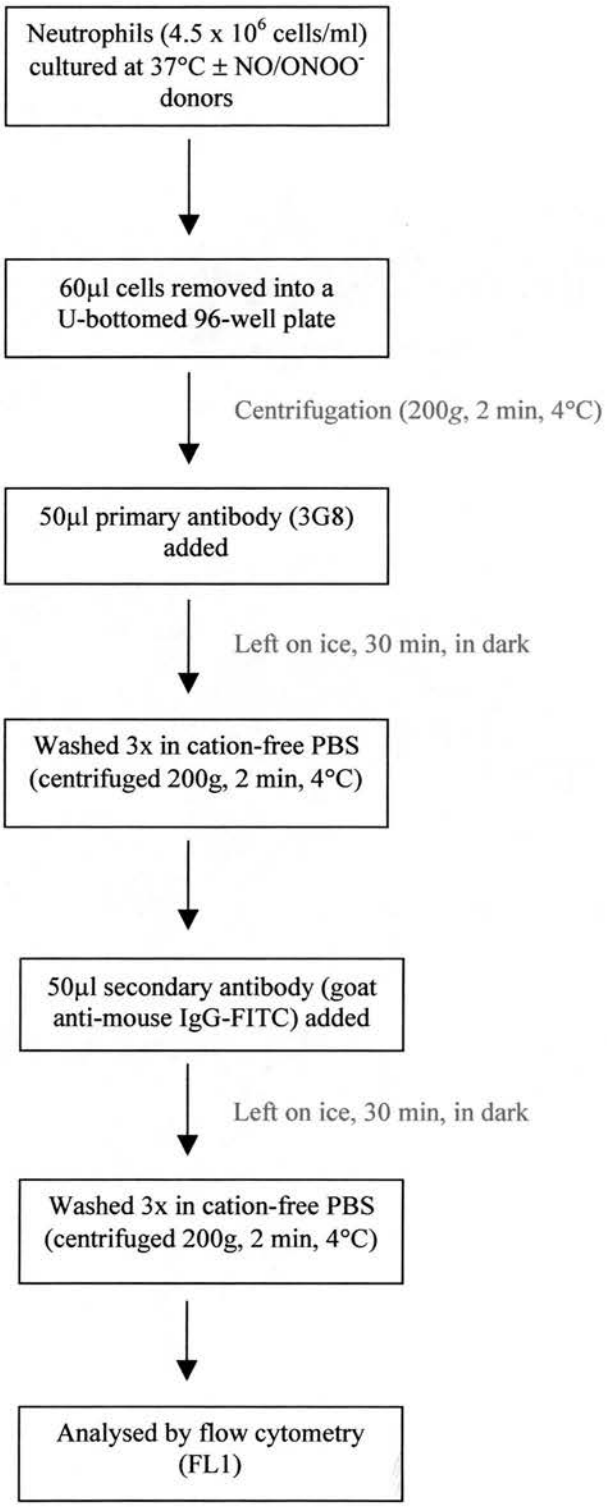


Figure 2.6 Flow diagram of assessment of apoptosis by CD16 shedding

general is not altered as neutrophils undergo apoptosis, as demonstrated by the use of an isotype control antibody during the development of the CD16 shedding assay (Dransfield *et al.*, 1994).

Neutrophils (60µl of recovered cells) were transferred to a U-bottomed 96-well Flexiwell plate and centrifuged (200*g*, 2 min). Following resuspension of the cells by gentle vortexing, 50 µl of a 1:10 dilution of the 3G8 hybridoma supernatant containing monoclonal Ab against CD16 was added. Following incubation (4°C, 30 min), the cells were washed 3x in PBS without Ca^{2+}/Mg^{2+}, before addition of 50 µl of a FITC-conjugated goat anti-mouse secondary antibody (1:40 dilution). Cells were incubated (4°C, 30 min) before being washed 3x as before, resuspended and analysed by flow cytometry using an EPICS XL2 (FL1), with CD16 'low' expressing cells representing the apoptotic population (see Chapter 4 for typical profiles). A schematic representation of the protocol is shown in Figure 2.6.

2.4.3 Annexin V binding

Annexin V is a phospholipid-binding protein with a high affinity for phosphatidylserine (PS). In viable cells, PS has an asymmetric distribution, being retained on the inner cell surface. During apoptosis, this asymmetry is lost, and PS becomes exposed on the outer surface of the cell, thus allowing annexin V binding, which can be measured using flow cytometry (Homburg *et al.*, 1995).

Fluorescein isothiocyanate (FITC)-conjugated annexin V (5 µl), was added to 15 ml of annexin V binding buffer (Hanks Balanced Salt Solution plus 2.5 ml of 1 M $CaCl_2$; final concentration 5 µM). Following incubation, 20 µl of recovered neutrophils were transferred to a FACS tube and 180 µl of the annexin V binding solution was added. Cells were incubated on ice for 10 min before flow cytometric analysis using an EPICS XL2 (FL1), with annexin V positive cells representing the apoptotic population. A schematic representation of the protocol is shown in Figure 2.7.

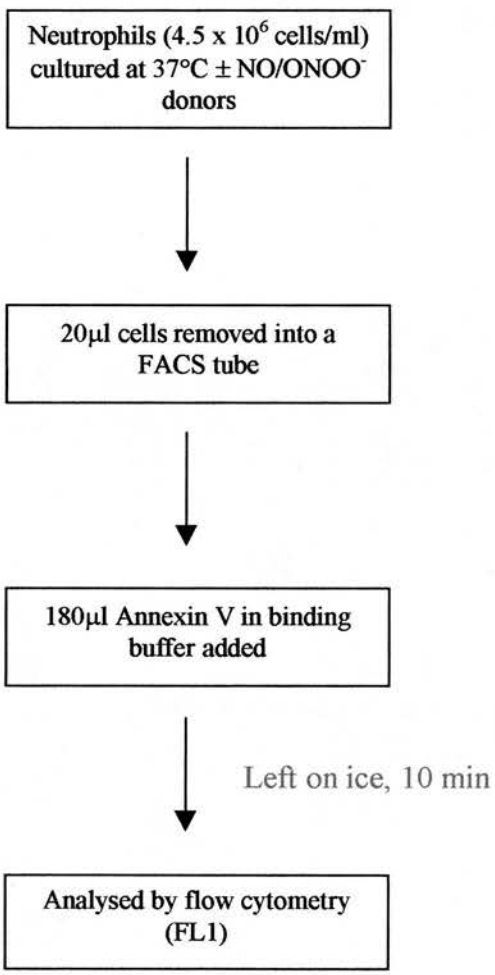


Figure 2.7 Flow diagram of assessment of apoptosis by Annexin V binding

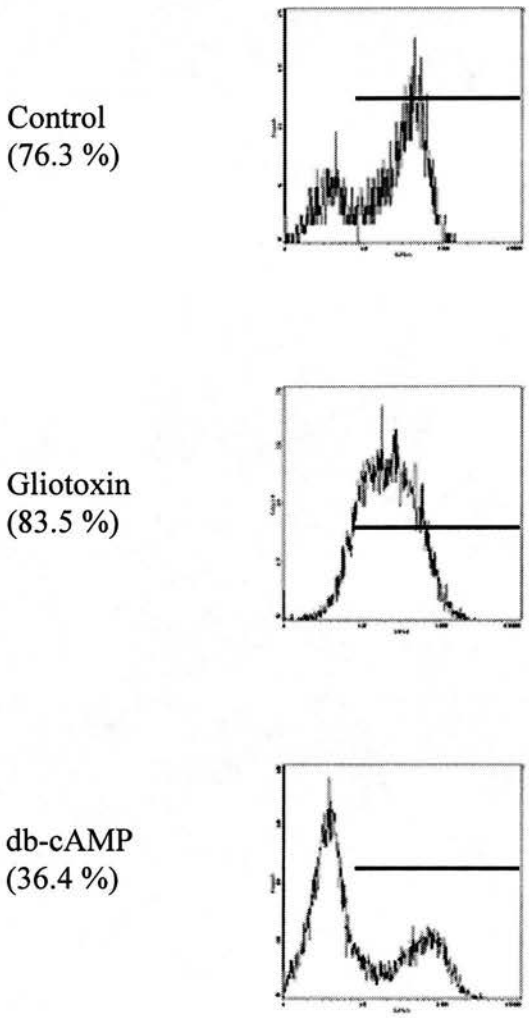


Figure 2.8 Control annexin V binding experiment. Neutrophils (4.5 x 106 cells/ml) were incubated for 20 h with PBS (control), gliotoxin (0.1 μg/ml) or db-cAMP (0.2 mM). Apoptosis levels were then assessed by annexin V binding. Horizontal lines represent the apoptotic population of neutrophils, and numbers in brackets show the levels of apoptosis detected by this method.

In order to verify that the annexin V assay is able to detect changes in the rate of neutrophil apoptosis, control experiments were carried out using the known inhibitor of neutrophil apoptosis, db-cAMP, and known inducer of apoptosis, gliotoxin. Neutrophils (4.5×10^6 cells/ml) were incubated for 20 hours with PBS (control), gliotoxin (0.1 μg/ml) or db-cAMP (0.2 mM), then apoptosis was assessed by Annexin V binding. Figure 2.8 shows that the annexin V assay was able to detect acceleration of neutrophil apoptosis by gliotoxin and delayed apoptosis mediated by db-cAMP.

2.4.4 Internucleosomal DNA fragmentation

Internucleosomal DNA fragmentation is considered to be a characteristic feature of apoptotic cell death. During apoptosis, DNA is cleaved by CAD/DFF40 into specific-sized fragments. This process can be assessed by measuring the DNA content of cells or by running DNA gels to look for the characteristic 'laddering' pattern of DNA from apoptotic cells (Wyllie, 1997).

2.4.4.1 Propidium Iodide Staining

The fluorescent molecule, propidium iodide (PI), intercalates into DNA and can be used to measure the amount of DNA in a given cell. This can be used to assess internucleosomal DNA fragmentation in apoptotic cells.

Neutrophils (100μl of recovered cells) were transferred to a U-bottomed 96-well Flexiwell plate and centrifuged (200*g*, 2 min). Following resuspension of the cells by gentle vortexing, 100 μl of 70% ethanol was added to fix and permeabilise the cells. Cells were then incubated on ice for 10 min before being washed 3x with PBS without Ca^{2+}/Mg^{2+}. 50 μl of RNase A (0.5 mg/ml) then 50 μl of PI (0.1 mg/ml) were then added to the cells, which were wrapped in foil at room temperature for 25 min before analysis by flow cytometry using an EPICS XL2 (FL3) to determine the

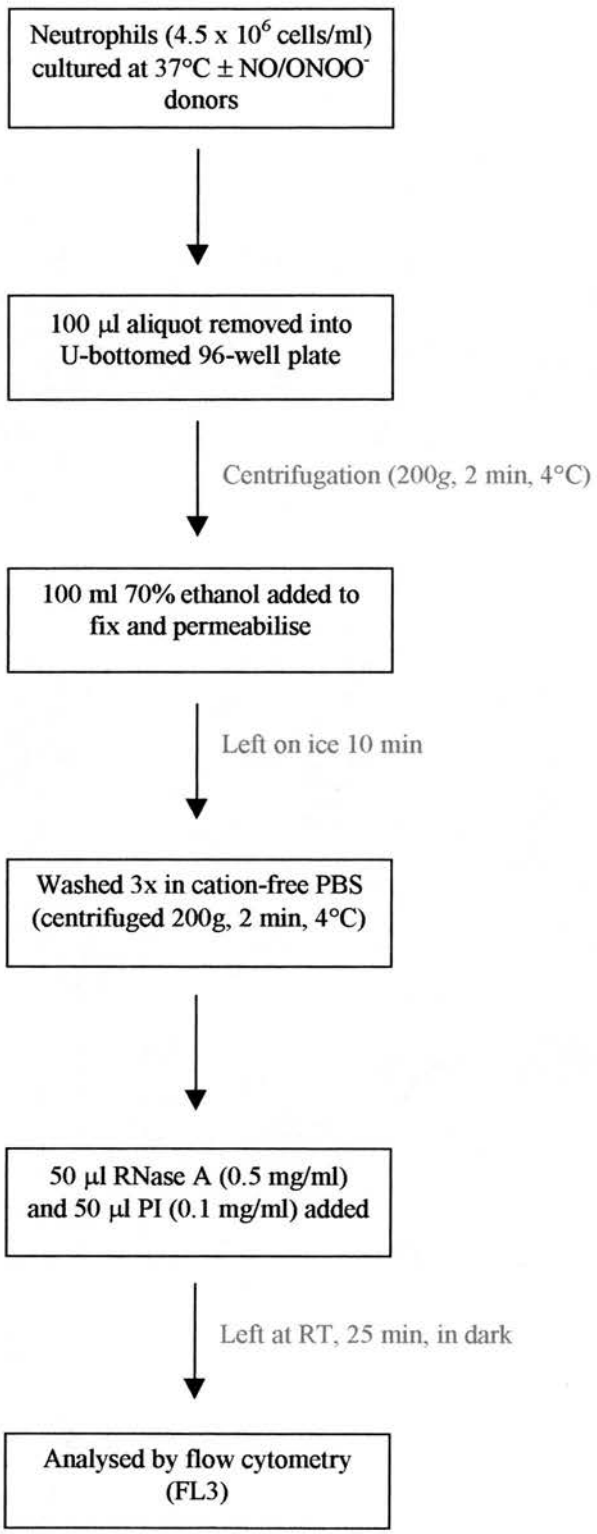


Figure 2.9 Flow diagram of assessment of apoptosis by PI intercalation

percentage of cells with hypodiploid DNA content. The permeabilisation of cells in all conditions was shown to be effective by Trypan Blue staining and also by the lack of a flow cytometry peak at the far left side of the histogram on analysis (coinciding with unlabelled cells), which would have represented gated cells exhibiting no PI staining at all (see Chapter 4 for representative flow cytometry histograms). A schematic representation of the protocol is shown in Figure 2.8.

2.4.4.2 DNA Gel Electrophoresis

Internucleosomal DNA fragmentation during apoptosis generates pieces of DNA that produce a distinctive 'laddered' appearance when separated by gel electrophoresis (Wyllie, 1997).

Neutrophils (5 x 10^6 cells per sample) were incubated for 8 or 20 h in the presence of PBS, $NO/ONOO^-$ donors, the known inducer of neutrophil apoptosis, gliotoxin, and the known neutrophil apoptosis inhibitor, db-cAMP. Cells were then harvested and centrifuged (14,000 rpm, 10s) and pellets resuspended in 50 µl of PBS without Ca^{2+}/Mg^{2+}. 500 µl of 7M guanidine hydrochloride was added to each sample to lyse the cells, then DNA was extracted using a Wizard Miniprep kit according to the manufacturer's instructions.

Miniprep Columns were dampened by addition of 50 µl of Column Wash Solution (90 mM NaCl, 9 mM Tris-Cl pH 7.4, 2.25 mM EDTA, 55% ethanol) then lysed cells were added to the columns. Columns were centrifuged at 14,000rpm for 1 min at room temperature, then the flowthrough was poured off and 750 µl of Wash Solution was added before centrifugation as before. After discarding flowthrough, 250 µl of Wash Solution was added before centrifugation at 14,000 rpm for 2 min. Spin Columns were then transferred to a fresh, sterile 2 ml Eppendorf tube and 50 µl of TE + RNase A added (10 mM Tris-Cl pH 8.0, 1 mM EDTA, 50 µg/ml RNase). The Eppendorf tubes were then centrifuged at 14,000 rpm for 1 min at room temperature to elute the DNA. DNA samples (23 µl DNA plus 7 µl loading buffer)

were then run on a 2% Sea Kem agarose gel containing 1 μl of ethidium bromide stock solution (500 μg/ml), in TBE (10 mM Trisma base, 10 mM boric acid, 1 mM EDTA) for 1 h at 100V and DNA was viewed under uv light.

2.5 Electrochemical Detection of NO Generation

An NO electrode (Iso-NO II, World Precision Instruments) was calibrated daily in pH 4 buffer at 37 °C using concentrations of 1×10^{-7} M – 3.2×10^{-6} M DEA/NO. This yielded free NO in the range 2×10^{-7} M – 6.4×10^{-6} M, as there are two molecules of NO per molecule of DEA/NO. NO release was measured in milliVolts (mV) by the electrode; plotting $\log_{10}$ mV vs $\log_{10}$ [NO] gave a straight line of equation, $y = mx + c$, from which subsequent NO release could be calculated (see Figure 3.2).

GEA 3162 (100 μM), SIN-1 (100 μM), DEA/NO (1 μM) or SPER/NO (1 μM) were then added to stirred, pre-warmed PBS (2 ml) in order to detect free NO liberated from these compounds. NO generation was measured for 20 min, then the electrode was rinsed in distilled H_2O before insertion into fresh PBS and addition of the next NO donor.

In order to investigate the masking effect of O_2^- on detection of NO from these compounds, these experiments were repeated in the presence of 50 U/ml Cu/Zn superoxide dismutase (SOD). Again, NO liberation was measured for 20 min before rinsing of the electrode and insertion into fresh prewarmed PBS containing 50 U/ml SOD (Lizasoain *et al.*, 1996).

2.6 Dihydrorhodamine 123 Studies

Dihydrorhodamine 123 (DHR 123) is a dye which is oxidised to its fluorescent form, rhodamine 123 by various reactive oxygen species, including $ONOO^-$, O_2^-, H_2O_2 and HOCl, but not by NO (Crow, 1997). This compound can

therefore be used to discriminate between agents that release NO only and those that generate NO and O_2^- simultaneously.

2.6.1 Direct Measurement of ROS generation from NO/$ONOO^-$ Donors

A volume of 100 µl of PBS, the neutrophil activating agent, phorbol 12-myristate 13-acetate (PMA; final concentration 10 nM), SIN-1 (1 mM), GEA 3162 (100 µM) or DEA/NO (1 mM) was added to 900 µl PBS in 2 ml Eppendorf tubes. DHR 123 was added to each tube to a final concentration of 1 µM. Tubes were incubated for 60 min (37°C, 5% CO_2), then 450 µl was transferred to a 0.5 ml cuvette, excited at 480 nm and the fluorescence emitted at 500 nm was read using a spectrofluorimeter (Perkin Elmer, UK).

2.6.2 Measurement of ROS in Neutrophils

Further DHR 123 studies were carried out in the presence of neutrophils in order to determine whether $ONOO^-$ may be generated by the interaction of drug-derived NO with O_2^- from neutrophils, or whether $ONOO^-$ is derived from the drug itself. This was determined by using the protein kinase C inhibitor, Ro-31-8220 (Dieter and Fitzke, 1991). Neutrophil generation of reactive oxygen species such as O_2^- proceeds through activation of protein kinase C (Wilson *et al.*, 1986). Therefore, if DHR 123 is activated by $ONOO^-$ produced from combination of NO with neutrophil-derived O_2^-, oxidation of DHR 123 should be inhibited in the presence of Ro-31-8220. On the other hand, fluorescence should be unchanged in the presence of Ro-31-8220 if the compound itself generates $ONOO^-$.

Isolated cells were resuspended in IMDM at a density of 5 x 10^6 cells/ml and DHR 123 was added to a final concentration of 1 µM. Neutrophils (135 µl) were added to a 15 µl volume of PBS, PMA (final concentration 10 nM), SIN-1 (1 mM), GEA 3162 (100 µM) or DEA/NO (1 mM) in a flat-bottomed 96-well plate in the

absence or presence of the protein kinase C inhibitor, Ro-31-8220 (1 μM). Following incubation (37°C, 5% CO_2) for 45 min or 2 h, samples were removed and assessed by flow cytometry (FL2) using an EPICS XL2 (Coulter Electronics, Luton, UK).

2.7 Western Blotting

Western blotting was carried out to investigate the breakdown of ICAD/DFF45 in neutrophils undergoing apoptosis. This breakdown allows CAD/DFF40 to translocate to the nucleus and cause internucleosomal DNA fragmentation.

2.7.1 Preparation of Whole Cell Neutrophil Lysates

Isolated neutrophils (5 x 10^6 cells/sample) were incubated in 24-well plates for 8 h in the presence of PBS, $NO/ONOO^-$ donors or the known inducer of apoptosis, gliotoxin (0.1 μg/ml; (Ward *et al.*, 2000). Whole cell neutrophil lysates were then made from these cells and also from freshly isolated neutrophils as a negative control for ICAD/DFF45 breakdown.

A number of protease inhibitors (7 mM AEBSF, 3 μM aprotinin, 0.42 mM leupeptin, 30 μM pepstatin A, 20 mM sodium orthovanadate, 10 mM benzamidine, 40 mM levamisole and 6 mM β-glycerophosphate) were added to 0.5 ml of whole cell lysis buffer (Sigma Protease Cocktail, 1:50 dilution in TBS) and the volume made up to 1 ml with TBS. These extremely high concentrations of protease inhibitors are required in the preparation of neutrophil lysates because neutrophils contain large amounts of protease enzymes that may cleave proteins on extraction if incompletely inhibited. Rigorous titration of protease inhibitor concentrations required for the preparation of neutrophil whole cell lysates has previously been carried out in the laboratory to minimise the risk of protein cleavage during the

extraction process. This was done by applying neutrophil lysates prepared in the presence of varying concentrations of protease inhibitors to Jurkat T cell lysates and blotting to determine the breakdown of I-κB. The concentrations of inhibitors that caused no I-κB breakdown in Jurkat lysates were considered adequate to completely inhibit neutrophil proteases and were used for subsequent lysate preparation.

Non-adherent apoptotic neutrophils were aspirated from the plate and transferred to 2 ml Eppendorf tubes. A volume of 50 µl of the protease inhibitor cocktail described above was added to each well and the well was gently scraped to harvest any adherent cells. This procedure was carried out on ice to minimise the activity of any protease liberated, alongside the presence of protease inhibitors. Cells were centrifuged (14,000 rpm, 10s) and supernatant removed, then cells were washed in PBS. Cells were resuspended in 100 µl of the protease inhibitor cocktail before addition of 10 µl of NP-40 to each sample and incubation for 10 min on ice. Eppendorf tubes were centrifuged (15,000 rpm, 20 min, 4°C) then supernatant removed and added to 50 µl of sample buffer (30% glycerol; 10% β-mercaptoethanol; 8% SDS; 0.25 M Tris HCl pH 6.8%, 0.02% bromophenol blue) prewarmed to 95°C. Lysates were then boiled at 95°C for 5 min before being stored at –20°C as 45 µl aliquots.

2.7.2 Western Blotting

Protein separation in previously prepared neutrophil lysates was carried out by SDS-PAGE. Lysates (40 µl) were loaded onto a 9% acrylamide gel (0.375 M Tris-HCl pH 8.8) and electrophoresed at 100 V through the stacking gel (0.125 M Tris-HCl pH 6.8, 9% acrylamide) and at 150 V through the separating gel for approximately 1 h, using a running buffer of 25 mM Tris, 192 mM glycine pH 8.3 and 0.1% SDS. Proteins were then transferred onto nitrocellulose membranes at 80 V for 1 h using a transfer buffer of 20% methanol, 250 mM Tris and 1.92 M glycine pH 8.3. Non-specific binding sites were blocked by incubation of nitrocellulose membranes in 5% casein solution in TBS 0.1% Tween-20 on a shaker for 1 h at room

temperature. Membranes were then washed 3x 3 min using TBS 0.1% Tween-20 before addition of primary antibody (ICAD/DFF45 mouse monoclonal antibody, 1:500 dilution in TBS 0.1% Tween-20) and incubation overnight at 4°C on a shaker. Following 3x 3 min washes with TBS 0.1% Tween-20, the secondary antibody (goat anti-mouse IgG-HRP) was added at a dilution of 1:1250 in TBS 0.1% Tween-20 and left for 1 h on a shaker at room temperature. Membranes were washed 3 x 3 min in TBS 0.1% Tween-20 before addition of ECL Plus electrochemiluminescence solution for 5 min, then exposed to film for 2 min before developing. Equal protein loading to the gels was assessed by Ponceau staining and always showed equal loading.

In order to determine appropriate antibody concentrations to use, whole cell neutrophil lysates were blotted using 1:500 and 1:1000 dilutions of the primary antibody against ICAD/DFF45 and with 1:1250 and 1:2500 dilutions of secondary antibody (goat anti-mouse HRP). The film was exposed for 1 and 2 min to determine the optimal exposure time. The optimal conditions for this antibody were found to be a 1:500 primary antibody dilution with a 1:1250 dilution of the secondary antibody, which detected the long (45 kDa) and short (40 kDa) splice variants of ICAD/DFF45, with a 2 min exposure time, as shown in Figure 2.10.

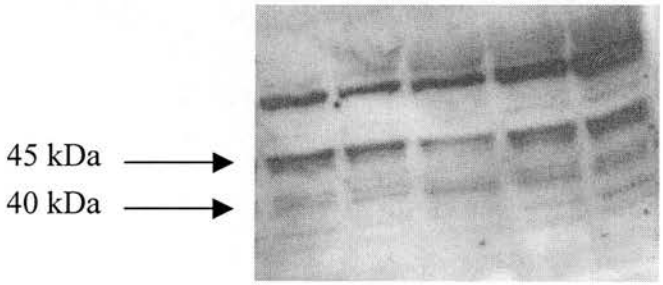


Figure 2.10 Control Western blot for the ICAD/DFF45 antibody. Whole cell neutrophil lysates were blotted using primary antibody at a 1:500 dilution and secondary antibody at 1:1250, and the film was exposed for 2 minutes.

2.8 Statistical Analysis

Results are plotted as mean ± S.E.M. Data were analysed for statistical significance by one-way ANOVA with either Student-Newman-Keuls (to compare each group with all others e.g. concentration in the concentration-response studies) or Dunnett's (to compare every group to control, e.g. gliotoxin in the absence or presence of DTT) post-hoc test. Where there were two variables, such as concentration and time in the time-course studies, two-way ANOVA with Bonferroni post-hoc test was used, and where two points were being compared, a two-tailed paired *t* test was used. Values of $p < 0.05$ were considered to be statistically significant.

2.9 Materials

Alexis Biochemicals (Nottingham, UK): Diethylamine Diazeniumdiolate (DEA/NO); GEA 3162; Spermine Diazeniumdiolate (SPER/NO)

Amersham Pharmacia Biotech, UK Ltd (Buckinghamshire, UK): Hybond C nitrocellulose membrane

Baxter Healthcare (Glasgow, UK): Diff-Quik physiological stain

Becton Dickinson (Oxford, UK): Cell culture plastic

Bender MedSystems (Vienna, Austria): FITC-labelled recombinant human Annexin V

BioVision: ICAD/DFF45 mouse monoclonal antibody

Flowgen (Ashby-de-la-Zouch): Sea Kem agarose

Gibco Life Technologies (Paisley, UK): Iscoves Modified Dulbecco's Medium (IMDM); L-Glutamine; Penicillin (50 U/ml); Streptomycin (50 U/ml); Trypsin EDTA

Pharmacia (Milton Keynes, UK): Dextran; Percoll

Promega (Southampton, UK): Wizard® Plus SV Minipreps DNA Purification System

Sigma (Poole, UK): 3-morpholinosydnonimine hydrochloride (SIN-1); Dithiothreitol (DTT); Dulbecco's Phosphate Buffered Saline (PBS) without Ca^{2+}/Mg^{2+}, sterile, endotoxin free pH 7.4; DMEM; Ethidium Bromide; Kodak Biomax Film; Propidium Iodide

Chapter 3: Characterisation of NO and $ONOO^-$ generation from NO donors

3.2 Introduction

A wide variety of NO donor compounds are available either for clinical use or as experimental tools. Each class of NO donors has unique chemical properties and differing mechanisms of NO generation, with some compounds liberating NO spontaneously and others requiring complex enzymatic degradation or the presence of intracellular thiols (Megson, 2000). Such differences may be exploited to examine the mechanisms through which biological effects are mediated; for example, compounds that spontaneously release free NO can be compared with S-nitrosothiols, that might also cause biological effects through transnitrosation.

NO generation from sydnonimines can be mediated by both enzymatic and nonenzymatic processes. For example, the prodrug molsidomine is metabolised in the liver to form SIN-1 (Figure 3.1D). This is then converted by hydrolysis to the active metabolite SIN-1A, from which NO is liberated without need for thiols or other cofactors. The rate of release is strongly influenced by pH, oxygen and light, and is non-linear due to the hydrolytic step required to generate SIN-1A (Feelisch *et al.*, 1989). In the presence of O_2, however, O_2^- is formed concomitantly with NO, which rapidly combine NO to form cytotoxic $ONOO^-$ (Feelisch *et al.*, 1989). Thus, these drugs are limited in their clinical potential, as oxidative damage is believed to be involved in early disease processes, such as atherogenesis (Bult, 1996).

Diazeniumdiolates contain two molecules of NO attached to a nucleophilic molecule through a nitrogen atom (Figure 3.1), which may be contained within a primary or secondary amine, polyamine, oxide or sulphite (Maragos *et al.*, 1991). NO is released predictably and spontaneously by first-order kinetics in aqueous solution, with no enzyme or thiol requirement, to activate sGC (Keefer *et al.*, 1996). Each dizeniumdiolate molecule is capable of liberating up to 2 molecules of NO. The exact amount of NO generated, and the rate at which it is released depends on the nature of the nucleophile molecule, the pH and temperature – the rate of release

rises with increasing acidity and temperature (Maragos *et al.*, 1991).

The half-lives of decomposition of the diazeniumdiolates range from 1 min to 1 day at physiological pH at 37°C (Keefer *et al.*, 1996). For example, diethylamine diazeniumdiolate (DEA/NO) has the NO linked to diethylamine (Figure 3.1A), and can be used to provide a rapid, brief spike of NO, as the half-life of this compound is just 2 min. Decomposition of DEA/NO liberates 1.5 molecules of NO per molecule of drug, but may also produce the potent carcinogen N-nitrosodiethylamine as a by-product (Keefer *et al.*, 1996). At the other end of the scale, the half-life of DETA diazeniumdiolate (DETA/NO) has been measured as 20 h, and this compound releases 2 molecules of NO from each molecule of drug. Intermediate to these two is spermine diazeniumdiolate (SPER/NO; Figure 3.1B), the half-life of which is generally believed to be ~ 40 min, although published values range from 10 to 90 min (Keefer *et al.*, 1996). It has been reported to release 1.9 molecules of NO per mole of drug, although this may be an overestimate, as measurements were made at pH 2, which ought to increase the amount of NO generated (Maragos *et al.*, 1991).

Mesoionic 3-aryl substituted oxatriazole-5-imine derivatives such as GEA 3162 and GEA 3175 are another class of NO donors. GEA 3162 (Figure 3.1C) decomposes spontaneously in physiological solution, although reducing agents may contribute to this process *in vivo* (Megson, 2000). Release of NO from GEA 3175 but not GEA 3162 is increased in the presence of human plasma, suggesting that decomposition of this compound may be accelerated by enzymatic degradation, perhaps degradation of the sulphonamide moiety, or by thiols, before it is able to release NO (Kankaanranta *et al.*, 1996). They may be preferred to existing drugs as no tolerance has been observed and O_2^- is not thought to be generated alongside NO (Megson, 2000), although it has been suggested that this group of compounds has the ability to donate $ONOO^-$ (Kankaanranta *et al.*, 1996).

Early investigations using various techniques, including NO-haemoglobin formation, ozone chemiluminescence, nitro blue tetrazolium reduction and nitrotyrosine formation suggested that the species generated from GEA 3162 is free NO and that no O_2^- is liberated alongside NO, therefore this compound was regarded

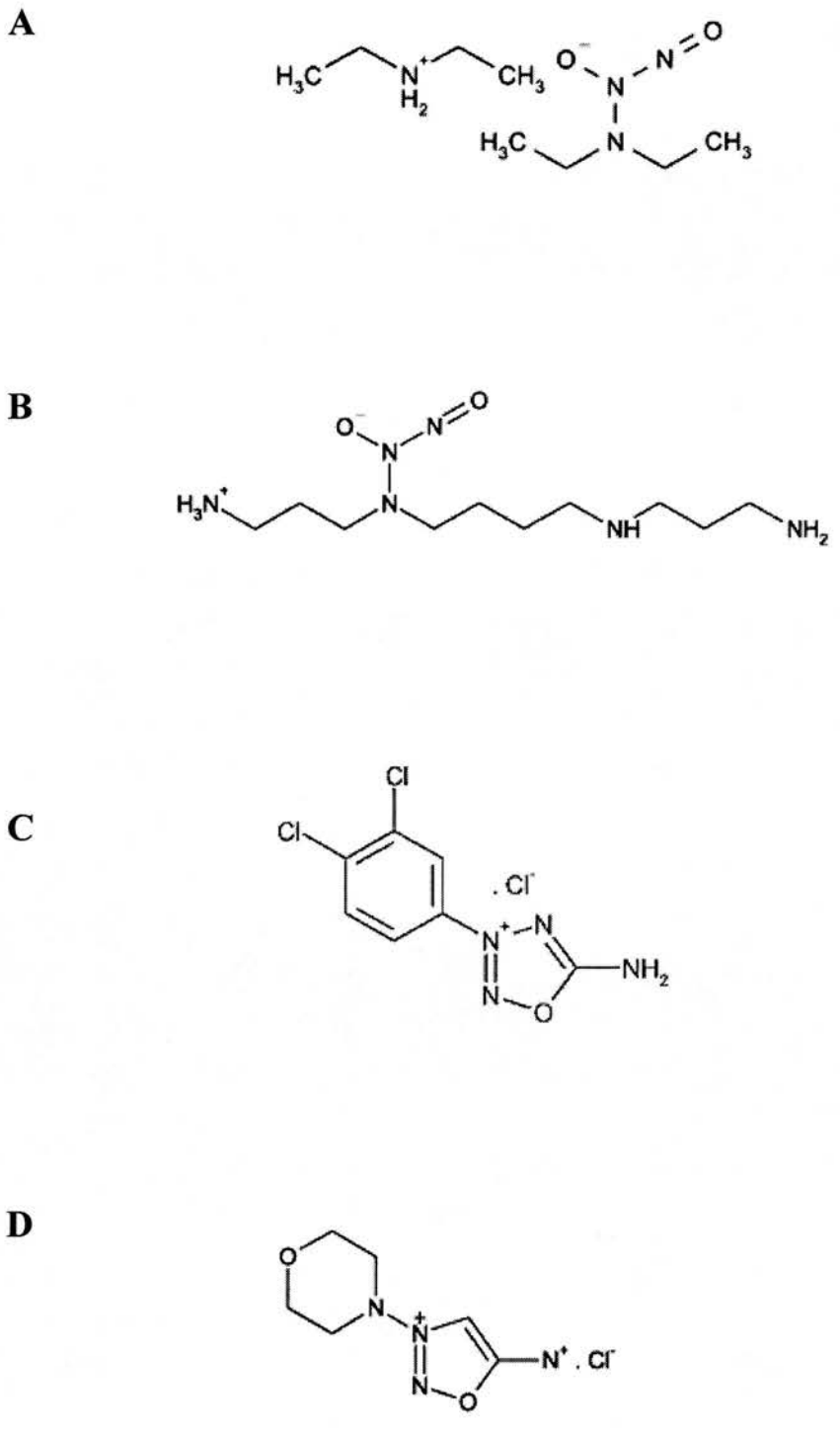


Figure 3.1 Structures of NO and $ONOO^-$ donors. Chemical structures of (A) diethylamine diazeniumdiolate (DEA/NO) (B) spermine diazeniumdiolate (SPER/NO) (C) 1,2,3,4-oxatriazolium, 5-amino-3-(3,4-dichorophenyl)-chloride (GEA 3162) and (D) 3-morpholinosydnonimine (SIN-1)

as a pure NO donor (Kankaanranta *et al.*, 1996; Holm *et al.*, 1998). In contrast, recent work using an NO electrode has demonstrated concomitant release of NO and O_2^- from this compound, making GEA 3162 a $ONOO^-$ donor (Schmidt *et al.*, 2001).

The aim of these investigations was to use an NO electrode and spectrofluorimetric and flow cytometric techniques to characterise the rate and extent of release of NO or $ONOO^-$ from four compounds, DEA/NO, SPER/NO, SIN-1 and GEA 3162 (Figure 3.1), for future biological studies in this thesis. In particular, it was intended to resolve the controversy surrounding GEA 3162 with regard to the NO-related species liberated from this compound.

3.2 Results

3..1 Measurement of release of NO from NO and $ONOO^-$ donors

Different NO-donating compounds have different rates of liberation of NO, which may influence the biological effects of these compounds. In order to characterise the amount and rate of NO generated by NO donors which were to be investigated in apoptosis studies, an NO-specific electrode was used. Following calibration of the electrode using DEA/NO in pH 4 phosphate buffer (Figure 3.2), NO donors were added to pre-warmed PBS buffer into which an electrode was inserted, and left for 20 min or until the level of NO being generated returned to baseline.

The diazeniumdiolate, DEA/NO, is known to be a fast-releasing NO donor that liberates NO spontaneously at physiological temperature and pH. Figure 3.3 shows a typical NO electrode trace obtained with DEA/NO in PBS over 20 min. Addition of this compound to a final concentration of 1 μM caused a rapid and predictable increase in the level of NO within the solution, reaching a maximal concentration of 1.42 ± 0.14 μM after approximately 3 min (Figure 3.4). As expected, levels of NO quickly fell to baseline levels; NO release was undetectable after approximately 15 min.

SPER/NO is another diazeniumdiolate compound, but the nature of the nucleophile (spermine) to which the NO is attached means that NO is released more slowly from this compound than from DEA/NO. A typical NO electrode trace for SPER/NO in PBS over 20 min is shown in Figure 3.3. These studies showed that 1 μM of SPER/NO (which would release ~ 2 μM of NO in total) spontaneously generated a maximum concentration of 0.64 ± 0.15 μM free NO over a 20 min period and that the release of NO was slower than that observed from DEA/NO (Figure 3.4). NO generation from SPER/NO reached a maximum after approximately 20 min, then slowly declined back to baseline until the signal was abolished after approximately 200 min (Figure 3.5).

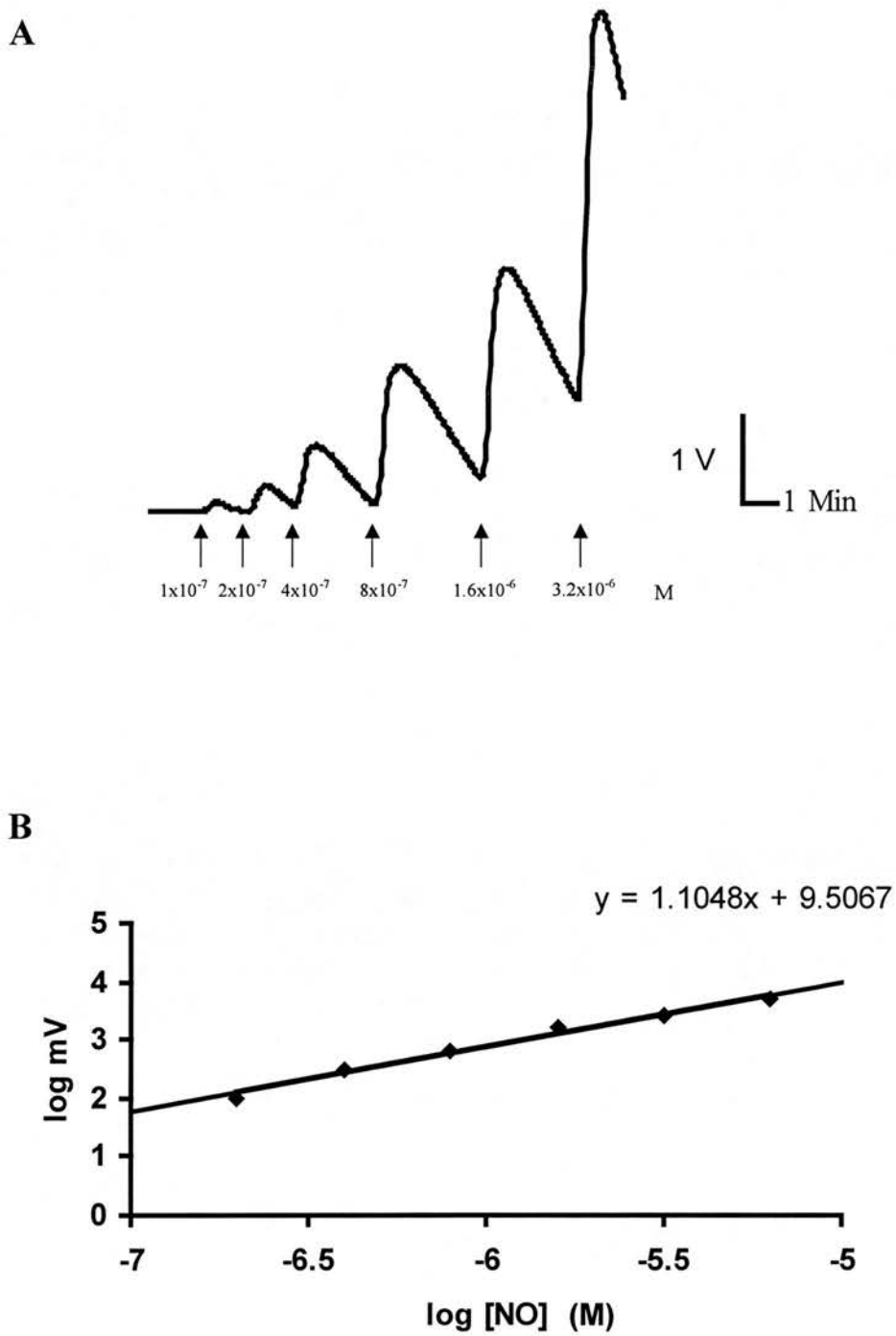


Figure 3.2 Typical NO electrode trace and calibration curve. (A) Diethylamine diazeniumdiolate (DEA/NO; 100 nM – 3.2 μM) was added cumulatively to pH4 buffer into which an NO electrode was immersed, yielding NO in the range 200 nM - 6.4 μM. (B) Resulting NO generation was measured in mV and plotted as a calibration curve.

In contrast to the diazeniumdiolates, neither SIN-1 nor GEA 3162 (100 μM) generated any detectable levels of NO in solution; representative traces are shown in Figure 3.3. Concentrations of NO detected from the four compounds in PBS over a 20 min period are shown in Figure 3.4.

However, in the presence of SOD (50 U/ml), free NO was detectable from both SIN-1 and GEA 3162. Typical electrode traces obtained with 50 U/ml SOD over 20 min are shown in Figure 3.6. NO release from GEA 3162 increased steadily in the presence of SOD over the 20 min period of measurement, and reached a maximum level of 2.71 ± 0.18 μM by 20 min (Figure 3.7). Release of NO from SIN-1 occurred to a lesser extent than that from GEA 3162 in the presence of SOD, with a maximum concentration of 1.41 ± 0.09 μM NO achieved after 20 min (Figure 3.7).

Preincubation with SOD (50 U/ml) had little effect on the amount of NO released from DEA/NO or SPER/NO (Figure 3.7). The profiles of NO release from both DEA/NO and SPER/NO are similar in the absence (Figure 3.3) and presence (Figure 3.6) of SOD. NO from DEA/NO reached a peak concentration of 1.1 ± 0.06 μM NO after just 3-4 min in the presence of SOD, while that from SPER/NO reached 0.88 ± 0.03 μM NO after 20 min. Although the height of the peak obtained with DEA/NO in the presence of SOD was very similar to that seen without SOD (Figure 3.6), it appears that addition of SOD to the buffer may prolong the duration of NO release, as the level of NO does not return fully to baseline (Figure 3.7).

In order to verify that the signals achieved from these compounds in the absence or presence of SOD were authentic NO, the known scavenger of NO, oxyhaemoglobin (Hb; 25 μM) was added to the cuvette. In all cases, the electrode signal was quenched by the addition of Hb, demonstrating that the signal was caused by NO release from these compounds (Figure 3.8).

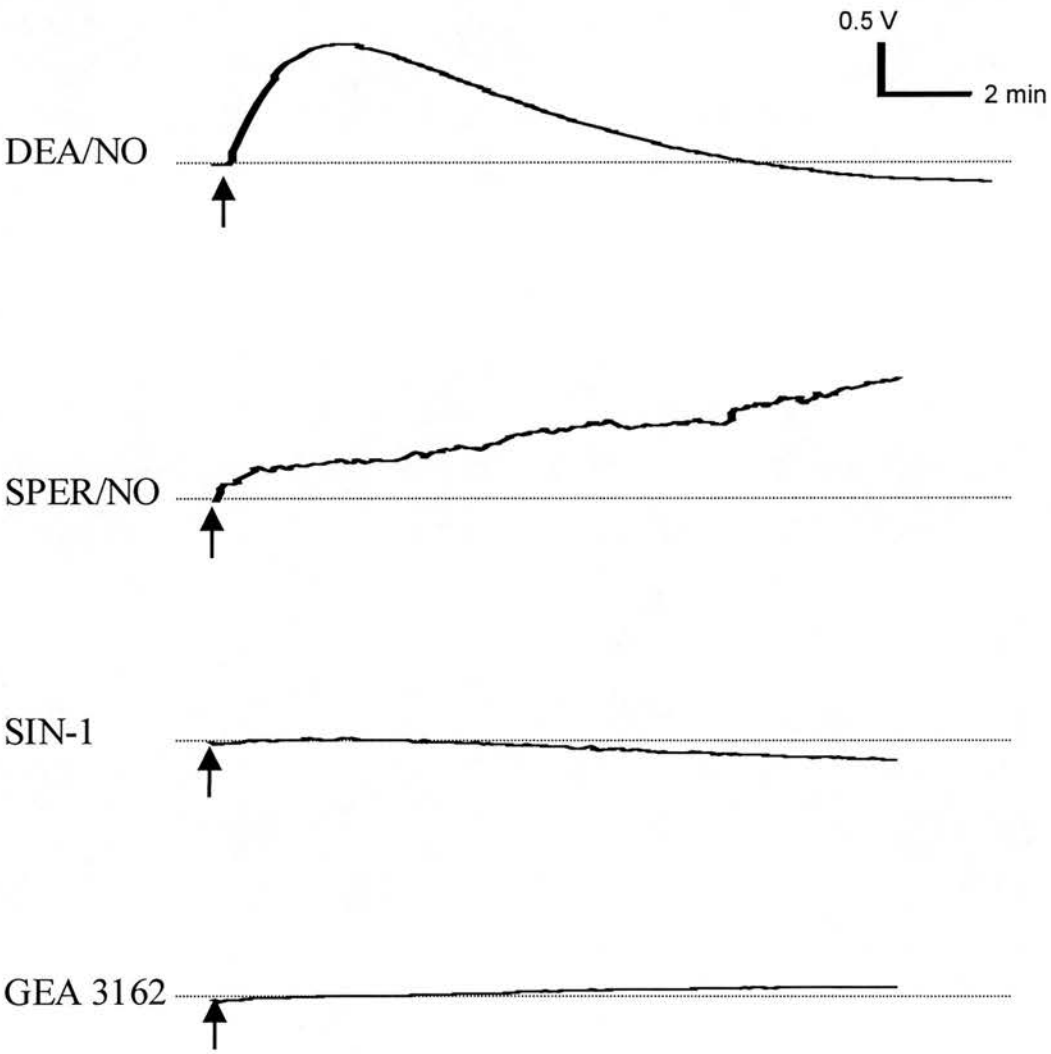


Figure 3.3 Spontaneous release of NO from DEA/NO and SPER/NO, but not GEA 3162 or SIN-1, in PBS. Typical electrode traces for spontaneous NO release from NO and $ONOO^-$ donors are shown. Diethylamine diazeniumdiolate (DEA/NO; 1 μM), spermine diazeniumdiolate (SPER/NO; 1 μM), 1,2,3,4-oxatriazolium, 5-amino-3-(3,4-dichorophenyl)-chloride (GEA 3162; 100 μM) or 3-morpholinosydnonimine (SIN-1; 100 μM) were added to PBS buffer into which an NO electrode was immersed, and NO generation was measured for 20 minutes. Arrows represent addition of NO/$ONOO^-$ donor.

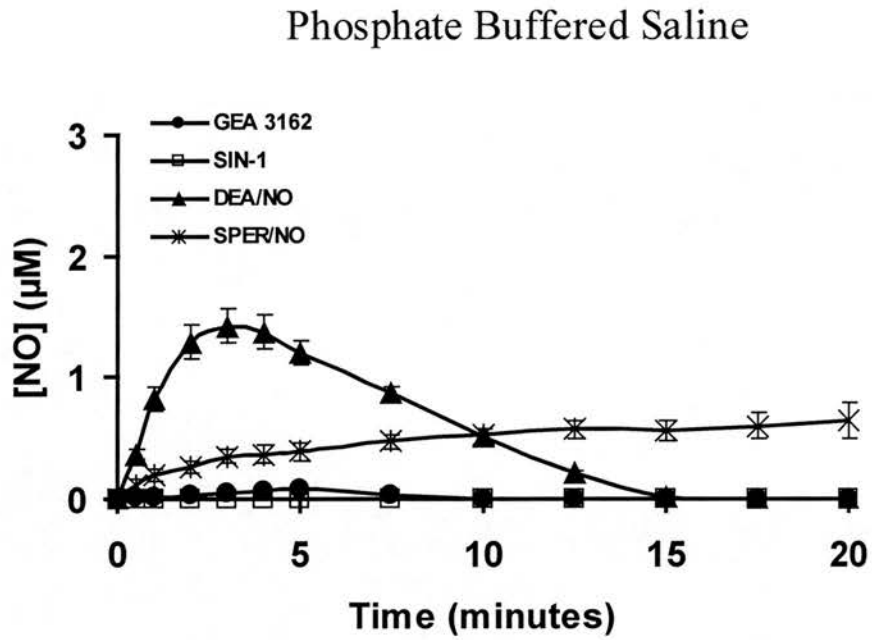


Figure 3.4 Amount and rate of NO released from NO and $ONOO^-$ donors in PBS over a 20-min period. Diethylamine diazeniumdiolate (DEA/NO; 1 μM), spermine diazeniumdiolate (SPER/NO; 1 μM), 1,2,3,4-oxatriazolium, 5-amino-3-(3,4-dichorophenyl)-chloride (GEA 3162; 100 μM) or 3-morpholinosydnonimine (SIN-1; 100 μM) were added to PBS buffer into which an NO electrode was immersed, and the concentration of NO generated was measured for 20 minutes. Results represent mean ± SEM from n = 3 experiments

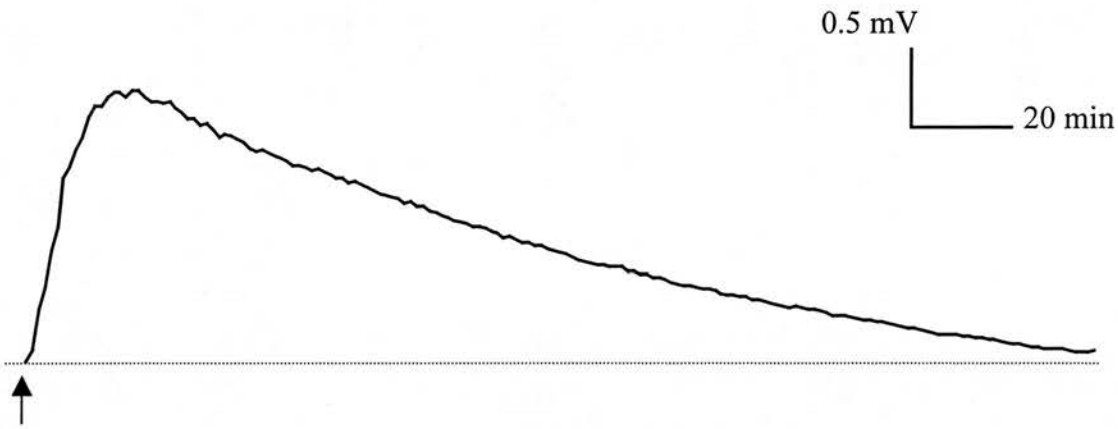


Figure 3.5 Decomposition of SPER/NO in PBS. NO electrode trace showing NO release from SPER/NO (1 μM) over a 215 min period. Spermine diazeniumdiolate (SPER/NO; 1 μM), was added to PBS buffer into which an NO electrode was immersed, and NO generation was measured until NO levels returned to baseline. Arrows represent addition of SPER/NO

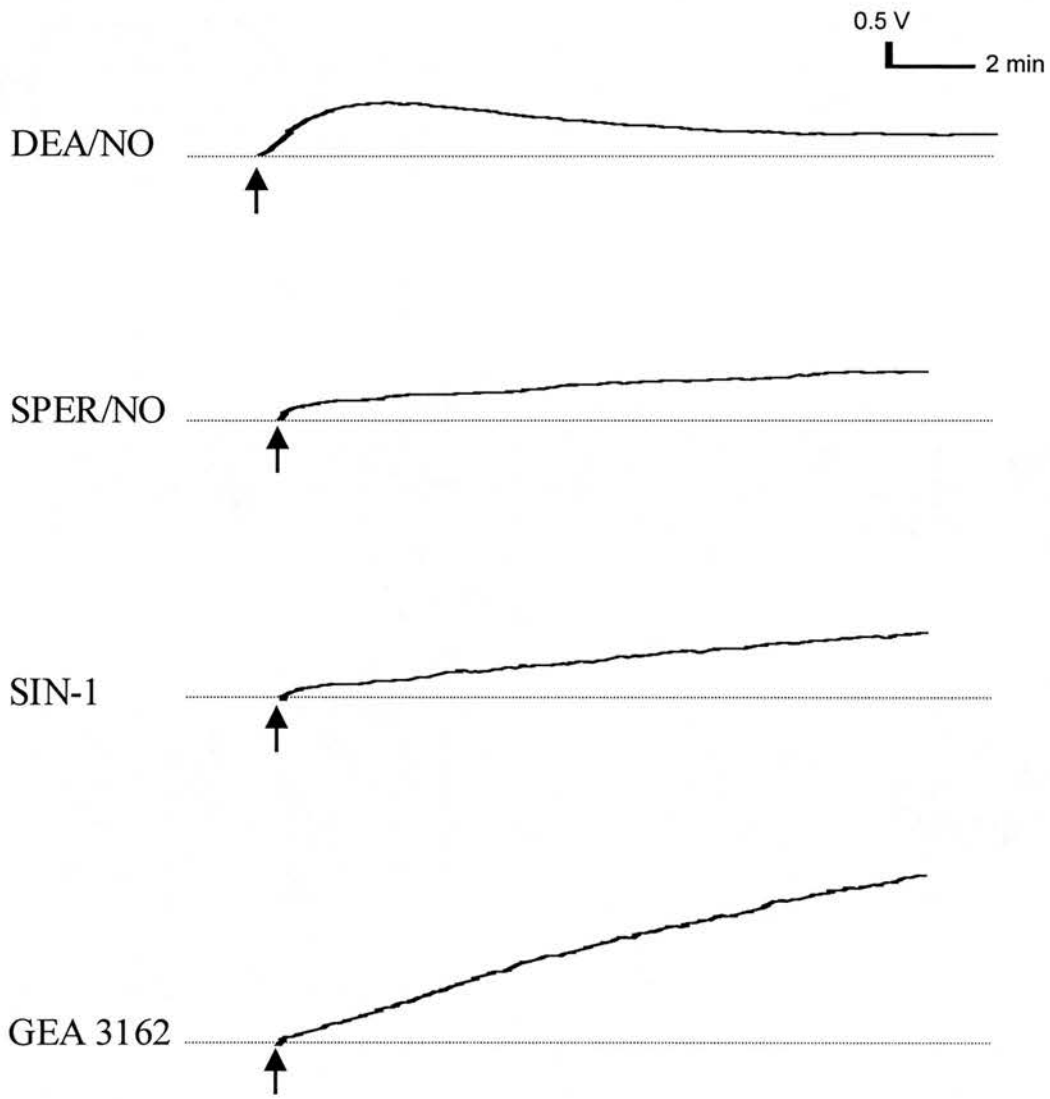


Figure 3.6 Unmasking of free NO from $ONOO^-$ donors by SOD. Typical electrode traces are shown for NO release from NO and $ONOO^-$ donors, in the presence of SOD (50 U/ml). Diethylamine diazeniumdiolate (DEA/NO; 1 μM), spermine diazeniumdiolate (SPER/NO; 1 μM), 1,2,3,4-oxatriazolium, 5-amino-3-(3,4-dichorophenyl)-chloride (GEA 3162; 100 μM) or 3-morpholinosydnonimine (SIN-1; 100 μM) were added to PBS buffer preincubated with SOD (50 U/ml) for 10 min, and NO generation was measured for 20 min. Arrows represent addition of NO/$ONOO^-$ donor.

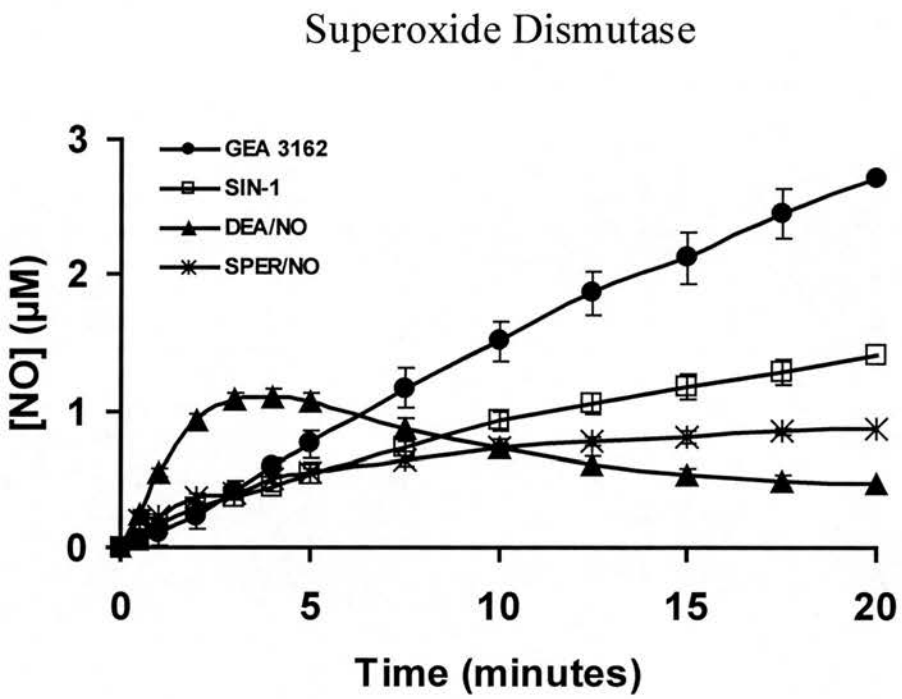


Figure 3.7 Extent and rate of free NO release from NO and $ONOO^-$ donors in the absence and presence of SOD. Diethylamine diazeniumdiolate (DEA/NO; 1 μM), spermine diazeniumdiolate (SPER/NO; 1 μM), 1,2,3,4-oxatriazolium, 5-amino-3-(3,4-dichorophenyl)-chloride (GEA 3162; 100 μM) or 3-morpholinosydnonimine (SIN-1; 100 μM) were added to PBS buffer or PBS buffer preincubated with SOD (50 U/ml) for 10 min, and NO generation was measured for 20 min. Results represent mean ± SEM from n = 3 experiments

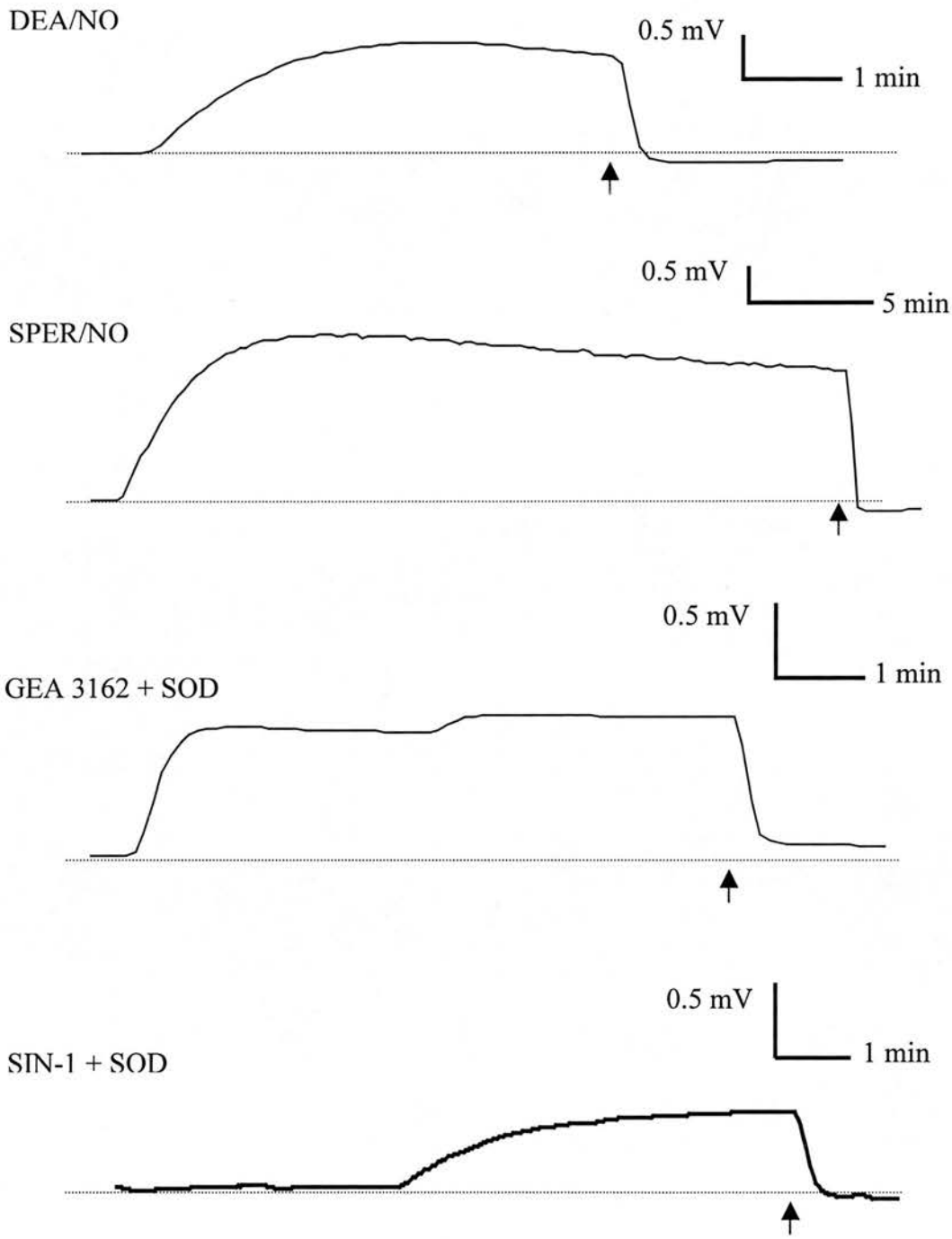


Figure 3.8 Quenching of NO signal by oxyhaemoglobin. Diethylamine diazeniumdiolate (DEA/NO; 1 μM) or spermine diazeniumdiolate (SPER/NO; 1 μM) were added to PBS buffer, and 1,2,3,4-oxatriazolium, 5-amino-3-(3,4-dichorophenyl)-chloride (GEA 3162; 100 μM) or 3-morpholinosydnonimine (SIN-1; 100 μM) were added to PBS buffer preincubated with 50 U/ml SOD. NO generation was measured, then oxyhaemoglobin (Hb, 25 μM) was added to the cuvette to demonstrate that the signal generated was NO (indicated by arrows).

3.2 Spectrofluorimetric detection of reactive nitrogen species liberated by NO/$ONOO^-$ donors

Dihydrorhodamine 123 (DHR 123) is a dye that can be oxidised to fluorescent rhodamine 123 by a number of reactive oxygen and nitrogen species, including O_2^-, $ONOO^-$, hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). However, free NO is unable to cause such oxidation. DHR 123 was therefore used as a tool to discriminate between compounds that liberate pure NO and those that generate $ONOO^-$. NO/$ONOO^-$ donors were incubated with DHR 123 for 1 h at 37°C then fluorescence was measured using a spectrofluorimeter.

These studies showed that fluorescence was low (22.8 ± 4.7 units) in control samples, which comprised DHR 123 in PBS (Figure 3.9). In contrast, in the presence of either 1 mM SIN-1 or 100 μM GEA 3162, fluorescence was high (803.2 ± 73.8 units and 756.7 ± 100.0 units respectively; $p < 0.05$, one-way ANOVA with Dunnett's post-hoc test), indicating a high level of conversion of DHR 123 to rhodamine 123 by oxidative species distinct from NO.

However, in the presence of the pure NO donor, DEA/NO (1 mM), fluorescence was slightly increased from control (52.5 ± 8.6 units), but this did not reach statistical significance ($p > 0.05$, one-way ANOVA with Dunnett's post-hoc test). None of the NO donors exhibited any autofluorescence in the absence of DHR 123 (all < 1 unit of fluorescence), thereby excluding autofluorescence as a possibility for the differences seen with these compounds.

3.2.3 *Flow cytometric detection of reactive nitrogen species liberated by NO/$ONOO^-$ donors*

The DHR 123 experiments were repeated in the presence of neutrophils (5 x 10^6 cells/ml) in order to determine whether a pure NO donor such as DEA/NO can form $ONOO^-$ through combination of drug-derived NO with neutrophil-derived O_2^-, through which its biological effects may be mediated.

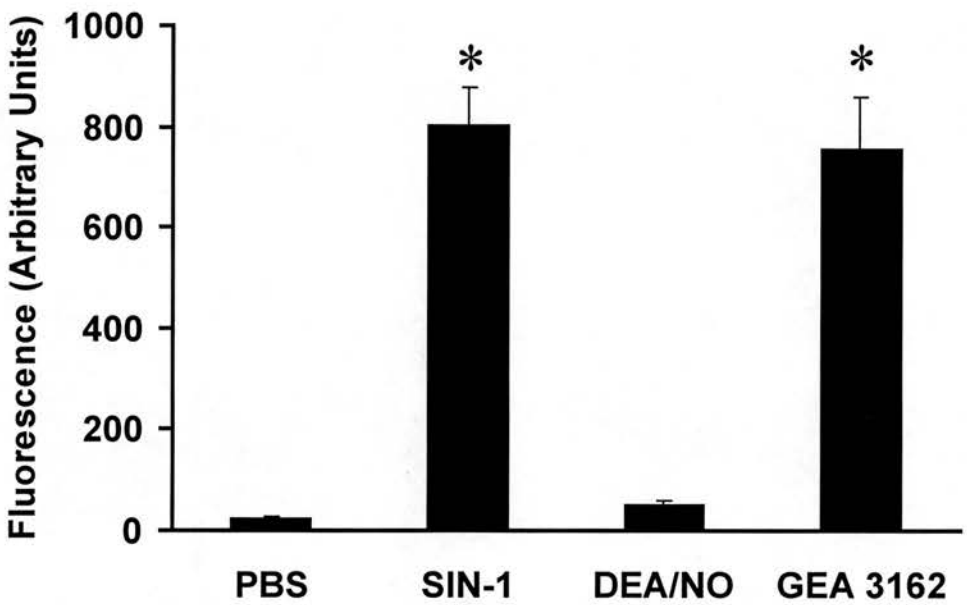


Figure 3.9 Spectrofluorimetric detection of the generation of oxidative species from NO or $ONOO^-$ donors. Diethylamine diazeniumdiolate (DEA/NO; 1 mM), 1,2,3,4-oxatriazolium, 5-amino-3-(3,4-dichorophenyl)-chloride (GEA 3162; 100 μM) or 3-morpholinosydnonimine (SIN-1; 1 mM) were incubated with DHR 123 for 1 hour before analysis of fluorescence levels using a spectrofluorimeter. Results represent mean ± SEM from n = 3 experiments and asterisks represent significant ($p < 0.05$, one-way ANOVA with Dunnett's post-hoc test) difference from control (PBS).

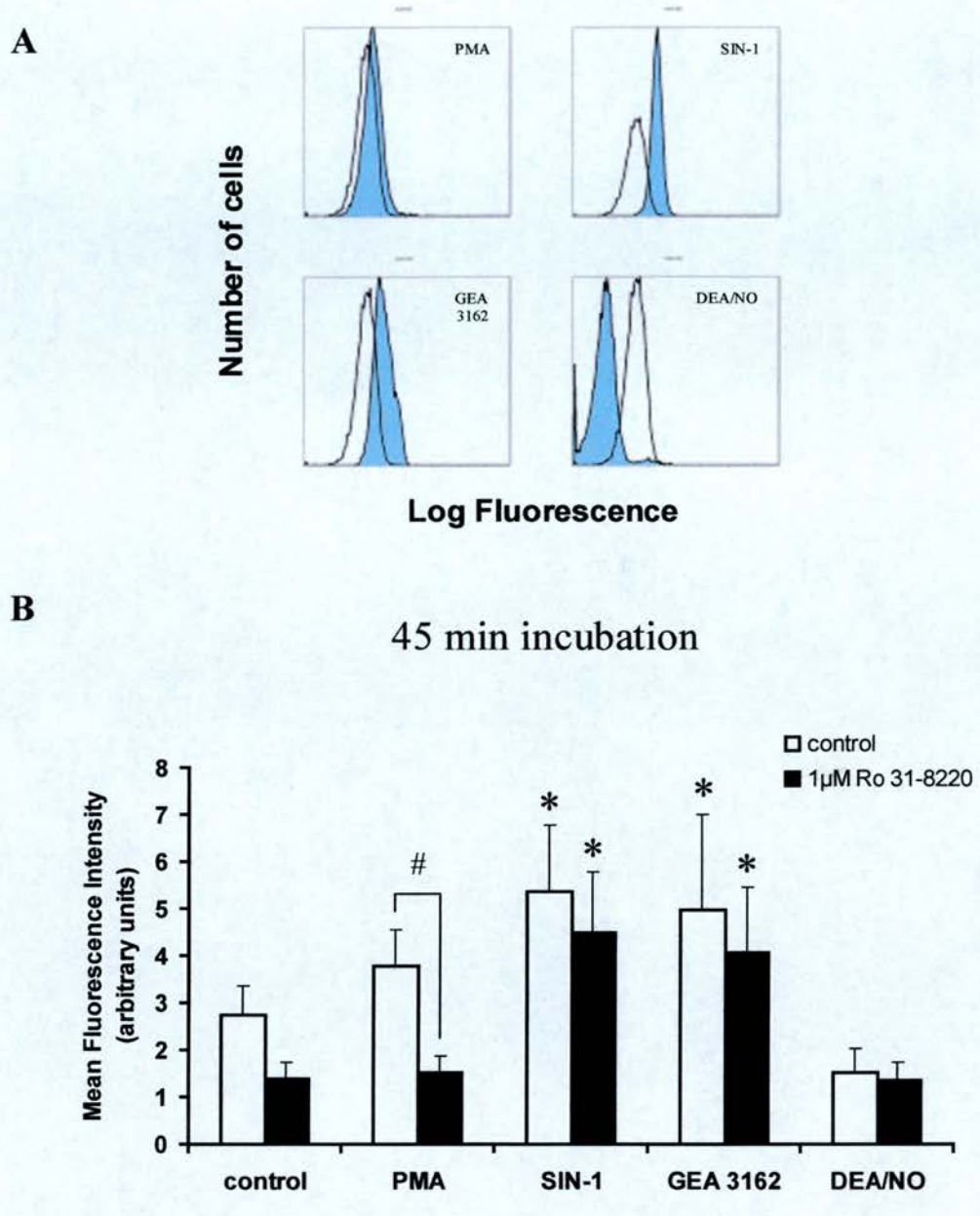


Figure 3.10 Oxidation of DHR 123 to rhodamine 123 by NO and ONOO⁻ donors in the presence of neutrophils, and contribution of cell-derived oxidants. PBS, the neutrophil activating agent PMA (10 nM), 3-morpholinosydnonimine (SIN-1; 100 μM), 1,2,3,4-oxatriazolium, 5-amino-3-(3,4-dichorophenyl)-chloride (GEA 3162; 100 μM) or diethylamine diazeniumdiolate (DEA/NO; 1 mM), were incubated with neutrophils in the presence of DHR 123 (1 μM) for 45 min before analysis of mean fluorescence levels using a flow cytometer. (A) Typical flow cytometry profiles showing fluorescence in treated cells (blue peaks) vs. control cells (transparent peaks) after 45 min (B) Graph of fluorescence in untreated and treated cells and effects of the PKC inhibitor, Ro 31-8220 (1 μM). Results represent mean ± SEM from n = 6 experiments. Analysis by two-way ANOVA with Bonferroni post-hoc test showed a significant ($p < 0.05$) difference between groups, and asterisks represent significant ($p < 0.05$) difference from control without Ro 31-8220. Hashes represent significant ($p < 0.05$) difference between fluorescence observed in the absence or presence of Ro 31-8220 for a given condition.

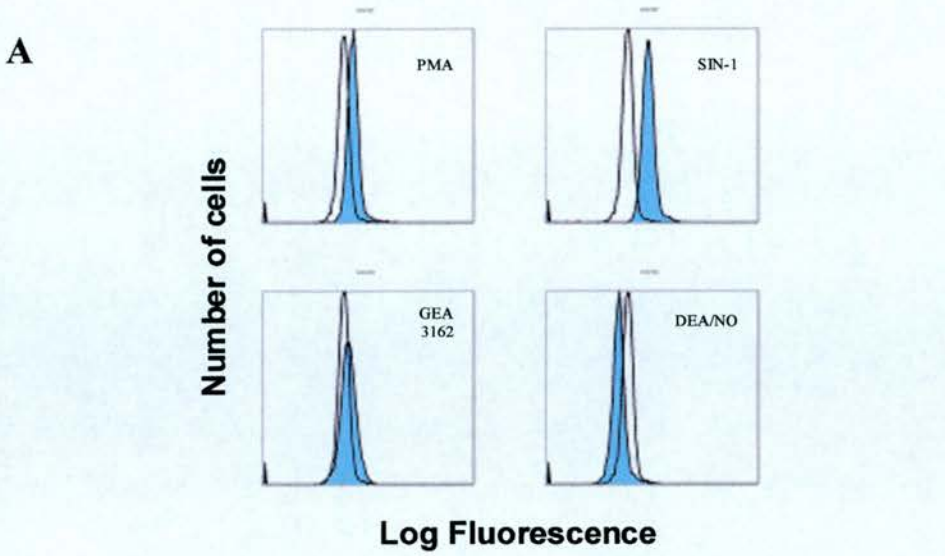


Figure 3.11 Oxidation of DHR 123 to rhodamine 123 by NO and ONOO⁻ donors in the presence of neutrophils, and contribution of cell-derived oxidants. PBS, the neutrophil activating agent PMA (10 nM), 3-morpholinosydnonimine (SIN-1; 100 μM), 1,2,3,4-oxatriazolium, 5-amino-3-(3,4-dichorophenyl)-chloride (GEA 3162; 100 μM) or diethylamine diazeniumdiolate (DEA/NO; 1 mM), were incubated with neutrophils in the presence of DHR 123 (1 μM) for 120 min before analysis of mean fluorescence levels using a flow cytometer. (A) Typical flow cytometry profiles showing fluorescence in treated cells (blue peaks) vs. control cells (transparent peaks) after 120 min (B) Graph of fluorescence in untreated and treated cells and effects of the PKC inhibitor, Ro 31-8220 (1 μM). Results represent mean ± SEM from $n = 6$ experiments. Analysis by two-way ANOVA with Bonferroni post-hoc test showed a significant ($p < 0.05$) difference between groups, and asterisks represent significant ($p < 0.05$) difference from control without Ro 31-8220. Hashes represent significant ($p < 0.05$) difference between fluorescence observed in the absence or presence of Ro 31-8220 for a given condition.

Neutrophils (5 x 10^6 cells/ml) were incubated in the presence of DHR and NO/$ONOO^-$ donors for 45 min before assessment by flow cytometry, in the absence and presence of the protein kinase C (PKC) inhibitor, Ro 31-8220 (1 μM). Generation of ROS from neutrophils is PKC-dependent, therefore use of Ro 31-8220 eliminates any fluorescence caused by cell-derived ROS, and oxidation of DHR 123 can be mediated only by drug-derived oxidant species. In this case, it would be expected that if SIN-1 and GEA 3162 are both $ONOO^-$ generators, the presence of Ro 31-8220 would have little effect on the amount of fluorescence seen in neutrophils treated with these compounds, only abolishing the oxidative burst-derived fluorescence. On the other hand, if fluorescence observed with DEA/NO represents $ONOO^-$ formed as a result of combination of NO from DEA/NO and neutrophil-generated O_2^-, fluorescence should be drastically reduced in the presence of Ro 31-8220.

Representative flow cytometry histograms for a 45-min incubation period with DHR 123 in the presence of NO/$ONOO^-$ donors are shown in Figure 3.10A. Analysis of the mean fluorescence generated showed a significant ($p < 0.05$) difference between groups, therefore a further analysis by Bonferroni post-hoc test was carried out to reveal which groups differed from control (Figure 3.10B).

Fluorescence from control (PBS-treated) cells was reduced by the presence of Ro 31-8220 (1.4 ± 0.4 units vs 2.7 ± 0.6 units control) after 45 min, and this effect was more pronounced in cells treated with the powerful PKC activator, PMA (1.5 ± 0.4 vs 3.8 ± 0.8 control units, $p < 0.05$, 2-way ANOVA with Bonferroni post-hoc test). Fluorescence was observed to be significantly different ($p < 0.05$) from control in cells treated with either SIN-1 (5.4 ± 1.4 vs. 2.7 ± 0.6 log units) or GEA 3162 (5.0 ± 2.0 vs. 2.7 ± 0.6 units). However, in contrast to control and PMA-treated cells, fluorescence was not attenuated by Ro 31-8220; with SIN-1 in the presence of Ro 31-8220, fluorescence was 4.5 ± 1.3 units, and with GEA 3162 plus Ro 31-8220, it was 4.1 ± 1.4 units ($p < 0.05$ from control, two-way ANOVA with Bonferroni post-hoc test).

In DEA/NO-treated cells, fluorescence was not increased from control; indeed it was slightly decreased in the presence of DEA/NO (1.5 ± 0.5 units vs 2.7 ± 0.6 units control; $p > 0.05$ from control, two-way ANOVA with Bonferroni post-hoc test). Furthermore, the presence of Ro 31-8220 had no effect on the level of fluorescence in DEA/NO-treated cells (1.4 ± 0.4 vs 1.5 ± 0.5 units; $p > 0.05$ from control, two-way ANOVA with Bonferroni post-hoc test).

Typical flow cytometry traces obtained following 2 h incubation are shown in Figure 3.11A. Analysis of the data by two-way ANOVA showed a significant difference ($p < 0.05$) between treatments, therefore a Bonferroni post-hoc test was carried out to determine where these differences occurred. A significant increase in fluorescence was seen with PMA (9.4 ± 3.1 units) and SIN-1 (10.7 ± 2.9 units) compared to control (5.0 ± 1.2 units; $p < 0.05$, two-way ANOVA with Bonferroni post-hoc test).

The trends of the results followed much the same pattern as for 45 min (Figure 3.11B), with fluorescence in control (2.1 ± 0.3 vs 5.0 ± 1.2 units) and PMA-treated (2.1 ± 0.4 vs 9.4 ± 3.1 units, $p < 0.05$, two-way ANOVA with Bonferroni post-hoc test) cells being abrogated by Ro 31-8220 ($p > 0.05$ vs. control, two-way ANOVA with Bonferroni post-hoc test), but not that seen in the presence of SIN-1 (8.9 ± 2.2 vs 10.7 ± 2.9 units, $p < 0.05$ vs. control, two-way ANOVA with Bonferroni post-hoc test). GEA 3162-treated cells showed no significant increase in the level of fluorescence compared to control (5.0 ± 1.0 vs.5.0 ± 1.2 units; $p > 0.05$, two-way ANOVA with Bonferroni post-hoc test). However, in contrast to control cells, the presence of Ro 31-8220 did not decrease the fluorescence observed in these cells (4.5 ± 0.9 vs 5.0 ± 1.0 units).

3.2.4 Effect of whole serum and human serum albumin on oxidation of DHR 123 by NO/$ONOO^-$ donors in human neutrophils

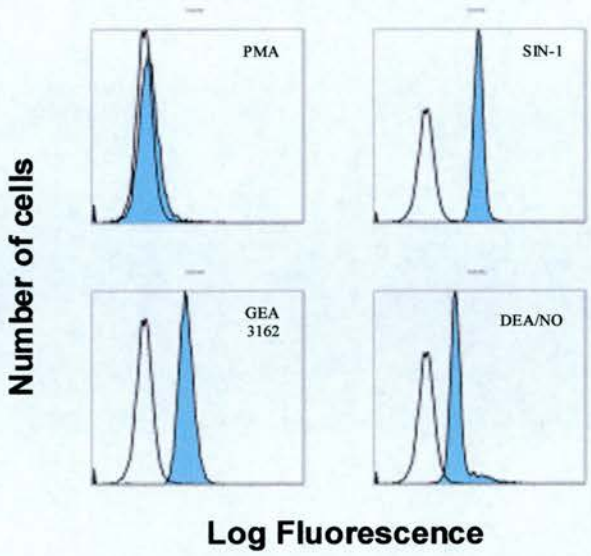
Different levels of DHR 123 oxidation were seen with neutrophils incubated

in the presence of autologous serum compared to those seen in the absence of serum in the same set of cells in concurrently-run experiments; typical flow cytometry histograms are shown in Figure 3.12A. Two-way ANOVA showed no significant differences between treatment groups ($p > 0.05$) in the presence of serum.

Preliminary studies showed that serum reduced the extent of fluorescence achieved, with control fluorescence of 1.9 ± 0.5 units after 2 h compared to 5.0 ± 1.2 units achieved in the absence of serum, and Ro 31-8220 failed to reduce the fluorescence (1.7 ± 0.3 vs 1.9 ± 0.5 units). PMA again increased fluorescence (8.6 ± 6.6 vs 1.9 ± 0.5 units), although not to the same extent as in medium alone, and this did not reach statistical significance due to the high variability of fluorescence achieved between experiments. This appears to be due to variability between donors of the responsiveness to PMA in the presence of serum, as increased fluorescence in response to PMA was observed in some replicates of the experiment but not others.

Again, increased fluorescence in the presence of 1 mM SIN-1 or 100 μM GEA 3162 could not be reduced back to control levels by Ro 31-8220. Fluorescence generated by SIN-1 was 6.6 ± 2.4 units in the presence of Ro 31-8220 compared to 6.5 ± 2.0 units with SIN-1 alone, and GEA 3162 gave a fluorescence of 6.4 ± 1.3 units with Ro 31-8220 vs. 7.4 ± 1.6 units without the PKC inhibitor.

In contrast, results obtained with 1 mM DEA/NO conflicted with those seen in the absence of serum for experiments carried out on the same set of cells on the same day. In the presence of serum, fluorescence obtained with DEA/NO was increased compared to control (5.4 ± 0.5 vs 1.9 ± 0.5 units), and was brought back down towards control levels (3.1 ± 0.4 units) by Ro 31-8220. Although two-way ANOVA showed no significant differences between groups, two-tailed paired *t* test of fluorescence obtained with DEA/NO versus that seen in control cells showed a significant increase in fluorescence by DEA/NO ($p < 0.05$). This is in contrast with data obtained in the absence of serum at 2 h, in which no significant difference was detectable between the two groups ($p > 0.05$, two-tailed paired *t* test). Although two-way ANOVA showed no significant differences in these preliminary studies, there



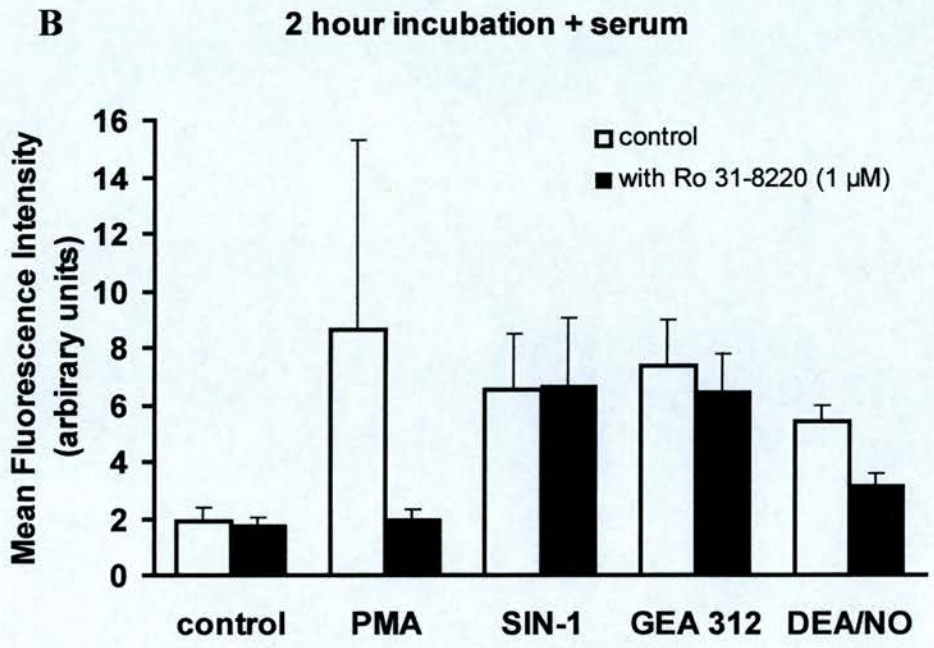


Figure 3.12 Effect of autologous serum on oxidation of DHR 123 to rhodamine 123 by NO and ONOO⁻ donors in the presence of neutrophils, and contribution of cell-derived oxidants. PBS, the neutrophil activating agent PMA (10 nM), 3-morpholinosydnonimine (SIN-1; 1 mM), 1,2,3,4-oxatriazolium, 5-amino-3-(3,4-dichorophenyl)-chloride (GEA 3162; 100 μM) or diethylamine diazeniumdiolate (DEA/NO; 1 mM), were incubated with neutrophils in 10% autologous serum in the presence of DHR 123 (1 μM) for 120 min before analysis of mean fluorescence levels using a flow cytometer. (A) Typical flow cytometry profiles showing fluorescence in treated cells (blue peaks) vs. control cells (transparent peaks) after 120 min (B) Graph of fluorescence in untreated and treated cells and effects of the PKC inhibitor, Ro 31-8220 (1 μM). Results represent mean ± SEM from n = 3 experiments. Data did not reach statistical significance.

was a clear trend towards increased fluorescence with DEA/NO in the presence of serum, and further replications would be expected to show a significant difference.

In order to further investigate the differential effects obtained with DEA/NO in the absence and presence of serum, studies were carried out to determine the role of DEA/NO-mediated modification of plasma albumin. Neutrophils (4.5×10^6 cells/ml) were incubated for 2 h in the absence and presence of serum (10%) and in the absence and presence of PMA (10 nM) and DEA/NO (1 mM). Neutrophils were also incubated in culture medium supplemented with human serum albumin (HSA; 0.4 % w/v) in the absence and presence of PMA or DEA/NO.

These studies also showed suppression of DHR 123 oxidation by the presence of serum in control cells (1.8 ± 0.7 units vs 2.4 ± 0.6 units in the absence of serum), although this failed to reach statistical significance, and the attenuation of oxidative burst stimulated by PMA (2.6 ± 0.8 units vs 4.2 ± 0.8 units in the absence of serum; $p < 0.05$, one-way ANOVA with Student-Newman-Keuls post-hoc test). Furthermore, the reduction in fluorescence from control by DEA/NO in culture medium alone, but increased fluorescence in the presence of serum was also observed. However, these results did not reach statistical significance, although a clear trend was observed in each replication of the experiment. DEA/NO-induced fluorescence was 1.6 ± 0.5 units vs 2.4 ± 0.6 units control in medium alone, but was 2.5 ± 1.0 vs 1.8 ± 0.7 units control with serum.

Supplementation of culture medium with albumin, a major serum protein, mimicked the effects of whole human serum, producing an inhibition (albeit not statistically significant) of spontaneous ROS generation (1.8 ± 1.0 units vs 2.4 ± 0.6 units in the absence of HSA; Figure 3.13B). In the presence of both DEA/NO and HSA, fluorescence increased to higher than control levels; 3.3 ± 0.8 units fluorescence was seen with DEA/NO and HSA compared to 1.8 ± 1.0 units in HSA-supplemented medium alone ($p < 0.05$, one-way ANOVA with Student-Newman-Keuls post-hoc test). This increased fluorescence observed with DEA/NO was also significantly different from that seen with DEA/NO in IMDM in the absence of HSA

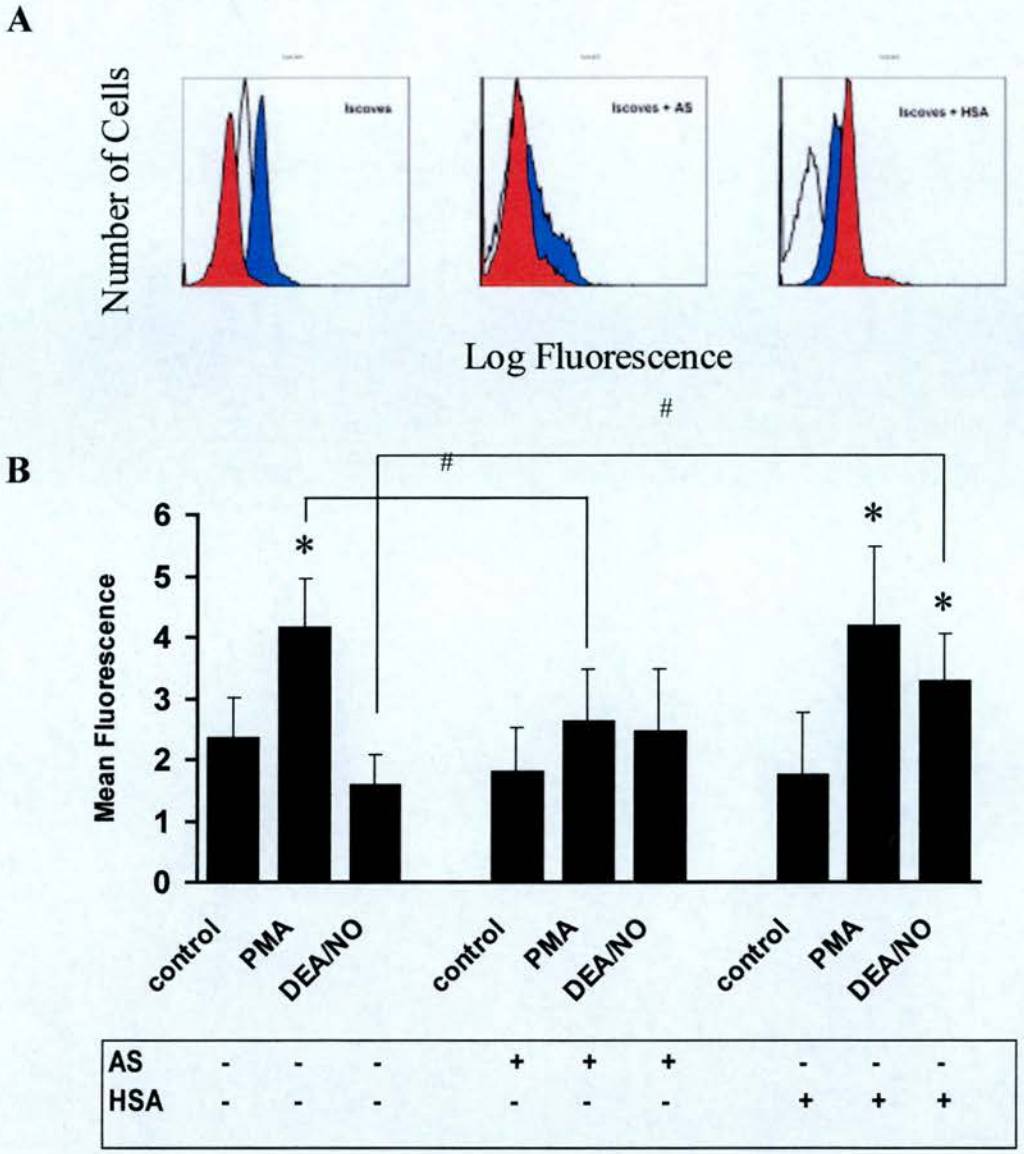


Figure 3.13 Contribution of human serum albumin (HSA) to autologous serum (AS)-mediated inhibition of ROS generation by neutrophils and reversal of inhibition by DEA/NO. PBS, the neutrophil activating agent PMA (10 nM) or diethylamine diazeniumdiolate (DEA/NO; 1 mM), were incubated with neutrophils in Iscove's MDM (IMDM) alone, or supplemented with 10 % AS or 0.4 % HSA in the presence of DHR 123 (1 μM) for 120 min before analysis of mean fluorescence levels using a flow cytometer. (A) Typical flow cytometry profiles showing fluorescence in PMA-treated cells (blue peaks) and DEA/NO-treated cells (red peaks) vs. control cells (white peaks) after 120 min (B) Graph of fluorescence in untreated and treated cells in IMDM, IMDM + 10% AS or IMDM + 0.4% HSA. Results represent mean ± SEM from n = 3 experiments. Asterisks represent significant ($p < 0.05$) difference from control (untreated) cells under the same culture conditions and hashes represent differences between the same treatment under different conditions by one-way ANOVA with Student-Newman-Keuls post-hoc test.

(3.3 ± 0.8 vs. 1.6 ± 0.5 units, $p < 0.05$, one-way ANOVA with Student-Newman-Keuls post-hoc test). Furthermore, the presence of HSA caused PMA-induced fluorescence to be significantly higher than that seen in control ($p < 0.05$, one- way ANOVA with Student-Newman-Keuls post-hoc test).

3.2 Discussion

Preliminary characterisation of the extent and rate of liberation of NO from various compounds is very important, as it has been shown that the rate of NO release can have profound implications for the biological effects of NO donors or their mechanisms of action. For example, high-flux diazeniumdiolates such as MAMA/NO inhibit cholesterol peroxidation through a chain prevention mechanism, whereas slower-releasing compounds such as PAPA/NO and SPER/NO act at the level of downstream radicals (Korytowski *et al.*, 2000). Furthermore, it has been shown that the rate of NO release from diazeniumdiolates influences the efficacy of inhibition of vascular smooth muscle cell proliferation; diazeniumdiolate compounds with short half lives (DEA/NO and PAPA/NO) were unable to inhibit proliferation, whereas longer-lasting diazeniumdiolate compounds (SPER/NO, DPTA/NO and DETA/NO) were effective, with efficacy of inhibition being positively correlated with the half life of NO release (Mooradian *et al.*, 1995). Furthermore, the extent of inhibition of platelet aggregation by diazeniumdiolates also correlated with the amount of NO delivered (Raulli, 1998) and induction of apoptosis in neuronal PC12 cells was only observed with NO donors of intermediate duration (NOR2 and NOR3) but not those of short (NOR1) or long (NOR4) duration (Yamamoto *et al.*, 2000).

Studies using an NO electrode showed that free NO was liberated spontaneously from the diazeniumdiolate compounds (1 μM). Thus, with DEA/NO at this concentration, all NO is released from this compound after 15 min, whereas SPER/NO takes approximately 200 min to fully liberate its attached NO. The maximal concentration of NO achieved with 1 μM DEA/NO was 1.42 ± 0.14 μM, whereas only 0.64 ± 0.15 μM was achievable from 1 μM SPER/NO over 20 min,

indicating that local concentrations of NO from DEA/NO in future apoptosis studies are likely to be higher than those using SPER/NO. These compounds can therefore be used to distinguish between effects of a short burst of high concentrations of NO (from DEA/NO) and more sustained release of lower concentrations of NO (from SPER/NO).

It is also crucial to know the nature of the NO-related species derived from so-called 'NO donors' as NO-related species may exert differential biological effects or similar effects to NO that are mediated through different mechanisms. For example, both NO and $ONOO^-$ from SIN-1 cause vasodilation in isolated blood vessels through elevation of cGMP, although $ONOO^-$ itself may cause vasodilation via a cGMP-independent mechanism (Trakranrungsie and Will, 2001). Furthermore, both NO and $ONOO^-$ decrease LTB_4 release from neutrophils via cGMP (Ney *et al.*, 1990). However, it has been reported that NO inhibits the oxidative burst in neutrophils, especially at low oxygen tensions (Iha *et al.*, 1996), but that $ONOO^-$ enhances ROS generation in response to neutrophil activating agents such as PMA (Iha *et al.*, 1996; Bednar *et al.*, 1996). Although both species inhibit respiration, they do so through different mechanisms (Lizasoain *et al.*, 1996).

In contrast to diazeniumdiolates, liberation of free NO from either SIN-1 or GEA 3162 was not detected by the NO electrode. Absence of an NO signal from these compounds was due to scavenging of free NO by O_2^- produced alongside NO (forming $ONOO^-$) as free NO could be unmasked by the presence of SOD, which converts O_2^- to H_2O_2 and therefore protects free NO from scavenging. These studies were carried out with only one concentration of SOD (50 U/ml). However, further studies by our group showed that for both GEA 3162 and SIN-1, the amount of NO unmasked followed a non-linear relationship with the concentration of SOD, with the NO concentration detected levelling off with higher concentrations of SOD. This suggests that the capacity for SOD to protect NO from O_2^- scavenging becomes saturated.

The reaction kinetics for the dismutation of O_2^- by SOD is considerably slower than the reaction of NO with O_2^- for a 1:1 ratio of NO and O_2^- generation

(Huie and Padmaja, 1993). Such kinetics suggest that, although a certain proportion of $ONOO^-$ generation is blocked by the presence of SOD, this scavenging would be incomplete and some $ONOO^-$ could still be generated by $ONOO^-$ donors, therefore the NO levels detectable using the NO electrode would not necessarily represent the same concentrations of $ONOO^-$ achievable with these compounds. GEA 3162 and SIN-1 are therefore both $ONOO^-$ generators rather than donors of pure NO. Although this has long been known for SIN-1, it is a novel finding for GEA 3162. However, given the striking structural similarity between these two compounds (Fig 3.1) along with previous reports of the potential for GEA 3162 to generate $ONOO^-$ (Kankaanranta *et al.*, 1996), it is not a surprising finding. The findings from these studies agree with those previously published for SIN-1 regarding the detection of free NO following O_2^- dismutation by SOD (Lizasoain *et al.*, 1996). However, they are at odds with published reports on the nature of the species generated by GEA 3162.

Previous studies using a number of different techniques but, importantly, not NO electrode studies have found that GEA 3162 generated NO in the absence of O_2^- release, making GEA 3162 a pure NO donor rather than a $ONOO^-$ donor (Kankaanranta *et al.*, 1996; Holm *et al.*, 1998). However, more recent evidence has suggested that GEA 3162 may indeed co-generate NO and O_2^- (Schmidt *et al.*, 2001).

In order to clarify this issue, these two compounds, DEA/NO or PBS were incubated in the presence of DHR 123 to distinguish between those that liberate pure NO and those that generate $ONOO^-$. Such studies are founded on the premise that DHR 123 can be oxidised to fluorescent rhodamine 123 by certain reactive oxygen species, but not by pure NO, therefore fluorescence should only be increased from control in the presence of $ONOO^-$ generating compounds and not in the presence of compounds that liberate pure NO. These studies concurred with the NO electrode findings, as both SIN-1 and GEA 3162 increased fluorescence, but no significant increase in fluorescence was obtained with the known pure NO generator, DEA/NO.

However, it is still conceivable that biological actions of DEA/NO in neutrophils are mediated through the generation of $ONOO^-$. DEA/NO is known to

liberate free NO in aqueous solution, and activated neutrophils are known to produce O_2^- as part of the oxidative burst following activation of these cells. Therefore, the potential exists for these species to combine to generate $ONOO^-$. In order to test this possibility, the DHR 123 experiments were also carried out in the presence of neutrophils, to determine if exposure of neutrophils to DEA/NO results in $ONOO^-$ being generated from combination of drug-derived NO and cell-derived O_2^-. This was done using the protein kinase C inhibitor, Ro 31-8220 (Dieter and Fitzke, 1991), to inhibit endogenously produced oxidants, so any remaining fluorescence observed in the presence of Ro 31-8220 must be oxidants generated from the drugs themselves. Using this method, it should be able to determine what component of DHR 123 oxidation in the presence of NO or $ONOO^-$ donors results from the oxidative burst from neutrophils. In this case, compounds that liberate $ONOO^-$ would generate a high fluorescence which would not be significantly reduced by inhibition of the neutrophil oxidative burst. In contrast, pure NO donors would only generate high fluorescence if the NO reacts with neutrophil-generated O_2^- to form $ONOO^-$, and this would be inhibitable by Ro 31-8220.

In the absence of serum, results correlated with those obtained using the NO electrode. GEA 3162 and SIN-1 were $ONOO^-$ generators, as the high levels of DHR 123 oxidation induced by these compounds were only slightly reduced by Ro 31-8220. The observed small reduction most likely represented the minor contribution of spontaneous ROS generation by neutrophils to oxidation of DHR 123. This demonstrates that the major oxidant species responsible for the generation of fluorescence is derived from the compounds themselves, and therefore this rules out these compounds as pure NO donors, as free NO by itself is incapable of oxidising DHR 123 to rhodamine 123, as previous studies have shown (Crow, 1997). These studies also confirmed the absence of oxidant production from DEA/NO, as fluorescence was not significantly increased compared to control. Indeed, there was a small reduction in DHR 123 oxidation by DEA/NO, suggesting an inhibitory effect of DEA/NO on ROS generation by neutrophils. This concurs with a number of studies that have proposed inhibitory actions of NO on neutrophil functions, including chemotaxis, degranulation, shape change and oxidative burst (Moilanen *et*

al., 1993; Ward *et al.*, 2000; Gluckman *et al.*, 2000). A number of studies have demonstrated an inhibitory effect of NO on the neutrophil respiratory burst *in vitro* (Clancy *et al.*, 1992; Forslund and Sundqvist, 1995; Iha *et al.*, 1996) and *in vivo* (Gessler *et al.*, 1996; Bloomfield *et al.*, 1997) and others have found an inhibitory action of $ONOO^-$ (Clements *et al.*, 2003). However, other studies have shown increased oxidative burst in response to $ONOO^-$ (Iha *et al.*, 1996; Bednar *et al.*, 1996), and others have demonstrated no effect of NO on ROS generation in an *in vivo* model of acute pulmonary inflammation (Kermarrec *et al.*, 1998).

Addition of 10% autologous serum to these experiments led to suppression of ROS generation by neutrophils in most experimental conditions versus matched experiments in the absence of serum. Although this effect was not found to be statistically significant over the three replications of the experiment, there was a consistent trend observed each time, and suggests that this may be a real effect that would be significant on further investigation. Control fluorescence was reduced compared to that seen in the absence of serum when neutrophils were incubated with DHR 123 for 2 h. This represents suppression of endogenous activation of the oxidative burst in the presence of serum, as Ro 31-8220 was unable to further reduce the extent of DHR 123 oxidation.

Additionally, the extent of stimulation of the oxidative burst by the neutrophil activating agent, PMA, was severely attenuated by the presence of serum with some, but not all, donors. This suggests that a factor (or factors) in serum protects neutrophils from becoming activated. As in culture medium alone, GEA 3162 and SIN-1 both increased fluorescence that was not reduced by addition of Ro 31-8220. The major difference between these studies and those carried out in the absence of serum, however, was observed with DEA/NO. Although this compound produced a small inhibition of ROS generation in medium alone, a significant increase in fluorescence above control levels was obtained in the presence of serum, which was reduced back to control levels by Ro 31-8220. This suggests either that DEA/NO is able to stimulate oxidative burst in the presence of serum or that it is able to release the natural brake on ROS generation exerted by serum. A number of factors make the latter possibility more likely; given the protective effect of serum against other

stimuli such as PMA, the lack of oxidative burst-stimulating effects of DEA/NO in medium alone and the anti-neutrophil effects of NO that have been widely reported (Granger and Kubes, 1996), it is unlikely that a pure NO donor would be able to promote ROS generation from neutrophils in the presence, but not in the absence, of serum. Furthermore, the good correlation between the level of fluorescence generated by DEA/NO in the presence of serum and that seen in control cells in medium alone suggests that reversal of the inhibition of the oxidative burst by serum is a more plausible explanation. Enhanced fluorescence observed with DEA/NO is not due to DEA/NO generating an oxidant species itself in the presence of serum, as Ro 31-8220 was able to reverse this effect, demonstrating a role for neutrophil-derived oxygen radicals in oxidation of DHR 123. Previous studies have suggested that albumin, a highly-abundant protein present in serum, is able to inhibit the oxidative burst in neutrophils (Nathan *et al.*, 1993). Furthermore, it is known that S-nitrosoalbumin is formed in plasma from S-nitrosation of albumin by derivatives of NO (Stamler *et al.*, 1992;Crane *et al.*, 2002). It is therefore possible that albumin in serum is responsible for the inhibition of DHR 123 oxidation and that modification of albumin by NO, through formation of S-nitrosoalbumin is sufficient to reverse this inhibition.

In order to test the hypothesis that DEA/NO may reverse the brake on endogenous ROS generation exerted by serum through modification of serum proteins such as albumin, experiments were carried out to compare DHR 123 oxidation under various conditions. Neutrophils were incubated with DHR 123 for 2 h in medium alone, in medium supplemented with 10% autologous serum or in medium supplemented with human serum albumin (HSA; 0.4% w/v), to which PBS, PMA (10 nM) or DEA/NO (1 mM) were added. This percentage of HSA was chosen because albumin is present in serum at levels of approximately 4% (Rutstein *et al.*, 1954), and 10% serum is added to neutrophils in serum-replete studies. Therefore, 0.4% HSA represents the approximate levels of albumin observed in the whole serum studies. If the hypothesis is valid, the inhibition of oxidative burst observed with serum should be mimicked by the presence of HSA in the culture medium. However, DEA/NO should reverse this inhibition and return the

fluorescence level to that of control. These studies showed that the presence of HSA reduced fluorescence in a manner similar to that observed with whole serum. This concurs with previous findings by Valerius et al, who found that O_2^- release from neutrophils was inversely proportional to levels of albumin, and suggested that this effect was due to inhibition of neutrophil adhesion (Valerius, 1983). In the presence of HSA, both DEA/NO and PMA increased fluorescence to levels above control. This suggests that the inhibition of ROS generation by serum is multifactorial, and not due solely to albumin, as this inhibition is more easily overcome by DEA/NO than is seen with whole serum. Therefore the hypothesis is valid and DEA/NO does reverse the albumin-mediated inhibition of the oxidative burst in neutrophils. It is likely that this effect is mediated by modification of albumin by NO from DEA/NO, in particular S-nitrosation of the serum protein seems a potential candidate to explain the abolition of the inhibitory effect of serum. The implications of these results are that in serum-replete apoptosis studies (Chapter 4), pro-apoptotic effects of DEA/NO may be mediated by $ONOO^-$, as this compound promotes the oxidative burst from neutrophils and NO from DEA/NO may react with O_2^- from the neutrophils to generate $ONOO^-$.

In summary, therefore, two independent indicators of NO or oxidant species generation both demonstrate that diazeniumdiolate compounds such as DEA/NO and SPER/NO release free NO alone, and can therefore be considered to be 'pure' NO donors. On the other hand, both SIN-1 and GEA 3162 release NO and O_2^- concomitantly, and should therefore be considered to be $ONOO^-$ generators. In future chapters, therefore, the diazeniumdiolates, DEA/NO and SPER/NO, will be referred to as NO donors and SIN-1 and GEA 3162 as $ONOO^-$ donors. Biological effects of DEA/NO are unlikely to be mediated by $ONOO^-$ in the absence of serum, despite ROS generation by neutrophils. However, in the presence of serum, this possibility cannot be excluded, as increased ROS production is observed in the presence of both DEA/NO and serum. This observation may be due to reversal of protective effects of serum on neutrophils by DEA/NO. Having carefully characterised the above compounds in terms of their NO or $ONOO^-$ generation, they were then used to investigate the regulation of apoptosis in neutrophils by NO and

$ONOO^-$, with comparisons being made between NO donors of differing rates of decomposition and compounds that liberate $ONOO^-$.

Chapter 4: Effects of $NO/ONOO^-$ Donors on the Rate of Neutrophil Apoptosis

4.1 Introduction

NO has been shown to be a critical regulator of apoptotic cell death in many cell types, and can be either pro- or anti-apoptotic. Low concentrations of NO tend to be protective and these effects are frequently attributed to increased cGMP and/or S-nitrosation of caspase proteases (Liu and Stamler, 1999). Inhibition or delay of apoptosis has been observed in trophoblasts (Dash *et al.*, 2003), neurones (Thippeswamy *et al.*, 2001; Ha *et al.*, 2003), endothelial cells (Suschek *et al.*, 1999) cardiomyocytes (Andreka *et al.*, 2001; Zingarelli *et al.*, 2002), hepatocytes (Kim *et al.*, 1997), keratinocytes (Weller *et al.*, 2003), B lymphocytes (Genaro *et al.*, 1995), chondrocytes (Oh and Chun, 2003) and eosinophils (Beauvais *et al.*, 1995).

NO has also been shown to promote apoptotic cell death, either directly through exogenously-supplied NO or as an essential mediator of apoptosis triggered by other stimuli. In contrast to inhibition of apoptosis, NO-mediated induction of apoptosis is usually reported to be independent of cGMP and is often attributed to the initiation of the stress pathway and the associated mitochondrial changes characteristic of this pathway, frequently through formation of $ONOO^-$. Direct induction of apoptosis has been demonstrated in eosinophils (Zhang *et al.*, 2003b), T lymphocytes (Valenti *et al.*, 2003), various colon cancer cell lines (Liu *et al.*, 2003), chondrocytes (Blanco *et al.*, 1995), cardiomyocytes (Taimor *et al.*, 2001; Chae *et al.*, 2001), neuroblastoma cells (Oh-Hashi *et al.*, 1999), insulin-secreting β islet cell-like cell lines (Bernabe *et al.*, 2001) and neurones (Ghatan *et al.*, 2000; Wang *et al.*, 2003; Zhang *et al.*, 2002). NO has been reported to mediate apoptosis induced by farnesyltransferase inhibitors in chronic myeloid leukaemia cells (Selleri *et al.*, 2003), that promoted by morphine in T lymphocytes (Wang *et al.*, 2001) and also mediates cytokine (LPS/IFN-γ)-induced death in macrophages (Scivittaro *et al.*, 1997).

Neutrophil apoptosis has been shown to be enhanced by exogenously-supplied NO by a number of groups (Fortenberry *et al.*, 1998; Blaylock *et al.*, 1998; Singhal *et al.*, 1999; Fortenberry *et al.*, 1999; Ward *et al.*, 2000). In order to understand the mechanism of action of the effects of NO or $ONOO^-$ donor drugs on neutrophil apoptosis, a selection of agents with specific properties were used. The aims of the experiments described in this chapter were to characterise the effects of two diazeniumdiolate NO donors with different rates of NO release, DEA/NO and SPER/NO, the oxatriazole 5-imine derivative compound, GEA 3162, and the $ONOO^-$ donor, SIN-1, on neutrophil apoptosis, using a number of different events to measure apoptotic cell death. Both concentration-response and time-course studies were used to examine the relative potencies and temporal effects of these compounds.

4.2 Results

4.2.1 Assessment of constitutive neutrophil apoptosis

Four independent events of neutrophil apoptosis were measured in these studies: the characteristic morphological changes of cell shrinkage and nuclear condensation, exposure of phosphatidylserine (PS) on the outer surface of apoptotic neutrophils, shedding of CD16 from the cell surface and internucleosomal DNA fragmentation. When these techniques were applied to constitutive neutrophil apoptosis, all four correlated well, and by 20 h the levels of apoptosis detected were 53.1 ± 2.8, 67.9 ± 2.7, 61.2 ± 5.9 and 61.5 ± 9.8 % apoptosis respectively ($p > 0.05$, one-way ANOVA; Figure 4.1). All four events occurred virtually simultaneously in spontaneous apoptosis. Levels of apoptosis remained low (approximately 1 %) in cells undergoing constitutive apoptosis until the 8 h time point, at which time 9.8 ± 6.4 % of cells exhibited morphological signs of apoptosis. Apoptosis then increased rapidly at later time points, producing a sigmoidal curve when apoptosis was plotted vs. time.

4.2.2 Concentration-response studies using NO/$ONOO^-$ donors

In order to determine appropriate concentrations of NO/$ONOO^-$ donors to use, concentration-response studies were carried out. Neutrophils were incubated in the absence or presence of NO (DEA/NO or SPER/NO; 0.1 μM – 1 mM) or $ONOO^-$ (GEA 3162; 0.1 μM – 100 μM) donors for 20 h, then apoptosis was assessed by morphology and Annexin V binding. These studies showed that low concentrations of the NO donors delayed neutrophil apoptosis compared to control. The inhibition obtained was small (< 10 %), but the results achieved statistical significance ($p < 0.05$, one-way ANOVA with Student-Newman-Keuls post-hoc test) at concentrations of 0.1 – 30 μM for DEA/NO by morphology and 0.1 – 300 μM by Annexin V binding. At the lowest concentration (0.1 μM), morphological apoptosis was 54.0 ±

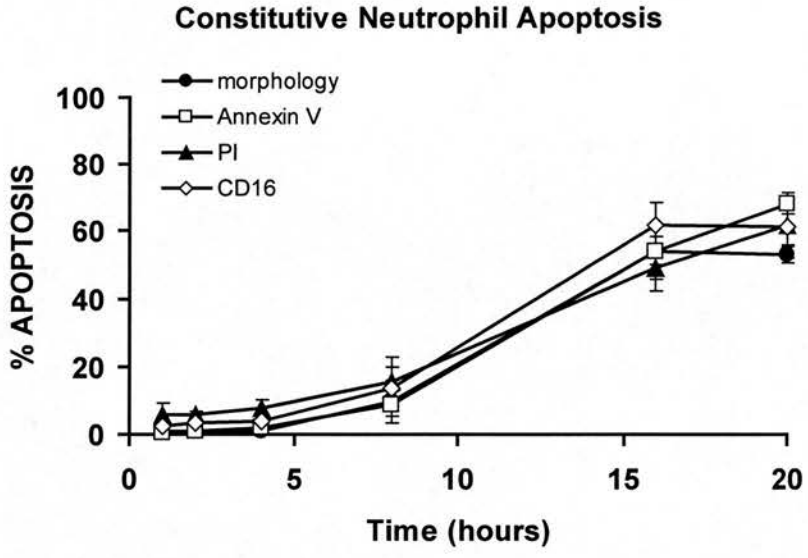


Figure 4.1 Correlation of 4 different techniques in the measurement of constitutive neutrophil apoptosis. Neutrophils (4.5 x 10^6 cells/ml) were incubated in Iscove's MDM supplemented with 10 % autologous serum for 1, 2, 4, 8, 16 or 20 hours, then apoptosis was assessed by morphology, Annexin V binding, PI intercalation and CD16 shedding. Results represent mean ± S.E.M. from n = 3 - 6 experiments.

4.9 % vs. 59.9 ± 4.4 % control, and PS exposure was 64.1 ± 3.2 % vs. 72.3 ± 2.6 % (Figure 4.2). Using SPER/NO, a significant inhibition ($p < 0.05$, one-way ANOVA with Student-Newman-Keuls post-hoc test) was seen at concentrations of 0.1 – 3 μM by morphology and 0.1 μM by Annexin V binding. Morphological apoptosis measured with 0.1 μM SPER/NO was 42.9 ± 2.7 % vs. 53.1 ± 2.8 % control, and PS exposure was 60.1 ± 1.9 % vs. 67.9 ± 2.7 % (Figure 4.3).

In contrast, higher concentrations of these compounds showed an acceleration of neutrophil apoptosis. Concentrations of 1 mM DEA/NO produced a significant ($p < 0.05$, one-way ANOVA with Student-Newman-Keuls post-hoc test) induction of apoptosis by morphology and by Annexin V binding. Neutrophils treated with 1 mM DEA/NO showed 100 % morphological apoptosis, compared to 59.9 ± 4.4 % apoptosis in PBS-treated cells, and 81.1 ± 5.7 % PS exposure compared to 72.3 ± 2.6 % control (Figure 4.2). A significant induction of neutrophil apoptosis was observed with concentrations of 300 μM – 1 mM SPER/NO when apoptosis was assessed by morphology and with 1 mM as determined by Annexin V binding ($p < 0.05$, one-way ANOVA with Student-Newman-Keuls post-hoc test). Neutrophils exposed to 1 mM SPER/NO for 20 h showed 89.5 ± 5.1 % morphological apoptosis vs. 53.1 ± 2.8 % control, and PS exposure in these cells was 82.0 ± 2.7 % compared to 67.9 ± 2.7 % in untreated cells (Figure 4.3).

When neutrophils were exposed to the $ONOO^-$ donor, GEA 3162, no significant reduction of the rate of apoptosis was seen at concentrations equivalent to that observed with the diazeniumdiolates; at the lowest concentration (0.1 μM), morphological apoptosis was measured as 49.3 ± 3.6 % compared to 53.1 ± 2.8 % control, and levels of PS exposure were 66.6 ± 2.2 % vs. 67.9 ± 2.7 % in control cells ($p > 0.05$, one-way ANOVA with Student-Newman-Keuls post-hoc test). However, a significant ($p < 0.05$, one-way ANOVA with Student-Newman-Keuls post-hoc test) acceleration of morphological neutrophil apoptosis was achieved with 30 and 100 μM of this compound. Using 100 μM GEA 3162, virtually all cells underwent apoptosis over 20 h, with death measured as 93.6 ± 3.0 % compared to 53.1 ± 2.8 % control (Figure 4.4A). However, no significant increase in apoptosis was detectable

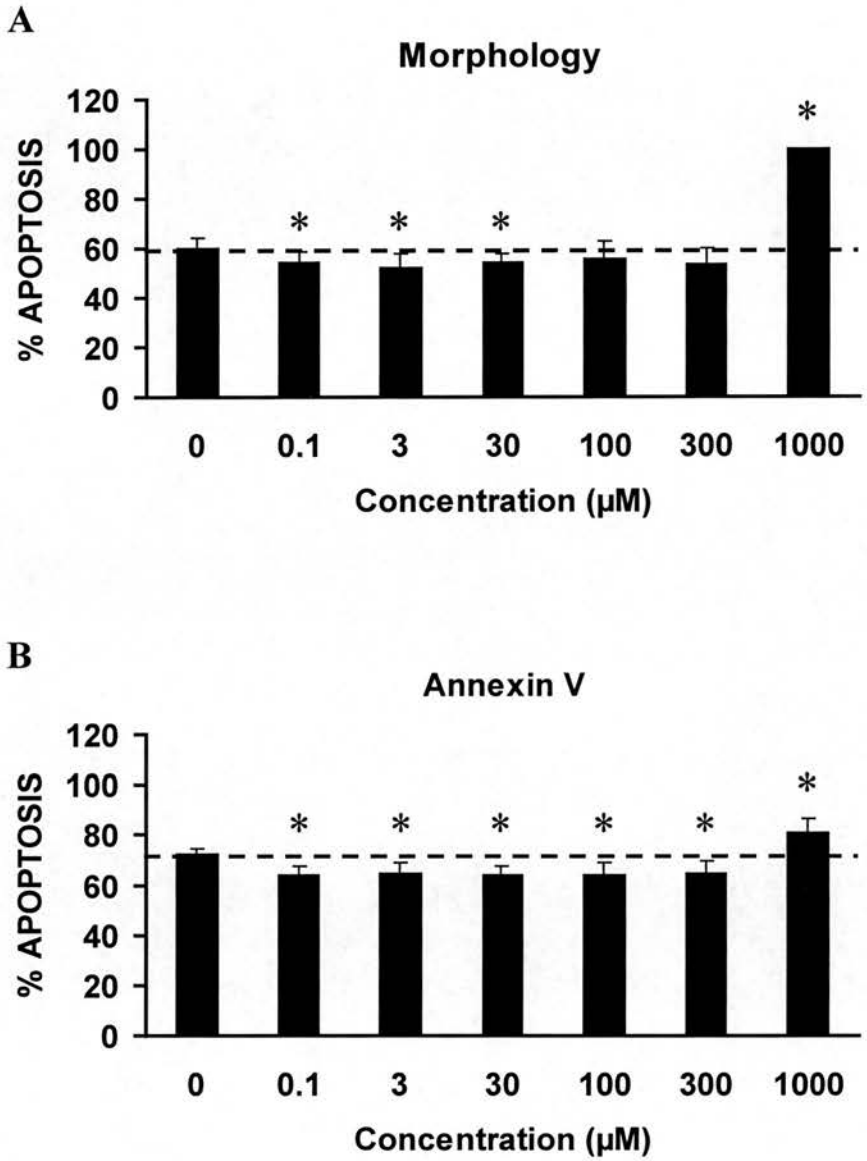


Figure 4.2 Concentration-response graphs for DEA/NO on neutrophil apoptosis assessed by morphology and Annexin V binding. Neutrophils (4.5 x 10^6 cells/ml) were incubated in Iscove's MDM supplemented with 10 % autologous serum for 20 hours in the absence or presence of DEA/NO (0.1 μM – 1 mM), then apoptosis was assessed by (A) morphology and (B) Annexin V binding. Results represent mean ± S.E.M. from n = 6 experiments. Asterisks represent significant ($p < 0.05$) difference from control (dashed line) by one-way ANOVA with Student-Neuman-Keuls post-hoc test.

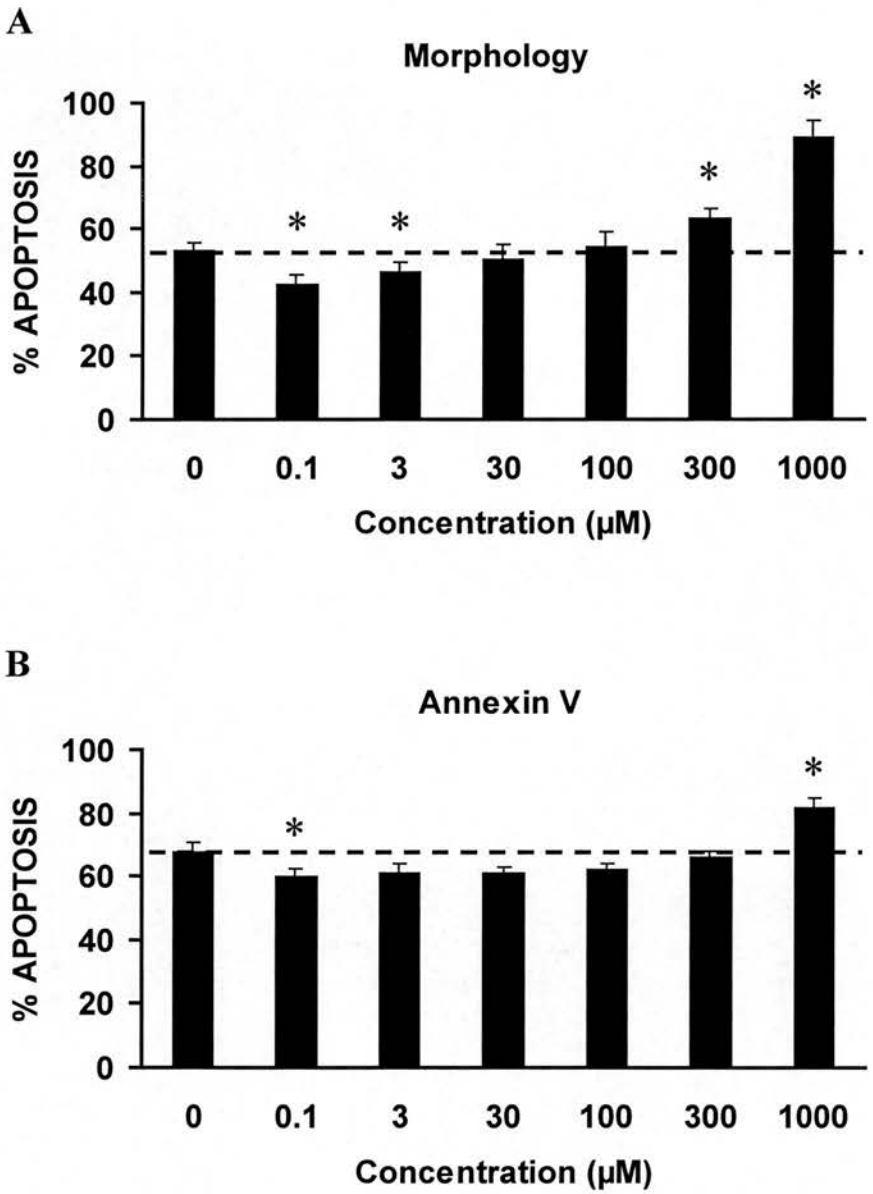


Figure 4.3 Concentration-response graphs for SPER/NO on neutrophil apoptosis assessed by morphology and Annexin V binding. Neutrophils (4.5 x 10^6 cells/ml) were incubated in Iscove's MDM supplemented with 10 % autologous serum for 20 hours in the absence or presence of SPER/NO (0.1 μM – 1 mM), then apoptosis was assessed by (A) morphology and (B) Annexin V binding. Results represent mean ± S.E.M. from n = 6 experiments. Asterisks represent significant ($p < 0.05$) difference from control (dashed line) by one-way ANOVA with Student-Neuman-Keuls post-hoc test.

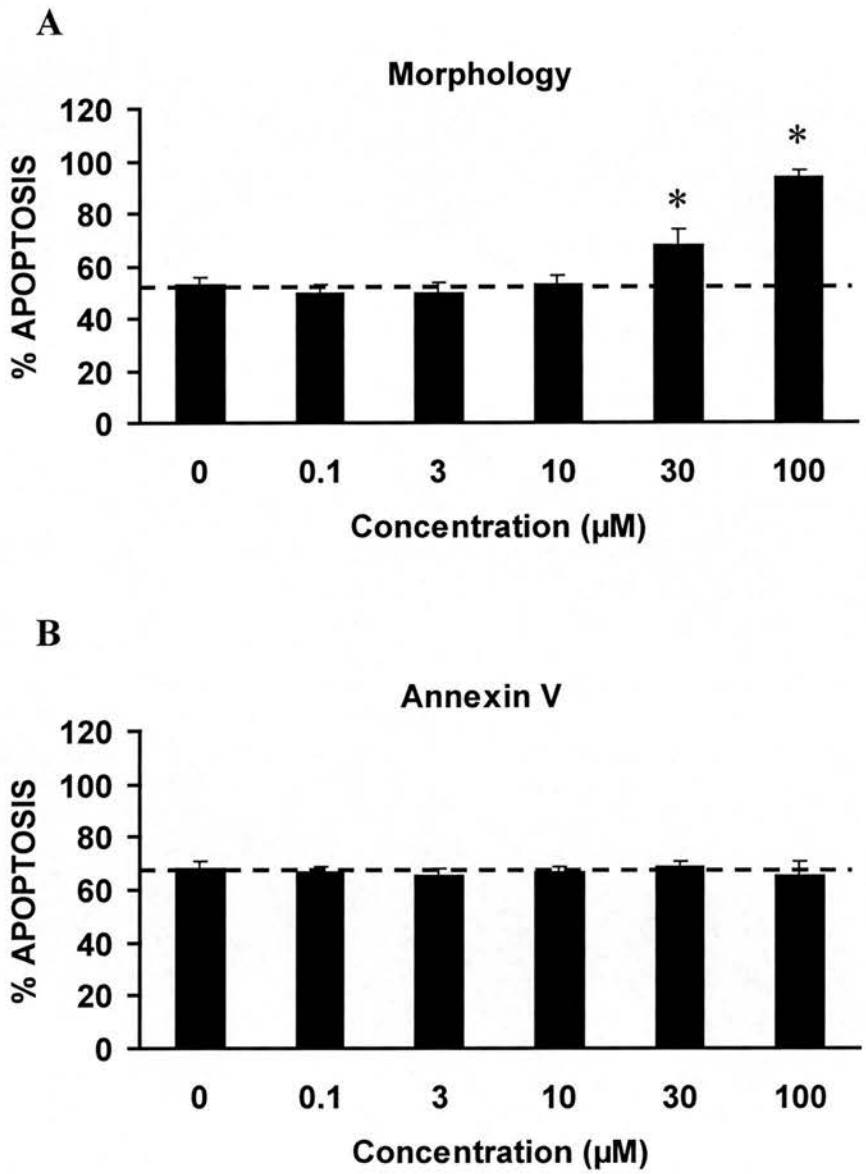


Figure 4.4 Concentration-response graphs for GEA 3162 on neutrophil apoptosis assessed by morphology and Annexin V binding. Neutrophils (4.5 x 10^6 cells/ml) were incubated in Iscove's MDM supplemented with 10 % autologous serum for 20 hours in the absence or presence of GEA 3162 (0.1 μM – 100 μM), then apoptosis was assessed by (A) morphology and (B) Annexin V binding. Results represent mean ± S.E.M. from n = 6 experiments. Asterisks represent significant ($p < 0.05$) difference from control (dashed line) by one-way ANOVA with Student-Neuman-Keuls post-hoc test.

when Annexin V binding was used as the measure of apoptosis ($p > 0.05$, one-way ANOVA with Student-Newman-Keuls post-hoc test); 100 μM GEA 3162 gave 65.5 ± 5.3 % PS exposure vs. control levels of 67.9 ± 2.7 % as assessed by this technique (Figure 4.4B).

4.2.3 Time-course studies using NO/$ONOO^-$ donors

Time-course studies were then performed to look at the changes characteristic of neutrophil apoptosis over time in the absence and presence of these compounds. Neutrophils were exposed to NO donors (DEA/NO and SPER/NO; 0.1 μM or 1 mM) or the $ONOO^-$ donor, GEA 3162 (30 or 100 μM) for 1, 2, 4, 8, 16 or 20 h and apoptosis measured by four different techniques; morphology, Annexin V binding, CD16 shedding and PI intercalation.

Studies using DEA/NO at concentrations of 0.1 μM and 1 mM demonstrated the biphasic effect of this compound on neutrophil apoptosis. Throughout the 20-h incubation period, the inhibition of morphological apoptosis evoked by the lower concentration of DEA/NO was minimal, with a difference from control only observed at time points of 16 h and later, and only representing a difference of a few percent. At 16 h, apoptosis was 42.5 ± 7.2 % compared to 54.0 ± 8.3 % control (Figure 4.5). Although the overall time course line for 0.1 μM DEA/NO did not vary significantly from control ($p > 0.05$, 2-way ANOVA), the inhibition observed at 20 h was significantly different from control ($p < 0.05$, two-tailed paired *t* test).

This was also the case for apoptosis measured by Annexin V binding, with PS exposure at 16 h being measured as 45.3 ± 6.1 % vs. 54.1 ± 4.3 % in untreated cells (Figure 4.6B), although the time course line failed to reach statistical significance ($p > 0.05$, 2-way ANOVA). However, data obtained at 20 h showed significant differences from control ($p < 0.05$, two-tailed paired *t* test). Typical flow cytometry traces (Figure 4.6A) show only a slight leftward shift of fluorescence from control at 8 and 20 h, representing inhibition of PS exposure. These traces

demonstrate the trends observed in each replication of this experiment, although the precise levels of apoptosis varied from experiment to experiment due to biological variation between donors.

However, assessment of apoptosis by CD16 shedding showed virtually identical levels of apoptosis to control throughout the 20-h period and no significant difference from control was detectable between the lines ($p > 0.05$, two-way ANOVA); at 20 h, control apoptosis was 61.2 ± 5.9 % and in the presence of DEA/NO, it was 58.2 ± 2.4 ($p > 0.05$, two-tailed paired *t* test; Figure 4.7B). This is clearly shown in virtually identical flow cytometry profiles for untreated and 0.1 μM DEA/NO-treated cells at both 8 and 20 h (Figure 4.7A).

The inhibition of neutrophil apoptosis by 0.1 μM DEA/NO was seen using PI intercalation as a measure of internucleosomal DNA fragmentation. Such inhibition was apparent at 16 h, and by 20 h apoptosis was 47.1 ± 10.7 % compared to control levels of 61.5 ± 9.8 % (Figure 4.8B). However, the time course lines did not quite reach statistical significance ($p > 0.05$, two-way ANOVA). Again, examples of histograms obtained by flow cytometry are shown in Figure 4.8A.

Acceleration of neutrophil apoptosis by 1 mM DEA/NO occurred much more quickly than the inhibition by 0.1 μM DEA/NO. Morphologically, large differences from control were seen by 4 h (34.9 ± 8.5 % vs. 1.0 ± 0.1 % apoptosis in control cells, and increased levels of apoptosis were maintained through the remainder of the culture period ($p < 0.0001$, two-way ANOVA; Figure 4.5).

Annexin V binding gave less conclusive results than morphology, with a clear difference not being seen until 8 h, and this difference was smaller than that seen by morphology; 29.4 ± 14.2 % compared to 8.9 ± 3.5 % control (Figure 4.6B), although a significant ($p < 0.0001$) difference from the control time course was detectable by 2-way ANOVA. Flow cytometry profiles show a distinct increase in highly-fluorescent neutrophils exposed to 1 mM DEA/NO compared to control at both 8 and 20 h (Figure 4.6A).

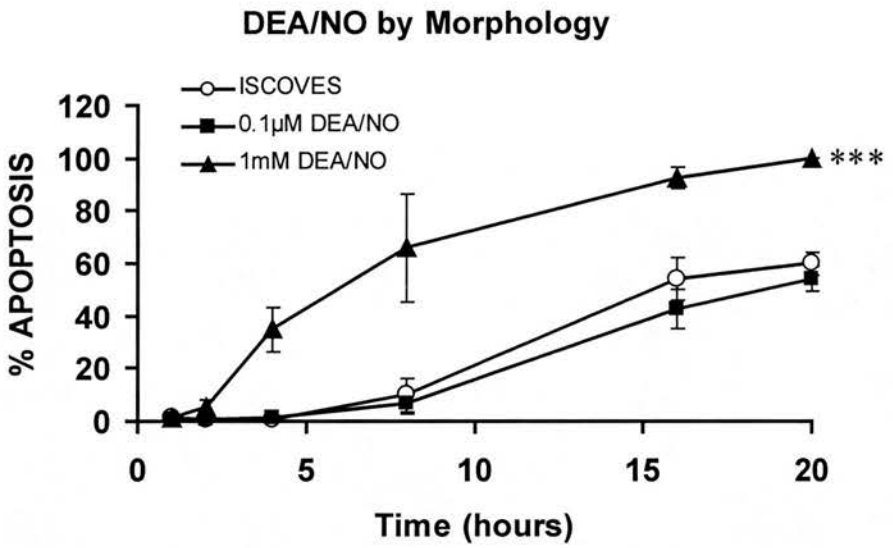
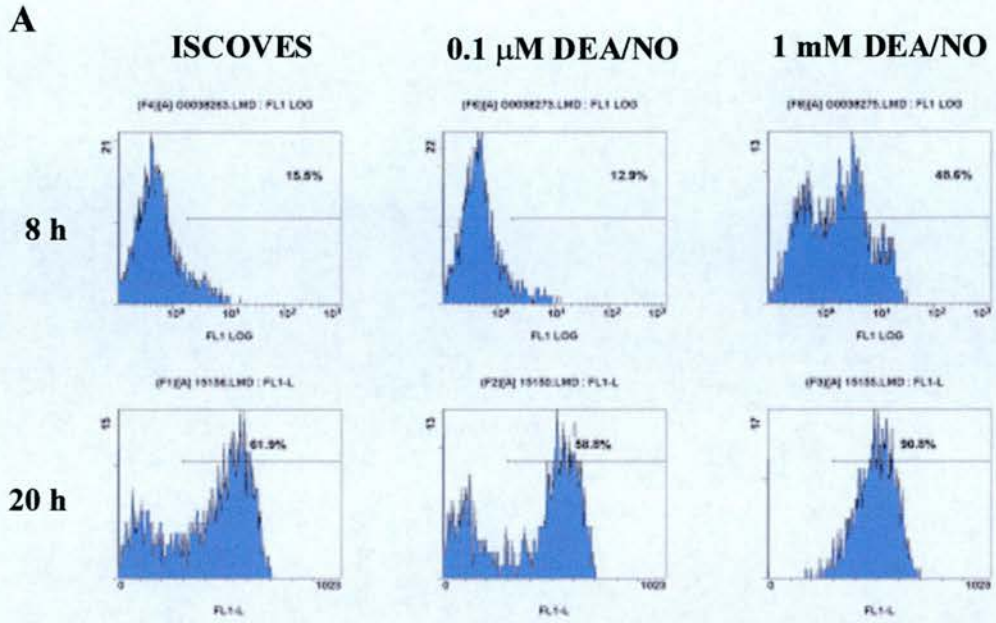


Figure 4.5 Time course studies for effects of DEA/NO on neutrophil apoptosis assessed by morphology. Neutrophils (4.5 x 10^6 cells/ml) were incubated in Iscove's MDM supplemented with 10 % autologous serum for 1 - 20 hours in the absence or presence of DEA/NO (0.1 μM or 1 mM), then apoptosis was assessed by morphology. Results represent mean ± SEM from n = 3 - 6 experiments. Asterisks represent significant ($p < 0.0001$) difference from control (open symbols) time-course by two-way ANOVA.



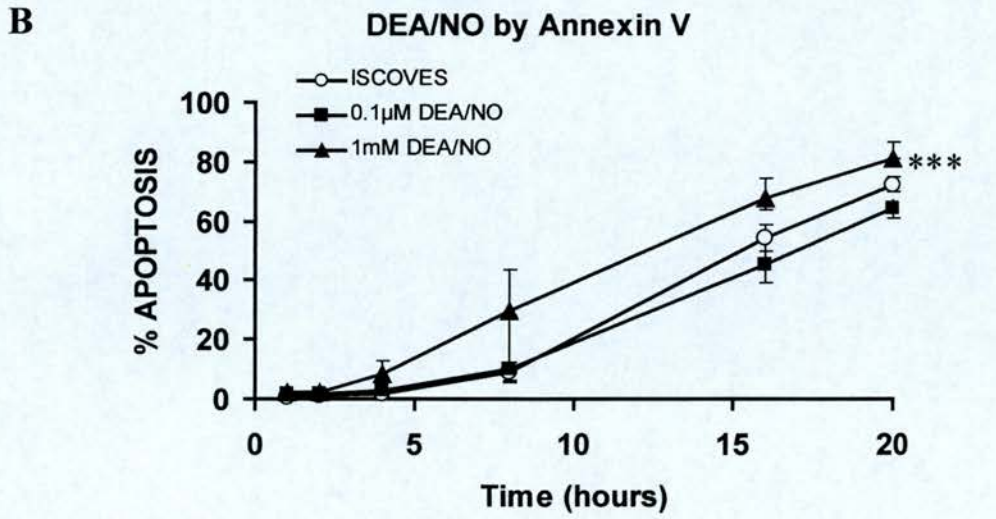


Figure 4.6 Time course studies for effects of DEA/NO on neutrophil apoptosis assessed by Annexin V binding. Neutrophils (4.5 x 10^6 cells/ml) were incubated in Iscove's MDM supplemented with 10 % autologous serum for 1 - 20 hours in the absence or presence of DEA/NO (0.1 μM or 1 mM), then apoptosis was assessed by Annexin V binding (A) Typical flow cytometry traces showing percentage Annexin V binding in neutrophils after 8 and 20 hours (B) Graph showing changes in PS exposure over time in control cells or neutrophils treated with DEA/NO. Results represent mean ± SEM from n = 3 - 6 experiments. Asterisks represent significant ($p < 0.0001$) difference from control (open symbols) time-course by two-way ANOVA.

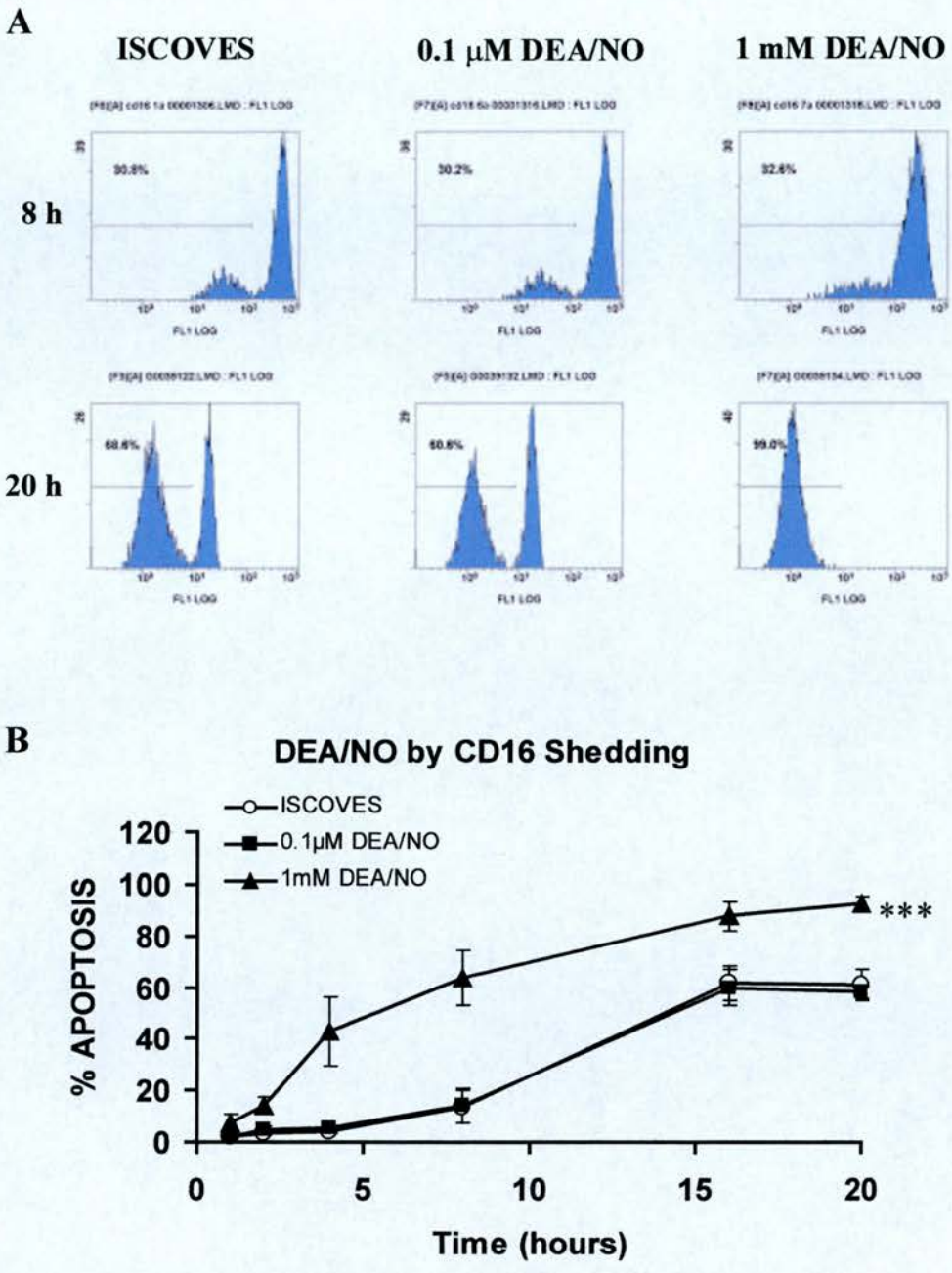


Figure 4.7 Time course studies for effects of DEA/NO on neutrophil apoptosis assessed by CD16 shedding. Neutrophils (4.5 x 10^6 cells/ml) were incubated in Iscove's MDM supplemented with 10 % autologous serum for 1 - 20 hours in the absence or presence of DEA/NO (0.1 μM or 1 mM), then apoptosis was assessed by CD16 shedding (A) Typical flow cytometry traces showing percentage CD16 shedding in neutrophils after 8 and 20 hours (B) Graph showing changes in CD16 shedding over time in control cells or neutrophils treated with DEA/NO. Results represent mean ± SEM from n = 3 - 6 experiments. Asterisks represent significant ($p < 0.0001$) difference from control (open symbols) time-course by two-way ANOVA.

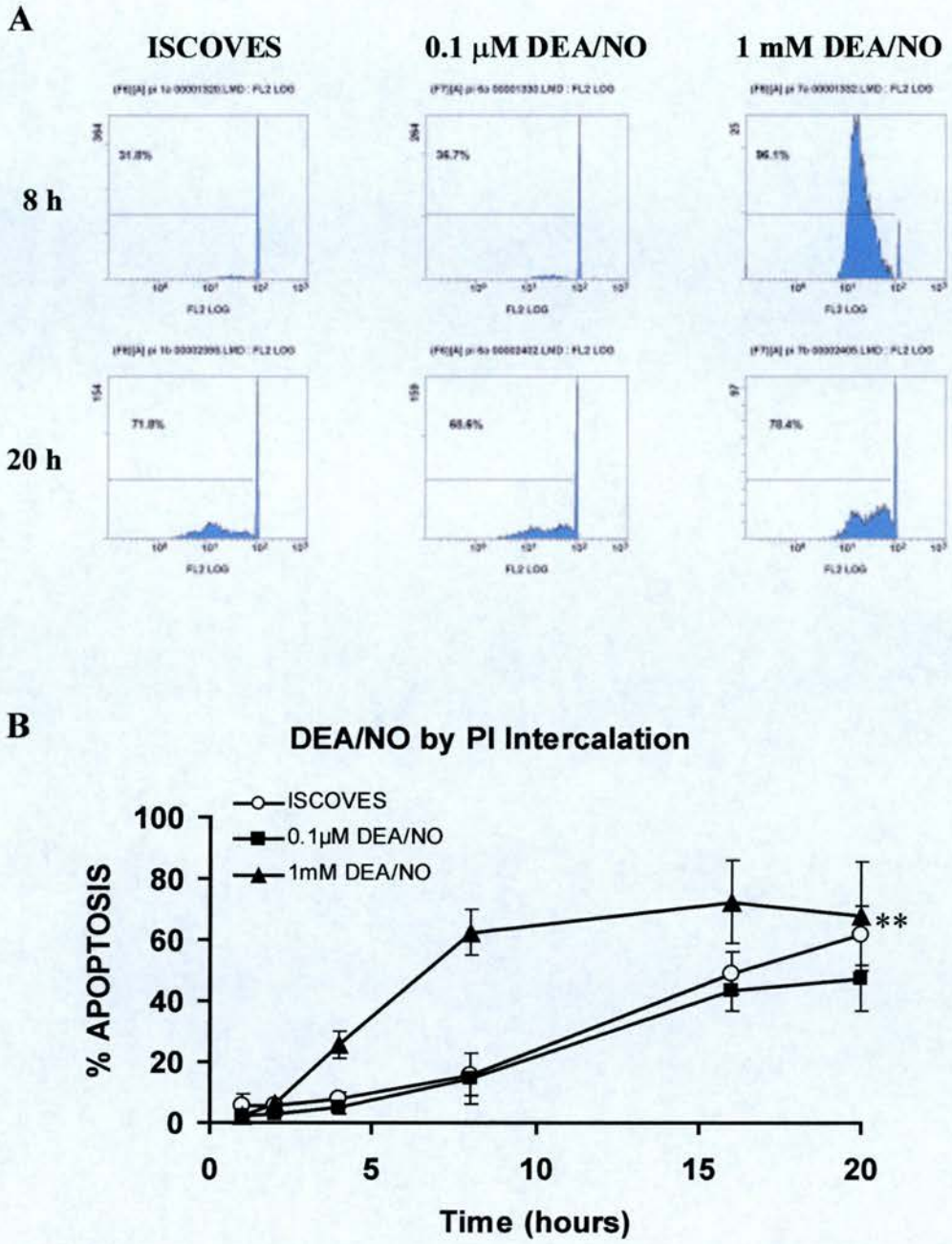


Figure 4.8 Time course studies for effects of DEA/NO on neutrophil apoptosis assessed by PI intercalation. Neutrophils (4.5 x 10^6 cells/ml) were incubated in Iscove's MDM supplemented with 10 % autologous serum for 1 - 20 hours in the absence or presence of DEA/NO (0.1 μM or 1 mM), then apoptosis was assessed by PI intercalation (A) Typical flow cytometry traces showing percentage hypodiploid cells in neutrophils after 8 and 20 hours (B) Graph showing changes in PI intercalation over time in control cells or neutrophils treated with DEA/NO. Results represent mean ± SEM from n = 3 - 6 experiments. Asterisks represent significant ($p < 0.01$) difference from control (open symbols) time course by two-way ANOVA.

Assessment of apoptosis by CD16 shedding correlated much more closely with morphological observations, with induction of apoptosis becoming apparent by just 2 h, with 14.0 ± 3.4 % of cells having shed surface CD16 compared to 3.5 ± 0.9 % control, and after 20 h, apoptosis was 92.2 ± 3.2 % vs. 61.2 ± 5.9 % control measured by this method (Figure 4.7B). The time course for 1 mM DEA/NO was significantly different from control ($p < 0.0001$, 2-way ANOVA). Examples of histograms achieved when apoptosis was assessed by this method are shown in Figure 4.7A, although a clear leftward shift in fluorescence is only evident at 20 h.

By propidium iodide intercalation, a clear difference between treated and untreated cells was again apparent by 4 h, with 25.6 ± 4.5 % of cells having hypodiploid DNA content, representing internucleosomal DNA fragmentation, compared to 7.7 ± 2.5 % in untreated cells. The acceleration of the rate of apoptosis by 1 mM DEA/NO was evident throughout the remainder of the culture period ($p < 0.01$, 2-way ANOVA), although the extent of this induction reduced towards 20 h, as apoptosis measured by this technique slowed down and was 'caught up' by control cells, so that by 20 h, 67.7 ± 17.6 % of DEA/NO-treated cells were measured as apoptotic compared to 61.2 ± 5.9 % in control cells (Figure 4.8B). The increase in internucleosomal DNA fragmentation is plainly evident in the flow cytometry histograms at both 8 and 20 h (Figure 4.8A).

Similar to the anti-apoptotic effects of low concentrations of DEA/NO, 0.1 μM SPER/NO inhibited neutrophil apoptosis over a comparable time course. As for DEA/NO, the inhibition of morphological apoptosis did not become apparent until 16 h of culture and beyond, and the time course was not significantly different from control ($p > 0.05$, two-way ANOVA) over the 20 h. After 16 h, 45.8 ± 7.3 % of SPER/NO-treated cells showed the characteristic morphology of apoptosis compared to 54.0 ± 8.3 % control (Figure 4.9), and at 20 h, a significant difference from control apoptosis was seen ($p < 0.05$, two-tailed paired *t* test).

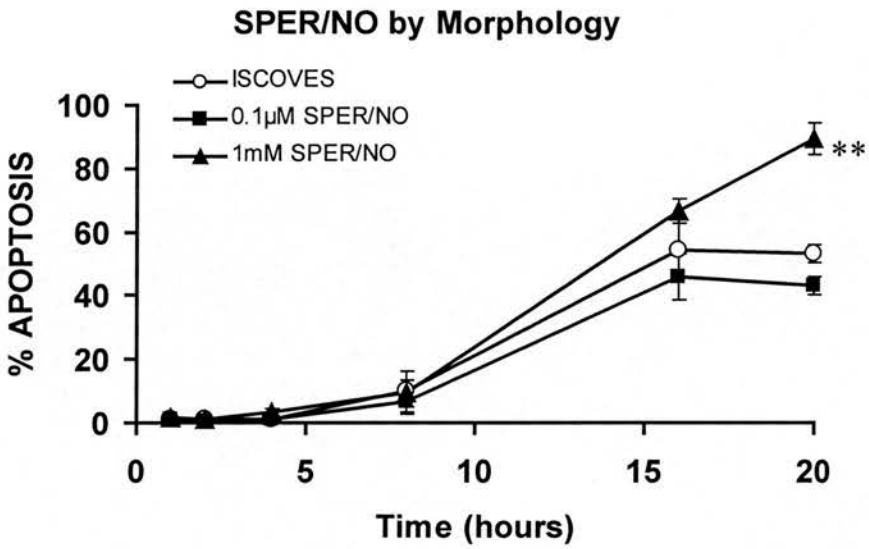


Figure 4.9 Time course studies for effects of SPER/NO on neutrophil apoptosis assessed by morphology. Neutrophils (4.5 x 10^6 cells/ml) were incubated in Iscove's MDM supplemented with 10 % autologous serum for 1 - 20 hours in the absence or presence of SPER/NO (0.1 μM or 1 mM), then apoptosis was assessed by morphology. Results represent mean ± SEM from n = 3 - 6 experiments. Asterisks represent significant ($p < 0.01$) difference from control (open symbols) time course by two-way ANOVA.

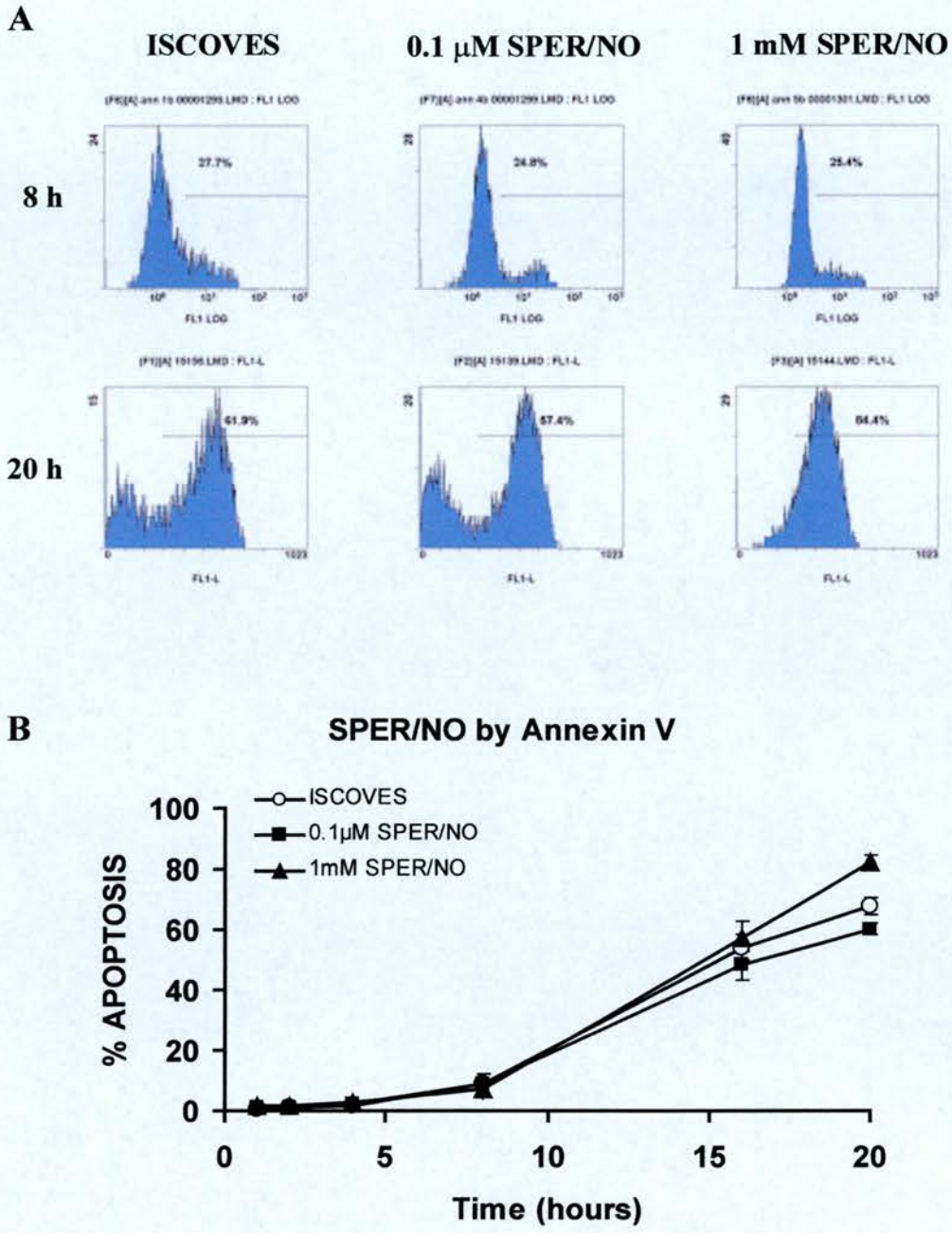


Figure 4.10 Time course studies for effects of SPER/NO on neutrophil apoptosis assessed by Annexin V binding. Neutrophils (4.5 x 10^6 cells/ml) were incubated in Iscove's MDM supplemented with 10 % autologous serum for 1 - 20 hours in the absence or presence of SPER/NO (0.1 μM or 1 mM), then apoptosis was assessed by Annexin V binding (A) Typical flow cytometry traces showing percentage PS exposure in neutrophils after 8 and 20 hours (B) Graph showing changes in PS exposure over time in control cells or neutrophils treated with SPER/NO. Results represent mean ± SEM from n = 3 - 6 experiments. Data did not reach statistical significance.

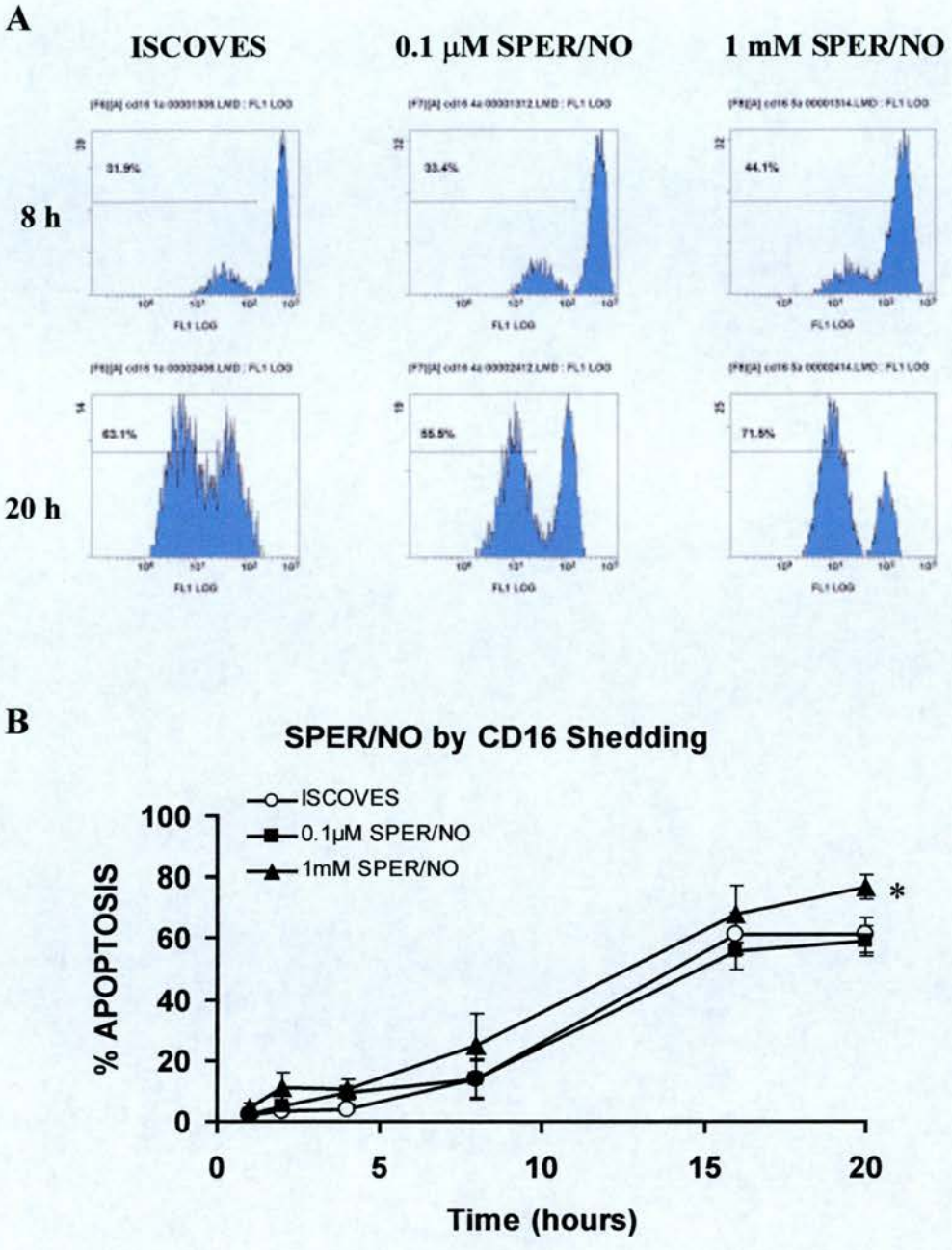


Figure 4.11 Time course studies for effects of SPER/NO on neutrophil apoptosis assessed by CD16 shedding. Neutrophils (4.5×10^6 cells/ml) were incubated in Iscove's MDM supplemented with 10 % autologous serum for 1 - 20 hours in the absence or presence of SPER/NO (0.1 μM or 1 mM), then apoptosis was assessed by CD16 shedding (A) Typical flow cytometry traces showing percentage CD16 shedding in neutrophils after 8 and 20 hours (B) Graph showing changes in CD16 shedding over time in control cells or neutrophils treated with SPER/NO. Results represent mean ± SEM from n = 3 - 6 experiments. Asterisks represent significant ($p < 0.05$) difference from control (open symbols) time course by two-way ANOVA.

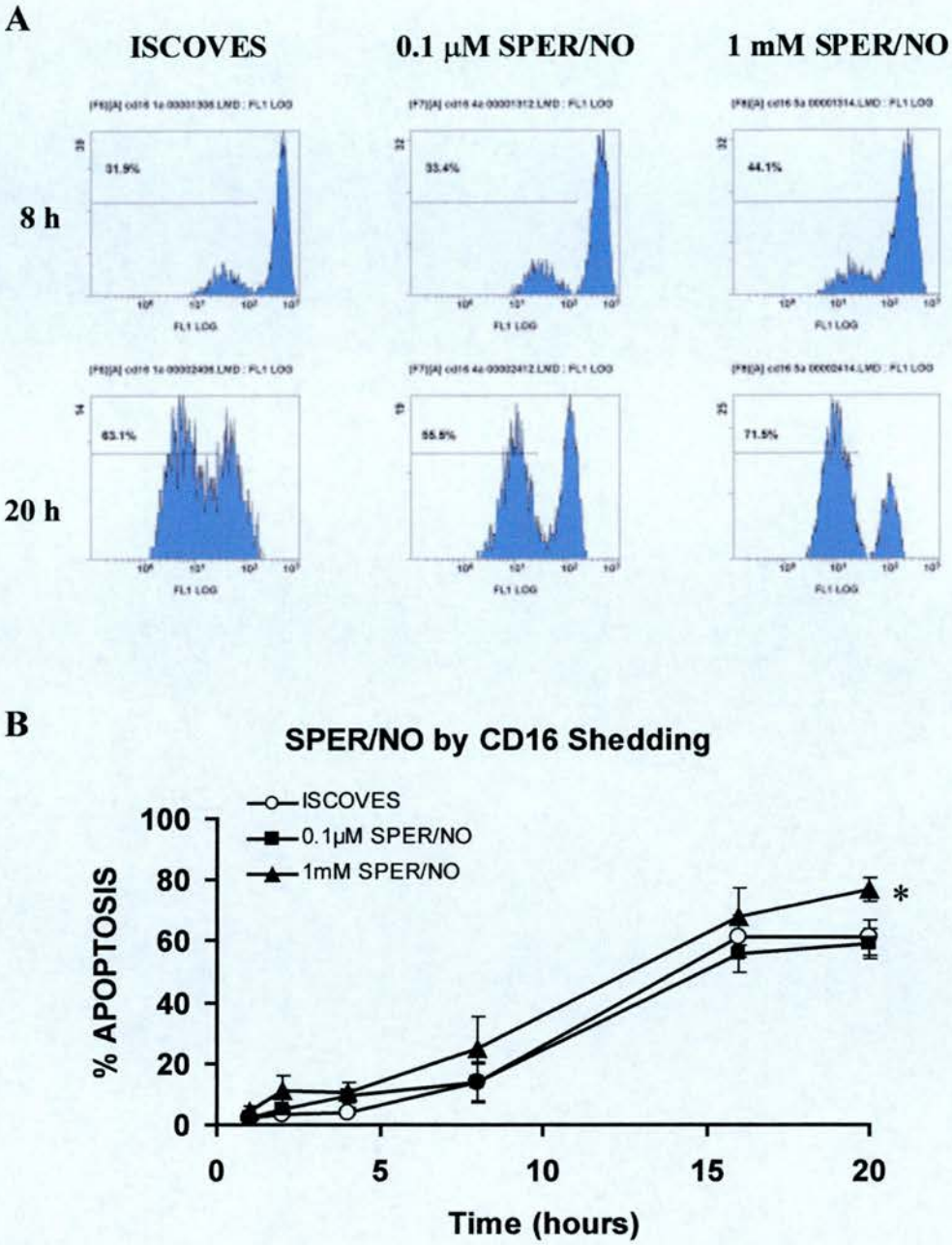


Figure 4.11 Time course studies for effects of SPER/NO on neutrophil apoptosis assessed by CD16 shedding. Neutrophils (4.5 x 10^6 cells/ml) were incubated in Iscove's MDM supplemented with 10 % autologous serum for 1 - 20 hours in the absence or presence of SPER/NO (0.1 μM or 1 mM), then apoptosis was assessed by CD16 shedding (A) Typical flow cytometry traces showing percentage CD16 shedding in neutrophils after 8 and 20 hours (B) Graph showing changes in CD16 shedding over time in control cells or neutrophils treated with SPER/NO. Results represent mean ± SEM from n = 3 - 6 experiments. Asterisks represent significant ($p < 0.05$) difference from control (open symbols) time course by two-way ANOVA.

Annexin V binding studies showed a small inhibition of apoptosis by 0.1 μM SPER/NO by 16 h (48.1 ± 4.5% vs. 54.1 ± 4.3 % control), which was not much more apparent, but was significant, by 20 h (60.1 ± 1.9 % vs. 67.9 ± 2.7 %; Figure 4.10B). Overall, the time course in 0.1 μM SPER/NO-treated neutrophils was not significantly different from that seen in control cells ($p > 0.05$, 2-way ANOVA), but at the 20-h time point a significant difference was seen ($p < 0.05$, two-tailed paired *t* test). Flow cytometry histograms show virtually no difference in PS exposure at 8 h, but an apparent inhibition by 20 h (Figure 4.10A).

No significant inhibition was observed using CD16 shedding as a measure of apoptosis ($p > 0.05$, two-way ANOVA), although a general inhibitory tend was visible, with SPER/NO-treated neutrophils showing 59.1 ± 5.2 % apoptosis after 20 h and control cells 61.2 ± 5.9 % (Figure 4.11B). The examples of flow cytometry histograms show no inhibition at 8 h but some inhibition at 20 h by this technique (Figure 4.11A).

Inhibition measured by PI intercalation showed the clearest evidence of inhibition, becoming apparent after 16 h (43.5 ± 7.5 % vs. 49.0 ± 7.0 % control) and reaching levels of 50.5 ± 10.2 % by 20 h, compared to 61.5 ± 9.8 % in control cells, although this data did not reach significance ($p > 0.05$, two-way ANOVA; Figure 4.12B). However, assessment of the 20-h time point vs. control by two-tailed paired *t* test showed a significant difference ($p < 0.05$). Flow cytometry histograms shown in Figure 4.12A demonstrate no difference in DNA fragmentation from control in 0.1 μM SPER/NO-treated cells at 8 h but a marked inhibition at 20 h.

The induction of neutrophil apoptosis initiated by 1 mM SPER/NO occurred at a slower rate than with an equivalent concentration of DEA/NO. Morphologically, increased apoptosis was observed after 16 h, with 66.4 ± 3.9 % of cells having undergone apoptosis compared to 54.0 ± 8.3 % apoptosis detectable in control cells. This effect was more pronounced by 20 h, at which time 89.5 ± 5.1 % of SPER/NO-treated cells were apoptotic vs. 53.1 ± 2.8 % control (Figure 4.9). The overall time course was found to be significantly different from control ($p < 0.01$, two-way

ANOVA).

PS exposure was again the least obvious event of apoptosis in NO-treated cells, with an increase in apoptosis only seen at 20 h (82.0 ± 2.7 % vs. 67.9 ± 2.7 % control; Figure 4.10B). The time course was found not to be significantly different from control over the 20 h ($p > 0.05$, 2-way ANOVA), but results obtained at the 20-h time point were significantly different from control ($p < 0.05$, two-tailed paired *t* test). This phenomenon is clearly shown in the examples of flow cytometry histograms, in which no difference from control is visible at 8 h, but a marked increase is evident at 20 h (Figure 4.10A).

A general pro-apoptotic trend for 1 mM SPER/NO was achieved when apoptosis was measured by CD16 shedding, reaching levels of 76.6 ± 3.8 % CD16 shedding compared to control levels of 61.2 ± 5.9 % at 20 h ($p < 0.05$, two-way ANOVA; Figure 4.11B). The leftward shift in fluorescence in cells exposed to 1 mM SPER/NO compared to control can be seen at both 8 and 20 h in the examples of flow cytometry histograms shown in Figure 4.11A.

However, unexpected results were obtained with 1 mM SPER/NO when apoptosis was assessed by PI intercalation. Figure 4.12B shows that internucleosomal DNA fragmentation was decreased compared to control throughout the 20-h time course, with levels of hypodiploid cells measured being remarkably similar to those observed with 0.1 μM SPER/NO. At the 20-h time point, control levels of apoptosis were 61.5 ± 9.8 %, whereas the percentage of cells with hypodiploid DNA content in SPER/NO-treated cells was 48.5 ± 13.5 % (Figure 4.12B). However, although this inhibition was clearly visible, it was not statistically significant ($p > 0.05$, two-way ANOVA). Flow cytometry histograms shown in Figure 4.12A illustrate the inhibition of DNA fragmentation by 1mM SPER/NO, which is particularly prominent at 8 h, although this effect was not seen on every replication of the experiment.

Concentrations of 30 and 100 μM were used to study the time-course of induction of neutrophil apoptosis by the $ONOO^-$ donor, GEA 3162, as these

concentration were shown to be effective at inducing apoptosis in the concentration-response studies. Morphological studies of apoptosis showed a concentration-dependent increase in the percentage of cells displaying the characteristic changes of apoptosis compared to control cells. With the lower concentration of GEA 3162, increased apoptosis from control was apparent at 8 h culture and beyond, with levels of apoptosis measured as 27.5 ± 12.5 % vs. 9.8 ± 6.4 % control at 8 h, rising to 67.5 ± 6.2 % vs. 53.1 ± 2.8 % control by 20 h ($p < 0.05$, two-way ANOVA). However, acceleration of apoptosis was visible by 4 h with 100 μM GEA 3162 (20.6 ± 11.9 % compared to 1.0 ± 0.1 % control). This effect reached a maximum of 93.6 ± 3.0 % vs. 53.1 ± 2.8 % control by the end of the culture period ($p < 0.0001$, two-way ANOVA; Figure 4.13).

As observed in the concentration-response studies, assessment of apoptosis by Annexin V binding showed no significant difference of GEA 3162-treated neutrophils from control cells at 20 h ($p > 0.05$, two-way ANOVA), for both concentrations of GEA 3162. However, increased PS exposure was observed at earlier time points with 100 μM GEA 3162, although this was a small effect compared to that observed by morphology; the pro-apoptotic effect was most clearly visible at 4 h, when 12.8 ± 9.7 % of cells had exposed PS, compared to 1.9 ± 0.5 % in control cells (Figure 4.14B). The enhancement of PS exposure at early time points followed by lack of effects at later time points is clearly shown in the flow cytometry histograms shown in Figure 4.14A, in which increased fluorescence is seen in cells treated with both 30 and 100 μM GEA 3162 at 8 h, but virtually identical histograms to control are seen after 20 h culture.

Once again, studies investigating the extent of CD16 shedding showed a good correlation with morphological observations of apoptosis. The increase in CD16 shedding compared to control was evident after 4 h culture, was maintained throughout the culture period, and was clearly concentration-dependent ($p < 0.05$, two-way ANOVA, for 30 mM GEA 3162; $p < 0.0001$, two-way ANOVA, for 100 μM GEA 3162). At the 4-h time point, control apoptosis was 4.1 ± 0.9 %, but apoptosis measured in neutrophils exposed to 30 μM GEA 3162 was 10.8 ± 6.0

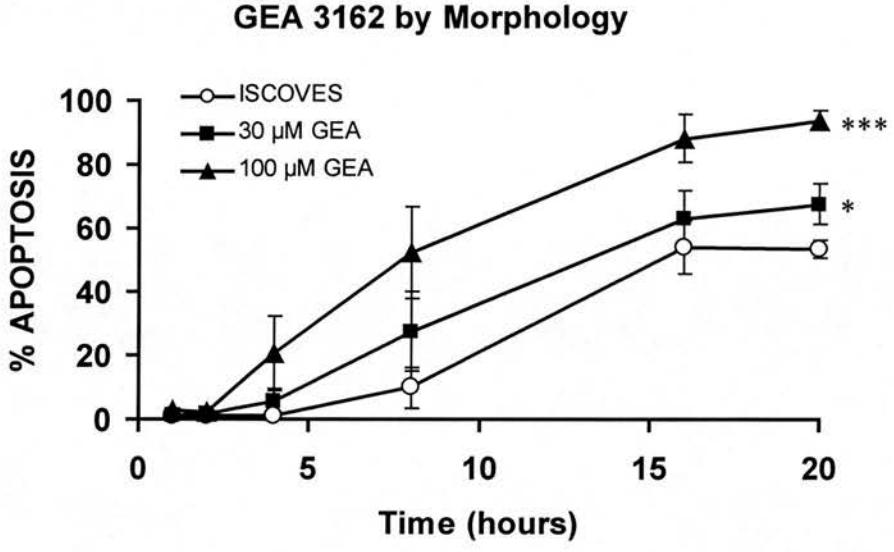


Figure 4.13 Time course studies for effects of GEA 3162 on neutrophil apoptosis assessed by morphology. Neutrophils (4.5×10^6 cells/ml) were incubated in Iscove's MDM supplemented with 10 % autologous serum for 1 - 20 hours in the absence or presence of GEA 3162 (30 or 100 μM), then apoptosis was assessed by morphology. Results represent mean ± SEM from n = 3 - 6 experiments. Asterisks represent significant (* $p < 0.05$; *** $p < 0.0001$) difference from control (open symbols) time course by two-way ANOVA.

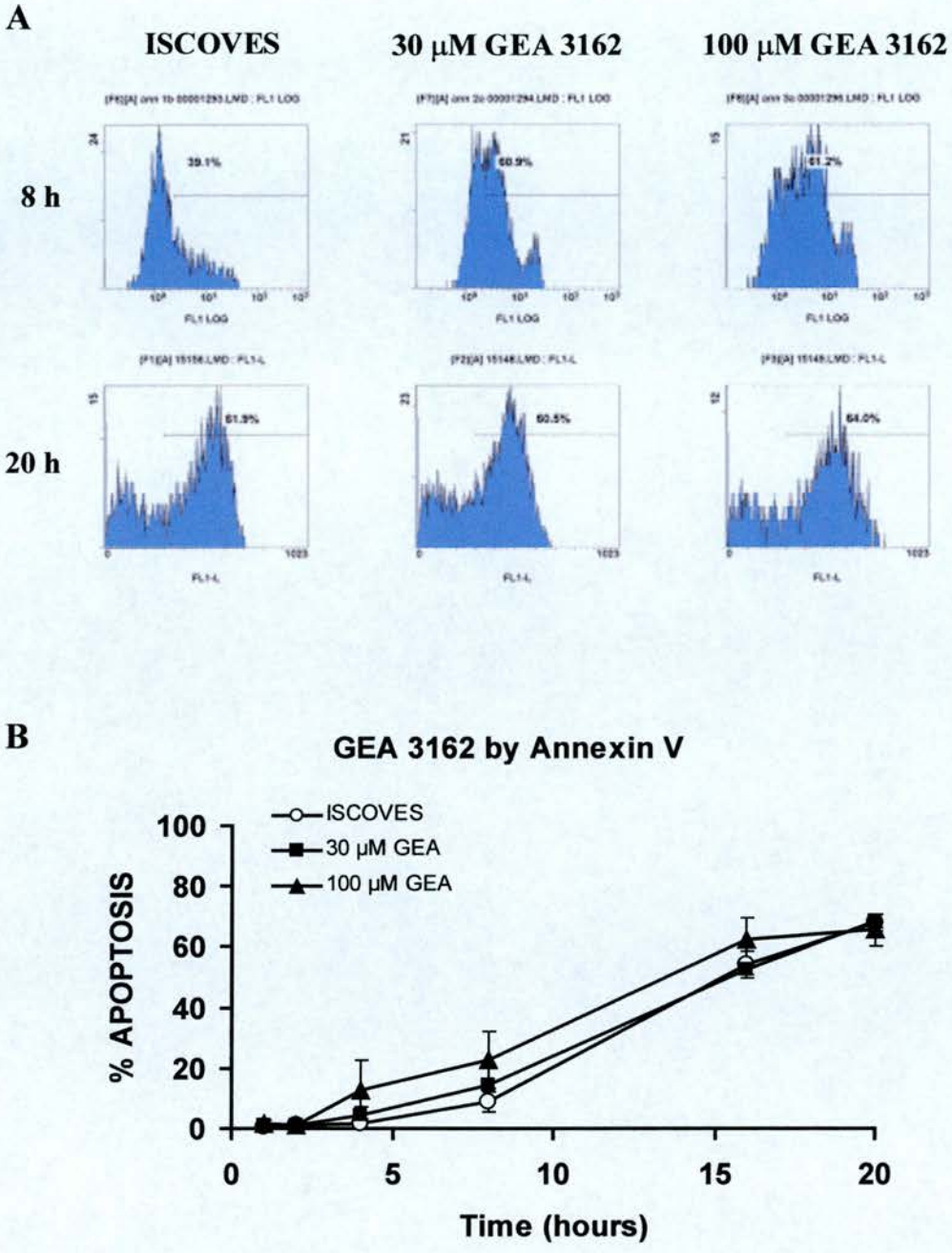


Figure 4.14 Time course studies for effects of GEA 3162 on neutrophil apoptosis assessed by Annexin V binding. Neutrophils (4.5 x 10^6 cells/ml) were incubated in Iscove's MDM supplemented with 10 % autologous serum for 1 - 20 hours in the absence or presence of GEA 3162 (30 or 100 μM), then apoptosis was assessed by Annexin V binding (A) Typical flow cytometry traces showing percentage Annexin V binding in neutrophils after 8 and 20 hours (B) Graph showing changes in PS exposure over time in control cells or neutrophils treated with GEA 3162. Results represent mean ± SEM from n = 3 - 6 experiments. Data did not reach statistical significance.

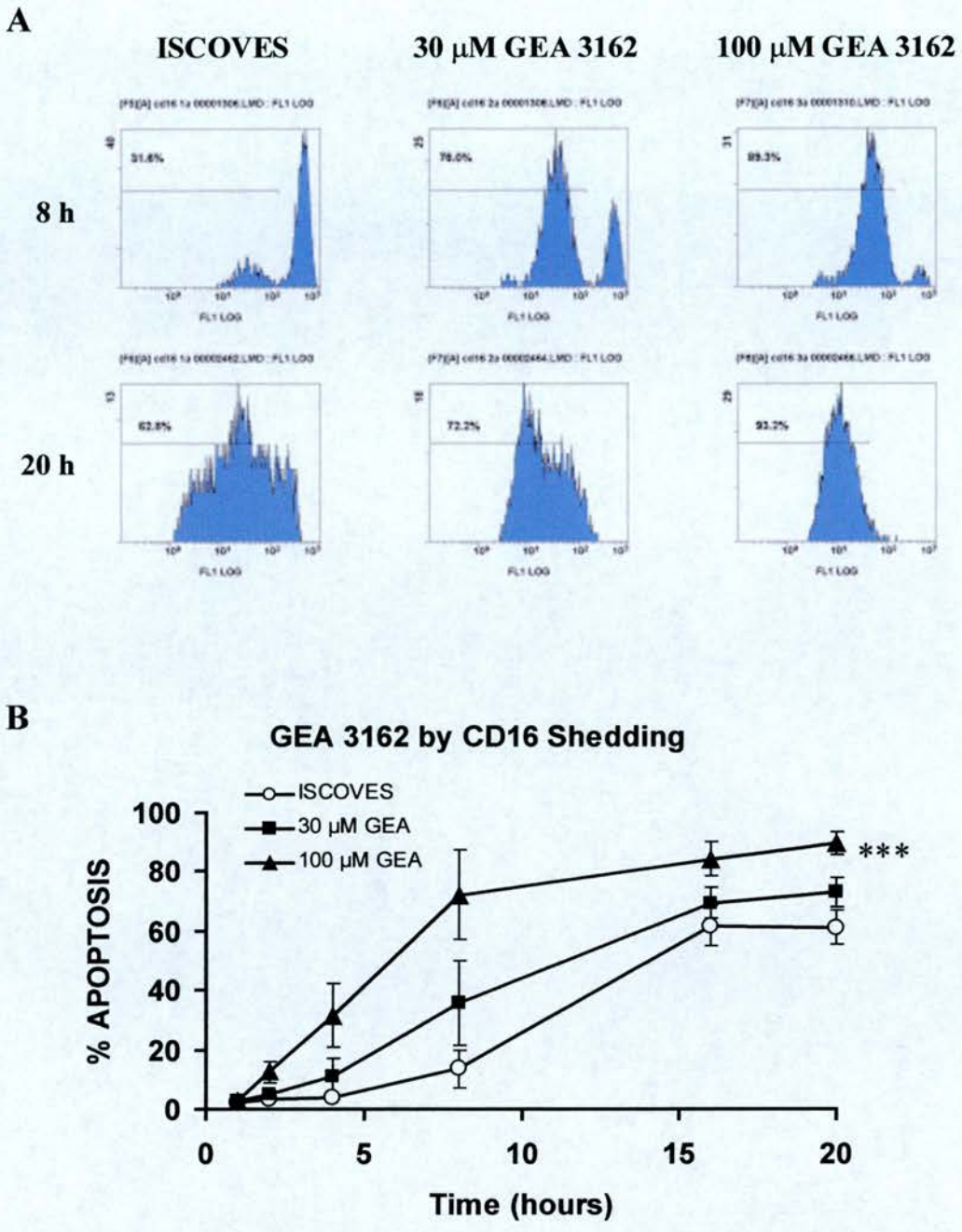


Figure 4.15 Time course studies for effects of GEA 3162 on neutrophil apoptosis assessed by CD16 shedding. Neutrophils (4.5 x 10^6 cells/ml) were incubated in Iscove's MDM supplemented with 10 % autologous serum for 1 - 20 hours in the absence or presence of GEA 3162 (30 or 100 μM), then apoptosis was assessed by CD16 shedding (A) Typical flow cytometry traces showing percentage CD16 shedding in neutrophils after 8 and 20 hours (B) Graph showing changes in CD16 shedding over time in control cells or neutrophils treated with GEA 3162. Results represent mean ± SEM from n = 3 - 6 experiments. Asterisks represent significant (* $p < 0.05$; *** $p < 0.0001$) difference from control (open symbols) by two-way ANOVA.

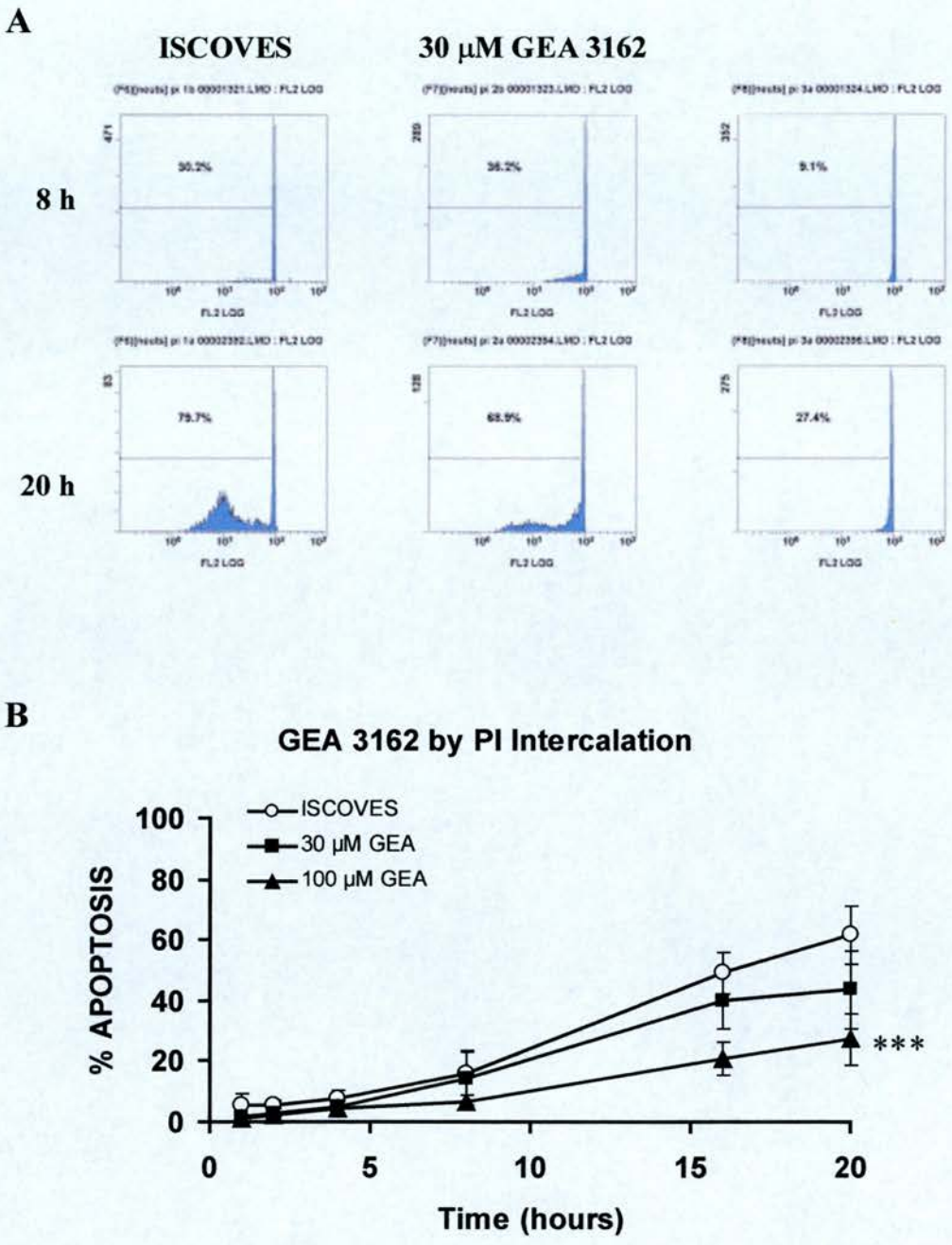


Figure 4.16 Time course studies for effects of GEA 3162 on neutrophil apoptosis assessed by PI intercalation. Neutrophils (4.5 x 10^6 cells/ml) were incubated in Iscove's MDM supplemented with 10 % autologous serum for 1 - 20 hours in the absence or presence of GEA 3162 (30 or 100 μM), then apoptosis was assessed by PI intercalation (A) Typical flow cytometry traces showing percentage hypodiploid cells in neutrophils after 8 and 20 hours (B) Graph showing changes in PI intercalation over time in control cells or neutrophils treated with GEA 3162. Results represent mean ± SEM from n = 3 - 6 experiments. Asterisks represent significant ($p < 0.0001$) difference from control (open symbols) time course by two-way ANOVA.

%, and that in cells treated with 100 μM of this compound was 31.5 ± 10.8 %. Differences from control levels of apoptosis were highest at the 8 h time point, when 30 μM GEA 3162 produced 35.6 ± 14.4 and 100 μM produced 72.0 ± 15.1 % apoptosis, whereas control apoptosis was just 13.6 ± 6.4 %. By 20 h, the induction of apoptosis was less marked; 73.2 ± 4.9 % and 89.5 ± 3.7 % apoptosis was achieved by 30 and 100 μM GEA 3162 respectively and untreated cells showed 61.2 ± 5.9 % CD16 shedding (Figure 4.15B). These effects are demonstrated in the typical flow cytometry histograms, in which GEA 3162 produces a concentration-dependent leftward shift in fluorescence, representing the shedding of CD16 from the cell surface, and this effect is more pronounced at 8 h than at 20 h (Figure 4.15A).

Interestingly, a paradoxical concentration-dependent inhibition of internucleosomal DNA fragmentation by GEA 3162 was observed by PI intercalation, which was more pronounced than that seen with SPER/NO. With 30 μM GEA 3162, the percentage of hypodiploid cells followed that seen in control cells closely until the 16 h time point when inhibition became apparent (40.1 ± 9.5 % vs. 49.0 ± 7.0 % control) and reached a maximum after 20 h (43.6 ± 12.9; Figure 4.16B). Although this time course failed to reach significance from control ($p > 0.05$, two-way ANOVA), the extent of DNA fragmentation in response to 30 mM GEA 3162 was significantly different from control ($p < 0.05$, two-tailed paired *t* test).

The inhibition seen with 100 μM GEA 3162 became evident after 8 h (6.5 ± 2.2 % compared to 15.7 ± 7.0 % in control cells), and by 20 h was 27.1 ± 8.5 %, whereas DNA fragmentation in control cells was 61.5 ± 9.8 %. The overall time course in response to 100 mM GEA 3162 was significantly different from control ($p < 0.0001$, two-way ANOVA; Figure 4.16B). The examples of flow cytometry histograms in Figure 4.16A show inhibition of internucleosomal DNA fragmentation by 100 μM GEA 3162 only at 8 h and both 30 and 100 μM GEA 3162 at 20 h.

4.2.4 Effects of the $ONOO^-$ donor, SIN-1, on neutrophil apoptosis

In order to test whether the effect seen with GEA 3162 is a general $ONOO^-$ effect or is specific to GEA 3162 itself, the known $ONOO^-$ donor, SIN-1 (1 - 3 mM) was used at a 20-h time point. A concentration-dependent enhancement of neutrophil apoptosis was seen in the presence of SIN-1; at 20 h, 1 mM of this compound caused 64.5 ± 7.4 % morphological apoptosis and 3 mM caused 74.9 ± 8.3 %, compared to control levels of 33.7 ± 4.3 % ($p < 0.05$, two-way ANOVA with Bonferroni post-hoc test). Addition of SOD (50 U/ml) reversed this effect, taking the level of apoptosis observed with 1 mM SIN-1 back down to 47.7 ± 4.1 % and that seen with 3 mM to 45.0 ± 2.9 % ($p > 0.05$, two-way ANOVA with Bonferroni post-hoc test; Figure 4.17).

Measurement of apoptosis by Annexin V binding produced the same results as seen by morphology; 1 mM SIN-1 produced 58.9 ± 5.9 % PS exposure and 3 mM produced 62.9 ± 1.8 %, compared to 30.2 ± 4.2 % control ($p < 0.05$, two-way ANOVA with Bonferroni post-hoc test). Again, this induction of apoptosis was reversible by 50 U/ml SOD, bringing apoptosis induced by 1 mM and 3 mM SIN-1 down to 38.6 ± 3.1 % and 44.9 ± 5.8 % respectively ($p > 0.05$ vs. untreated cells, two-way ANOVA with Bonferroni post-hoc test; Figure 4.18B). Typical flow cytometry histograms for assessment of apoptosis by Annexin V binding are shown in Figure 4.18A.

Similarly, assessment of apoptosis by CD16 shedding showed the same effects of SIN-1 on neutrophil apoptosis. Exposure of neutrophils to 1 mM SIN-1 gave 63.8 ± 5.3 % apoptosis and to 3 mM SIN-1 gave 74.5 ± 7.8 %, whereas control apoptosis as measured by this technique was 35.2 ± 3.2 % ($p < 0.05$, two-way ANOVA with Bonferroni post-hoc test). Once again, the presence of 50 U/ml SOD reversed the increased apoptosis observed with SIN-1; apoptosis with 1 mM and 3 mM SIN-1 in the presence of SOD was 44.4 ± 4.2 % and 41.8 ± 3.4 % respectively ($p > 0.05$ vs. control, two-way ANOVA with Bonferroni post-hoc test; Figure 4.19B). Flow cytometry histograms shown in Figure 4.19A demonstrate this effect.

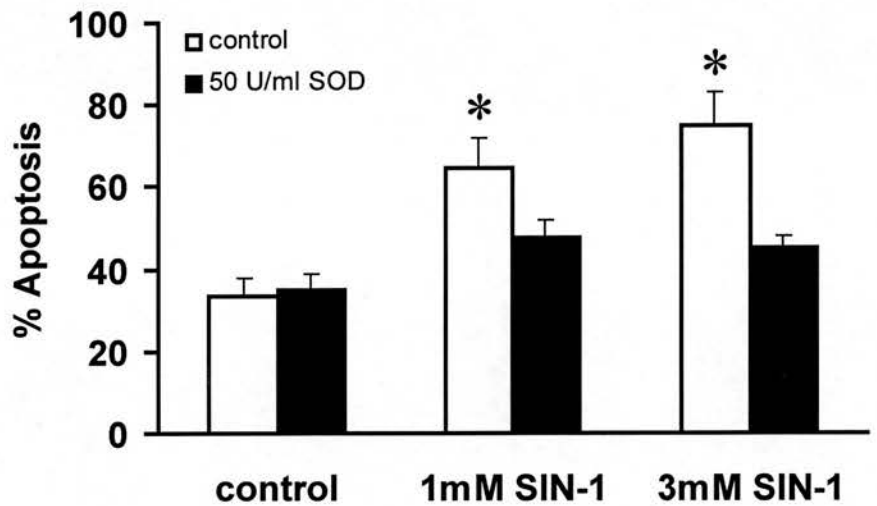


Figure 4.17 Effect of SIN-1 on morphological apoptosis in human neutrophils. Neutrophils (4.5 x 10^6 cells/ml) were incubated in Iscove's MDM supplemented with 10 % autologous serum for 20 hours in the absence or presence of SIN-1 (1 or 3 mM) and the absence or presence of SOD (50 U/ml), then apoptosis was assessed by morphology. Results represent mean ± SEM from n = 5 experiments. Two-way ANOVA revealed significant ($p < 0.05$) differences between treatment groups, and asterisks represent significant ($p < 0.05$) difference from control (without SOD) by Bonferroni post-hoc test.

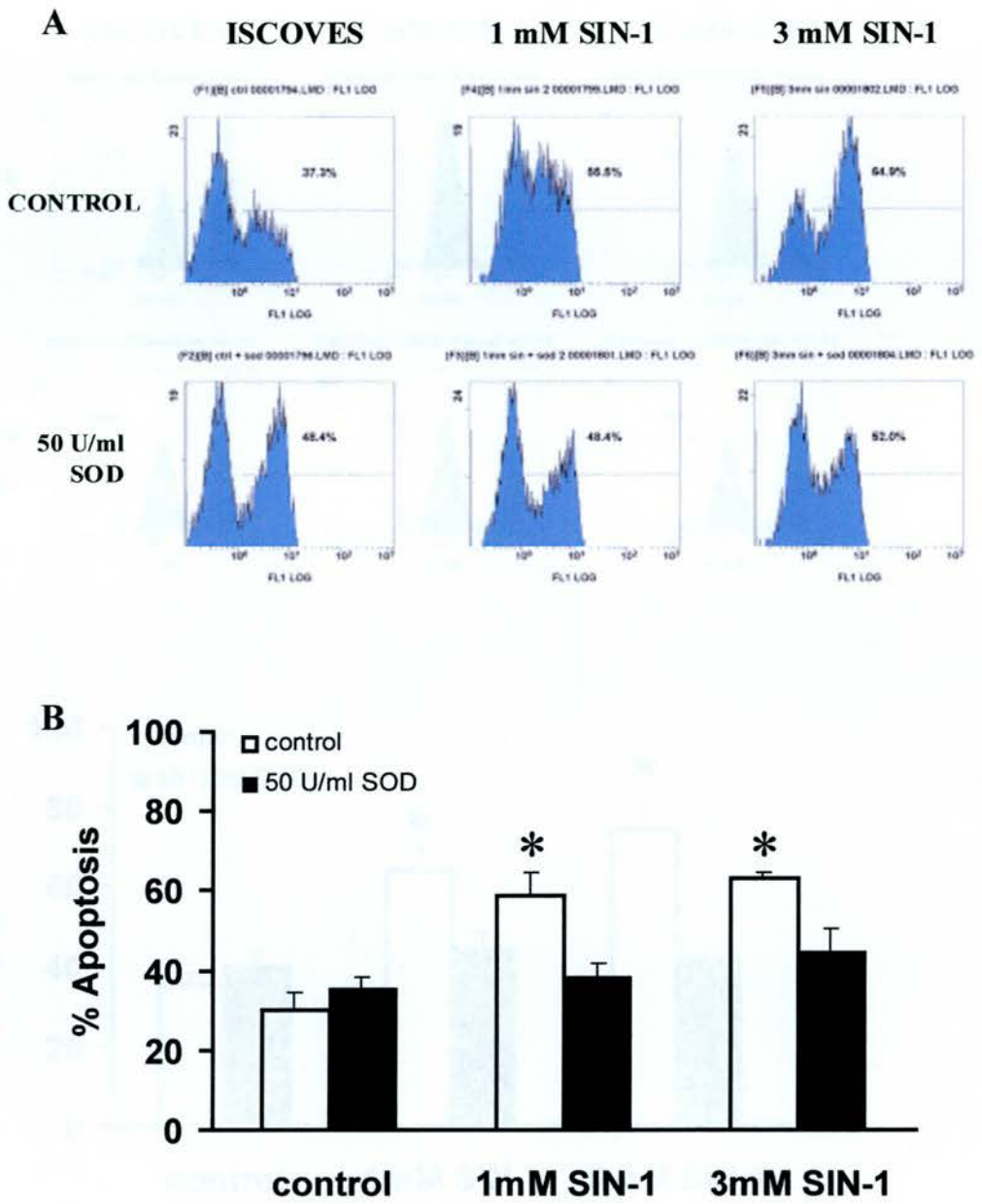


Figure 4.18 Effect of SIN-1 on Annexin V binding in human neutrophils. Neutrophils (4.5 x 10^6 cells/ml) were incubated in Iscove's MDM supplemented with 10 % autologous serum for 20 hours in the absence or presence of SIN-1 (1 or 3 mM) and the absence or presence of SOD (50 U/ml), then apoptosis was assessed by Annexin V binding (A) Typical flow cytometry traces showing percentage PS exposure in neutrophils after 20 hours (B) Graph showing changes in PS exposure in control cells or neutrophils treated with SIN-1 ± SOD. Results represent mean ± SEM from n = 5 experiments. Two-way ANOVA revealed significant ($p < 0.05$) differences between treatment groups, and asterisks represent significant ($p < 0.05$) difference from control (without SOD) by Bonferroni post-hoc test.

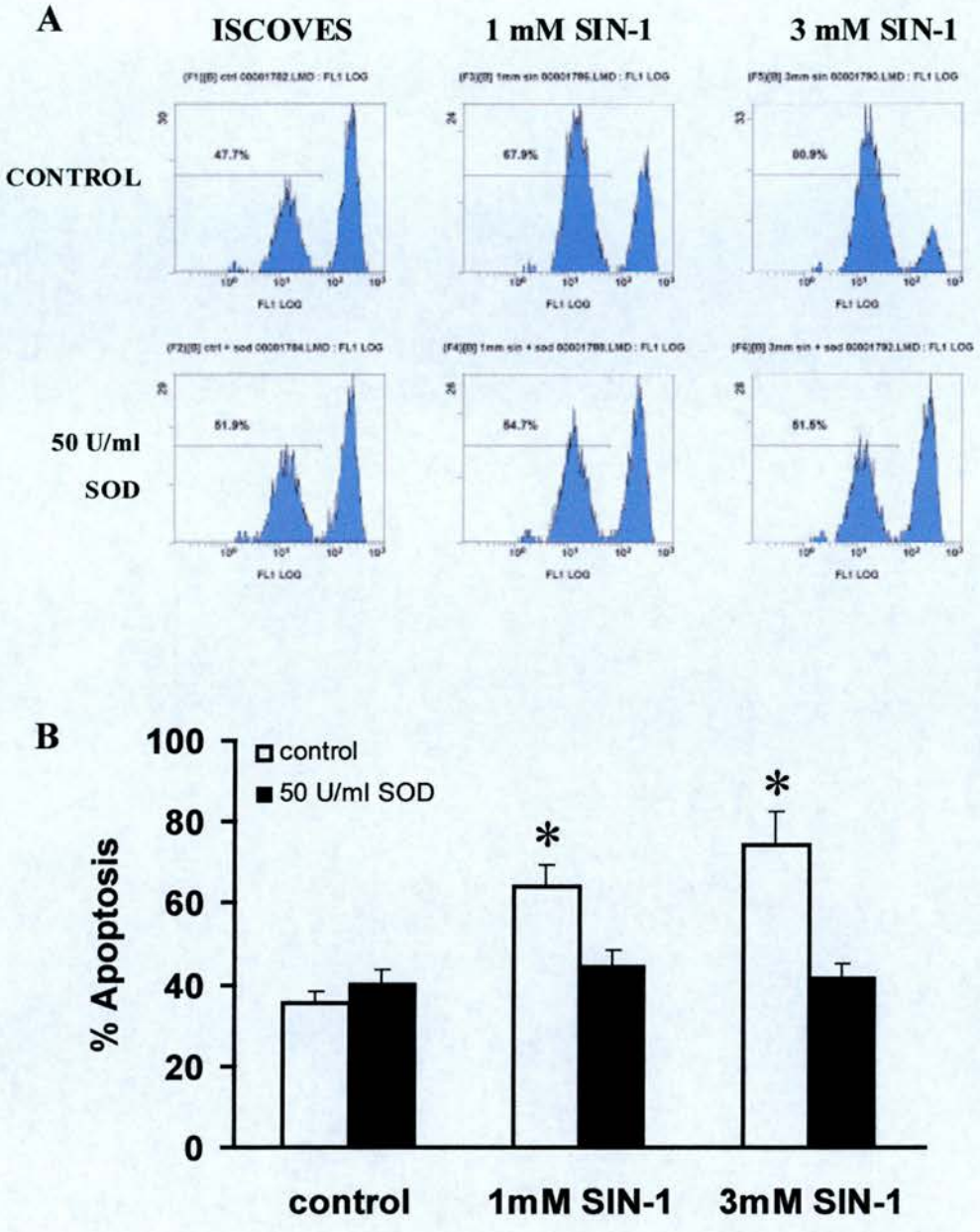


Figure 4.19 Effect of SIN-1 on CD16 shedding in human neutrophils. Neutrophils (4.5×10^6 cells/ml) were incubated in Iscove's MDM supplemented with 10 % autologous serum for 20 hours in the absence or presence of SIN-1 (1 or 3 mM) and the absence or presence of SOD (50 U/ml), then apoptosis was assessed by CD16 shedding (A) Typical flow cytometry traces showing percentage CD16 shedding in neutrophils after 20 hours (B) Graph showing changes in CD16 shedding in control cells or neutrophils treated with SIN-1 ± SOD. Results represent mean ± SEM from n = 5 experiments. Two-way ANOVA revealed significant ($p < 0.05$) differences between treatment groups, and asterisks represent significant ($p < 0.05$) difference from control (without SOD) by Bonferroni post-hoc test.

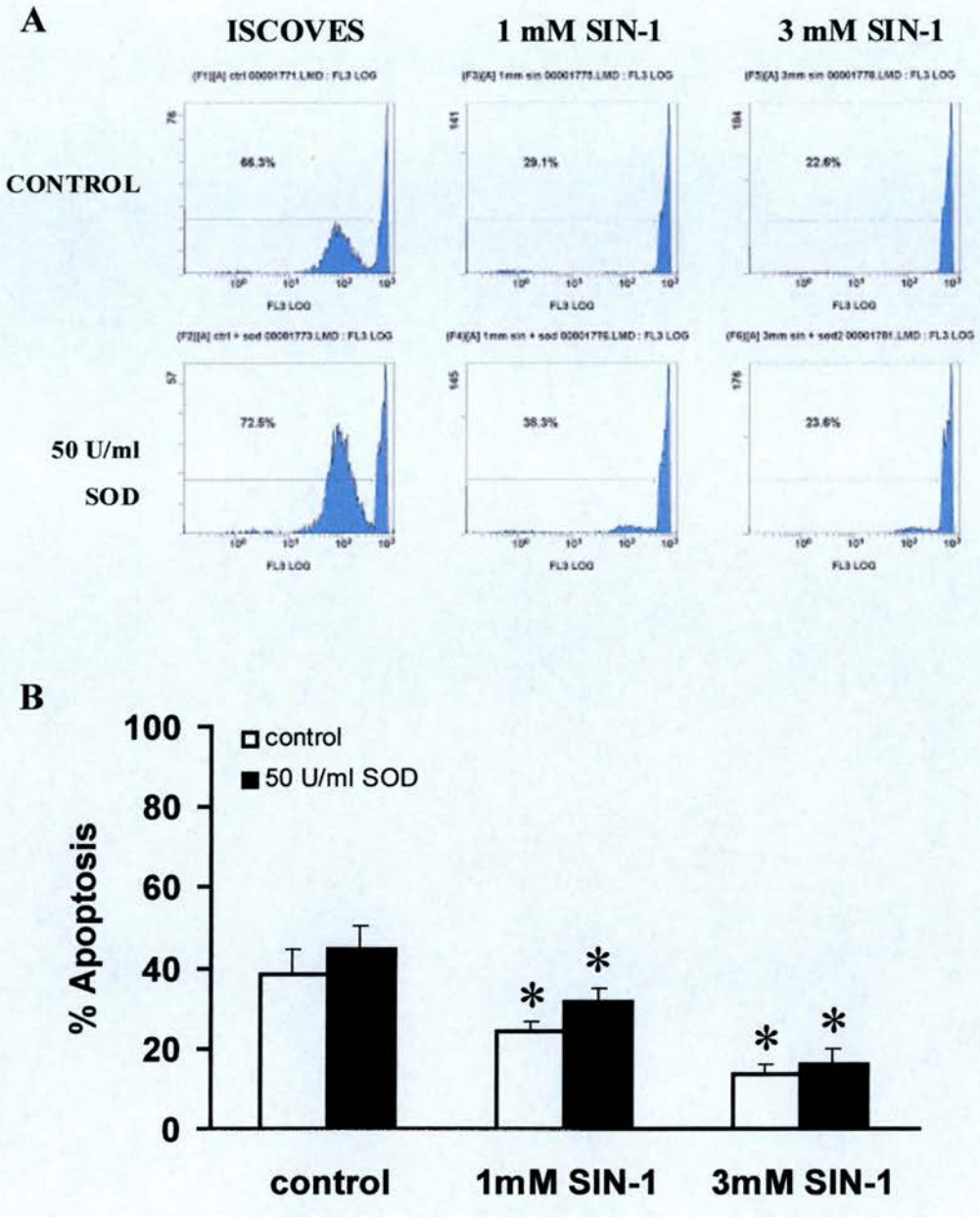


Figure 4.20 Effect of SIN-1 on PI intercalation in human neutrophils. Neutrophils (4.5×10^6 cells/ml) were incubated in Iscove's MDM supplemented with 10 % autologous serum for 20 hours in the absence or presence of SIN-1 (1 or 3 mM) and the absence or presence of SOD (50 U/ml), then apoptosis was assessed by PI intercalation (A) Typical flow cytometry traces showing percentage hypodiploid neutrophils after 20 hours (B) Graph showing changes in PI intercalation in control cells or neutrophils treated with SIN-1 ± SOD. Results represent mean ± SEM from n = 5 experiments. Two-way ANOVA revealed significant ($p < 0.05$) differences between treatment groups, and asterisks represent significant ($p < 0.05$) difference from control (without SOD) by Bonferroni post-hoc test.

Results obtained using PI intercalation as a measure of apoptosis provided the most interesting observations; SIN-1 had the same inhibitory effect as GEA 3162 on internucleosomal DNA fragmentation. A concentration-dependent decrease in the percentage of hypodiploid cells was seen with SIN-1; 24.4 ± 2.4 % and 13.8 ± 2.3 % apoptosis was seen with 1 mM and 3 mM of this compound respectively, compared to 38.5 ± 6.1 % in untreated cells ($p < 0.05$ two-way ANOVA with Bonferroni post-hoc test). However, unlike other indices of apoptosis, the presence of SOD failed to fully reverse the effects of SIN-1. In the presence of SOD (50 U/ml), apoptosis levels were 31.3 ± 3.7 % and 15.8 ± 4.2 % with 1 mM and 3 mM SIN-1 respectively (Figure 4.20), and the inhibition of DNA fragmentation remained significantly different from control ($p < 0.05$, two-way ANOVA with Bonferroni post-hoc test).

4.3 Discussion

These experiments were carried out in order to compare the effects of pure NO donors and $ONOO^-$ donors on neutrophil apoptosis and to examine the rates at which these effects occur. Firstly, concentration-response studies were conducted to examine how effective these compounds were at modifying the rate of neutrophil apoptosis. Time course studies were then carried out to compare the rate at which these compounds exerted their effects, and to determine whether NO and $ONOO^-$ have different mechanisms of action. These studies were carried out using morphology, Annexin V binding, CD16 shedding and propidium iodide (PI) intercalation, as measures of apoptosis in order to determine whether events associated with apoptosis proceed at different rates or simultaneously.

The dichotomous effects of NO on apoptosis are demonstrated in these studies through the use of the diazeniumdiolate compounds, DEA/NO and SPER/NO. Low concentrations of these compounds inhibited the rate of neutrophil apoptosis (0.1 - 30 μM for DEA/NO and 0.1 - 3 μM for SPER/NO), as assessed by morphology. Although several previous studies have demonstrated pro-apoptotic effects of NO on neutrophils, this is the first time that an inhibitory effect has been

shown. However, although significant, the inhibitory effect of these compounds (particularly DEA/NO) is minimal, both in terms of the extent of inhibition (< 10%) and the rate at which these effects are observed (16 h and later). This is especially true when compared to the extent of inhibition observed with other inhibitors of neutrophil apoptosis such as db-cAMP, LPS and GM-CSF. Overall, DEA/NO is relatively inactive as an inhibitor of neutrophil apoptosis, and SPER/NO only slightly more active. Both compounds produce a much more profound accelerating effect on neutrophil apoptosis and are therefore more active in inducing apoptosis at higher concentrations than they are at inhibiting this process.

Time-course studies failed to demonstrate a significant difference from control by two-way ANOVA for either of these compounds by any of the techniques used. However, this may be due to the delayed effects of these compounds, with inhibition not being observed until 16 h or later. Indeed, statistical analysis of the 20-h time points (two-tailed paired *t* test) demonstrated a significant ($p < 0.05$) difference between drug-treated and control cells at this time point for the majority of techniques.

In contrast, neutrophil apoptosis was promoted by high concentrations of these compounds (1 mM for DEA/NO and 300 μM – 1 mM for SPER/NO, as assessed by morphology). This concurs with previous reports of biphasic actions of NO. It has previously been proposed that low NO concentrations are likely to protect cells from apoptosis, whereas higher concentrations are pro-apoptotic (Dimmeler and Zeiher, 1997; Kim *et al.*, 1999). The induction of apoptosis by high concentrations of DEA/NO occurs much more rapidly than the inhibition seen with low concentrations, suggesting that the differential effects of this compound do not represent opposing effects on the same pathway. This is in agreement with the published literature; protection from apoptosis by NO has been reported to be mediated by cGMP in eosinophils (Beauvais *et al.*, 1995), B lymphocytes (Genaro *et al.*, 1995), hepatocytes (Kim *et al.*, 1997), neural cells (Fiscus, 2002), astrocytes (Takuma *et al.*, 2002), monocytic cells (Barsacchi *et al.*, 2002), lymphoblastoid cells (Gomes *et al.*, 2002), keratinocytes (Weller *et al.*, 2003) and trophoblast cells (Dash *et al.*, 2003), whereas promotion of apoptosis has been shown to be cGMP-

independent in smooth muscle cells (Nishio *et al.*, 1996), endothelial cells (Shen *et al.*, 1998), neutrophils (Ward *et al.*, 2000) and eosinophils (Zhang *et al.*, 2003b). It is possible that low concentrations stimulate the formation of cGMP, which mediates anti-apoptotic actions of NO, but that higher concentrations initiate alternative pro-apoptotic pathways that override the cGMP effect and drive the cells into programmed cell death.

This hypothesis is supported by observations in astrocytes, in which a biphasic effect of the NO donor, [N-ethyl-2-(1-ethyl-hydroxy-2-nitrosohydrazino)-ethanamine] (NOC12), on apoptosis was seen. Although inhibition of cGMP abolished the protective effects of low concentrations in this cell type, it exacerbated the toxic effects of higher concentrations, suggesting that cGMP activation partially abrogates the pro-apoptotic effects of NO (Takuma *et al.*, 2002). Additionally, cGMP-mediated activation of JNK by NO has been implicated as having a role in delaying and reducing the extent of NO-induced apoptosis in cardiac myocytes (Andreka *et al.*, 2001), and sGC activation by NO has also been implicated in limiting NO-induced damage in neural cells (Fiscus, 2002). On the other hand, both inhibition and induction of neutrophil apoptosis achieved using 0.1 μM and 1 mM SPER/NO respectively were only visible at time points of 16 h and beyond by most techniques. Therefore, the pro- and anti-apoptotic effects of SPER/NO may reflect contrasting actions on the same pathway.

The slower effects of the diazeniumdiolates do not become apparent until 16 h of culture, therefore it is possible that these effects are mediated through regulation of gene expression, as the NO released from these compounds would not persist until these times in culture, particularly in the case of the fast-releasing diazeniumdiolate, DEA/NO. This is also true of the pro-apoptotic actions of 1 mM SPER/NO. However, the fast effects of 1 mM DEA/NO are seen by 4 h, making it possible that the induction of apoptosis by this compound is independent of gene transcription.

The rate at which NO is released from the diazeniumdiolates affects the pro-apoptotic actions of these compounds; fast release of NO from DEA/NO (from which NO generation is likely to be complete in under 1 h) causes a rapid induction

of apoptosis, whereas the more sustained release of lower concentrations of NO from the same concentration of SPER/NO (approximately a 12-h duration of NO release) promotes apoptosis at a much slower rate.

In contrast, the rate of NO release does not influence the rate at which the inhibition of neutrophil apoptosis occurs, with 0.1 μM of both DEA/NO and SPER/NO producing inhibition of apoptosis at 16 h and later. However, the slower NO release from 0.1 μM SPER/NO is more effective at inhibiting neutrophil apoptosis than the rapid burst of NO from 0.1 μM DEA/NO. The inhibition of apoptosis at 20 h is more pronounced with SPER/NO than with DEA/NO; a difference of only 5.9 % from control morphological apoptosis is achieved with 0.1 μM DEA/NO, whereas a difference of 10.2 % apoptosis is seen with the same concentration of SPER/NO, showing that SPER/NO is a slightly more effective inhibitor of neutrophil apoptosis. On the other hand, an inhibitory effect of DEA/NO is observed with higher concentrations of DEA/NO; with this compound, inhibition of morphological apoptosis is seen with concentrations of 30 μM and below, whereas with SPER/NO, the concentration has to go down to 3 μM before a statistically significant difference from control is seen.

The induction of apoptosis by the diazeniumdiolates occurs at lower concentrations of SPER/NO than DEA/NO; a significant difference was seen with 300 μM of this compound compared to 1 mM with DEA/NO. However, 1 mM DEA/NO produced 100 % apoptosis of neutrophils by 20 h, whereas the same concentration of SPER/NO only gave 89.5 % apoptosis. The time course of the induction of apoptosis by 1 mM DEA/NO is more highly significant than that seen with 1 mM SPER/NO for apoptosis measured by morphology ($p < 0.0001$ vs. $p < 0.01$) and CD16 shedding ($p < 0.0001$ vs. $p < 0.05$). Therefore, very high concentrations of DEA/NO have a more effective pro-apoptotic effect than seen with SPER/NO and a large burst of NO is marginally more effective than chronic exposure to NO.

In contrast to the 'pure' NO donors, $ONOO^-$ released from GEA 3162 did not inhibit neutrophil apoptosis, even at low concentrations, suggesting that this effect of

NO is not shared by $ONOO^-$. However, GEA 3162 induced apoptosis at concentrations lower than with the diazeniumdiolates (30 μM and above), and this effect was concentration-dependent, as results obtained with 100 μM GEA 3162 were more highly significant than with 30 μM GEA 3162 when induction of apoptosis was assessed by morphology ($p < 0.0001$ vs. $p < 0.05$) or CD16 shedding ($p < 0.0001$ vs. $p < 0.05$). This is consistent with previous findings for this compound on neutrophil apoptosis (Ward *et al.*, 2000). The rate of induction of apoptosis followed similar kinetics to 1 mM DEA/NO, with a difference from control being seen by 4 h when apoptosis was measured by most methods. Indeed, the levels of morphological apoptosis detected at all time points were remarkably similar for 100 μM GEA 3162 and 1 mM DEA/NO, with the diazeniumdiolate producing marginally higher levels of apoptosis.

For diazeniumdiolate compounds, the four independent events of neutrophil apoptosis (morphological changes, PS exposure, CD16 shedding and DNA fragmentation) occurred virtually simultaneously, with no single event occurring noticeably before the others. This is in agreement with observations using cultured cardiomyocytes treated with agonistic anti-Fas antibody, in which nuclear condensation and DNA fragmentation occurred simultaneously (Takemura *et al.*, 2001). However, it is at odds with observations in rat ventricular myocytes, in which PS exposure preceded DNA fragmentation in response to staurosporine (Rucker-Martin *et al.*, 1999) and in a number of cell types, including neutrophils, in which PS exposure was found to trail other apoptotic events, such as CD16 shedding (Frey, 1997). Staurosporine treatment of eosinophils led to apoptotic morphological changes which were visible before evidence of PS exposure (Walsh *et al.*, 1998) and studies in HL-60 cells showed that PS exposure was followed by morphological observations of apoptosis and DNA fragmentation sequentially in response to etoposide or cisplatin (Kravtsov *et al.*, 1999). The temporal regulation of apoptotic events therefore appears to be dependent upon both the cell type and the initiating stimulus.

The inhibition of DNA fragmentation produced by GEA 3162 occurred at

later time points than the induction of the other apoptotic events. By Annexin V binding, increased apoptosis was only observed at early time points and was not different from control at later time points. The reason for this discrepancy is unclear, but it is consistent with results obtained by Frey, in which aged neutrophils showed complete CD16 shedding, whilst PS exposure only occurred in a subset of neutrophils (Frey, 1997). It is notable that in some conditions, Annexin V binding gave the least apparent indication of modification of apoptosis by NO or $ONOO^-$ donors, in that differences were smaller or did not appear until later time points than with other methods. This suggests that a proportion of neutrophils are relatively resistant to the exposure of PS on the outer cell surface, and that this technique is not ideal for the assessment of neutrophil apoptosis. For GEA 3162 and 1 mM DEA/NO, CD16 shedding correlated most closely with morphological observations, and for 1 mM SPER/NO, Annexin V binding gave levels of apoptosis closest to morphology. Inhibition of apoptosis by 0.1 μM DEA/NO was most profoundly apparent using PI intercalation to assess apoptosis, whereas with SPER/NO, morphological assessment of apoptosis gave the clearest indication of inhibition.

A paradoxical inhibition of DNA fragmentation was observed using SPER/NO and GEA 3162. However, the shorter-lasting NO donor, DEA/NO, failed to inhibit this process. The differential effects of the two diazeniumdiolates is likely to be related to the half-lives of these two compounds. It is possible that NO from diazeniumdiolates has to combine with neutrophil-derived O_2^- to form peroxynitrite in order to exert an inhibitory effect. It is unlikely that O_2^- levels would be very high in cultured neutrophils during the time that it takes DEA/NO to completely release all its bound NO (less than 1 h), therefore there is little potential for $ONOO^-$ generation as the presence of these two molecules is unlikely to coincide temporally. In contrast, NO from SPER/NO is generated over a 12-h period, during which time the levels of spontaneously-generated O_2^- in neutrophils could become sufficiently high to allow significant $ONOO^-$ formation, as free NO and O_2^- could potentially be present simultaneously within the neutrophils. Alternatively, $ONOO^-$ formation could occur with both DEA/NO and SPER/NO, but the inhibitory effect may occur at the level of a relatively late event of the DNA fragmentation pathway, such as

inhibition of active caspase 3, and therefore be dependent upon the availability of $ONOO^-$ at the time of activation of this enzyme, which is feasible with SPER/NO but less so with DEA/NO.

The inhibitory effect on DNA fragmentation was more profound with the $ONOO^-$ donor, GEA 3162 than with SPER/NO. This observation is consistent with the above hypothesis, as GEA 3162 is a generator of $ONOO^-$ through simultaneous release of NO and O_2^-, therefore NO levels are matched by levels of O_2^- and $ONOO^-$ formation will be maximal. On the other hand, NO from SPER/NO would have to combine with O_2^- from neutrophils to form $ONOO^-$. Thus, the formation of $ONOO^-$ using SPER/NO is likely to be limited by the relative concentrations of the two species; it is highly unlikely that a large proportion of the free NO from SPER/NO would be converted to $ONOO^-$, therefore $ONOO^-$ levels would be lower than with GEA 3162, and inhibition would be less pronounced.

The known $ONOO^-$ donor, SIN-1, was used to examine whether this effect is exclusive to GEA 3162, or is a common phenomenon of neutrophil exposure to $ONOO^-$. These studies confirmed the inhibition of DNA fragmentation by $ONOO^-$ donors, despite enhancement of various other characteristic events of neutrophil apoptosis. As for GEA 3162, the inhibitory effect of SIN-1 on internucleosomal DNA fragmentation was concentration-dependent. This inhibition was not fully reversible by SOD, which converts O_2^- to H_2O_2, thus reducing the availability of O_2^- for combination with NO to form $ONOO^-$. This likely suggests that only a small amount of $ONOO^-$ is required to be formed in order to inhibit DNA fragmentation. As previously mentioned, the reaction kinetics for the formation of $ONOO^-$ from NO and O_2^- are 3 times faster than those for the dismutation of O_2^- by SOD. Therefore, even in the presence of SOD, some $ONOO^-$ is still likely to be formed, and this may be sufficient to influence the DNA fragmentation pathway. These surprising findings demonstrate that different events of neutrophil apoptosis may be dissociated from each other and, therefore, may be differentially regulated. Inhibition of DNA fragmentation has previously been reported by one group, who noted that low concentrations of $ONOO^-$ promote DNA fragmentation through inhibition of poly (ADP-ribose) synthetase (PARS) in HL60 cells, via a caspase 3 dependent

mechanism, but that concentrations of 60 μM $ONOO^-$ actually inhibit this process (Virag *et al.*, 1998).

Internucleosomal DNA fragmentation has been reported to be a definitive event of apoptotic cell death, and several studies have used this event as the sole determinant of the level of apoptosis. However, the present studies show that several different apoptotic events should routinely be measured, as assessment of the extent of DNA fragmentation may not accurately reflect the level of this form of cell death. The dissociation of DNA fragmentation from other events of apoptosis has previously been reported in other cell types, under various experimental conditions. Xu et al demonstrated the dissociation of apoptotic events from DNA fragmentation in a hepatoma cell line. DNA fragmentation induced by c-myc was dependent upon zinc and was only observed in serum-free conditions (Xu *et al.*, 1996). In contrast, zinc has been shown to inhibit endonuclease activity in peripheral blood lymphocytes, in which treatment with H_2O_2 induced apoptotic cell death without DNA fragmentation (Marini and Musiani, 1998). It has also been shown that nuclear condensation characteristic of apoptosis does not require DNA fragmentation in order to occur. Transformed Jurkat T cells or TF-1 cells overexpressing caspase-resistant ICAD exhibited nuclear condensation in the absence of DNA fragmentation in response to staurosporine, etoposide, UV or gamma radiation (Jurkats) or GM-CSF withdrawal in TF-1 cells (Sakahira *et al.*, 1999; McIlroy *et al.*, 1999). Neuronal PC12 cells treated with activated microglia undergo apoptosis, and overexpression of Bcl-2 in these cells caused morphological changes of cell death without DNA fragmentation, which was ascribed to a non-apoptotic form of cell death (Tanabe *et al.*, 1999). DNA laddering was absent in three strains of MCF-7 breast cancer cells treated with doxorubicin, despite increased apoptosis (Gooch and Yee, 1999). Oligonucleosomal DNA fragmentation can also be inhibited in hematopoietic cells exposed to hyperosmotic shock by inhibition of Cl^- efflux (Rasola *et al.*, 1999). PC12 neurons with caspase 3 knocked out were induced to undergo apoptosis by K^+ deprivation, although in contrast to wild type cells, DNA fragmentation and chromatin condensation were absent from apoptosis in these cells, suggesting that caspase 3 is required for these apoptotic events in this cell type, but not for apoptotic

cell death per se (D'Mello *et al.*, 2000). Furthermore, caspase 3 was shown to be critical for DNA fragmentation and nuclear condensation in MCF-7 breast carcinoma cells in response to H_2O_2, although caspase 3 null cells still exposed PS exposure and underwent apoptotic death, providing further evidence for the dissociation of DNA fragmentation from other apoptotic events (Kim *et al.*, 2000). Upregulation of catalase led to inhibition of caspase 3 activation and DNA fragmentation in PC12 cells in response to H_2O_2 but was not sufficient to inhibit death (Jiang *et al.*, 2001). Apoptosis in the absence of DNA fragmentation was also observed in cultured cardiomyocytes in response to an agonistic anti-Fas antibody when Ca^{2+}/Mg^{2+} dependent endonuclease was blocked using excess zinc (Takemura *et al.*, 2001). The neuroblastoma cell line, IMR-5, also underwent apoptosis without DNA fragmentation on exposure to staurosporine, and this was attributed to loss of CAD protein during apoptosis, as DNA fragmentation was observed on overexpression of human recombinant CAD (Yuste *et al.*, 2001). Therefore, several authors have advised against single parameter detection of apoptotic cell death (Frey, 1997; Gooch and Yee, 1999; Kravtsov *et al.*, 1999). However this is the first time that such a phenomenon has been shown and studied in detail in neutrophils.

To summarise, 'pure' NO donors exert differential effects upon neutrophil apoptosis, in which low concentrations delay but high concentrations enhance this form of cell death, although the inhibitory effect is modest (< 10%). The rate of NO release has an impact on the biological effects of the diazeniumdiolates; the fast-releasing drug, DEA/NO, inhibits apoptosis to a lesser extent than seen with the slower release from SPER/NO, but a significant effect is seen with higher concentrations. In contrast, the induction of apoptosis requires a higher concentration of DEA/NO than SPER/NO, but accelerates apoptosis to a greater extent. Only the pro-apoptotic effect is observed with the $ONOO^-$ donor, GEA 3162. Unexpectedly, both GEA 3162 and the longer-lasting pure NO donor, SPER/NO produce an inhibition of DNA fragmentation compared to control cells, a phenomenon previously reported in a number of different cell types, but not previously in neutrophils.

Chapter 5: Mechanisms of Action of NO /$ONOO^-$ donors

5.1 Introduction

This thesis has demonstrated both pro- and anti-apoptotic effects of NO donors, and pro-apoptotic effects of $ONOO^-$ donors in neutrophils, however the mechanisms through which these compounds act remain to be elucidated. A number of mechanisms have been proposed to account for the regulatory effects of NO and $ONOO^-$ *in vitro*, as discussed in previous chapters.

The DNA fragmentation pathway proceeds downstream of caspase 3 activation through the stress or death receptor-mediated pathways, but the sole role for caspase 3 in this pathway is in the activation of a 40 kDa endonuclease, known as caspase-activated DNase (CAD), or DNA fragmentation factor 40 (DFF40) (Liu *et al.*, 1997). CAD/DFF40 exists as a cytoplasmic complex with the inhibitor of caspase-activated DNase (ICAD), which is known as DNA fragmentation factor 45 (DFF45) in humans. It is now known that ICAD/DFF45 acts as a chaperone molecule as well as an endogenous inhibitor of CAD/DFF40 (Enari *et al.*, 1998), and its presence is essential during translation to ensure the correct folding and effective DNase activity of CAD/DFF40 (Uegaki *et al.*, 2000).

CAD/DFF40 is a protein of 342 amino acids and contains a nuclear localisation signal (NLS), whereas ICAD exists as long and short forms, which differ by the presence or absence of a NLS due to alternative splicing (Liu *et al.*, 1997;Samejima and Earnshaw, 2000). These two molecules interact via their CAD domains, which are homologous regions of approximately 80 amino acids at the N-termini of these two molecules via oppositely-charged amino acids within the beta sheets of their respective CAD domains (Uegaki *et al.*, 2000; Otomo *et al.*, 2000).

Active caspase 3 is able to proteolytically cleave ICAD/DFF45 at two sites (Liu *et al.*, 1997; Sakahira *et al.*, 1998). This releases the endonuclease, CAD/DFF40, from inhibition and this molecule translocates to the nucleus, where it degrades DNA into the internucleosomal fragments that produce a characteristic

laddering pattern by gel electrophoresis. However, it has been suggested that CAD/DFF40 may instead induce activity of a Ca^{2+}/Mg^{2+}-dependent endonuclease and have no DNase activity of its own (Liu *et al.*, 1997).

It has been shown that caspase 7 is also able to cleave ICAD/DFF45, although the physiological significance of this is questionable (Wolf *et al.*, 1999). Others have demonstrated that ICAD/DFF45 can be cleaved by two caspases, including caspase 3, but that cleavage by caspases other than caspase 3 fails to activate CAD/DFF40 (Tang and Kidd, 1998). Once ICAD/DFF45 has been cleaved by capsases, however, caspase activity is no longer required for the execution of DNA fragmentation (Samejima *et al.*, 1998). A 40 kDa human endonuclease, named CPAN, that is inhibited by ICAD/DFF45 has been described, with the authors suggesting that this may represent the human homologue of CAD/DFF40 (Halenbeck *et al.*, 1998).

Both caspases and CAD/DFF40 contain reduced cysteines that may be essential for the correct functioning of these proteins (Mohr *et al.*, 1997;Sakahira *et al.*, 2000), and NO and NO-related species have been shown to inhibit caspase activity through thiol modification (Mohr *et al.*, 1997). Dithiothreitol (DTT) is a reducing agent that is able to maintain protein thiols in their reduced state. Therefore, if GEA 3162 acts through oxidative modification of reduced cysteines contained within essential proteins of the DNA fragmentation pathway, the effect would be inhibited in the presence of DTT.

The induction of apoptosis by NO has been proposed to be mediated by $ONOO^-$ (Liaudet *et al.*, 2000). This could be possible in neutrophils due to release of O_2^- from these cells that could combine with NO from NO donors to generate $ONOO^-$. Scavenging of O_2^- would therefore prevent DEA/NO-mediated neutrophil apoptosis if $ONOO^-$ generation is necessary. Cu/Zn SOD dismutates extracellular O_2^- to H_2O_2, therefore addition of SOD alongside DEA/NO would inhibit the induction of neutrophil apoptosis if $ONOO^-$ generation occurs outside the cell.

The principal aim of these experiments was to make some progress towards

the elucidation of the mechanism by which $ONOO^-$ from GEA 3162 is able to inhibit DNA fragmentation in neutrophils undergoing apoptosis. This was done using the PI intercalation method of assessing apoptosis, and Western blotting. Further experiments were carried out to investigate the possibility that DEA/NO-induced apoptosis is mediated by $ONOO^-$, using the multiple techniques for assessing apoptosis described in Chapter 4.

5.2 Results

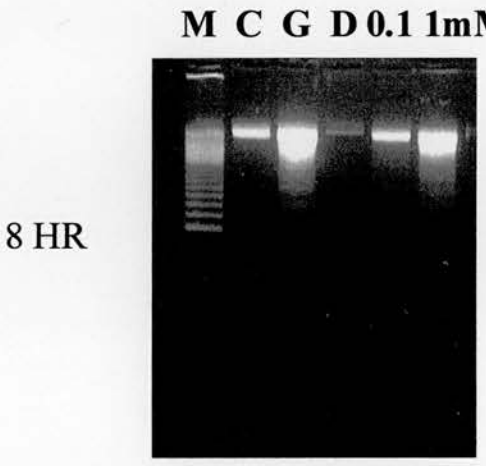
5.2.1 Verification of the effects of NO/$ONOO^-$ donors on DNA fragmentation in human neutrophils

The inhibitory effects of GEA 3162 and SPER/NO, but not of DEA/NO were verified using gel electrophoresis to detect the characteristic 'laddering' pattern of fragmented DNA in apoptotic cells. DNA was isolated from neutrophils treated with PBS (control); the fungal metabolite and known inducer of neutrophil apoptosis, gliotoxin (positive control; 0.1 μg/ml); the cell permeable analogue of cAMP and known inhibitor of neutrophil apoptosis, db-cAMP (negative control; 0.2 mM) or NO/$ONOO^-$ donors at concentrations known to inhibit or induce apoptosis for 8 or 20 h. Morphological apoptosis was measured alongside the gel electrophoresis of DNA, in order to verify that the compounds were having the expected effects on neutrophil apoptosis. In all cases, DNA fragmentation was visibly increased in gliotoxin-treated cells and reduced in cells exposed to db-cAMP, and these results were borne out by morphological observations of apoptosis; gliotoxin always increased apoptosis, while db-cAMP always inhibited apoptosis compared to control.

The extent of DNA laddering was very similar to control in cells treated with 0.1 μM DEA/NO but increased compared to control (PBS-treated) cells in neutrophils exposed to 1 mM DEA/NO at both 8 (Figure 5.1A) and 20 (Figure 5.1B) h. Apoptosis measured morphologically was 18.0 % with 0.1 μM and 91.4 % with 1 mM DEA/NO at 8 h compared to 16.3 % control, and at 20 h control apoptosis was 45.2 % compared to 25.9 % and 100 % apoptosis with 0.1 μM and 1 mM DEA/NO respectively.

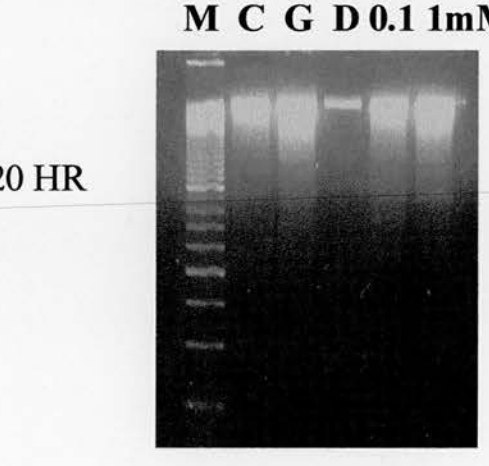
In cells treated with SPER/NO, an inhibition of DNA laddering was seen with both 0.1 μM and 1 mM of this compound at 8 h, with no fragmentation at all being visible with 1 mM, although morphological apoptosis was 21.8 % with 0.1 μM and 22.4 % with 1 mM SPER/NO, compared to 17.4 % control (Figure 5.2A). At 20 h, differences in the extent of laddering were not clearly visible with either concentration of SPER/NO, in contrast to morphological apoptosis, which was 54.8

A



	Control	Gliotoxin	db-cAMP	0.1 μM DEA/NO	1 mM DEA/NO
% Morphological Apoptosis	16.3	84.8	0.8	18.0	91.4

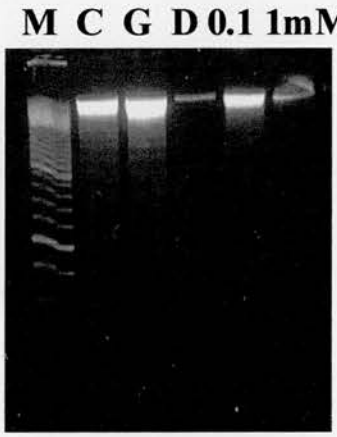
B



	Control	Gliotoxin	db-cAMP	0.1 μM DEA/NO	1 mM DEA/NO
% Morphological Apoptosis	45.2	100	2.8	25.9	100

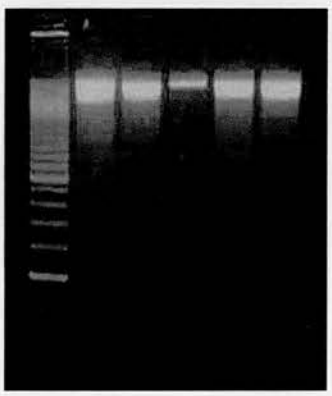
Figure 5.1 Effects of DEA/NO on DNA fragmentation. Neutrophils (4 x 10^6 cells) were incubated for (A) 8 or (B) 20 hours with PBS (control; C), gliotoxin (0.1 μg/ml; G), db-cAMP (0.2 mM; D) or 0.1 μM or 1 mM DEA/NO. DNA was then extracted and run on a 2% agarose gel containing ethidium bromide and visualised under u.v. light, along with molecular weight markers (M). Morphological apoptosis of the neutrophil population was assessed by cytospin alongside DNA fragmentation.

A



	Control	Gliotoxin	db-cAMP	0.1 μM SPER/NO	1 mM SPER/NO
% Morphological Apoptosis	17.4	79.2	1.1	21.8	22.4

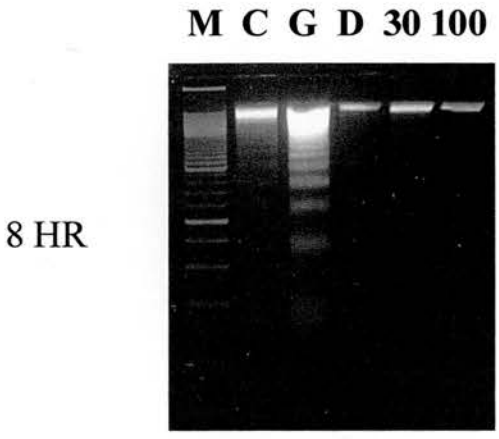
B



	Control	Gliotoxin	db-cAMP	0.1 μM SPER/NO	1 mM SPER/NO
% Morphological Apoptosis	54.8	100	29.9	54.8	75.8

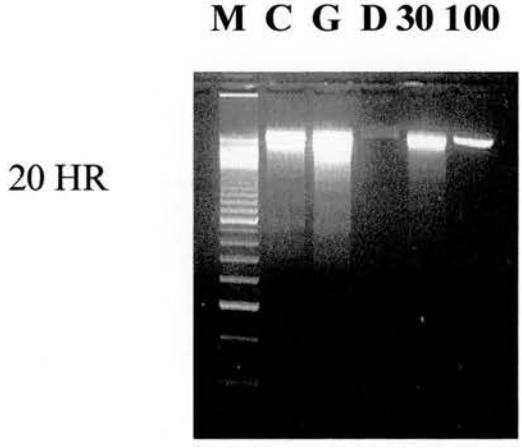
Figure 5.2 Effects of SPER/NO on DNA fragmentation. Neutrophils (4 x 10^6 cells) were incubated for (A) 8 or (B) 20 hours with PBS (control; C), gliotoxin (01 μg/ml; G), db-cAMP (0.2 mM; D) or 0.1 μM or 1 mM SPER/NO. DNA was then extracted and run on a 2% agarose gel containing ethidium bromide and visualised under u.v. light, along with molecular weight markers (M). Morphological apoptosis of the neutrophil population was assessed by cytospin alongside DNA fragmentation.

A



	Control	Gliotoxin	db-cAMP	30 μM GEA 3162	100 μM GEA 3162
% Morphological Apoptosis	13.6	100	2.4	55.3	90.0

B



	Control	Gliotoxin	db-cAMP	30 μM GEA 3162	100 μM GEA 3162
% Morphological Apoptosis	48.6	100	2.8	50.2	100

Figure 5.3 Effects of GEA 3162 on DNA fragmentation. Neutrophils (4 x 10^6 cells) were incubated for (A) 8 or (B) 20 hours with PBS (control; C), gliotoxin (0.1 μg/ml; G), db-cAMP (0.2 mM; D) or 30 or 100 μM GEA 3162. DNA was then extracted and run on a 2% agarose gel containing ethidium bromide and visualised under u.v. light, along with molecular weight markers (M). Morphological apoptosis of the neutrophil population was assessed by cytospin alongside DNA fragmentation.

% with 0.1 μM and 75.8 % with 1 mM SPER/NO, and 54.8 % in control cells (Figure 5.2B).

No DNA fragmentation was detectable in neutrophils treated with either 30 or 100 μM GEA 3162 at 8 h, despite morphological observations of 55.3 % and 90.0 % apoptosis respectively vs. 13.6 % control in these cells (Figure 5.3A). At 20 h, a small amount of laddering was visible with 30 μM GEA 3162, although this was clearly less evident than that seen in control cells. However, a total lack of DNA fragmentation was still observed with 100 μM GEA 3162 at this time point. Morphologically, 50.2 % apoptosis was seen with 30 μM of this compound, and 100 % with 100 μM, compared to 48.6 % control at 20 h (Figure 5.3B).

5.2.2 Effects of DTT on inhibition of DNA fragmentation by GEA 3162

In order to test the hypothesis that the inhibition of DNA fragmentation in cells treated with GEA 3162 occurs through the oxidative modification of critical proteins in the DNA fragmentation pathway, neutrophils were pre-incubated with the reducing agent, dithiothreitol (DTT) before being exposed to GEA 3162 for 20 h. Apoptosis was then assessed by PI intercalation and again, morphological changes of apoptosis were measured to verify the pro-apoptotic effects of GEA 3162 on apoptosis. Figure 5.4A shows that treatment with GEA 3162 alone caused a concentration-dependent inhibition of DNA fragmentation as measured by PI intercalation. DNA fragmentation was seen in 61.6 ± 4.5 % of cells treated with 30μM GEA 3162 and in 49.5 ± 4.2 % of cells exposed to 100 μM ($p < 0.05$, one-way ANOVA with Student-Newman-Keuls post-hoc test) compared to 69.0 ± 4.1 % control. However, the presence of DTT (100 or 300 μM) reversed this inhibition in a concentration-dependent manner back to control levels. Neutrophils pre-treated with 100 μM DTT showed 66.4 ± 3.9 % DNA fragmentation in response to 30 μM GEA 3162 and 55.9 ± 6.3 % with 100 μM of this compound, and treatment with 300 μM DTT led to 68.4 ± 5.1 % and 71.7 ± 3.8 % DNA fragmentation in response to 30 and 100 μM GEA 3162 respectively ($p > 0.05$ from control, one-way ANOVA with

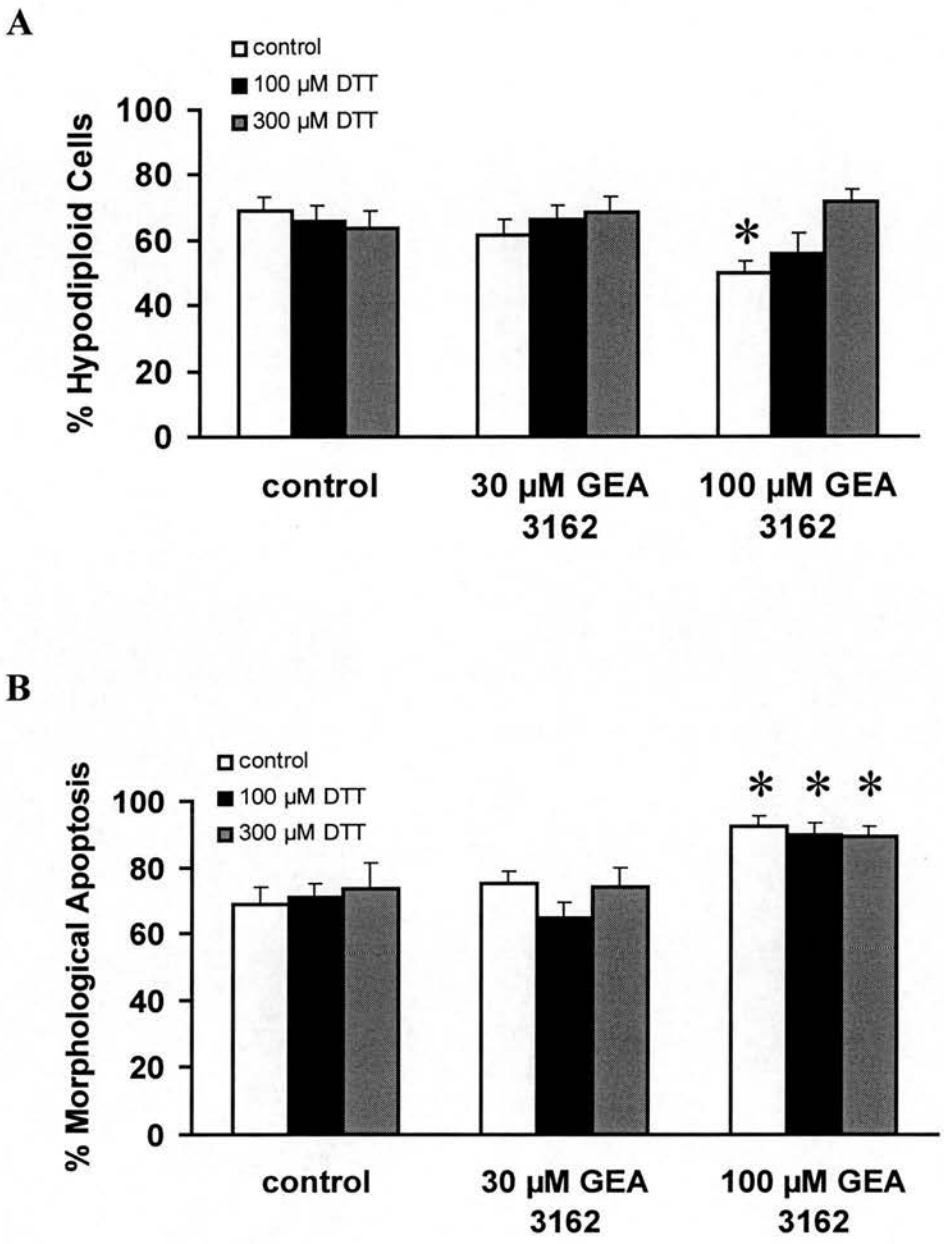


Figure 5.4 Effects of DTT on GEA 3162-induced inhibition of DNA fragmentation. Neutrophils (4.5 x 10^6 cells/ml) were incubated for 20 hours with PBS (control) or 30 or 100 µM GEA 3162 in the absence or presence of dithiothreitol (DTT; 100 – 300 µM). Apoptosis was then assessed by (A) propidium iodide intercalation and (B) morphology. Results represent mean ± S.E.M. from n = 7 experiments. Asterisks represent significant ($p < 0.05$) difference from control by one-way ANOVA with Student-Newman-Keuls post-hoc test.

Student-Newman-Keuls post-hoc test). DTT had no effect on the levels of DNA fragmentation in untreated cells (65.7 ± 4.8 % and 63.8 ± 5.5 % with 100 and 300 μM DTT respectively, compared to 69.0 ± 4.1 % in cells not exposed to DTT; $p > 0.05$, one-way ANOVA with Student-Newman-Keuls post-hoc test).

In order to ensure that DTT itself does not affect the rate of apoptosis in neutrophils, morphological assessment of apoptosis was carried out alongside PI intercalation. These studies showed that the concentration-dependent induction of neutrophil apoptosis by GEA 3162 was unaffected by the presence of DTT (Figure 5.4B). Morphological apoptosis induced by 30 and 100 μM GEA 3162 was 75.2 ± 3.4 % and 95.2 ± 3.1 % respectively compared to 68.9 ± 5.2 % control ($p < 0.05$ from control with 100 mM GEA 3162, one-way ANOVA with Student-Newman-Keuls post-hoc test). In the presence of DTT (100 or 300 μM), levels of apoptosis were 64.7 ± 4.6 % and 73.9 ± 5.9 % respectively in cells exposed to 30 μM GEA 3162, and 89.4 ± 4.1 % and 88.9 ± 3.3 % respectively in response to 100 μM GEA 3162. Apoptosis in cells exposed to DTT remained significantly different from control apoptosis ($p < 0.05$, one-way ANOVA with Student-Newman-Keuls post-hoc test) in the 100 μM GEA 3162-treated cells.

5.2.3 Effects of DTT on gliotoxin-induced neutrophil apoptosis

These experiments were also carried out to determine the effects of DTT on apoptosis in neutrophils induced to undergo apoptosis by gliotoxin. Following 20 h exposure to gliotoxin, PI intercalation showed that 88.5 ± 5.0 % of cells had hypodiploid DNA content compared to 62.5 ± 8.3 % control ($p < 0.05$, one-way ANOVA with Dunnett's post-hoc test; Figure 5.5A). Morphologically, gliotoxin induced 97.4 ± 1.5 % of cells to undergo apoptosis by 20 h compared to 61.6 ± 5.7 control ($p < 0.05$, one-way ANOVA with Dunnett's post-hoc test; Figure 5.5B). In contrast to the effects seen with GEA 3162, however, the presence of DTT (100 or 300 μM) slightly inhibited DNA fragmentation in response to this compound in a concentration-dependent manner; with 100 μM DTT, 83.9 ± 6.5 % of gliotoxin-

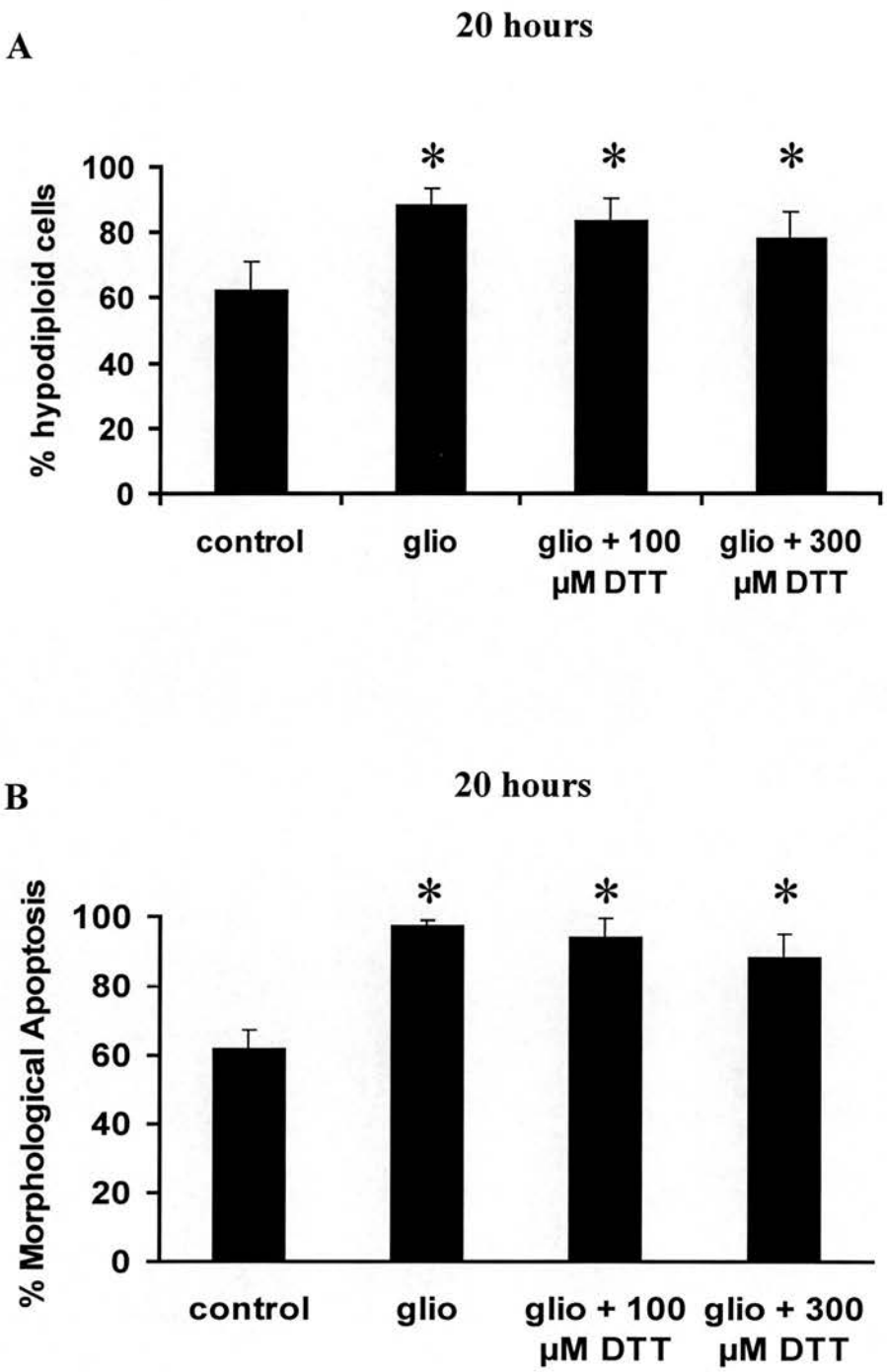


Figure 5.5 Effects of DTT on gliotoxin-induced neutrophil apoptosis. Neutrophils (4.5 x 10^6 cells/ml) were incubated for 20 hours with PBS (control) or gliotoxin (0.1 µg/ml) in the absence or presence of dithiothreitol (DTT; 100 – 300 µM). Apoptosis was then assessed by (A) propidium iodide intercalation and (B) morphology. Results represent mean ± S.E.M. from *n* = 6 experiments. Asterisks represent significant ($p < 0.05$) difference from control by one-way ANOVA with Dunnett's post-hoc test.

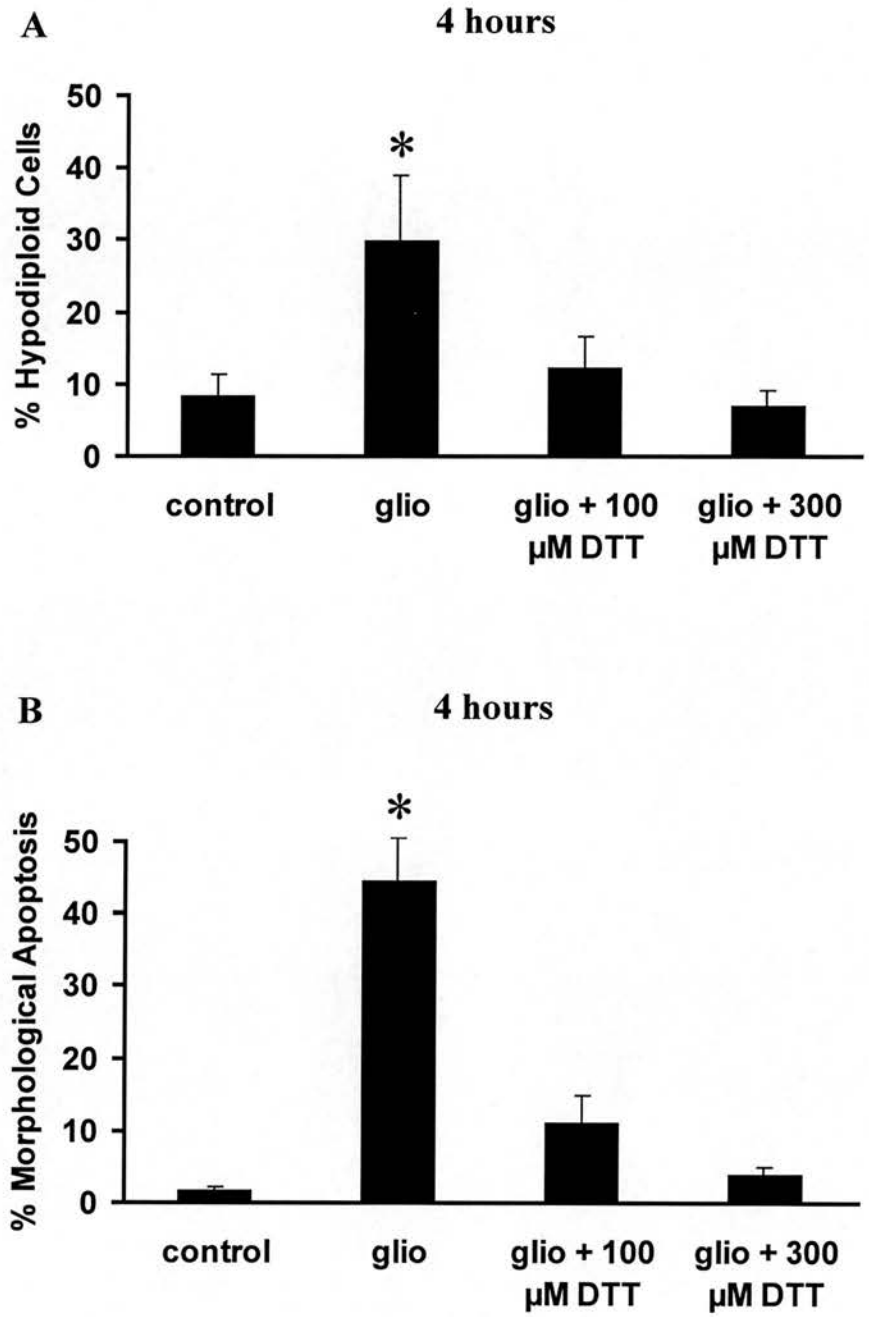


Figure 5.6 Effects of DTT on gliotoxin-induced neutrophil apoptosis. Neutrophils (4.5×10^6 cells/ml) were incubated for 4 hours with PBS (control) or gliotoxin (0.1 µg/ml) in the absence or presence of dithiothreitol (DTT; 100 – 300 µM). Apoptosis was then assessed by (A) propidium iodide intercalation and (B) morphology. Results represent mean ± S.E.M. from $n = 4$ experiments. Asterisks represent significant ($p < 0.05$) difference from control by one-way ANOVA with Dunnett's post-hoc test.

treated cells had hypodiploid DNA content, and with 300 μM DTT, the level was 78.4 ± 7.8 %, but these levels remained significantly different from control levels ($p < 0.05$, one-way ANOVA with Dunnett's post-hoc test).

This inhibitory effect of DTT was not confined to DNA fragmentation; in contrast to the lack of effect of DTT on morphological apoptosis induced by GEA 3162, this reducing agent slightly decreased the extent of morphological apoptosis in cells exposed to gliotoxin. In the presence of 100 μM DTT, apoptosis was 94.2 ± 5.3 %, and with 300 μM DTT, it was 88.5 ± 6.6 % ($p < 0.05$ from control, one-way ANOVA with Dunnett's post-hoc test), whereas in gliotoxin-treated cells in the absence of DTT, apoptosis was 97.4 ± 1.5 % and that in untreated cells was 61.6 ± 5.7 %.

The effects of DTT on gliotoxin-induced neutrophil apoptosis were also examined at 4 h, when gliotoxin induces less than 100 % apoptosis, so that any increase in DNA fragmentation in the presence of DTT, as seen with GEA 3162, would be detectable. At this time point, gliotoxin produced 29.9 ± 9.0 % DNA fragmentation compared to 8.3 ± 3.1 % in control cells ($p < 0.05$, one-way ANOVA with Dunnett's post-hoc test; Figure 5.6A). As seen with the later time point, DTT reduced the levels of DNA fragmentation in gliotoxin-treated cells, in contrast to the effects observed with GEA 3162, and this effect was more pronounced than that observed at 20 h. In the presence of 100 μM DTT, 12.1 ± 4.6 % of neutrophils had hypodiploid DNA content, and with 300 μM DTT, this figure was as low as 6.9 ± 2.2 %, i.e. DNA fragmentation was reduced to control levels ($p > 0.05$ from control, one-way ANOVA with Dunnett's post-hoc test).

Consistent with the results achieved at 20 h, the presence of DTT also inhibited morphological induction of apoptosis by gliotoxin at 4 h (Figure 5.6B). Cells exposed to gliotoxin showed 44.6 ± 5.9 % apoptosis by morphology, compared to 1.8 ± 0.3 % control ($p < 0.05$, one-way ANOVA with Dunnett's post-hoc test), but levels of apoptosis were reduced to 11.1 ± 3.8 % and 3.9 ± 1.1 % with 100 and 300 μM DTT respectively ($p > 0.05$, one-way ANOVA with Dunnett's post-hoc test).

5.2.4 Effects of NO/$ONOO^-$ donors on breakdown of ICAD/DFF45

Breakdown of the inhibitor and chaperone molecule of CAD/DFF40, ICAD/DFF45, was assessed by Western blotting. Whole cell neutrophil lysates were made from freshly-isolated neutrophils and following 8 h culture in the presence of gliotoxin (positive control), PBS (control), or NO/$ONOO^-$ donors at concentrations known to induce neutrophil apoptosis. Lysates were assessed for ICAD/DFF45 levels using a mouse anti-human ICAD/DFF45 monoclonal antibody followed by a goat anti-mouse IgG antibody conjugated to HRP, and developed using ECL fluid. Figure 5.7 shows the resulting Western blot, in which levels of ICAD/DFF45 were shown to be reduced in neutrophils treated with gliotoxin (Lane 2), 30 or 100 μM GEA 3162 (Lanes 4 & 5) and with 1 mM DEA/NO (Lane 6). In contrast, levels were unchanged from control, or possibly slightly higher than control, in cells exposed to 1 mM SPER/NO (Lane 7).

5.2.5 Effect of SOD on DEA/NO-mediated Neutrophil Apoptosis

In order to investigate the possibility that neutrophil apoptosis induced by DEA/NO is mediated by the formation of $ONOO^-$, cells were coincubated with SOD (50 U/ml) to scavenge any O_2^- released from neutrophils during incubation. Measurement of apoptosis by all four techniques (morphology, Annexin V binding, CD16 shedding and PI intercalation) showed no effect of SOD (50 U/ml) on control levels of apoptosis ($p > 0.05$, one-way ANOVA with Dunnett's post-hoc test). DEA/NO (1 mM) induced apoptosis as measured by morphology (98.7 ± 1.3 % vs 51.8 ± 10.2 % control, $p < 0.05$, one-way ANOVA with Dunnett's post-hoc test; Figure 5.8). However, the presence of 50 U/ml SOD failed to affect the rate of apoptosis in cells exposed to DEA/NO, with apoptosis in the presence of SOD measured as 98.8 ± 1.2 % compared to 98.7 ± 1.3 % in the absence of SOD, and remained significantly different from control ($p < 0.05$, one-way ANOVA with Dunnett's post-hoc test).

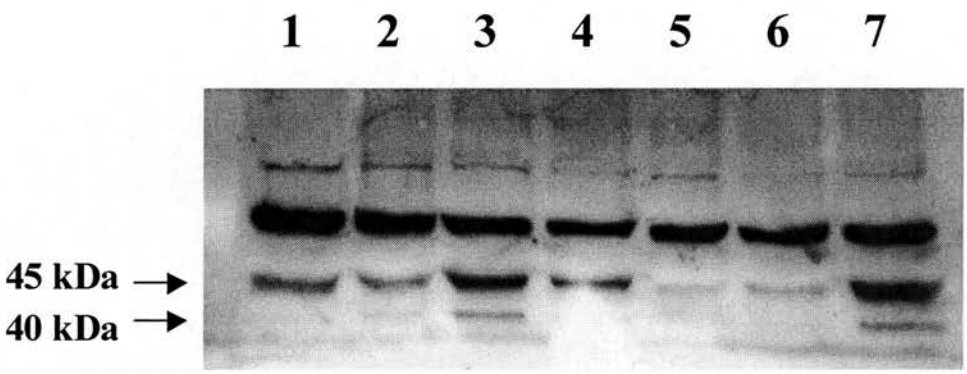


Figure 5.7 Western blotting for ICAD/DFF45. Whole cell lysates were made from freshly isolated neutrophils (Lane 1) or those treated for 8 hours with 0.1 μg/ml gliotoxin (Lane 2), PBS control (Lane 3), 30 μM GEA 3162 (Lane 4), 100 μM GEA 3162 (Lane 5), 1 mM DEA/NO (Lane 6) or 1 mM SPER/NO (Lane 7). Blots were probed with antibody against ICAD/DFF45 (1:500 dilution) followed by goat anti-mouse IgG-HRP secondary antibody (1:1250 dilution) and developed onto photographic film using electrochemiluminescence (ECL) fluid. Representative blot of two blots performed from one set of whole cell lysates.

Similarly, apoptosis measured by Annexin V binding showed a lack of effect of SOD (Figure 5.9). Annexin V binding in neutrophils exposed to 1 mM DEA/NO was 92.4 ± 3.2 %, whereas in control cells it was 63.0 ± 2.5 % ($p < 0.05$, one-way ANOVA with Dunnett's post-hoc test). In the presence of SOD (50 U/ml), apoptosis in response to DEA/NO was 94.8 ± 2.2 % and this was still significantly different from control, despite the presence of SOD ($p > 0.05$, one-way ANOVA with Dunnett's post-hoc test).

CD16 shedding in DEA/NO-treated cells was measured as 99.3 ± 0.3 %, but was only 55.4 ± 5.9 % in untreated cells ($p < 0.05$, one-way ANOVA with Dunnett's post-hoc test; Figure 5.10). Once again, the addition of SOD (50 U/ml) failed to significantly inhibit the acceleration of apoptosis by DEA/NO; in the presence of SOD, apoptosis was 98.6 ± 0.5 % ($p < 0.05$ from control, one-way ANOVA with Dunnett's post-hoc test).

Measurement of apoptosis by PI intercalation again showed enhanced apoptosis in response to 1 mM DEA/NO, with 80.3 ± 6.6 % of cells having hypodiploid DNA content compared to 54.5 ± 7.9 % control, although this just failed to reach significance ($p > 0.05$, one-way ANOVA with Dunnett's post-hoc test). Coincubation with 50 U/ml SOD once again failed to inhibit the pro-apoptotic effect of DEA/NO, with apoptosis measured as 78.9 ± 5.2 % ($p > 0.05$ from control, one-way ANOVA with Dunnett's post-hoc test; Figure 5.11).

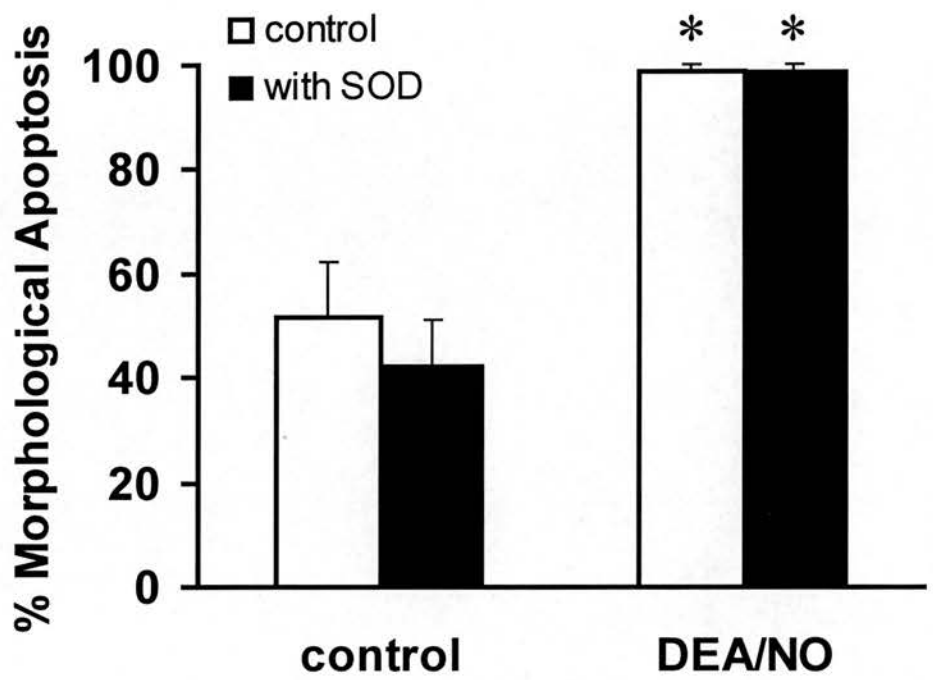


Figure 5.8 Effects of SOD on DEA/NO-induced neutrophil apoptosis as assessed by morphology. Neutrophils (4.5 x 10^6 cells/ml) were incubated for 20 hours with PBS (control) or DEA/NO (1 mM) in the absence or presence of SOD (50 U/ml). Apoptosis was then assessed by morphology. Results represent mean ± S.E.M. from n = 3 experiments. Asterisks represent significant ($p < 0.05$) difference from control by one-way ANOVA with Dunnett's post-hoc test.

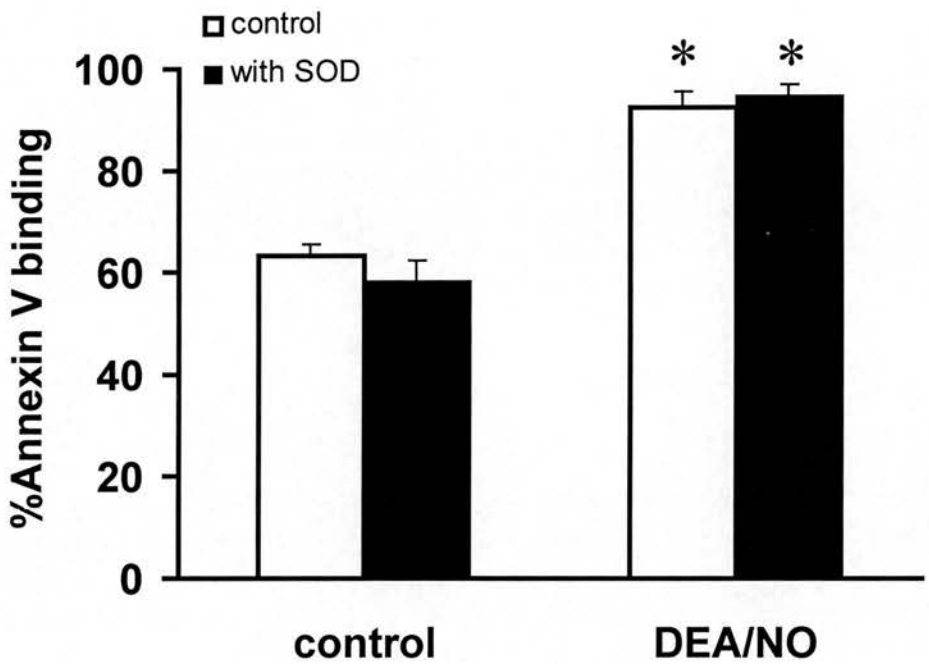


Figure 5.9 Effects of SOD on DEA/NO-induced neutrophil apoptosis as assessed by Annexin V binding. Neutrophils (4.5 x 10^6 cells/ml) were incubated for 20 hours with PBS (control) or DEA/NO (1 mM) in the absence or presence of SOD (50 U/ml). Apoptosis was then assessed by Annexin V binding. Results represent mean ± S.E.M. from $n = 3$ experiments. Asterisks represent significant ($p < 0.05$) difference from control by one-way ANOVA with Dunnett's post-hoc test.

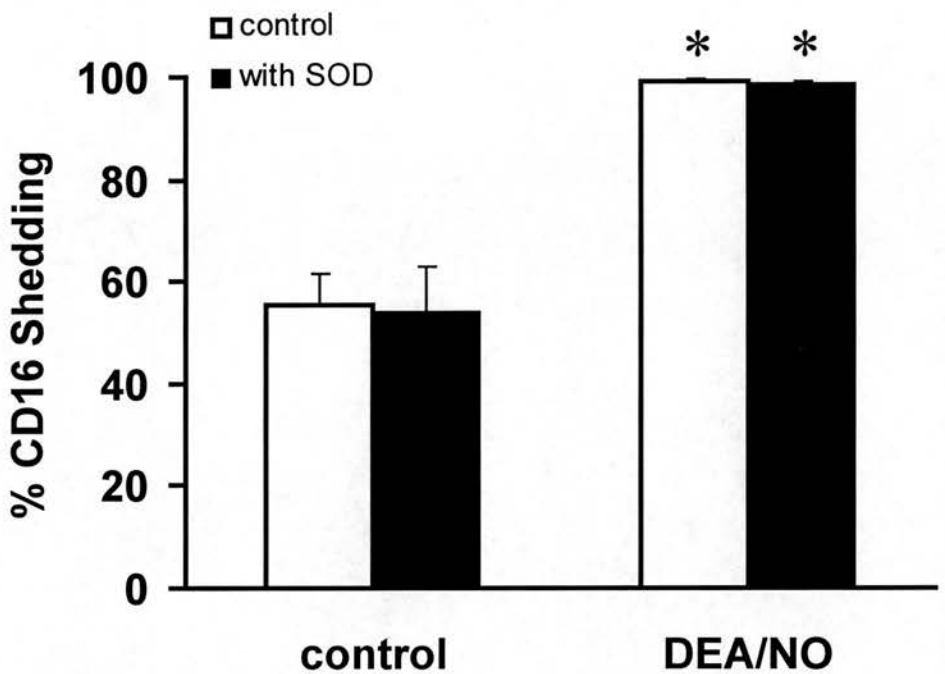


Figure 5.10 Effects of SOD on DEA/NO-induced neutrophil apoptosis as assessed by CD16 shedding. Neutrophils (4.5 x 10^6 cells/ml) were incubated for 20 hours with PBS (control) or DEA/NO (1 mM) in the absence or presence of SOD (50 U/ml). Apoptosis was then assessed by CD16 shedding. Results represent mean ± S.E.M. from n = 3 experiments. Asterisks represent significant ($p < 0.05$) difference from control by one-way ANOVA with Dunnett's post-hoc test.

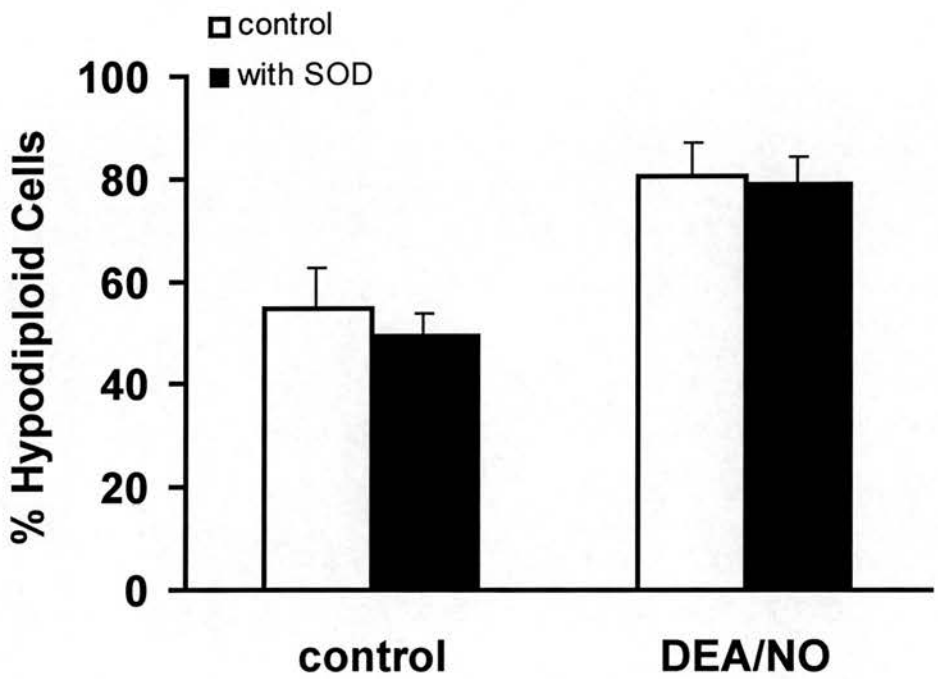


Figure 5.11 Effects of SOD on DEA/NO-induced neutrophil apoptosis as assessed by PI intercalation. Neutrophils (4.5×10^6 cells/ml) were incubated for 20 hours with PBS (control) or DEA/NO (1 mM) in the absence or presence of SOD (50 U/ml). Apoptosis was then assessed by PI intercalation. Results represent mean ± S.E.M. from $n = 3$ experiments.

5.3 Discussion

Experiments using gel electrophoresis as an alternative measure of DNA fragmentation confirmed the results previously obtained using propidium iodide intercalation. DNA gels run following 8 h neutrophil incubation with $NO/ONOO^-$ donors showed no difference from control or a small reduction in laddering with low (0.1 μM) concentrations of the diazeniumdiolates and with a high (1 mM) concentration of SPER/NO. In contrast, cells treated with a high (1 mM) concentration of DEA/NO exhibited increased DNA laddering compared to control, whereas those exposed to GEA 3162 (30 or 100 μM) showed no evidence of DNA laddering.

Following 20 h incubation, no difference from control laddering was observed in neutrophils exposed to 0.1 μM DEA/NO or SPER/NO (0.1 μM or 1 mM), and DNA fragmentation was increased in cells treated with 1 mM DEA/NO. Cells treated with 30 μM GEA 3162 exhibited some DNA laddering, although this was clearly less marked than in control cells. In the presence of 100 μM GEA 3162, no DNA laddering was seen, even at 20 h.

These results were consistent with observations made using propidium iodide, in which low concentrations of the diazeniumdiolates showed a small decrease in DNA fragmentation, and high concentrations of DEA/NO increased DNA fragmentation, which were consistent with morphological observations of apoptosis, but high concentrations of SPER/NO and GEA 3162 showed a paradoxical inhibition of DNA fragmentation despite an increase in morphological apoptosis. This shows that the unexpected results obtained with PI intercalation were real effects of the $NO/ONOO^-$ donors, rather than peculiarities of this assay. It also confirms the concentration-dependent nature of the inhibition of fragmentation by GEA 3162; at 20 h, a small amount of laddering was seen with 30 μM GEA 3162, although this was clearly less than control, but no laddering whatsoever was seen with 100 μM GEA 3162. This suggests that a concentration of 30 μM GEA 3162 is sufficient to delay the onset of DNA fragmentation, but not to completely block this

process, but that 100 μM is able to block the DNA fragmentation pathway almost completely.

Having confirmed the ability of GEA 3162 to inhibit DNA fragmentation in neutrophils, the mechanism by which this occurs was investigated. The DNA fragmentation pathway contains several proteins containing reduced cysteines within their active sites. It has previously been shown that NO can S-nitrosate caspases to inactivate them (Mohr *et al.*, 1997;Li *et al.*, 1997), however it is unlikely that caspases such as caspase 3 are the target for the inhibitory effect of GEA 3162, as inactivation of this enzyme would affect multiple events of apoptosis, such as chromatin condensation and cell shrinkage, rather than just DNA fragmentation. However, the mouse homologue of the downstream protein of the DNA fragmentation pathway, CAD/DFF40, contains several reduced cysteines within its structure (Mukae *et al.*, 1998;Enari *et al.*, 1998). The amino acid sequence and 3D structure of CAD/DFF40 are shown in Figure 5.12, with cysteine residues coloured yellow. These cysteines could be potential targets for oxidative modification by NO or NO-related species, thus inactivating the enzyme and preventing DNA fragmentation. Alternatively, inactivation of the protein could occur via nitrotyrosine formation within the protein by $ONOO^-$.

As $ONOO^-$ is a powerful oxidant species, which is highly reactive and highly unstable, with a half-life of 1.9 seconds (Muscara and Wallace, 1999), neutrophils were pre-treated with the reducing agent, dithiotreitol (DTT; 100-300 μM) to determine whether this agent could block the inhibitory effect of GEA 3162 on DNA fragmentation over a 20-h culture period. DTT has been used in many studies, in particular as a strategy to maintain protein cysteine residues in their reduced state, and to prevent or reverse S-nitrosation of proteins (Mohr *et al.*, 1997;Arstall *et al.*, 1998).

Assessment of DNA fragmentation by PI intercalation showed that the inhibition of DNA fragmentation by GEA 3162 was concentration-dependent, although the effect was less pronounced than observed in the time-course studies, with only the inhibition produced by 100 mM GEA 3162 reaching statistical

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1
MLQKPKSVKLRALRSPRKFGVAGRSCQEVLRKGCLRFQLPERGSRLCLYE
51
DGTELTEDYFPSVPDNAELVLLTLGQAWQGYVSDIRRFLSAFHEPQVGLI
101
QAAQQLLCDEQAPQRQRLLADLLHNVSQNIAAETRAEDPPWFEGLESRFQ
151
SKSGYLRYSCESRIRSYLREVSSYPSTVGAEAQEEFLRVLGSMCQRLRSM
201
QYNGSYFDRGAKGGSRLCTPEGWFSCQGPFDMDSCLSRHSINPYSNRESR
251
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B

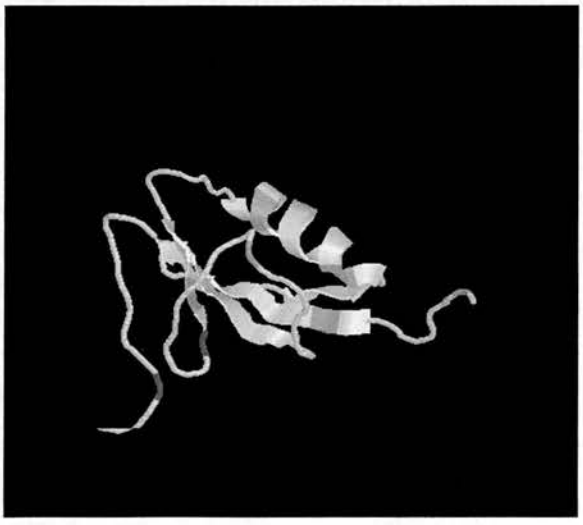

Figure 5.12 Amino acid sequence and 3D structure of Caspase Activated DNase (CAD/DFF40). (A) Amino acid sequence of human CAD/DFF40 [Adapted from Mukae et al., Proc. Nat. Acad. Sci., 95, 9123-9128 (1998)]. (B) 3D structure of CAD/DFF40, with regions coloured yellow representing cysteine residues. [From Protein Data Bank]

significance ($p < 0.05$, one-way ANOVA with Student-Newman-Keuls post-hoc test). This appeared to be an effect of the pre-incubation period (15 min), as parallel experiments conducted on the same set of neutrophils without the pre-incubation period showed profound inhibition of DNA fragmentation in response to GEA 3162 (data not shown). This suggests that leaving the neutrophils for a period of time following isolation conferred a degree of resistance to the inhibitory effects of GEA 3162. It was also observed that constitutive apoptosis was lower in pre-incubated cells than in those incubated immediately following isolation.

Despite the reduced inhibitory effect of GEA 3162, the presence of DTT was shown to be effective in overcoming the blockade of DNA fragmentation in response to this compound. Inhibition mediated by both 30 and 100 μM GEA 3162 was reversed to control levels in a concentration-dependent manner by DTT. This confirms that the inhibitory effect of GEA 3162 is mediated by an oxidative mechanism, and supports the hypothesis of oxidative modification of critical proteins of the DNA fragmentation pathway by $ONOO^-$ liberated from GEA 3162.

In order to determine whether DTT affects the apoptotic process itself, thus accounting for the increased DNA fragmentation observed in cells exposed to DTT, cells treated with GEA 3162 in the absence or presence of the reducing agent were also subjected to morphological assessment of apoptosis. These studies confirmed that the presence of DTT had no effect on the levels of either constitutive or GEA 3162-mediated apoptosis, with no significant difference being observed between levels of apoptosis in the absence or presence of DTT in any of the experimental conditions ($p > 0.05$, one-way ANOVA with Student-Newman-Keuls post-hoc test). This indicates that constitutive apoptosis itself is not dependent upon the generation of reactive oxygen species, as has been suggested (Watson *et al.*, 1996), and that the initiation of apoptosis by GEA 3162 is also independent of oxidative modifications of proteins. However, it also suggests that the mechanism by which GEA 3162 inhibits the DNA fragmentation pathway does rely on an oxidative process, possibly S-nitrosation of protein, which remains to be elucidated.

In order to demonstrate that the effects of DTT observed with GEA 3162 are

specific to apoptosis induced by this compound and not common to all apoptotic stimuli, the experiment was repeated in neutrophils exposed to the fungal metabolite, gliotoxin, for 20 h. These studies demonstrated the acceleration of neutrophil apoptosis by gliotoxin compared to control, with most of the cells being apoptotic by 20 h. However, in contrast to cells exposed to GEA 3162, DTT did not increase the levels of DNA fragmentation as assessed by PI intercalation. Indeed, a small reduction in DNA fragmentation was observed. Morphological assessment of apoptosis in these cells also demonstrated a possible inhibitory effect of DTT on gliotoxin-induced apoptosis.

A 4 h time point with gliotoxin was also investigated because any increase in DNA fragmentation levels in the presence of DTT would not be detectable at 20 h, as the majority of gliotoxin-treated cells showed evidence of hypodiploid DNA content at this time point. At 4 h of incubation, a significant ($p < 0.05$, one-way ANOVA with Dunnett's post-hoc test) increase in DNA fragmentation was observed in gliotoxin-treated cells compared to control, but this was substantially less than 100 % of cells, allowing any DTT-mediated increase in DNA fragmentation to be observed. However, at this time point, the inhibition of the pro-apoptotic effects of gliotoxin by DTT were more marked, reducing the percentage of cells with hypodiploid DNA content back to control levels. Similar findings were made with morphological studies of apoptosis, demonstrating that DTT has a protective effect against gliotoxin-mediated neutrophil apoptosis, but does not appear to have any pro-apoptotic effects on apoptosis, or even DNA fragmentation, by itself.

Such data imply that gliotoxin induces neutrophil apoptosis via an oxidative mechanism, as the reducing agent was able to inhibit both morphological and DNA fragmentation events of apoptosis induced by this compound. This is consistent with previous findings using gliotoxin as a pro-apoptotic stimulus; an oxidative mechanism has been shown to be involved in gliotoxin-induced apoptosis in thymocytes (Beaver and Waring, 1995) and macrophages (Suen *et al.*, 2001).

It has been reported that the mechanism of activation of CAD/DFF40 bears a remarkable similarity to the mechanism of activation of the transcription factor, NF-

κB (Enari *et al.*, 1998), and NO has been shown to inhibit NF-κB activation though induction and stabilisation of the inhibitor molecule, IκB, that is the equivalent of ICAD/DF45 in the DNA fragmentation pathway (Peng *et al.*, 1995). Therefore, Western blotting studies were carried out to determine whether NO/$ONOO^-$ donors are able to inhibit the proteolytic cleavage of ICAD/DFF45 in neutrophils.

Cells were exposed to NO/$ONOO^-$ donors, gliotoxin (positive control) or PBS (control) for 8 h, as this is the time when the most marked differences in DNA fragmentation are observed with some compounds, such as DEA/NO. Whole cell lysates were made from freshly-isolated neutrophils and those incubated for 8 h, and analysed by Western blot for levels of ICAD/DFF45. Both long (45 kDa) and short (40 kDa) splice variants of ICAD/DFF45 were detectable in neutrophils.

Reduced levels of ICAD/DFF45 compared to control were detected in gliotoxin-treated neutrophils as expected, as this compound induces neutrophil apoptosis alongside enhanced DNA fragmentation, as described in the previous section. Neutrophils exposed to GEA 3162 (30 or 100 μM) showed a concentration-dependent breakdown of ICAD/DFF45, and DEA/NO-treated cells showed clearly visible ICAD/DFF45 breakdown. However, SPER/NO-treated cells showed no breakdown of ICAD/DFF45, and the band was possibly larger than that seen in control cells.

Although it was to be expected that any ICAD/DFF45 breakdown in SPER/NO-treated cells would be minimal, as accelerated apoptosis is not seen until 16 h with this compound, the large band suggests that the inhibition of DNA fragmentation by SPER/NO may be mediated through inhibition of ICAD/DFF45 breakdown, and therefore stabilisation of the ICAD/CAD complex. This would represent a similar mechanism of inhibition of CAD/DFF40 activation as has been previously reported for NF-kB (Peng *et al.*, 1995). Levels of ICAD/DFF45 would also have to be tested at 16 or 20 h to establish whether this hypothesis holds true.

An apparent decrease in ICAD/DFF45 levels was observed in neutrophils treated with DEA/NO, which is the expected result as this compound enhances DNA

fragmentation, which is particularly obvious at the 8 h time point. Therefore, acceleration of neutrophil apoptosis by DEA/NO proceeds through the established DNA fragmentation pathway

In contrast, ICAD/DFF45 levels decreased with increasing concentrations of GEA 3162, demonstrating that the inhibition of DNA fragmentation evoked by this compound does not involve inhibition of the cleavage of ICAD/DF45. As expected, therefore, GEA 3162-derived $ONOO^-$ does not inhibit the apoptotic pathway at the level of caspase 3, or other caspases able to activate CAD/DFF40, as this would have resulted in an inhibition of ICAD/DFF45 breakdown. The inhibition therefore must occur downstream of ICAD/DFF45 cleavage, for example through inhibition of the activity of CAD/DFF40 through oxidative modification of its reduced cysteines after its release from inhibition by ICAD/DFF45 cleavage. Alternatively, GEA 3162-derived $ONOO^-$ may alter DNA itself, leaving it more resistant to internucleosomal cleavage by CAD/DFF40, or it may modify chromatin-associated proteins such as histone H1, high mobility group proteins or topoisomerase II, that have been reported to be associated with the ICAD/CAD complex and to potentiate CAD/DFF40 activity (Zhang and Xu, 2002). In particular, histone H1 associates with active CAD/DFF40 and confers DNA binding and endonuclease activity on CAD/DFF40 (Liu *et al.*, 1999), therefore histone H1 modification could prevent its interaction with CAD/DFF40 and therefore inhibit DNA fragmentation. Furthermore, CAD/DFF40 oligomerises to form a complex before binding to DNA (Liu *et al.*, 1999), therefore oxidative modification of active CAD/DFF40 via its reduced cysteines could potentially inhibit complex formation and hence DNA binding and cleavage.

The lack of internucleosomal DNA fragmentation in neutrophils exposed to $NO/ONOO^-$ donors such as GEA 3162 and SPER/NO may have implications for their potential therapeutic use. It has been suggested that the absence of DNA fragmentation could lead to large amounts of DNA escaping from apoptotic cells and either provoking an autoimmune response or being taken up by neighbouring cells and potentially leading to the development of cancer (Zhang and Xu, 2002).

The cytotoxic effects of NO are often attributed to the formation of $ONOO^-$

(Liaudet *et al.*, 2000), although this is not always necessarily the case, as others have shown a direct cytotoxic effect of NO without the need for $ONOO^-$ formation (Fricker *et al.*, 1999). Apoptosis experiments were carried out using SOD to determine whether DEA/NO-induced apoptosis is mediated by $ONOO^-$ generated by NO liberated from DEA/NO combining with O_2^- released from neutrophils during incubation. However, these experiments showed no effect of SOD on DEA/NO-mediated neutrophil apoptosis, suggesting that there is no role for $ONOO^-$ in this process. This is in contrast to observations with the $ONOO^-$ donor, GEA 3162, in which SOD has been shown to inhibit the induction of neutrophil apoptosis by this compound (Ward *et al.*, 2000).

However, SOD is not cell permeable, therefore would only be effective if the combination of NO and O_2^- occurred outside the cells. Intracellular SOD mimetics are available, and could be used to scavenge intracellular neutrophil-generated O_2^- in order to inhibit $ONOO^-$ formation within the cells. It is possible that NO is able to pass into the neutrophils before generating $ONOO^-$ by interacting with O_2^- contained within the neutrophils. However, this is unlikely to occur, because the NO from DEA/NO would all be released very early during the incubation period, probably before the neutrophils generate any significant levels of ROS. Furthermore, were DEA/NO to act via $ONOO^-$ formation, it would be expected that this compound would have the same inhibitory effects on DNA fragmentation as $ONOO^-$ generators, rather than enhancing DNA fragmentation.

To summarise, therefore, the inhibition of internucleosomal DNA fragmentation by GEA 3162 occurs via an as yet uncharacterised oxidative mechanism. However, the induction of morphological neutrophil apoptosis by this compound is not redox-sensitive. The inhibition of internucleosomal DNA fragmentation does not occur at the level or upstream of ICAD/DFF45 breakdown, as a concentration-dependent increase in this process is seen on exposure to GEA 3162 (30 – 100 μM). However, the lesser degree of inhibition observed with SPER/NO (0.1 μM - 1 mM) may occur through inhibition of ICAD/DFF45 cleavage, as a large band corresponding to ICAD/DFF45 was observed by Western blotting, although

this remains to be confirmed. There is no evidence in these studies to suggest that DEA/NO-induced neutrophil apoptosis is mediated by $ONOO^-$ generation. However, this hypothesis could still possibly hold true, as SOD only scavenges extracellular O_2^-, and this would require further investigation using intracellular SOD mimetics or a O_2^- specific spin trapping agent.

Chapter 6: Summary and future directions

6.1 Summary and Conclusions

Many factors are able to positively or negatively influence the rate of neutrophil apoptosis *in vitro*, which may have implications *in vivo* by determining the duration of an inflammatory response. Apoptosis is a physiological, active process which serves to eliminate unwanted or damaged cells without eliciting an inflammatory reaction. Apoptosis also facilitates non-inflammatry phagocytic cell clearance by macrophages. Thus, neutrophil apoptosis is an important process in the resolution of inflammation, as the alternative form of cell death, necrosis, provokes profound inflammation. Furthermore, phagocytic clearance of necrotic cells leads to release of pro-inflammatory factors from macrophages, therefore leading to exacerbated inflammation and tissue damage, and contributing to the pathogenesis of chronic inflammation. NO is an important molecule in inflammation, contributing to the bactericidal actions of inflammatory cells as well as possessing powerful vasodilator properties. Additionally, NO can regulate the lifespan of neutrophils, by affecting the rate at which these cells undergo apoptotic cell death. The aim of this thesis was to characterise the regulation of neutrophil apoptosis by NO and NO-related species *in vitro*.

These studies have demonstrated that diazeniumdiolate compounds, DEA/NO and SPER/NO, spontaneously generate free NO in aqueous solution; release of NO from DEA/NO occurs much more rapidly than from SPER/NO, although equivalent concentrations of these two compounds generate the same amount of NO overall. Consequently, DEA/NO generates a brief spike of high concentrations of NO, whereas SPER/NO produces a prolonged flux of lower concentrations of NO. NO from these compounds was unable to oxidise DHR 123 to rhodamine 123, a process used to distinguish between compounds that release free NO vs. those that release oxidative species such as peroxynitrite ($ONOO^-$). In contrast, the sydnonimine, SIN-1, and the oxatriazole-5-imine derivative, GEA 3162, failed to liberate free NO, except in the presence of SOD, which dismutates superoxide anion (O_2^-) to hydrogen peroxide, and were both able to oxidise DHR 123. Thus, these two compounds both

simultaneously generate NO and O_2^-, which rapidly react to form the powerful oxidant species, peroxynitrite $ONOO^-$. These results were confirmed by the demonstration of oxidation of DHR 123, in contrast to pure NO. Therefore, SIN-1 and GEA 3162 are $ONOO^-$ generators, rather than pure NO donors.

Neutrophil apoptosis studies showed that both diazeniumdiolate compounds have a biphasic effect on apoptosis. Whereas low concentrations of these compounds delay the rate of apoptosis, high concentrations promote this form of cell death. A more sustained release of NO from SPER/NO gives a more profound inhibitory effect than the rapid spike of the same total amount of NO from DEA/NO, and enhances apoptosis at lower concentrations than DEA/NO. However, the pro-apoptotic effects of DEA/NO occur much more rapidly than that evoked by SPER/NO and the highest concentration of DEA/NO tested induces a higher level of apoptosis than the equivalent concentration of SPER/NO. In contrast, the $ONOO^-$ donor, GEA 3162, shows no anti-apoptotic effects at equivalent concentrations to those that give an inhibitory effect with the diazeniumdiolates. However, pro-apoptotic effects are observed at lower concentrations of GEA 3162 than observed with the diazeniumdiolates.

An unexpected inhibition of DNA fragmentation alongside enhancement of other apoptotic events is observed with the pure NO donor, SPER/NO and more markedly with the $ONOO^-$ donor, GEA 3162. The established $ONOO^-$ donor, SIN-1, also gives this paradoxical dissociation of DNA fragmentation from other apoptotic events, showing that the inhibition evoked by GEA 3162 is a general $ONOO^-$-mediated effect, rather than an effect that is peculiar to GEA 3162. Further studies showed that the inhibition of DNA fragmentation is an oxidative effect as it could be abolished by addition of a reducing agent. However, Western blotting studies showed that the breakdown of ICAD/DFF45 is not affected by GEA 3162.

The promotion of neutrophil apoptosis is important for the resolution of acute inflammation and prevention of chronic inflammatory conditions. Limiting the lifespan of this cell type would consequently limit the amount of histotoxic and pro-inflammatory substances released into the local environment. This thesis has shown

that NO and related species, such as $ONOO^-$, are able to push neutrophils into apoptotic cell death, and may therefore be useful therapeutically. It is important to consider, however, that an upregulation of neutrophil apoptosis must not overwhelm the phagocytic capacity of the tissue, or apoptotic neutrophils would undergo secondary necrosis, thus exacerbating inflammation and tissue damage. Any profound induction of neutrophil apoptosis, therefore, would have to be accompanied by a similar upregulation of phagocytosis by macrophages. Further work is required to investigate the effects of NO and $ONOO^-$ donors on neutrophil apoptosis, and their subsequent clearance by macrophages, in order to determine which class of compound offers the most promising therapeutic potential, as described below.

6.2 Future Directions

6.2.1 Mechanisms of NO and $ONOO^-$-mediated inhibition or induction of neutrophil apoptosis

The effects of various NO and $ONOO^-$-releasing compounds have been thoroughly characterised in this thesis, but mechanisms of action have not been elucidated. Future studies would therefore focus on the mechanisms through which NO and $ONOO^-$ regulate neutrophil apoptosis.

A cutting edge proteomics approach could be taken to determine the up- or down-regulation of critical apoptotic proteins in response to exposure to these compounds. This technology involves alkylation of cysteine-containing proteins with isotope-coded affinity tags (ICAT) containing either a light or a heavy isotope of deuterium and a biotin affinity tag. One sample is labelled with the isotopically light reagent and the other with the heavy reagent, then samples are mixed and treated with trypsin to form peptides that are easily analysed by mass spectrometry (MS). Following cation exchange chromatography, mixtures are passed over a monomeric avidin-agarose column, which binds ICAT-labelled peptides through their biotin tag. Eluted peptides are then isolated by microcapillary liquid chromatography electrospray ionisation tandem mass spectrometry (LC/MS/MS).

The mass spectrometer can distinguish light and heavy isotope-labelled peptides due to their mass difference and the relative amounts in each sample can be measured. It is able to analyse a specific peptide of interest, even if many other peptides are present in the sample.

The advantage of this method over classical proteomics approaches is that it allows both determination of the identity of the peptides of interest and quantitative analysis of their relative abundance between samples in a single step. In particular, the gene products whose expression is induced by the transcription factors, NF-κB and AP1 could be investigated.

Furthermore, as the MAP kinase pathways are believed to have a role in apoptotic signalling in many cell types, it would be interesting to conduct studies to analyse activation levels of the 3 principal MAP kinase pathways; the ERK, JNK and p38 MAP kinase pathways. Kinase assays could be used to determine the activities of various kinase enzymes within these pathways, and Western blotting techniques could be used to measure the presence of kinases in their unphosphorylated (inactive) or phosphorylated (active) forms. Results obtained using this technique could then be verified through the use of pharmacological inhibitors of relevant signalling pathways.

This approach may allow identification of signalling pathways involved in promotion or inhibition of neutrophil apoptosis in response to NO or $ONOO^-$. Additionally, the levels of pro- and anti-apoptotic Bcl-2 family proteins that are known to be expressed in neutrophils, levels of active caspases and NF-κB activation could also be assessed using Western blotting techniques. Where appropriate antibodies are available, activation of MAP kinases and caspases, and localisation of Bcl-2 family proteins, could be visualised by immunofluorescence. Our group has successfully used the HIV-TAT system to introduce dominant negative signalling proteins into neutrophils (HIV-TAT-I-κB) and macrophages (HIV-TAT-cas). This system could potentially be exploited to definitively prove the involvement of particular signalling molecules in neutrophil apoptosis. These studies taken together would allow a picture to be built up of the mechanisms through which neutrophil

apoptosis is regulated by NO and $ONOO^-$, and time course studies would give an indication of the temporal regulation and integration of these pathways to produce models for the induction or inhibition of neutrophil apoptosis.

6.2.2 Apoptosis studies in cytokine- or adhesion-activated neutrophils

Studies on the regulation of neutrophil apoptosis have so far been carried out on non-activated cells. However, it is well established that, *in vivo*, adhesion of neutrophils to the endothelium and to extracellular matrix leads to neutrophil activation and a delay in the rate at which these cells undergo apoptosis. This may be a factor in the persistence of extravasated neutrophils within inflammatory sites, which prolongs the inflammatory response and may contribute to the pathogenesis of chronic inflammatory conditions. Therefore, it is important to examine the effects of the NO/$ONOO^-$ releasing compounds on adhesion-activated neutrophils. This could be done by repeating the experiments already carried out following culture of neutrophils on a monolayer of endothelial cells, which should reduce the rate of constitutive apoptosis. The hypothesis is that NO/$ONOO^-$ donors are able to override the survival signals promoted by adhesion of neutrophils to the endothelial cells. Similar to the experiments in non-activated cells, the signalling pathways involved could be determined using the proteomics, Western blotting and pharmacological approaches described above.

Furthermore, in order to examine the potential effects of these compounds within inflammatory sites, it would be useful to undertake studies to examine whether the NO/$ONOO^-$ donors are able to override survival signals evoked by pro-inflammatory mediators that are likely to be present at inflammatory sites, such as GM-CSF and LPS. Neutrophils could be exposed to such mediators before, simultaneously and after addition of the NO/$ONOO^-$ donors in order to determine whether the timing of exposure to these compounds is critical in determining the extent to which they are able to overcome the survival pathways induced by inflammatory mediators.

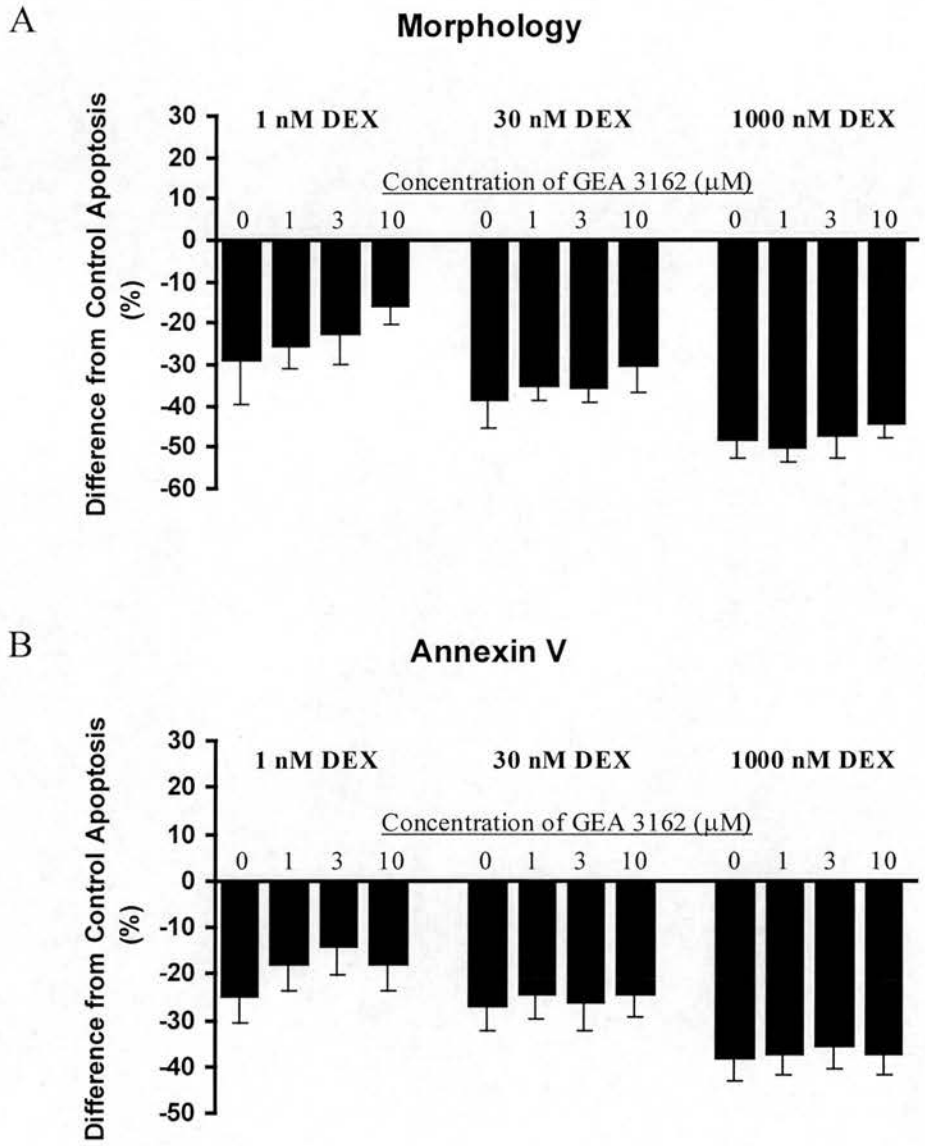

Figure 6.1 Effects of GEA 3162 on the dexamethasone-mediated delay of neutrophil apoptosis. Neutrophils (5 x 10^6 cells/ml) were exposed to 1, 30 or 1000 nM dexamethasone in the absence and presence of GEA 3162 (1-10 μM) for 20 hours, then apoptosis was assessed by (A) Morphology and (B) Annexin V binding. Results represent mean ± S.E.M. from n = 4 experiments. Baseline (control) levels of apoptosis were 72.5 ± 4.2 % by morphology and 56.5 ± 2.5 % by Annexin V binding.

Other agents that are able to delay neutrophil apoptosis, such as synthetic glucocorticoids, could also be assessed. Preliminary studies have demonstrated the ability of low concentrations of GEA 3162 (that do not in themselves induce apoptosis) to reduce the inhibition of neutrophil apoptosis seen with exposure to low concentrations of the synthetic glucocorticoid, dexamethasone, although it has no effect with higher concentrations of dexamethasone (Figure 6.1). These studies could be extended to determine the mechanism of this reversal; the hypothesis is that this compound S-nitrosates the glucocorticoid receptor, thereby preventing binding of dexamethasone. It has previously been shown that NO can S-nitrosate critical cysteine residues in the glucocorticoid receptor to inhibit glucocorticoid binding (Galigniana *et al.*, 1999).

6.2.3 Mechanisms of inhibition of DNA fragmentation

The inhibition of internucleosomal DNA fragmentation by $ONOO^-$ and longer-lasting NO donors should be further investigated, using the hypothesis that oxidative modifications of important proteins in the DNA fragmentation pathway are responsible for the inhibition. Western blotting could be carried out using a 'NitroGlo' kit to examine the level of S-nitrosation in treated neutrophils compared to untreated cells. Potential target proteins of the DNA fragmentation pathway may then be purified and evaluated on an individual basis. Furthermore, to determine whether GEA 3162 can directly inhibit the activity of Caspase-Activated DNase (CAD/DFF40), a plasmid vector for the expression of CAD/DFF40 and ICAD/DFF45 developed in the laboratory of Bill Earnshaw at the University of Edinburgh could be transfected into *E. coli*, and the CAD-ICAD complex would be purified. The complex could then be treated with active caspase 3 in order to degrade the ICAD, leaving the active CAD, which would then be exposed to PBS (control) or GEA 3162 for varying periods of time before being applied to isolated DNA from neutrophils and left for a period of several hours. The DNA would then be run by gel electrophoresis to look for the characteristic laddering pattern generated by CAD, as an indication of the activity of CAD.

In order to test the hypothesis that pure NO donors with a slower rate of NO release are able to inhibit DNA fragmentation via $ONOO^-$ formation, but that faster-releasing compounds are unable to do so, it would be interesting to test a range of diazeniumdiolate compounds with varying rates of release. If the hypothesis holds true, the extent of inhibition of DNA fragmentation seen with these compounds should correlate with the half-life of decomposition. Furthermore, in order to investigate whether inhibition of DNA fragmentation is not observed with faster-releasing diazeniumdiolates because NO and O_2^- are not present simultaneously within cells, neutrophils could be stimulated with an activating agent such as PMA to provoke early generation of O_2^- which should coincide with the timescale of NO release, thus promoting $ONOO^-$ generation. DNA fragmentation could then be assessed by propidium iodide intercalation to determine whether diazeniumdiolates, such as DEA/NO, that do not inhibit DNA fragmentation in their own right are able to do so following stimulation with PMA.

6.2.4 *Effects of NO/$ONOO^-$ donors on neutrophil apoptosis in PBS or culture medium without serum*

Recent studies have suggested that in the presence of HEPES (which is present at a concentration of 25 mM in Iscove's MDM culture medium), NO from diazeniumdiolates is undetectable as $ONOO^-$ is preferentially formed through oxidation of HEPES (Keynes *et al.*, 2003). Therefore it is possible that the pro-apoptotic effects of SPER/NO and DEA/NO seen in Iscove's MDM plus 10 % autologous serum are mediated by $ONOO^-$ formation, rather than by NO. Furthermore, it has been shown that buffer components such as HEPES have the ability to convert $ONOO^-$ to NO-donating compounds, but that this effect is not observed in phosphate buffer (Schmidt *et al.*, 1998), suggesting that apoptosis induced by $ONOO^-$ donors may be due, at least in part, to generation of free NO. others have reported that SIN-1-mediated cytotoxicity is due to H_2O_2 formation in the presence of HEPES, but is due to $ONOO^-$ in its absence (Lomonosova *et al.*, 1998). The studies described in this thesis have all been carried out in Iscove's

MDM, but have not been carried out in HEPES-free medium. In order to elucidate any potential influence of serum proteins or buffer components such as HEPES on neutrophil apoptosis induced *in vitro* by NO/$ONOO^-$ donors, apoptosis studies could be carried out in Iscove's MDM without serum, in PBS and in PBS supplemented with 10 % autologous serum.

6.2.5 *Effects of NO/$ONOO^-$ donors on apoptosis in other cell types*

The apoptosis studies could be extended into other cell types that are relevant to inflammatory disease processes within the cardiovascular system, such as endothelial cells and macrophages. Ideally, a NO/$ONOO^-$ donor would be found that is able to induce apoptosis in neutrophils but does not affect endothelial cell apoptosis at the same concentration. These studies may have implications for protection against or regression of inflammatory conditions such as post-angioplasty restenosis or myocardial ischaemia-reperfusion injury. Potential beneficial effects of these compounds could be tested using animal models of inflammation.

6.2.6 *Clearance of apoptotic neutrophils following treatment with NO/$ONOO^-$ donors*

Phagocytosis studies could be carried out to examine whether neutrophils that have been induced to undergo apoptosis by NO/$ONOO^-$ donors are still effectively cleared by macrophages. For example, these studies have shown that PS exposure on the outer surface of GEA 3162-treated cells is not different from control cells at a 20-h time point. This lack of enhancement of PS exposure may have adverse effects *in vivo* if the clearance of these cells is not similarly upregulated, as cells may undergo pro-inflammatory secondary necrosis. A flow cytometric assay could be used to examine the uptake of these cells by human macrophages differentiated in culture from isolated monocytes, to determine whether this is increased from control in GEA 3162-treated cells.

These studies should provide further insights into the differences that exist between distinct classes of NO and $ONOO^-$ donors, in particular differences in mechanisms of action. Elucidation of the apoptotic pathways induced by these compounds could allow particular pathways to be harnessed therapeutically. These studies should also provide information as to which type of NO or $ONOO^-$ donor is most likely to prove effective in an inflammatory situation, for example, which compound proves most effective at over-riding survival signals initiated by pro-inflammatory mediators or neutrophil adhesion, and with which compounds are apoptotic neutrophils most effectively phagocytosed following apoptosis. These studies could therefore determine which NO or $ONOO^-$ donors should be most rigorously pursued in terms of further research or future development of potential therapeutic compounds.

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