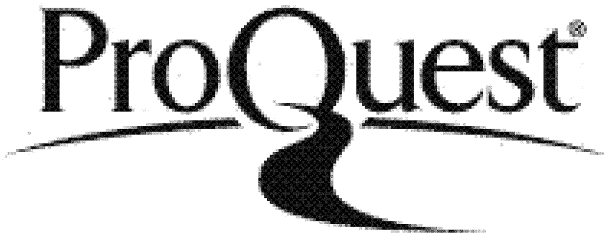
Modulation of Vascular Tone by Neutrophils *in vivo*

Jonathan Morton

A thesis submitted to Cardiff University for the degree of Doctor of Philosophy



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for Mum & Dad

PUBLICATIONS

PEER-REVIEWED PAPERS

- Anning, P. B., Coles, B., Wang, H., Morrow, J. D., Dey. S. K., *Morton, J.*, Marnett, L. J. & O'Donnell, V. B. (2006). Removal of nitric oxide is required for hypertensive and prothrombotic effects of COX-2 inhibition *in vivo*. *Blood,* 108, 4059–62.
- Davies, R., Coles, B., Anning, P.B., *Morton, J.*, Sivasankar, B., Morgan, B. P. & O'Donnell, V. B. (2007). Characterisation of vascular function in mice deficient in the complement inhibitor CD59a. *Immunology*, 121, 518–25.
- *Morton, J.*, Coles, B., Wright, K., Coffey, M. J., Anning, P. B., Morgan, B. P., Kühn, H., Kuban, R-J., Hobbs, A. J., Jones, S.A., O'Donnell, V. B. (2007). Circulating neutrophils regulate gene expression of smooth muscle and preserve functional integrity of blood vessels in healthy mice. *(in preparation).*

ABSTRACTS

- *Morton, J.*, Anning, P. B., Coles, B. & O'Donnell, V. B. (2005). Modulation of vascular tone by neutrophils *in vivo*. (Abstract). *Free Radical Biology & Medicine*, 39 (Suppl. 1), S89.
- *Morton, J.*, Anning, P. B., Coles, B. & O'Donnell, V. B. (2004). Regulation of nitric oxide bioavailability and vascular tone *in vitro* and *in vivo* by neutrophils. (Abstract). *Free Radical Biology & Medicine,* 37 (Suppl. 1), S87.

SUMMARY

The vascular endothelium regulates vasomotor tone and blood fluidity through the release of paracrine factors, such as nitric oxide (NO). Acutely activated neutrophils have been shown to consume vasodilatory NO *in vitro* through the NAD(P)H oxidase-dependent generation of superoxide. As such, neutrophils may contribute to the endothelial dysfunction and increased vascular tone commonly observed in inflammatory vascular diseases. Herein, the experiments in this thesis set out to examine a role for neutrophils in the regulation of vascular tone.

Initial experiments aimed to assess the capacity of inflammatory-primed neutrophils to consume NO *in vitro*. Inflammatory priming of human neutrophils resulted in significantly accelerated rates of NO disappearance from aerobic buffer *in vitro* in an NAD(P)H oxidase-dependent manner. Thus, neutrophil priming may have a detrimental effect on vascular homeostasis *in vivo*.

With this in mind, subsequent chapters aimed to elucidate a role for neutrophils in regulating vascular tone *in vivo* using immunodepletion techniques. Following neutrophil depletion, mice exhibited a gradual fall in systolic blood pressure over 3 days, and phenylephrine-induced vasoconstriction was significantly attenuated in thoracic aortae isolated from neutropenic animals compared to controls. There effects were due, at least in-part, to the interferon-γ-dependent upregulation of inducible nitric oxide synthase (iNOS) bioactivity in the thoracic aortae of neutropenic mice. Thus, under physiological conditions, neutrophils may modulate vascular tone by negatively regulating the bioactivity of pro-inflammatory enzymes, such as iNOS.

Finally, the effect of acute neutropenia on angiotensin (Ang) II-induced hypertension *in vivo* was examined. Neutrophil depletion prevented the onset of Ang II-induced hypertension, indicating that neutrophils may be directly involved in the development of hypertension associated with this octapeptide *in vivo*.

In conclusion, the data described in this thesis highlight the diversity of neutrophil activities *in vivo* and *in vitro* and how changes in neutrophil phenotype may impact upon vascular physiology.

ABBREVIATIONS

-/-	Homozygous knockout (genotype)
2,3-dinor-keto $PGF_{1\alpha}$	2,3-dinor-6-keto prostaglandin $F_{1\alpha}$
5-HT	Serotonin
AA	Arachidonic acid
ACE	Angiotensin-converting enzyme
ACh	Acetylcholine
ADP	Adenosine diphosphate
Ang I	Angiotensin I
Ang II	Angiotensin II
AP-1	Activating protein-1
AT_1	Angiotensin type-1 receptor
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
ATZ	Aminotriazole
BH_4	6(R)-5,6,78-tetrahydrobiopterin
BP	Blood pressure
Ca^{2+}	Calcium ion
$[Ca^{2+}]_i$	Intracellular free calcium concentration
CaM	Calmodulin
C/EBP	CCAAT-enhancer box binding protein
cGMP	Cyclic guanosine 3'5'-monophosphate
COX	Cyclooxygenase
CSE	Control standard endotoxin
CuZn-SOD	Copper-zinc superoxide dismutase
CYP	Cytochrome P-450
cyt *c*	Cytochrome *c*
DAG	Diacylglycerol
DEA-NONOate	Diethylamine NONOate
DMSO	Dimethyl sulphoxide
DPI	Diphenyleneidonium

DTT	Dithiothreitol
e^-	Electron
ECL	Enhanced chemiluminescence
EDHF	Endothelium-derived hyperpolarising factor
EDRF	Endothelium-derived relaxing factor
ELISA	Enzyme-linked immunosorbant assay
EMSA	Electrophoretic mobility shift assay
eNOS/NOS-3	Endothelial nitric oxide synthase
EPO	Eosinophil peroxidase
$E_{red}LOO^{\bullet}$	Lipid peroxyl radical intermediate
ET-1	Endothelin I
F_2-IP	F_2-isoprostane
FAD	Flavin adenine dinucleotide
FCS	Foetal calf serum
fMLP	*N*-formyl-L-methionyl-L-leucyl-L-phenylalanine
FMN	Flavin mononucleotide
GAS	Interferon-γ-activated site
GC/MS	Gas chromatography-mass spectrometry
GM-CSF	Granulocyte-monocyte colony-stimulating factor
GTP	Guanosine triphosphate
H^+	Proton
H_2O_2	Hydrogen peroxide
Hb	Haemoglobin
$HO^{\bullet}$	Hydroxyl radical
HOCl	Hypochlorous acid
HR	Heart rate
HRP	Horseradish peroxidase
hsp90	Heat shock protein 90
HUVEC	Human umbilical vein endothelial cell
IFN-γ	Interferon-γ
ICAM-1	Intracellular cell adhesion molecule-1
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
iNOS/NOS-2	Inducible nitric oxide synthase

i.p.	intraperitoneal
IP_3	Inositol 1,4,5-triphosphate
IRF-1	Interferon regulatory factor-1
IVIG	Intravenous administration immunoglobulin preparation
JAK	Janus kinase
k_{obs}	first order rate constant
LAL	*Limulus* amebocyte lysate
L-NAME	L-nitroarginine-methyl ester
LOOH	Lipid hydroperoxide
LOONO	Organic peroxynitrite species
LOX	Lipid hydroxide
LPO	Lactoperoxidase
LPS	Endotoxin/lipopolysaccharide
LTB_4	Leukotriene B_4
mAb	Monoclonal antibody
MCP-1	Monocyte chemoattractant protein-1
MEM	Minimum essential medium
MIP-1α	Macrophage inflammatory peptide-1α
MLCK	Myosin light-chain kinase
MPO	Myeloperoxidase
mtNOS	Mitochondrial nitric oxide synthase
Na^+	Sodium ion
NA	Noradrenaline
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate)
NaF	Sodium fluoride
NEDA	*N*-(1-naphtyl)-ethylenediamine
NF-κB	Nuclear factor κB
NO	Nitric oxide
NO^+	Nitrosium cation
NO_2	Nitrogen dioxide
NO_2^-	Nitrite anion
NO_3^-	Nitrate anion
NOS	Nitric oxide synthase
NO_x	Nitric oxide metabolites

nNOS/NOS-1	Neuronal nitric oxide synthase
O_2	Molecular oxygen
$O_2^{\bullet-}$	Superoxide
$ONOO^-$	Peroxynitrite
oxyHb	Oxyhaemoglobin
PAF	Platelet –activating factor
PBS	Phosphate-buffered saline
PE	Phenylephrine
PECAM-1	Platelet-endothelial cell adhesion molecule-1
PGI_2	Prostacyclin
PGIS	Prostacyclin synthase
PKA	Protein kinase A
PKG	Protein kinase G
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulfonyl fluoride
PMN	Polymorphonuclear leukocyte
pNA	p-nitroalanine
RASMC	Rat aortic smooth muscle cell
RBC	Red blood cell
R-NHCl	Chloramine
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
Ser	Serine
sGC	Soluble guanylate cyclase
SHR	Spontaneously hypertensive rat
SIE	Signal transducter and activator of transcription (STAT)-inducing element
SOD	Superoxide dismutase
SR	Sarcoplasmic reticulum
STAT1	Signal transducer and activator of transcription
TNF-α	Tumour necrosis factor-α
TxA_2	Thromboxane A_2

VCAM-1	Vascular cell adhesion molecule-1
VSMC	Vascular smooth muscle cell
WBC	White blood cell
WKY	Wistar-Kyoto rat
WT	Wild-type (genotype)
w/v	Weight/volume
XD	Xanthine dehydrogenase
XO	Xanthine oxidase

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction to the vascular system

The vascular system is comprised of arteries, arterioles, and capillaries designed to maintain the constant flow of blood rich in oxygen and nutrients to the body's tissues, as well as venules and veins, which carry deoxygenated blood and metabolic end products away for excretion. The ability of blood vessels to carry out these functions is largely dependent on their compliance or elasticity (i.e. the degree to which the luminal volume increases as the pressure increases); this is particularly important in the arterial system (i.e. arteries and arterioles), which absorbs the pulsations in cardiac output in order to provide a steady flow of blood to the tissues. The greater the compliance of the system, the more effectively fluctuations in arterial pressure are dampened out with each heartbeat (Rang *et al.* 1999).

Although the functional and structural characteristics of blood vessels vary according to size, all vessels contain an endothelium, a continuous monolayer of cells on the luminal surface (Figure 1.1) (Bryan *et al.* 2005). In addition, all vessels, except the capillaries, contain a layer of connective tissue and smooth muscle cells, which are principally responsible for the maintenance of vessel compliance through their contraction and relaxation. A reduction in arterial compliance, or 'hardening' of the arteries, due to

increased vascular smooth muscle tone, is observed in atherosclerosis, hypertension and ageing, and thus represents an important risk factor for vascular disease (Liao *et al.* 1999; Zieman *et al.* 2005). Therefore, the examination of the mechanisms responsible for regulating vascular smooth muscle tone is an area of extensive study in medical research.

1.2 Mechanisms for the development of vascular smooth muscle tone

1.2.1 Activation of contraction/relaxation

Vascular smooth muscle contraction and relaxation is initiated by an increase or decrease in intracellular free calcium concentration ($[Ca^{2+}]_i$), respectively (Filo *et al.* 1965; Somlyo & Himpens, 1989). Ca^{2+} binds calmodulin (CaM), forming a regulatory Ca-CaM complex, which binds to and activates myosin light-chain kinase (MLCK). Upon activation, MLCK phosphorylates a serine (Ser) at position 19 of the regulatory chain (MLC_{20}) of myosin, which in-turn allows a myosin adenosine triphosphatase (ATPase) to be activated by actin and the muscle to contract (Figure 1.2) (Somlyo & Somlyo, 1994; Sweeney *et al.* 1994). In contrast, a fall in $[Ca^{2+}]_i$ inactivates MLCK and permits dephosphorylation of MLC_{20} by myosin light-chain phosphatase, thus deactivating the actomysin ATPase and causing relaxation (Somlyo & Somlyo, 1994).

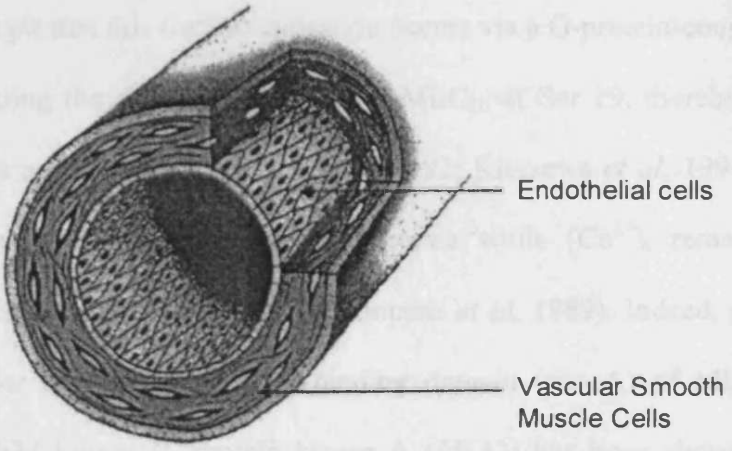

Figure 1.1 Schematic representation of a blood vessel, showing endothelial and vascular smooth muscle cells

(From Bryan *et al.* 2005)

1.2.2 Regulation of Ca^{2+} sensitivity

The ratio between smooth muscle contractile force and $[Ca^{2+}]_i$ varies according to stimuli, with generally greater force:$[Ca^{2+}]_i$ ratios being observed following receptor activation by agonists than by a depolarisation-induced increase in $[Ca^{2+}]_i$, indicating a 'Ca^{2+}-sensitising' effect of agonists (Bradley & Morgan, 1987; Rembold & Murphy, 1988). It is thought that this Ca^{2+}-sensitisation occurs via a G-protein-coupled mechanism (RhoA), preventing the dephosphorylation of MLC_{20} at Ser 19, thereby increasing the level of force at a given $[Ca^{2+}]_i$ (Gong *et al.* 1992; Kitazewa *et al.* 1991; Tansey *et al.* 1994). Conversely, contractile force can decline while $[Ca^{2+}]_i$ remains unchanged, indicating a decrease in Ca^{2+}-sensitivity (Himpens *et al.* 1989). Indeed, phosphorylation of a specific Ser residue in the CaM-binding domain (site A) of MLCK by protein kinases (e.g. CaM kinase II, protein kinase A (PKA)) has been shown to reduce the affinity of this enzyme for Ca^{2+}-CaM, thereby diminishing its phosphorylating activity and leading to muscle relaxation (Tansey *et al.* 1994).

1.2.3 Excitation-contraction coupling

The resting membrane potential of smooth muscle is, like that of other cells, negative with respect to the extracellular space (–40 to –70 mV). As such, depolaristion of smooth muscle cells can result in Ca^{2+} influx through voltage-gated Ca^{2+} channels, thereby promoting muscle contraction (Somlyo & Himpens, 1989). The plasma membranes of smooth muscle cells also contain ligand-gated ion channels, which allow Ca^{2+} entry in

response to hormones and neutrotransmitters (e.g. adenosine triphosphate (ATP) released from autonomic nerves), and G-protein coupled receptors, which promote the release of Ca^{2+} from the sarcoplasmic reticulum (SR) via the phospholipase C (PLC)-dependent generation of secondary messengers such as inositol 1,4,5-trisphosphate (IP_3) (Figure 1.2) (McDonald *et al.* 1994).

$[Ca^{2+}]_i$ can be reduced, causing relaxation, through several mechanisms. Although the rate and extent of Ca^{2+} pumping into the SR via Ca^{2+}-ATPase alone is sufficient to cause relaxation, additional mechanisms of Ca^{2+} removal include a plasma membrane Ca^{2+}-ATPase and, to a lesser extent, the sodium/calcium (Na^+/Ca^{2+}) exchanger (Bond *et al.* 1984; Carafoli & Guerini, 1993; Juhaszova *et al.* 1994). Furthermore, inhibition of Ca^{2+} influx, through hyperpolarisation or by Ca^{2+}-entry blockers (e.g. dihydropyridines), can also reduce $[Ca^{2+}]_i$ and cause relaxation (Nelson *et al.* 1990; Rang *et al.* 1999; Somlyo & Somlyo, 1994).

1.3 Regulation of vascular smooth muscle tone by the sympathetic nervous system

The total and regional peripheral resistance of the vasculature is regulated by the sympathetic nervous system, which influences vascular tone mainly through changes in the release of catecholamines (e.g. noradrenaline (NA) and adrenaline) from sympathetic nerve terminals and the adrenal medulla (Guimarães & Moura, 2001). NA

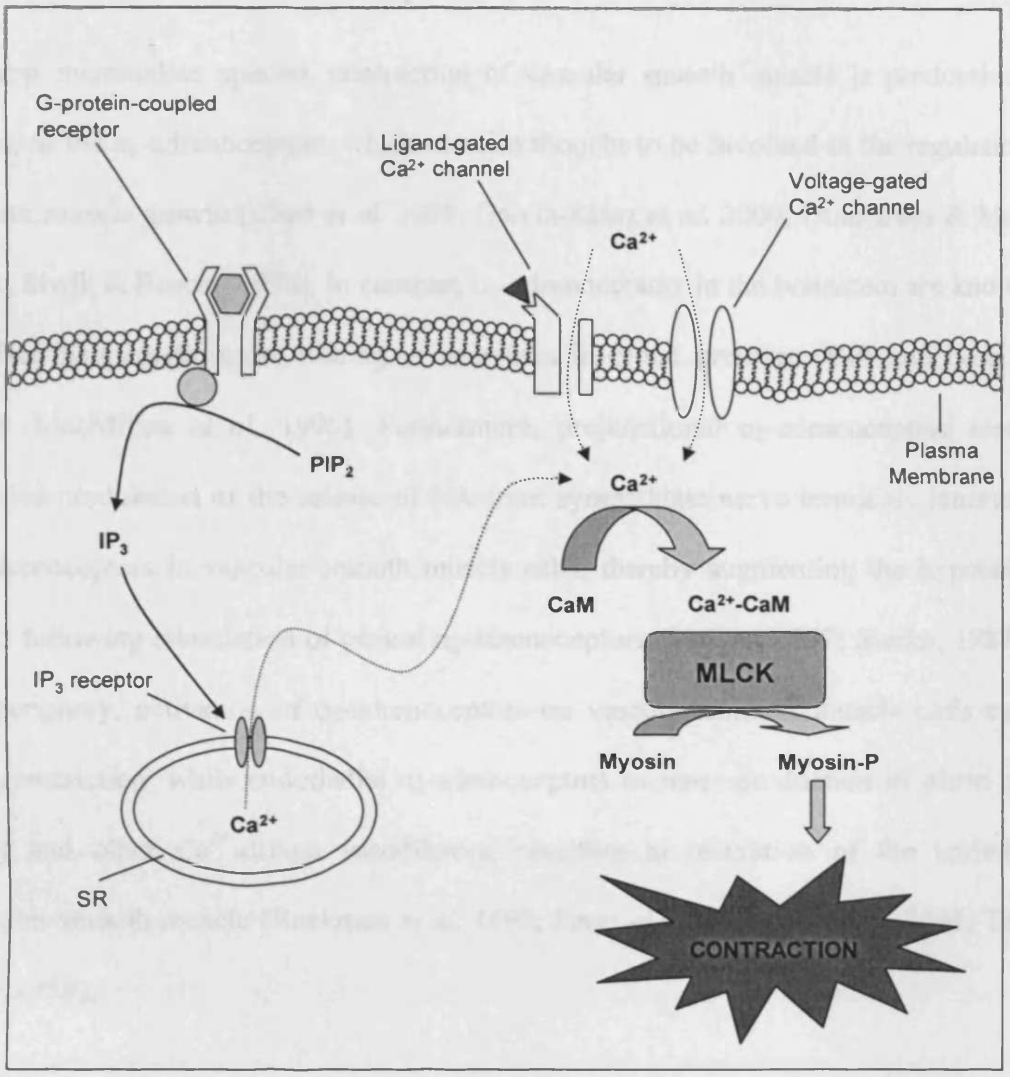

Figure 1.2 Excitation-contraction coupling in vascular smooth muscle cells

In smooth muscle, contraction can be produced either by Ca^{2+} entry through voltage- or ligand-gated Ca^{2+} channels, or by IP_3-mediated Ca^{2+} release from the SR. (SR, sarcoplasmic reticulum; CaM, calmodulin; IP_3, inositol 1,4,5-trisphosphate; MLCK, myosin light-chain kinase) (Adapted from Rang *et al.* 2003).

and adrenaline exert their effects through adrenoceptors, of which nine subtypes have been identified (α_{1A}, α_{1B}, α_{1D}, $\alpha_{2A/D}$, α_{2B}, α_{2C}, β_1, β_2, and β_3) (Guimarães & Moura, 2001).

In most mammalian species, contraction of vascular smooth muscle is predominantly mediated via α_1-adrenoceptors, which are also thought to be involved in the regulation of smooth muscle growth (Chen *et al.* 1995; García-Sáinz *et al.* 2000; Guimarães & Moura, 2001; Siwik & Brown, 1996). In contrast, α_2-adrenoceptors in the brainstem are known to mediate sympathetic tone, leading to decreases in blood pressure (BP) and heart rate (HR) (MacMillan *et al.* 1996). Furthermore, prejunctional α_2-adrenoceptors mediate negative modulation of the release of NA from sympathetic nerve terminals innervating α_1-adrenoceptors in vascular smooth muscle cells, thereby augmenting the hypotensive effect following stimulation of central α_2-adrenoceptors (Langer, 1997; Starke, 1987). In the periphery, activation of α_2-adrenoceptors on vascular smooth muscle cells causes vasoconstriction, while endothelial α_2-adrenoceptors increase production of nitric oxide (NO) and other Ca^{2+}-driven vasodilators, resulting in relaxation of the underlying vascular smooth muscle (Bockman *et al.* 1993; Feres *et al.* 1998; Kanagy, 2005; Thorin *et al.* 1998).

β-adrenoceptors are thought to play an important role in the regulation of vascular tone, at least in-part, by enhancing local NO production (Queen & Ferro, 2006). Indeed, during exercise, activation of β-adrenoceptors is known to contribute to the increased blood flow to skeletal muscle (Guimarães & Moura, 2001). β_1-adrenoceptors are predominantly found in the heart, where they increase HR and force of contraction, as well as increasing

the rate of relaxation (Queen & Ferro, 2006). In contrast, β_2-adrenoceptors are located on vascular smooth muscle cells (VSMCs) and endothelial cells, where they cause vasodilation, and on platelets, where they can inhibit platelet aggregation (Queen & Ferro, 2006). There is also evidence for the existence of β_3-adrenoceptors in the human heart which may inhibit myocardial contractility, although this has yet to be confirmed fully (Emorine *et al.* 1994; Gauthier *et al.* 1996; Moniotte *et al.* 2000).

Some studies have highlighted a role for changes in adrenoceptor signalling in the development/maintenance of hypertensive states. For example, an increase in α_{1D}-adrenoceptor expression has been associated with vascular supersensitivity in hypertension (Ibarra *et al.* 2000; Villalobos-Molina *et al.* 1999; Xu *et al.* 1998). Furthemore, in genetic linkage studies, a functional deletion polymorphism in the α_{2B}-adrenoceptor gene on chromosome 2 has been associated with the development of hypertension, due to attenuated NO release from the endothelium (Von Wowern *et al.* 2003; Von Wowern *et al.* 2004; Zhu *et al.* 2002). In contrast, little is known about how β-adrenoceptor-signalling is altered in hypertension, and the data that is available is conflicting (Arribas *et al.* 1994; Cockcroft *et al.* 1994). Nevertheless, β-adrenoceptor function is known to decline with increasing age, while receptor numbers remain relatively constant (Conlon *et al.* 1995; Dohi *et al.* 1995; Guimarães & Moura, 2001).

1.4 The role of the endothelium in the regulation of vascular smooth muscle tone

Despite first appearances, it is now known that the vascular endothelium acts not just a passive barrier between the plasma and extracellular fluid, but as an active endocrine tissue that regulates vasomotor tone, blood fluidity, vascular smooth muscle growth, and local inflammation through the release of a number of paracrine factors, including vasoactive prostanoids, NO and the vasoconstrictor endothelin (Huang & Vita, 2006). In addition, the plasma membrane of endothelial cells contains several enzymes (e.g. angiotensin-converting enzyme (ACE)) and transport mechanisms that act on circulating hormones to alter their activity or aid their entry into the cell where they may be broken down (e.g. serotonin, adenosine) (Vane *et al.* 1990). Through these mechanisms, the healthy endothelium maintains a vasodilatory, antithrombotic, and anti-inflammatory state and, as such, plays an essential role in regulating vascular physiology under basal conditions.

1.4.1 Nitric oxide

The importance of the endothelium in the maintenance of a healthy vascular system was first recognised in 1980, when Furchgott and Zawadski showed that purposeful removal of the endothelium prevented vessel relaxation in response to established vasodilator stimuli (e.g. acetylcholine (ACh) and bradykinin). It was concluded that stimulation of endothelial cells by these agents induced the release of a substance which Furchgott

named endothelium-derived relaxing factor (EDRF). This was later identified as the hydrophilic and lipophilic gas nitric oxide (NO) (Palmer *et al.*, 1987).

NO is continuously generated and/or released by endothelial cells and has been proven to play a central role in vascular homeostasis through the constant modulation of vascular smooth muscle tone and proliferation, platelet adhesion and aggregation and leukocyte function (Ignarro, 1989, Jeremy *et al.* 1999; Nath & Powledge, 1997). As such, interference with the basal release of NO, as observed in conditions in which there is local damage to the endothelium (e.g. atherosclerosis), can result in smooth muscle proliferation, increased platelet adhesion and increased intrinsic tone or responsiveness to endogenous contractile factors, leading to vasospasm or stroke (Ignarro, 1989; Jeremy *et al.* 1999).

NO mediates its actions through the formation of cyclic guanosine 3'5'-monophosphate (cGMP) from guanosine triphosphate (GTP) following the activation of soluble guanylate cyclase (sGC) (Moncada *et al*, 1991). NO activates sGC by binding to a prosthetic haem group on its surface (Carvajal *et al.*, 2000). In turn, cGMP exerts its intracellular effects by interacting with a group of intracellular cGMP receptors, which include cGMP-regulated ion channels, cGMP-binding phosphodiesterases, and cGMP-dependent protein kinases (PKGs) (Carvajal *et al.*, 2000; Lincoln & Cornwell, 1993). The resulting action of cGMP depends mostly on the cell-specific expression of these various proteins (Lincoln & Cornwell, 1993). In the vasculature, cGMP binds to and activates PKGs, since these serine/threonine protein kinases are found at high concentrations in these

tissues (Francis & Corbin, 1994). Once activated PKG phosphorylates several intracellular target proteins and accounts for many of the intracellular events induced by NO (Carvajal *et al.*, 2000). In particular, PKG is thought to induce smooth muscle relaxation either by reduction of cytosolic Ca^{2+} levels or by reducing the sensitivity of contractile proteins to Ca^{2+} in smooth muscle cells (Carvajal *et al.* 2000; Lincoln & Cornwell, 1993).

Despite its clear importance in the vasculature, NO is thought of as a ubiquitous substance mediating many biological processes. For instance, it is present in the nervous tissue, where it is involved with signal transduction in central and peripheral neurons (Bredt *et al.*, 1991; Narayanan & Griffith, 1994). It is also used by immune cells, in particular macrophages, as a 'killer molecule' to destroy microorganisms and cells which become cancerous (Hibbs *et al.*, 1988). The instability of NO results in its rapid degradation, and as such it is thought largely to act in close proximity to it site of synthesis.

1.4.1.1 Nitric oxide synthesis

NO is formed from L-arginine by a nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen (O_2)-dependent five electron oxidation reaction catalysed by nitric oxide synthase (NOS) (Figure 1.3). In addition to L-arginine, O_2 and NADPH, NOS requires the cofactors flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), haem, thiol and 6(R)-5,6,7,8-tetrahydrobiopterin (BH_4)

(Anggard, 1994; Griffith & Stuehr, 1995). There are at least three isoforms of NOS found in different tissues around the body (Forstermann *et al.*, 1993; Sessa, 1994). These isoforms are named according to the systems in which they were first identified and referred to as nNOS (or NOS-1, predominantly found in neuronal tissue), iNOS (or NOS-2, important in immune cells), and eNOS (or NOS-3, found in endothelial cells) (Alderton *et al.* 2001). eNOS and nNOS are constitutive enzymes and Ca^{2+}/CaM-dependent (Janssens *et al.*, 1992; Lamas *et al.*, 1992). Conversely, iNOS is Ca^{2+}/CaM-independent and is expressed in activated immune cells and other tissues in response to various inflammatory cytokines (e.g. interleukin (IL)-1, IL-2, tumour necrosis factor-α (TNF-α) or interferon-γ (IFN-γ)) (Griffith & Stuehr, 1995; Kleinert *et al.* 2003). Both naming systems are now known to be oversimplications, since all three isoforms can be induced or found constitutively expressed in a variety of cells. In addition, a potential fourth isoform, which shows marked similarity to iNOS, has been postulated in mitchondria (mtNOS) (Alderton *et al.* 2001; Bates *et al.* 1995; Eissa *et al.* 1996; Tatoyan & Giulivi, 1998).

As part of its structure, NOS has distinct C-terminal reductase and N-terminal oxygenase domains (Abu-Soud *et al.*, 1997; Stroes *et al.*, 1998). The oxygenase domain contains binding sites for haem, BH_4 and L-arginine, whereas the reductase domain binds FMN, FAD, and NADPH (Abu-Soud *et al.*, 1997). The presence of the haem group is mandatory for NOS dimerisation, while L-arginine and BH_4 facilitate the formation and stability of the dimer. In addition, the crystal structure of NOS shows a zinc tetrathiolate centre which is thought to stabilise the formation of the dimer and maintain the BH_4

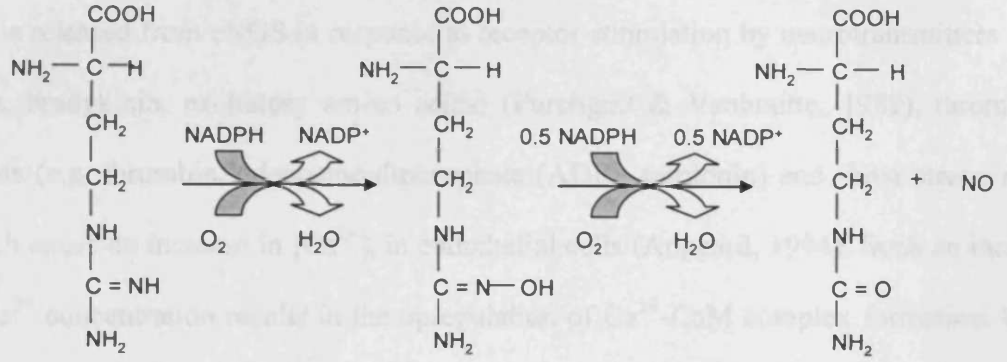

Figure 1.3 Generation of of NO and L-citrulline from L-arginine and O_2
(From Griffith & Stuehr, 1995).

binding site (Rosen *et al.* 2002). Between the two domains is a consensus sequence to which the Ca^{2+}/CaM complex can bind. It is thought that this Ca^{2+}/CaM-binding region acts as a hinge between the reductase and oxygenase domains, causing them to align on Ca^{2+}/CaM binding and in doing so activating the enzyme (Abu-Soud & Stuehr, 1993; Griffith & Stuehr, 1995; Abu-Soud *et al.*, 1997).

NO is released from eNOS in response to receptor stimulation by neurotransmitters (e.g. ACh, bradykinin, excitatory amino acids) (Furchgott & Vanhoutte, 1989), thrombotic agents (e.g. thrombin, adenosine diphosphate (ADP), serotonin) and shear stress, all of which cause an increase in $[Ca^{2+}]_i$ in endothelial cells (Anggard, 1994). Such an increase in Ca^{2+} concentration results in the upregulation of Ca^{2+}-CaM complex formation. Upon binding of a Ca^{2+}-CaM complex to eNOS there is a flow of electrons from NADPH, shuttled through the reduced flavins (FMN & FAD), to the haem group of the oxygenase domain. In the presence of O_2 this promotes conversion of L-arginine to NO and L-citrulline (Griffith & Stuehr, 1995; Abu-Soud *et al.*, 1997). The latter occurs via a two-step redox process: L-arginine is first hydroxylated to the intermediate N^G-hydroxy-L-arginine, which then undergoes oxidative cleavage to yield NO and L-citrulline (Klatt *et al.*, 1993; Stuehr *et al.*, 1991).

1.4.2 Vasoactive prostanoids

In 1976, several years prior to the discovery of NO, Moncada and colleagues described an anticlotting agent that was also capable of relaxing smooth muscle. This entity,

originally dubbed PGX, was later identified as prostacyclin (PGI_2), a lipid soluble and highly unstable mediator produced by endothelial cells following mechanical or chemical stimulation (Moncada *et al.* 1977; Vane *et al.* 1990). Some endogenous chemical stimulants of PGI_2 synthesis include substances derived from plasma, such as bradykinin and thrombin, and those liberated from stimulated platelets, such as serotonin, platelet-derived growth factor, IL-1, and adenine nucleotides (Vane *et al.* 1990).

PGI_2 production is initiated by the enzyme phospholipase A_2 (PLA_2), which liberates arachidonic acid (AA) from membrane phospholipids (Figure 1.4). The enzyme cyclooxygenase (COX, also known as prostaglandin G/H synthase) then converts the AA into the unstable cyclic endoperoxides PGG_2 and PGH_2 (Dogné *et al.* 2005). In endothelial cells, PGH_2 is then transformed to PGI_2 enzymatically by PGI_2 synthase (PGIS). PGI_2 causes relaxation predominantly by acting on G-protein-coupled IP receptors which trigger the adenylyl cyclase/cAMP transduction system, leading to PKA activation and phosphorylation of MLCK (see Figure 1.2). In addition, exogenous PGI_2 has also been reported to induce vasorelaxation through the hyperpolarisation of vascular smooth muscle cells (Dogné *et al.* 2005; Ignarro *et al.* 1985; Kukovetz *et al.* 1979; Parkington *et al.* 2004).

In addition to PGI_2 synthesis in the endothelium, other vasoactive mediators may also be synthesised from AA via the COX pathway (see Figure 1.4). Indeed, there are multiple COX isoforms that are encoded by different genes and expressed at various locations throughout body. COX-1 is typically expressed in platelets, although some studies

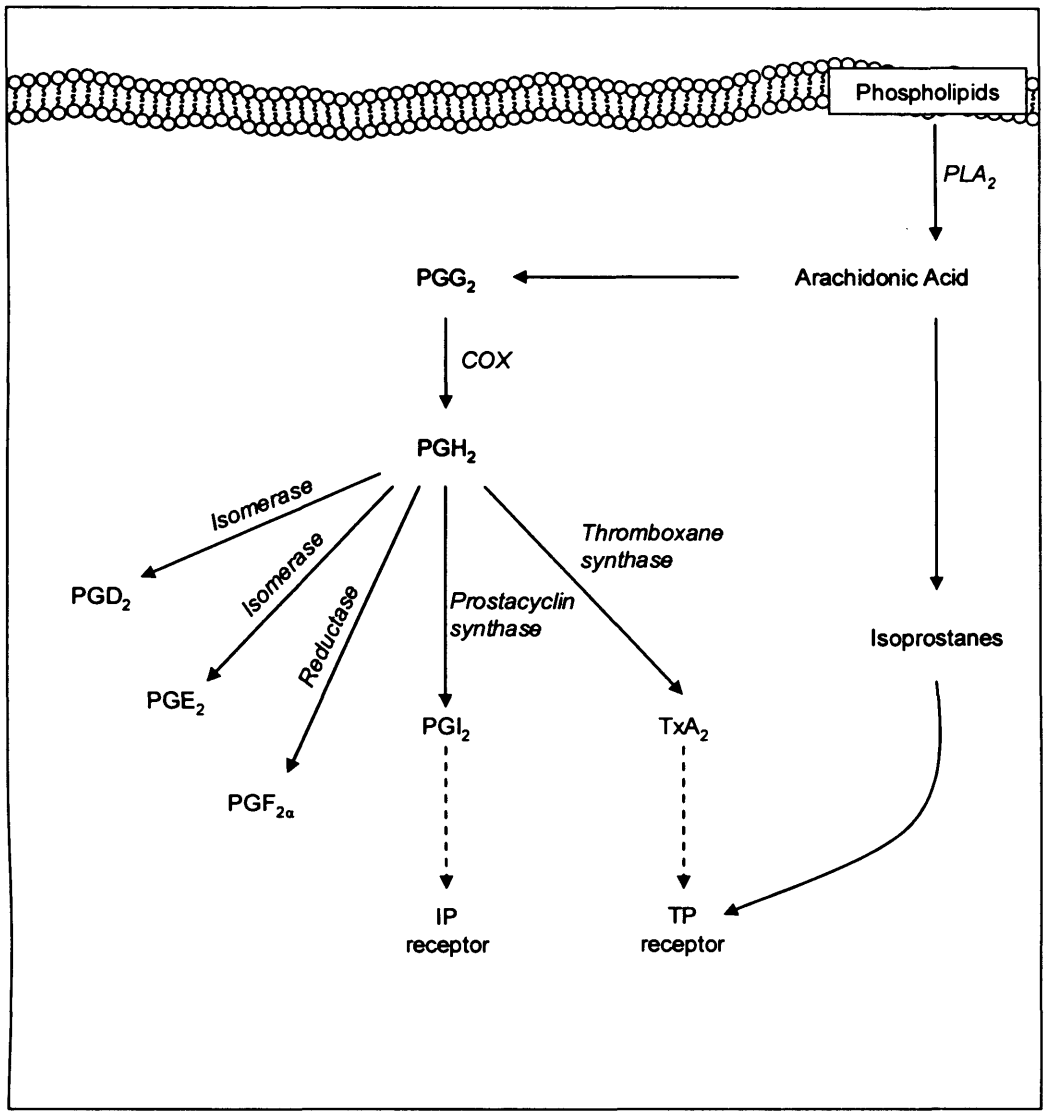

Figure 1.4 Metabolism of arachidonic acid by COX

Intracellular metabolism of arachidonic acid by COX results in the formation of unstable cyclic endoperoxides (PGG_2 and PGH_2). PGH_2 may then be transformed enzymatically into a range of vasoactive compounds. Alternatively, arachidonic acid may be matabolised by oxidation via a non-enzymatic lipid peroxidation pathway to yield F_2-isoprostanes (F_2-IPs) (Adapted from Dogné *et al.* 2005).

suggest that this enzyme is constitutively expressed throughout the vasculature (Davidge, 2001). In contrast, while COX-2 expression is induced mainly at sites of inflammation, it has also been shown to be expressed constitutively in several tissues such as the vascular endothelium, kidney, and brain (Dogné *et al.* 2005; Doroudi *et al.* 2000; McAdam *et al.* 1999; Wang *et al.* 1993). There is some evidence for a third isoform, COX-3 or COX-1b, which is a splice variant of COX-1, in canine tissues, although the existence of this enzyme has yet to be conclusively proven (Chandrasekharan *et al.* 2002).

COX-1 in platelets, monocytes and VSMCs is responsible, along with the enzyme thromboxane synthase, for the synthesis of thromboxane A_2 (TxA_2) (see Figure 1.4). TxA_2 acts via G-protein-coupled TP receptors and is a strong activator of platelets, a vasoconstrictor and a smooth-muscle-cell mitogen, and therefore represents direct opposition to the effects of COX-2-derived PGI_2 (Dogné *et al.* 2005; McAdam *et al.* 1999). In addition, oxidation of AA via a non-enzymatic pathway (lipid peroxidation) produces a family of F_2-isoprostanes (F_2-IPs), which might also act as ligands of TP receptors and, therefore, promote vasoconstriction and platelet activation (Dogné *et al.* 2005). Because TxA_2 and PGI_2 are biological opposites, the balance between these two mediators is crucial for the maintenance of vascular tone and haemostasis in both the healthy and diseased vasculature.

However, the relative importance of PGI_2 in the regulation of vascular tone is still the subject of much speculation. A recent study by Anning and colleagues (2006) demonstrated that despite causing a significant reduction in urinary excretion of the PGI_2

metabolite 2,3-dinor-6-ketoPGF$_{1\alpha}$, oral administration of the selective COX-2 inhibitor celecoxib (400 mg.kg^{-1}.day^{-1}) did not alter systolic BP in healthy mice. Furthermore, neither celecoxib nor the non-specific COX inhibitor indomethacin had any effect on endothelium-dependent vasodilation responses in isolated thoracic aortae (Anning *et al.* 2006). In contrast, in the absence of NO, following NOS inhibition with L-NAME, celecoxib and indomethacin provoked a significant increase in vascular tone, both *in vivo* and *in vitro* (Anning *et al.* 2006). Therefore, it appears that under physiological conditions PGI_2 plays in minor role in regulating vascular tone, whereas the PGI_2 is enhanced in the absence of NO. As such, there is growing evidence for a detrimental effect of selective COX-2 inhibitors (coxibs) prescribed in inflammatory cardiovascular diseases associated with reduced NO bioavailability, as these drugs cause the selective depletion of PGI_2, significantly shifting the balance towards pro-thrombotic TxA_2 signalling. For example, a recent placebo-controlled trial of rofecoxib revealed a 2-fold increase in myocardial infarction and stroke with this COX-2 inhibitor (Fitzgerald, 2004). Conversely, preferential inhibition of COX-1, such as with a low dose of aspirin, reduces the levels of pro-thrombotic TxA_2 but has little effect on PGI_2, thereby providing protection against cardiovascular events in high risk patients (Dogné *et al.* 2005).

Nevertheless, it is important to note that many vascular pathologies are associated with an increase in oxidative stress and dyslipidemia, which affect COX signalling as well as NO. For instance, reactive oxygen species (ROS) inactivate PGIS, the enzyme responsible for the formation of PGI_2, and promote the formation of vasoconstrictive F_2-IPs (Pratico *et al.* 1998; Zou *et al.* 2002). Alternatively, ROS or lipid hydroperoxides may activate COX

directly, once again resulting in increased levels of vasoconstrictive mediators (e.g. PGH_2/TxA_2) (Ling *et al.* 2005). Therefore, a combination of anti-oxidant therapy (e.g. vitamin C, superoxide dismutase (SOD)) and pharmacological blockade of the TP receptor would appear to be a more effect method for preserving vascular homeostasis in vascular pathologies than classical suppressors of TxA_2 synthesis such as low dose aspirin. However, this approach has yet to be put to the test *in vivo*.

1.4.3 Endothelium-derived Hyperpolarising Factor

In several blood vessels, endothelium-dependent relaxations are accompanied by the hyperpolarisation of smooth muscle cells (15-30 mV) (Bryan *et al.* 2005; Busse *et al.* 2002). This hyperpolarisation induces relaxation by reducing the open probability of voltage-gated Ca^{2+} channels, and also the turnover of IP_3, thereby decreasing $[Ca^{2+}]_i$ (Nelson *et al.* 1990). These responses can be partially or totally resistant to COX or NOS inhibitors, and can occur independently of rises in intracellular cAMP or cGMP levels (Garland & McPherson, 1992; Nagao & Vanhoutte, 1992; Busse *et al.* 2002). Thus, it has been postulated that the observed smooth muscle cell hyperpolarisation is due to an unidentified endothelial factor dubbed 'endothelium-derived hyperpolarisation factor' (EDHF) (McGuire *et al.* 2001). The contribution of EDHF-mediated responses to endothelium-dependent relaxation appears to increase as the vessel size decreases, with the exception of the coronary and renal vascular beds in which EDHF plays a major role even in conduit arteries (Busse *et al.* 2002; Shimokawa *et al.* 1996). However, the precise

nature of this vasoactive mediator has yet to be fully defined (see Bryan *et al.* 2005 and Busse *et al.* 2002 for a comprehensive review).

1.4.4 Endothelin-1

Endothelin-1 (ET-1), an endothelium-derived vasoconstrictor, was discovered by Yanagisawa and colleagues in 1988. Since then, two further structurally and pharmacologically separate endothelin isopeptides have been identified in humans and other mammalian species, simply labelled ET-2, and ET-3. However, only ET-1 is known to be made by endothelial cells (Vane *et al.* 1990). ET-1 is secreted by endothelial cells and, in inflammatory states, by vascular smooth muscle cells, in response to angiotensin II (Ang II), NA, ROS, thrombin, cytokines and growth factors (Schiffrin, 2005; Tonnessen *et al.* 1998; Yanagisawa *et al.* 1988).

1.4.5 Angiotensin II

Ang II is the main peptide hormone of the renin-angiotensin system, which plays a central role in the control of vascular tone, as well as regulating Na^+ excretion and fluid volume (Rang *et al.* 1999). This octapeptide is derived from the protein precursor angiotensinogen by the sequential actions of proteolytic enzymes, culminating in the conversion Ang I to Ang II by ACE, which is commonly found on the surface of endothelial cells (Goodfriend *et al.* 1996). Within the vasculature, Ang II exerts vasoconstrictive, pro-thrombotic, inflammatory and fibrotic effects, as well as promoting

vascular smooth muscle proliferation and hypertrophy; effects which are mediated by G-protein-coupled AT_1 receptors (Goodfriend *et al.* 1996; Schulman *et al.* 2006). Binding of Ang II to AT_1 results in PLC activation, generating diacylglycerol (DAG) and IP_3, thereby causing Ca^{2+} release from intracellular stores. Ang II also increases Ca^{2+} entry through channels in the cell membrane, promoting smooth muscle contraction (Goodfriend *et al.* 1996).

In addition to its direct vasoconstrictive effect, Ang II may act to alter vascular tone indirectly by promoting the removal of NO from the vasculature by ROS (see section 1.5.1.3) (Jaimes *et al.* 1998; Lessegue & Clempus, 2003; Pagano *et al.* 1998; Touyz *et al.* 2002). Furthermore, studies indicate that Ang II regulates the expression of eNOS and NO production *in vitro*, while NO down-regulates AT_1 receptor expression in VSMCs at the transcriptional level (Ichiki *et al.* 1998; Watanabe *et al.* 2005; Yan *et al.* 2003). Ang II has also been shown to stimulate the synthesis and release of ET-1 by endothelial cells, an effect in direct opposition to the inhibitory action of NO on ET-1 production (Sasser *et al.* 2002). Indeed, it has been proposed that ET-1 is responsible for the pressor response observed in Ang II-induced hypertension (Ortiz *et al.* 2001). However, given that a fall in NO bioavailability is required before ET-1 synthesis is upregulated, the balance between Ang II and NO appears to be of primary importance in regulating vascular tone.

Ang II-induced hypertension is also associated with an increase in leukocyte (e.g. monocytes and neutrophils) adhesion to the endothelium (Paragh *et al.* 2002). Indeed, stimulation of endothelial cells and VSMCs with Ang II *in vitro* has been shown to result

in increased expression in vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin, as well as the chemokines monocytes-chemoattractant protein-1 (MCP-1) and IL-8; molecules which promote leukocyte accumulation, adherence and migration across the endothelial layer (Libby *et al.* 2002; Nickening *et al.* 2002; Yan *et al.* 2003). The importance of this process in the pathogenesis of Ang II-induced hypertension is currently unknown, although inhibition of ACE has been shown to ameliorate endothelial injury *in vitro* via inhibition of neutrophil-endothelial cell interaction (Guba *et al.* 2000). Furthermore, monocytes and neutrophils can enhance NO removal from buffer *in vitro* via the production of ROS, suggesting that these cells may contribute to be vasoactive effects of Ang II infusion (Clark *et al.* 2002; Hancock & Jones, 1987). However, a role for leukocytes in Ang II-mediated hypertension has yet to be investigated *in vivo*.

1.5 Endothelial dysfunction

As already mentioned, vascular endothelial cells play a central role in maintaining cardiovascular homeostasis in health by secreting a number of mediators that regulate platelet aggregation, coagulation, fibrinolysis, leukocyte activation and vessel tone. As such, an imbalance in one or more of these processes is known as endothelial dysfunction, and is implicated in the pathophysiology of several cardiovascular disorders, including atherosclerosis, hypertension, heart failure and diabetes mellitus (Cai & Harrison, 2000; Ray & Shah, 2005).

Endothelial dysfunction commonly presents as an impairment of endothelium-dependent vasodilation, often due to a loss of NO bioactivity in the vessel wall (Cai & Harrison, 2000). As such, clinical assessment of systemic endothelium-dependent vasodilator function is used as a tool for predicting adverse cardiovascular events and long-term outcome in patients with ischaemic heart disease, hypertension and heart failure (Fichtlscherer *et al.* 2004; Halcox *et al.* 2002). The decrease in NO bioavailability observed in endothelial dysfunction may caused by reduced expression of eNOS by endothelial cells, as has been observed in atherosclerotic human vessels (Wilcox *et al.* 1997). Alternatively, NOS bioactivity may be suppressed due to insufficient supplies of its substrate L-arginine and/or cofactors (e.g. BH_4) (Pou *et al.* 1992).

In recent years, a large body of work has focussed on the accelerated removal of NO from the vasculature as another potential mechanism for endothelial dysfunction in cardiovascular disease. However, despite its clear importance in endothelium-dependent vasodilation *in vitro*, the consequences of reduced NO bioavailability *in vivo* are likely to be more complex. This was recently demonstrated by Anning and colleagues (2006), who observed an upregulation of PGI_2 signalling and gradual normalisation of systolic BP following NOS inhibition *in vivo*, suggesting that endothelial function may be partially maintained by PGI_2 in the absence of NO. As such, these alternative pathways should be taken into account when considering the effect that specific mechanisms of NO removal may have on endothelial function *in vivo*. For instance, while it is acknowledged that in vascular pathologies characterised by oxidative stress both NO and PGI_2 signalling are likely to be attenuated (see section 1.4.2), the direct catalytic consumption of NO does

not inhibit PGI_2 signalling and is therefore likely to have a less significant impact on vascular physiology. Furthermore, few studies have considered how accelerated NO removal may promote leukocyte adherence to the endothelium and how this may impact upon vascular homeostasis. Indeed, an important function of NO *in vivo* is to inhibit the expression of adhesion molecules on the surface of endothelial cells and neutrophils. This is particularly relevant given the marked infiltration of immune cells into vascular tissue in inflammatory vascular disease and the propensity for activated leukocytes to remove NO from buffer *in vitro* (Clark *et al.* 2002; Hancock & Jones, 1987).

With this in mind, the following section will examine the current theories regarding accelerated NO disappearance in the vasculature, and their relevance in the pathogenesis of endothelial dysfunction *in vivo*.

1.5.1 Metabolism and removal of NO

As a free radical, NO reacts rapidly with a select range of molecules that have orbitals with unpaired electrons, which are typically other free radicals, and with transition metals like haem iron (e.g. oxyhaemoglobin (oxyHb) and sGC) (Beckman & Koppenol, 1996). These molecules may be enzyme products that can react with and consume NO (e.g. ROS), or enzyme intermediates that catalytically consume NO as part of their catalytic cycle (e.g. haem peroxidase and lipoxygenase (LOX) intermediates).

1.5.1.1 Haem peroxidase enzymes

The ability of NO to rapidly react with haemoproteins such as those found in sGC and haemoglobin (Hb) is well known. In addition, a variety of studies have documented the ability of NO to bind to the haem moiety of peroxidase enzymes (Abu-Soud & Hazen, 2000; Ischiropoulos *et al.* 1996). The haem peroxidases are a superfamily of enzymes that include myeloperoxidase (MPO), horseradish peroxidase (HRP), eosinophil peroxidase (EPO) and lactoperoxidase (LPO).

NO can interact with haem peroxidases in a number of ways. At high concentrations, NO can reversibly bind to the haem moiety of the enzyme (Fe(III)), reversibly inhibiting peroxidase activity through the formation of a six-coordinate nitrosyl complex (Figure 1.5A). This is known as the 'inactive' catalytic cycle. In contrast, at lower NO concentrations the 'active' catalytic cycle predominates, whereby NO facilitates the peroxidase activity of the enzyme by serving as a one electron peroxidase cosubstrate for enzyme intermediates Compound I and Compound II, and is consumed by this process. Indeed, concentration-effect studies in pre-constricted isolated tracheal rings showed that increasing concentrations of haem peroxidases attenuated NO-dependent relaxation and reduced the maximal response to NO, consistent with the notion of these peroxidases serving as a catalytic sink for NO (Abu-Soud *et al.* 2001). Furthermore, another mammalian peroxidase enzyme, COX, has also been found to catalytically consume NO by a mechanism similar to that described here (Figure 1.5B) (O'Donnell *et al.* 2000).

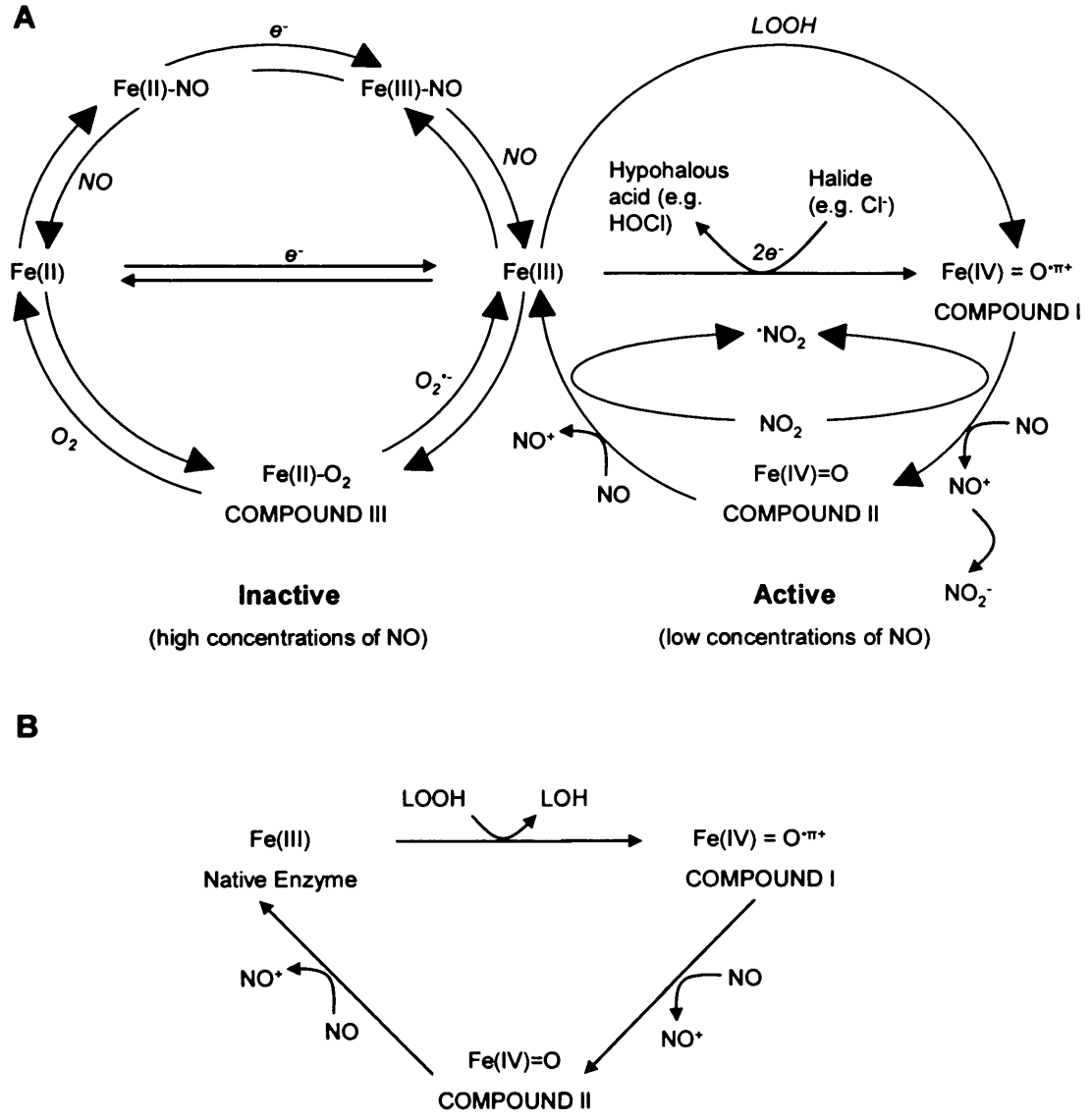

Figure 1.5 NO-peroxidase interactions

Panel A, Comprehensive model of NO interaction with mammalian peroxidases

(Adapted from Abu-Soud *et al.* 2001).

Panel B, Mechanism of peroxidase-catalysed NO consumption by COX

(From O'Donnell *et al.* 2000.)

NO^+, nitrosonium cation; NO_2-, nitrite; NO_2, nitrogen dioxide; LOOH, lipid hydroperoxide; LOH, lipid hydroxide; $O_2^{\bullet-}$, superoxide, O_2, molecular oxygen

Consumption of NO by haem peroxidases has been implicated in a variety of pathological states. For example, MPO and EPO are thought to contribute to the reduction of NO bioactivity observed in asthma, since levels of these peroxidases and their substrates were elevated in inflamed airways of asthmatic subjects (Abu-Soud *et al.* 2001). However, this is purely circumstantial and is likely to be due to inflammatory activation of neutrophils and eosinophils/basophils, which express MPO and EPO, respectively. Furthermore, a role for catalytic removal of NO by haem peroxidases *in vivo* has yet to be established. The fact that these enzymes only actively consume NO under conditions where NO bioavailability is already low suggests that this pathway occurs secondary to some other mechanism of NO removal. Indeed, NO consumption by haem peroxidases is dependent upon sufficient concentrations of hydrogen peroxide (H_2O_2), which is commonly formed following the dismutation of superoxide ($O_2^{\bullet-}$); a ROS that directly reacts with NO at diffusion-limited rates. Therefore, it is proposed that haem peroxidases only play a minor role in the removal of NO from the vasculature under inflammatory conditions, following prior NO consumption by ROS (e.g. $O_2^{\bullet-}$).

COX-mediated NO consumption has been associated with the development of hypertension, as infusion of indomethacin corrects the impaired ACh-dependent vasodilation and increase in forearm blood flow observed in hypertensive patients. Approximately half of this restoration was prevented by infusion with a NOS inhibitor, indicating that a proportion of the protective effect of indomethacin was due to increased NO bioavailability (Taddei *et al.* 1997). However, while COX-1-dependent NO consumption in platelets has been shown *in vitro*, the relevance of this enzyme in NO

consumption *in vivo*, particularly with regard to the regulation of vascular tone, has yet to be investigated (O'Donnell *et al.* 2000; Williams *et al.* 2005). Similarly, COX-2 consumes iNOS-derived NO in cytokine-treated J774.2 macrophages *in vitro*, but this mechanism has not been examined *in vivo* (Clark *et al.* 2005). Nevertheless, the co-expression of COX-2 and iNOS under inflammatory conditions suggests that COX-2-dependent NO consumption may serve as a protective mechanism which limits NO toxicity during inflammation, rather than as a vasoactive pathway. This could be investigated further by using pharmacological inhibitors to compare of the rates of iNOS-dependent NO generation and COX-2-dependent NO removal in inflammatory-activated cells *in vitro* in order to determine the net effect on NO bioavailability.

1.5.1.2 Lipoxygenase

The lipoxygenases (LOXs) are a family of non-haem iron-containing enzymes that catalyse the oxidation of unsaturated fatty acids at various sites around the body. For example, 12-LOX can be found in the vascular endothelium, VSMCs and platelets, while 5-LOX, 12/15-LOX and 15-LOX (the human equivalent of 12/15-LOX) are prediminantly found in leukocytes (Kim *et al.* 1995; Nassar *et al.* 1994; O'Donnell *et al.* 1999). NO consumption by these enzymes occurs via a reaction with an enzyme-bound lipid peroxyl radical intermediate ($E_{red}LOO^{\bullet}$) formed during enzyme turnover, resulting in the generation of an organic peroxynitrite species (LOONO), which then dissociates from the enzyme and is hydrolysed to lipid hydroperoxide (LOOH) and nitrite (NO_2^-) (see O'Donnell *et al.* 1999). Murine fibroblast PA317 cells transfected with 15-LOX,

primary porcine monocytes expressing 12/15-LOX, and rat A10 smooth muscle cells transfected with murine 12/15-LOX have all been shown to consume NO *in vitro* in the presence of lipid substrate (Coffey *et al.* 2001; O'Donnell *et al.* 1999). Furthermore, catalytic NO consumption by purified rabbit reticulocyte 15-LOX prevented activation of sGC in a cell-free system, and NO removal by 12/15-LOX in primary porcine monocytes completely blocked cGMP generation in these cells, indicating that these enzymes may contribute to vascular dysfunction by serving as catalytic sinks for NO in the vasculature (Coffey *et al.* 2001; O'Donnell *et al.* 1999).

Both 12/15-LOX and 15-LOX have been implicated in atherosclerosis, since 15-LOX mRNA, protein and lipid products have been found in atherosclerotic lesions in humans (Folcik *et al.* 1995; Kuhn *et al.* 1994; Yla-Herttuala *et al.* 1991). Furthermore, 12/15-$LOX^{-/-}$ mice are resistant to Ang II-induced hypertension *in vivo* and exhibit a significant upregulation of eNOS expression and plasma NO metabolites compared to wild-type mice (Anning *et al.* 2005). These data indicate that 12/15-LOX may alter vascular tone by inhibiting eNOS expression *in vivo*, rather than consuming NO directly. As such, the localisation of 12/15-LOX on circulating monocytes may implicate these cells as key mediators in the pathogenesis of inflammatory vascular disease, via the attenuation of eNOS expression.

1.5.1.3 Reactive oxygen species

ROS are a family of molecules, including O_2 and its derivatives, produced in aerobic cells. Examples include free radicals, such as $O_2^{\bullet-}$ and hydroxyl radicals ($HO^{\bullet}$), H_2O_2 and hypochlorous acid (HOCl); all of which contribute to oxidant stress (Cai & Harrison, 2000). Due to the reactive nature of these molecules, it is thought that the generation of ROS within the vasculature may contribute to the genesis of endothelial dysfunction via rapid reaction with NO (Li & Shah, 2004). Indeed, several studies have reported that levels of some free radical scavengers such as vitamin E and SOD are depressed in hypertensive patients, while pretreatment of cholesterol-fed rabbits with liposome-entrapped SOD restores ACh-induced relaxation in isolated femoral arteries close to those of control animals (Bayorh *et al.* 2004; White *et al.* 1994). Thus, increases in ROS generation within the vasculature appear to play a critical role in the pathology vascular disease.

A major mechanism of NO removal from the vasculature is its diffusion-limited reaction with $O_2^{\bullet-}$ to form peroxynitrite ($ONOO^-$) (see Figure 1.6A). Given the rapid nature of this interaction, there is likely always some $O_2^{\bullet-}$ reacting with NO within endothelial cells and in the extracellular space, although under physiological conditions endogenous antioxidant defences act to maintain what seems to be a tenuous balance between $O_2^{\bullet-}$ and NO (Cai & Harrison, 2000). $O_2^{\bullet-}$ is produced by the reduction of O_2 by one electron, resulting in a ROS with one unpaired electron that rapidly combines with NO (Figure 1.6A) (Beckman & Koppenol, 1996). Potential sources of $O_2^{\bullet-}$ include the mitochondrial

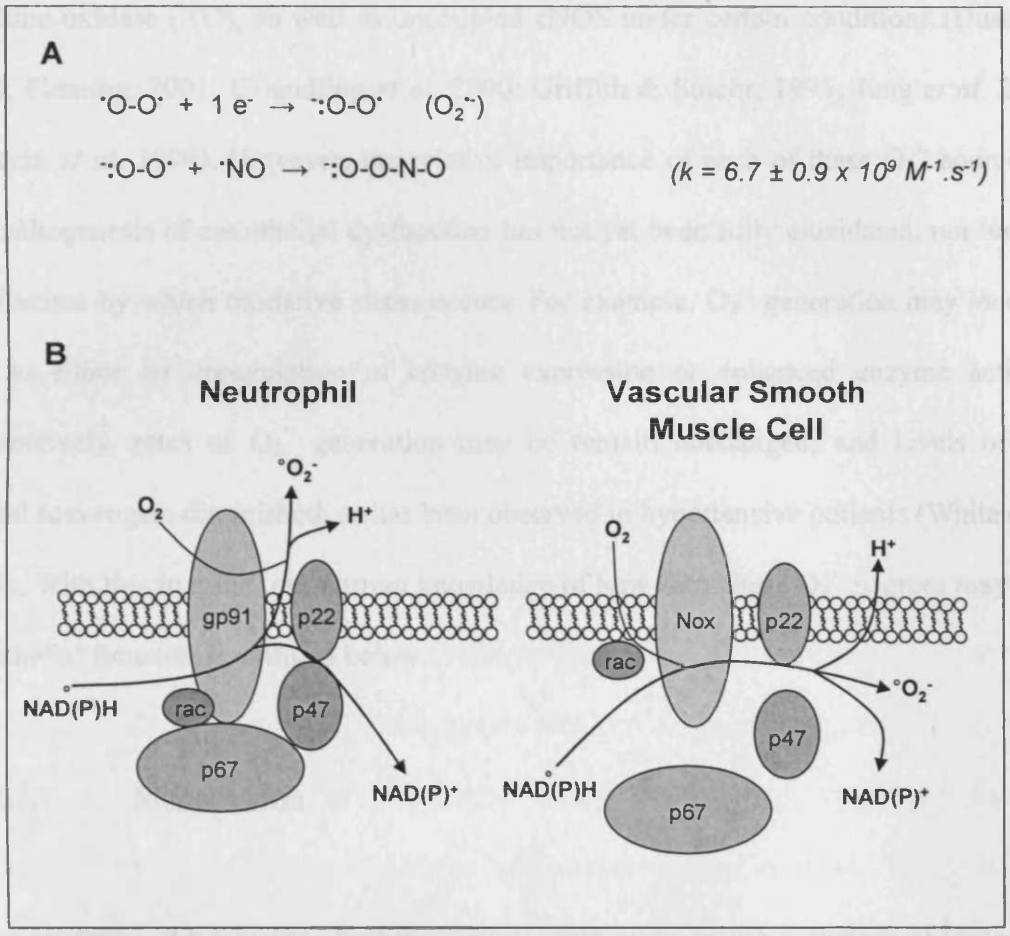

Figure 1.6 NO consumption by NAD(P)H oxidase

Panel A, Generation of $O_2^{\cdot-}$ by reduction of O_2, and scavenging of NO by $O_2^{\cdot-}$

(From Beckman & Koppenol, 1996).

Panel B, Structure of NAD(P)H oxidase

Left, Functional structure of neutrophil NAD(P)H oxidase. $gp91^{phox}$ and $p22^{phox}$ form the electron transfer component of the oxidase, and $p47^{phox}$ and $p67^{phox}$ are cytosolic components that interact with these 2 proteins to modulate its activity. The low molecular weight G protein rac also serves a regulatory function.

Right, Components of the neutrophil oxidase that have been identified in VSMCs. The functional interaction among these subunits remains to be determined.

(Adapted from Griendling *et al.* 2000).

electron transport chain, cytochrome P-450 (CYP) enzymes, NAD(P)H oxidase and xanthine oxidase (XO), as well as uncoupled eNOS under certain conditions (Duschen, 2004; Fleming, 2001; Griendling *et al.* 2000; Griffith & Stuehr, 1995; Jung *et al.* 2003; Marczin *et al.* 1996). However, the relative importance of each of these $O_2^{\bullet-}$ sources in the pathogenesis of endothelial dysfunction has not yet been fully elucidated, nor has the mechanism by which oxidative stress occurs. For example, $O_2^{\bullet-}$ generation may increase due to either an upregulation of enzyme expression or enhanced enzyme activity. Alternatively, rates of $O_2^{\bullet-}$ generation may be remain unchanged, and levels of free radical scavengers diminished, as has been observed in hypertensive patients (White *et al.* 1994). With this in mind, our current knowledge of how each these $O_2^{\bullet-}$ sources may alter endothelial function is outlined below.

1.5.1.3.1 Mitochondria

In simple terms, the mitochondrial respiratory chain involves the reduction of O_2 to H_2O via the action of a multicomponent nicotinamide adenine dinucleotide (NADH) dehydrogenase complex (Lenaz, 1998). However, on average up to 4% of O_2 is incompletely reduced by this pathway, resulting in $O_2^{\bullet-}$ formation at various sites in the NADH dehydrogenase complex. This mechanism of $O_2^{\bullet-}$ generation appears to be particularly prominent in situations of metabolic perturbation, such as hypoglycaemia and hypoxia, where enhanced $O_2^{\bullet-}$ production by mitochondria in endothelial cells is at least partially responsible for an increase in endothelial permeability (Du *et al.* 2000; Pearlstein *et al.* 2002). In addition, oxidative damage of mitochondria themselves can

further promote mitochondrial ROS production, leading to mitochondrial DNA damage and depletion of factors essential for ATP production (Ballinger *et al.* 2000; Duschen, 2004; Yan & Sohal, 1998). As such, ROS production by the mitochondrial respiratory chain can compromise several metabolic processes that influence both endothelial and VSMC function, thereby promoting atherogenesis (Knight-Lozano *et al.* 2002). However, despite a deleterious effect on cell metabolism, no direct effect of mitochondrial $O_2^{\bullet-}$ production on NO bioavailability has been demonstrated either *in vivo* or *in vitro*. Therefore, the relevance of mitochondrial $O_2^{\bullet-}$ generation in the pathogenesis of endothelial dysfunction requires further investigation. This could be carried out by comparing the rate of NO disappearance from aerobic buffer in the presence of human umbilical vein endothelial cells (HUVECs) under control or hypoglycemic conditions. The specific effect of mitochondrial $O_2^{\bullet-}$ release on NO disappearance could be evaluated using pharmacological inhibitors of NAD(P)H oxidase (e.g. apocynin) and xanthine oxidase (e.g. allopurinol), as well as the mitochondrial electron transport chain itself (e.g. rotenone). In presence of apocynin and allopurinol, any sustained elevation in the rate of NO disappearance over background levels would indicate a role for mitochondrial $O_2^{\bullet-}$ release in regulating NO bioavailability *in vitro*.

1.5.1.3.2 Cytochrome P-450

Although traditionally regarded as hepatic enzymes, CYP enzymes have been also recently been implicated in vascular regulation through the generation of vasodilator metabolites (e.g. EDHF), vasoconstrictors (e.g. 20-HETE), and ROS (Fisslthaler *et al.*

2003; Fleming, 2001). Specifically, vascular CYP enzymes can generate $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$ during the CYP reaction cycle when the electrons for the reduction of the central haem iron are transferred to the activated bound O_2 molecule in an NADPH-dependent reaction (Fleming, 2001). Both cultured and native porcine coronary endothelial cells have been shown to produce $O_2^{\bullet-}$ *in vitro* in a CYP-dependent manner, and increased CYP expression has been reported in both hypertension and hypercholesterolemia (Fleming *et al.* 2001; Li & Shah, 2004). However, like haem peroxidises, CYP enzymes are inhibited by NO under physiological conditions, due to NO binding to the central haem iron, and are therefore unlikely to promote endothelial dysfunction unless some other mechanism of NO removal is also upregulated. Furthermore, because CYP enzymes can generate both EDHF and ROS, vascular responsiveness is likely to be at least partially maintained despite the loss of NO bioavailability. Nevertheless, one of the reported consequences of increased CYP-dependent $O_2^{\bullet-}$ generation in porcine coronary artery endothelial cells *in vitro* is an elevation in VCAM-1 expression (Fleming, 2001). Therefore, while CYP enzymes are unlikely to alter vascular tone directly, they may promote the recruitment of inflammatory cells from the circulation, thereby promoting inflammatory vascular disease.

1.5.1.3.3 NAD(P)H oxidase

NAD(P)H oxidase was first described in neutrophils as a membrane-associated multi-component enzyme that catalyses the 1-electron reduction of O_2 using NADPH as the electron donor (Figure 1.6B) (Babior, 1999). This enzyme has also been found in

eosinophils and macrophages and plays an important role in the host defence system (Bolscher *et al.* 1990; Brozna *et al.* 1988). Leukocyte NAD(P)H oxidase consists of 4 major subunits: a plasma membrane-spanning heterodimeric flavohaemprotein (cytochrome b_{558}) composed of a large $gp91^{phox}$ subunit and a $p22^{phox}$ subunit, and 2 cytosolic components $p47^{phox}$ and $p67^{phox}$ (Figure 1.6B). Additional components include $p40^{phox}$ and the small G proteins rac and rap1A (Lassègue & Clempus, 2003). Rac participates in assembly of the active complex and rap1A copurifies with the enzyme and may participate in deactivation. The function of the $p40^{phox}$ subunit, which is not essential for oxidase activity, has yet to be fully elucidated (Griendling *et al.* 2000; Lassègue & Clempus, 2003). Upon enzyme activation, the cytosolic components translocate to and assemble with the membrane cytochrome b_{558} in a highly regulated process. In the activated enzyme complex, the flavin-containing catalytic subunit functions as an electron transport system which uses NADPH as a donor of electrons that are ultimately transferred to O_2, resulting in the generation of $O_2^{\bullet-}$ (Babior *et al.* 2002; Ray & Shah, 2005).

Similar enzymes have also been discovered within vascular endothelial cells, VSMCs and fibroblasts (Bayaraktutan *et al.* 2000). The molecular composition of these non-phagocytic enzymes differs from that found in neutrophils in that several homologues of the $gp91^{phox}$ catalytic subunit have been identified, each encoded by separate genes (Ray & Shah, 2005). These homologues are now referred to as Nox proteins (termed Nox for NADPH oxidase), and include Nox1 (formerly Mox1 or $p65^{mox}$), Nox2 ($gp91^{phox}$), Nox4

and Nox5 (Figure 1.6B) (Cheng *et al.* 2001; Suh *et al.* 1999). Vascular cells may express multiple Nox proteins with distinct functions and mechanisms of regulation.

Interestingly, vascular NAD(P)H oxidase expressed in endothelial cells and VSMCs differs functionally to that found in neutrophils in several ways. Firstly, vascular NAD(P)H oxidases are low-output, slow-release enzymes ($nmol.min^{-1}.mg\ protein^{-1}$) which appear to be constitutively active, whereas leukocyte NAD(P)H oxidase lacks constitutive activity but releases a 'burst' of $O_2^{\bullet-}$ upon activation ($\mu mol.min^{-1}.mg\ protein^{-1}$) (Rajagopalan *et al.* 1996). Secondly, activation of neutrophil NAD(P)H oxidase results in the release of $O_2^{\bullet-}$ into the extracellular space in order to carry out the microbicidal functions of the cell, whereas in vascular smooth muscle cells $O_2^{\bullet-}$ production appears to be mainly intracellular and is thought to play a signalling role under physiological conditions, stimulating smooth muscle cell growth and regulating vascular tone (Figure 1.6B) (Bauldry *et al.* 1992; Griendling *et al.* 1994; Rajagopalan *et al.* 1996; Watson *et al.*1991). Thirdly, vascular NAD(P)H oxidase appears to prefer NADH rather than NADPH as an electron donor (Bayraktutan *et al.* 1998; Brandes *et al.* 1997; Jones *et al.* 1996; Mohazzab *et al.* 1994). The reasons for these underlying differences are unclear, although they may be related to the expression of Nox subunits other than $gp91^{phox}$ in vascular cells. Alternatively, the subunits of vascular NAD(P)H oxidase may undergo different post-translational modifications to those in neutrophil NAD(P)H oxidase, which may alter enzyme assembly.

The difference in substrate preference between vascular and neutrophil NAD(P)H oxidases may be due to the method of detection. $O_2^{\bullet-}$ production in non-phagocytic cells is often measured using lucigenin, an acridylium dinitrite compound that emits light on reduction and interaction with $O_2^{\bullet-}$ (Gyllenhammar, 1987). This method has come under scrutiny because of the potential for lucigenin to undergo redox cycling and generate artefactual $O_2^{\bullet-}$, particularly in the presence of NADH (Li *et al.* 1998). Although the reason for this remains unclear, experiments with the flavoprotein inhibitor diphenyleneiodonium (DPI) suggest that NADPH-dependent $O_2^{\bullet-}$ is predominantly attributable to a flavoprotein, such as that found in the catalytic unit of NAD(P)H oxidase, while virtually none of the NADH-dependent chemiluminescence in intact endothelial cells involves a flavoprotein (Li & Shah, 2001). Therefore, NADH-dependent production of $O_2^{\bullet-}$ by vascular NAD(P)H oxidase appears to be purely artefactual, and is unlikely to be observed in neutrophils due to the fact that the rate of NADPH-(flavoprotein)-dependent $O_2^{\bullet-}$ generation is significantly greater in these cells.

The ability of NAD(P)H oxidase to act as a source of $O_2^{\bullet-}$ has led to intense research into its involvement in vascular dysfunction and atherosclerosis (Cai & Harrison, 2000; Meyer & Schmitt, 2000). For example, it has been shown that $O_2^{\bullet-}$ production by vascular NAD(P)H oxidase leads to endothelial dysfunction in human blood vessels *in vitro* and is increased in the presence of cardiovascular risk factors such as diabetes and hypercholesterolemia (Guzic *et al.* 2000). Interestingly, leukocyte–endothelial cell interactions have been found to be upregulated in hypercholesterolemic mice via a mechanism involving $O_2^{\bullet-}$ production by both vascular and leukocyte NAD(P)H oxidases

(Stokes *et al.* 2001). This suggests that leukocyte NAD(P)H oxidase may also play a role in the pathogenesis of inflammatory vascular disease. Indeed, this has previously been suggested following the observation that circulating neutrophils from hypertensive patients exhibit enhanced $O_2^{\bullet-}$ generation *in vitro* (Pontremoli *et al.* 1989). However, to date little effort has been made to elucidate the potential mechanisms by which infiltrating leukocytes may contribute to disease progression. In particular, it is not known whether $O_2^{\bullet-}$ generation by activated neutrophils alters endothelial function directly, or if the primary role of these cells is to recruit other cytotoxic immune cells (e.g. monocytes). As such, the relative contribution of neutrophil versus vascular NAD(P)H oxidases in the pathogenesis of vascular dysfunction requires further examination.

1.5.1.3.4 Xanthine oxidase

Like NAD(P)H oxidase, XO has been shown to consume NO indirectly through the production of $O_2^{\bullet-}$, using xanthine/hypoxanthine as substrates for the reduction of O_2 (Chung *et al.* 1997; Porras *et al.* 1981). This enzyme is expressed at high levels on the luminal surface of the endothelium following the post-translational modification of xanthine dehydrogenase (XD); either through reversible thiol oxidation of sulfhydryl residues on XD, or via irreversible proteolytic cleavage of a segment of XD during hypoxia, ischemia, or in the presence of various proinflammatory mediators (e.g. TNF-α) (Cai & Harrison, 2000; Li & Shah, 2004; Meneshian & Bulkley, 2002). As such, it is thought that XO activity may increase in response to oxidative stress. Indeed, in a recent

study, Ang II-induced increases in XO-dependent $O_2^{\bullet-}$ production by cultured endothelial cells were prevented by NAD(P)H oxidase inhibition (Landmesser *et al.* 2007).

Increased $O_2^{\bullet-}$ production by XO has been implicated in endothelial dysfunction in heavy smokers and in hypercholesterolemic rabbits (Guthikonda *et al.* 2003; Ohara *et al.* 1993). Furthermore, it is believed that XO-derived $O_2^{\bullet-}$ may act indirectly to further enhance local ROS production, by promoting the local accumulation and activation of neutrophils – and therefore, neutrophil NAD(P)H-oxidase – as part of the microvascular inflammatory response (Meneshian & Bulkley, 2002). Indeed, XO is activated by proinflammatory mediators such as TNF-α, interleukins, complement C5a, and lipopolysaccharide (LPS); all of which have been shown to promote neutrophil activation *in vitro* (see section 1.6). However, this concept has yet to be demonstrated in the vasculature, either *in vivo* or *in vitro*.

1.5.1.3.5 Uncoupled eNOS

eNOS can exist in two forms. A 'coupled' form, in which it oxidises L-arginine to NO, and an 'uncoupled' form, in which electrons are lost that would otherwise be used to generate product from substrate. In the uncoupled state, O_2 is the usual acceptor of the stray electrons, giving rise to $O_2^{\bullet-}$ (Cosentino & Katusic, 1995; Griffith & Stuehr, 1995; Stuehr *et al.*, 2001). Indeed, it is of interest that molecular cloning of NOS has shown that it shares a close amino acid sequence homology with cytochrome P-450 reductase,

another source of $O_2^{\bullet-}$ described earlier in this section (see section 1.5.1.3.2) (Bredt *et al.*, 1991; Cosentino & Luscher, 1998).

An increase in oxidative stress is a vital step in the genesis of endothelial dysfunction, since ROS may damage essential cofactors or sensitive amino acid residues within eNOS, making the enzyme unstable (Griffith & Stuehr, 1995; Verhaar *et al.* 2002). Indeed, in experimental hypertension, $O_2^{\bullet-}$ generation by NAD(P)H oxidase leads to eNOS uncoupling and amplification of ROS production (Landmesser *et al.* 2003). Experiments by Stroes *et al.* (1998) using electron spin resonance have suggested that $O_2^{\bullet-}$ is mainly produced by the haem (oxygenase) moiety of eNOS. The main evidence for this is that $O_2^{\bullet-}$ production is diminished when Ca^{2+} is depleted by exposure to EDTA (a Ca^{2+} chelator). Since Ca^{2+}/CaM acts to promote the shuttling of electrons from the flavin moiety to the haem moiety, it seems that this electron transfer is essential for $O_2^{\bullet-}$ production (Abu-Soud *et al.* 1994; Stroes *et al.*, 1998).

The balance between levels of coupled and uncoupled eNOS in the vasculature seems to be dependent upon the level of BH_4 present in the cell, since this cofactor appears to 'couple' the reduction of O_2 to L-arginine oxidation, as well as maintaining the stability of the NOS dimer (Tiefenbacher, 2001; Vásquez-Vivar *et al.* 2003). Indeed, levels BH_4 have been found to be reduced in the aortae of diabetic rats and hypercholesterolemic apolipoprotein E-knockout mice, and in plasma of spontaneously hypertensive rats (SHRs) (Hong *et al.* 2001; Laursen *et al.* 2001; Meininger *et al.* 2000). This decreased availability of BH_4 is thought to be due to oxidative stress, since BH_4 is known to be

highly susceptible to oxidation degradation by ROS such as $ONOO^-$ (Landmesser *et al.* 2003; Laursen *et al.* 2001). Alternatively, BH_4 depletion may be due to reduced expression of GTP cyclohydrolase I, the rate-limiting enzyme in BH_4 synthesis, as has been shown in coronary endothelial cells of diabetic rats (Meninger *et al.* 2000). However, the mechanisms behind this reduced GTP cyclohydrolase I expression have not yet been investigated.

The importance of eNOS uncoupling in the pathogenesis of cardiovascular disease *in vivo* has been demonstrated via intra-aterial infusion of BH_4, which improved endothelium-dependent vasodilation in chronic smokers (Heitzer *et al.* 2000). However, this does not discount a direct antioxidant effect of BH_4. Nevertheless, endothelial function has also been shown to be corrected *in vivo* following increased gene expression of GTP cyclohydrolase I, thus providing more convincing evidence for the *in vivo* relevance of eNOS uncoupling (Alp *et al.* 2003; Alp *et al.* 2004).

More recently, the association of heat-shock protein-90 (hsp90) with eNOS has been cited as another possible determinant of eNOS activity (Koshida *et al.*, 2003), since agents that inhibit hsp90 (e.g. geldanamycin, radicicol) have been found to attenuate NO-dependent signalling by uncoupling eNOS activity (Garcia-Cardena *et al.*, 1998; Pritchard *et al.*, 2001; Ou *et al.*, 2003). Furthermore, hsp90 has been shown to enhance NO production from nNOS (Song *et al.*, 2001). These reports suggest that hsp90 may determine whether NO or $O_2^{\bullet-}$ is generated when eNOS is activated. Indeed, studies by Garcia-Cardena *et al.* (1998) and Pritchard *et al.* (2001) both concluded that hsp90

association with eNOS enhances NO formation. Thus, hsp90, like BH_4, may potentially be viewed as a future therapeutic target in maintaining endothelial function or reversing endothelial dysfunction.

To summarise, there is now a large body of evidence that suggests that endothelial dysfunction is caused by the accelerated inactivation of NO, particularly by ROS. However, this knowledge has yet to result in the introduction of new therapies; various antioxidants (e.g. vitamin C, vitamin E and the carotenoids) have been tested in clinical trials for the prevention of cardiovascular end-points, but have been largely unsuccessful (Heart Protection Study Collaborative Group, 2002; Yusuf *et al.* 2000). It is surprising that, as yet, little attention has been paid to the potential role of immune cells in the pathogenesis of endothelial dysfunction, given that a number of inflammatory vascular diseases, such as atherosclerosis, are characterised by both the infiltration of immune cells into vascular tissue and a loss of vasodilation response. This, coupled with the propensity for inflammatory-activated neutrophils to release large amounts of $O_2^{\bullet-}$ into the extracellular space, forms the basis for further investigation into the role of neutrophils in the regulation of vascular tone.

1.6 Neutrophils and vascular tone

1.6.1 Introduction to neutrophils

Neutrophils, or polymorphonuclear leukocytes (PMNs), are members of the leukocyte family, comprised of neutrophils, basophils, eosinophils, monocytes, and lymphocytes. They are produced in the bone marrow and circulate in the blood as spherical cells about 7–15 μm in diameter, with a segmented nucleus made up of 3–5 lobules connected by a thin strand of chromatin (Figure 1.7) (Abbas & Lichtman, 2003; Brown + Larson, 2001). An adult human produces more than 1 x 10^{11} neutrophils per day, making up about two-thirds of the leukocyte population in the peripheral circulation, although each cell only circulates for approximately 8–12 hours before undergoing programmed cell death (Abbas & Lichtman, 2003; Yamashiro *et al.* 2001).

Neutrophils play a fundamental role in the innate immune response (Figure 1.8). Specifically, PMNs are the first blood-borne nucleated cells to infiltrate sites of injury or infection following the local release of inflammatory mediators (e.g. histamine), cytokines (e.g. IL-1, TNF-α) and oxygen-derived free radicals (e.g. $O_2^{\bullet-}$), which promote the transformation of the normal endothelial wall into a pro-adhesive surface for the anchorage of circulating neutrophils (Abbas & Lichtman, 2003; Dallegri & Ottonello, 1997; Wagner & Roth, 2000). Briefly, under normal conditions, circulating neutrophils routinely attach to the endothelium and 'roll' along the vessel wall via the reversible binding of transmembrane glycoprotein adhesion molecules called selectins, which are

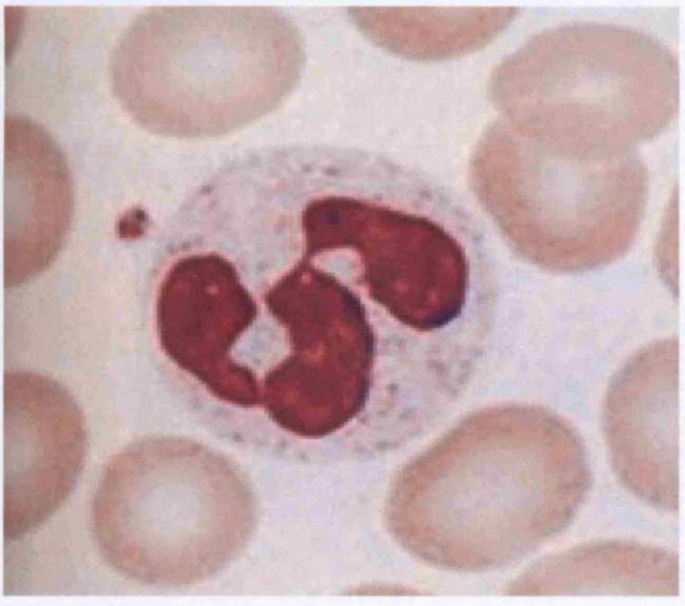

Figure 1.7 Neutrophil surrounded by several red blood cells
Note that the nucleus is made up of several lobes connected by a fine strand of chromatin. The cytoplasm stains pink with many fine, neutrophilic granules (From www.geocities.jp/kirinrara1947/neutrophil.jpg).

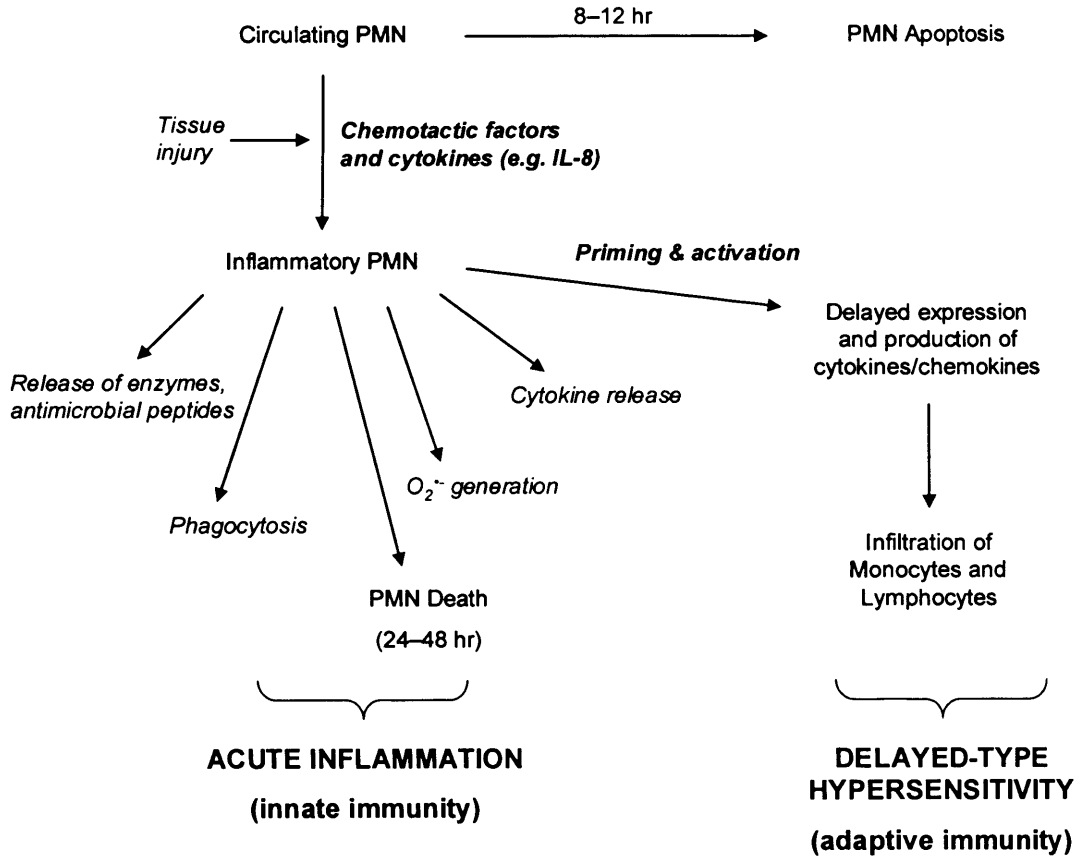

Figure 1.8 Schematic describing the role of neutrophils in the innate and acquired immune response

(Adapted from Yamashiro *et al.* 2001).

found on the surface of both PMNs (L-selectin) and endothelial cells (P-selectin and E-selectin). These reversible selectin-ligand interactions allow PMNs to associate with and respond to stimuli presented on the endothelial cell surface. Under inflammatory conditions, neutrophil rolling ceases and PMNs become firmly attached to the endothelium through integrins (e.g. CD11/CD18) on the surface of PMNs and intercellular adhesion molecules (e.g. ICAM-1, VCAM-1 and platelet–endothelial cell adhesion molecule-1 (PECAM-1)) on endothelial cells (Figure 1.9; see Wagner & Roth, 2000 for a comprehensive review). The net result of this process is the migration of neutrophils from the circulation, across the endothelial cell layer, and their accumulation around the infectious microbes in the sub-endothelial space. Neutrophil migration is directed by gradients of locally generated chemoattractants (Baggiolini *et al.* 1989; Ben-Baruch *et al.* 1995). These include the complement component C5a, formyl oligopeptides (e.g. *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP)) generated by tissue-invading bacteria, platelet-activating factor (PAF) produced by endothelial cells, platelets and macrophages, and leukotriene B_4 (LTB_4) generated by macrophages (Wagner & Roth, 2000). Also of importance are a series of chemokines belonging to the *CXC* supergene family, including IL-8, produced by macrophages, fibroblasts, endothelial cells and epithelial cells in response to TNF-α, IL-1 or LPS (Baggiolini *et al.* 1995).

Once recruited to sites of inflammation, neutrophils respond to injurious agents by phagocytosis and the release of antimicrobial products (e.g. $O_2^{\bullet-}$ and H_2O_2). Phagocytosis is a cytoskeleton-dependent process in which the neutrophil binds to and engulfs the microbe in its cell surface membrane to form an intracellular vesicle called a phagosome.

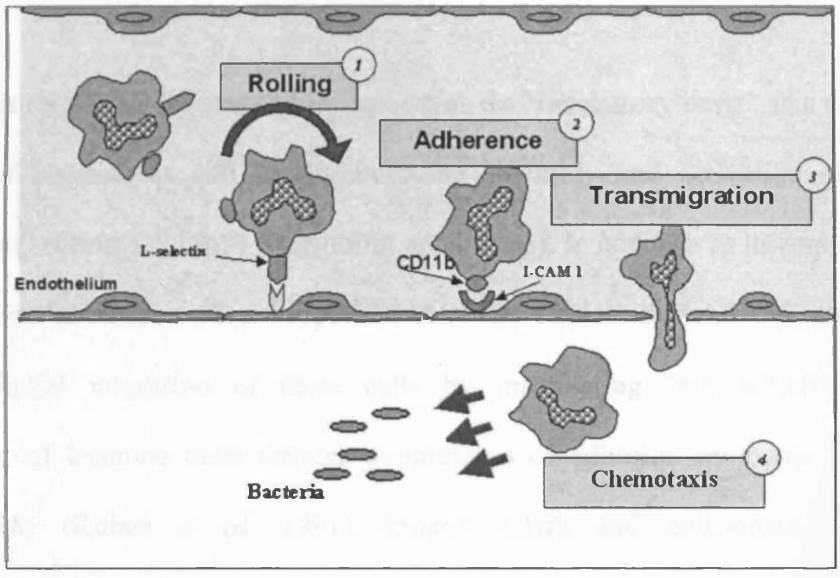

Figure 1.9 Schematic representation of leukocyte transendothelial migration

(1) Selectins mediate weak tethering and rolling of blood leukocytes, such as neutophils, on the endothelium. (2) Integrins (e.g. CD11b) mediate firm adhesion of neutrophils. (3) Chemokines increase the affinity of neutrophil integrins and stimulate the transmigration of the cells through the endothelium to the site of infection. (4) Blood neutrophils, monocytes, and activated T lymphocytes use essentially the same mechanisms to migrate to sites of infection. (Adapted from Seeley *et al.*, 2003).

Fusion of phagosomes with lysosomes results in the formation of phagolysosomes, where the microbicidal mechanisms are concentrated (Abbas & Lichtman, 2003). These include hydrolytic enzymes (e.g. lysozyme) and various oxidants such as $O_2^{\bullet-}$, H_2O_2, hypochlorous acid ($HOCl^-$) and chloramines (R-NHCl) (Babior, 1984; Dallegri & Ottonello, 1997).

The generation of $O_2^{\bullet-}$ by neutrophils, known as the 'respiratory burst', is a characteristic property of phagocytes and results from the assembly and activation of NAD(P)H oxidase (see section 1.5.1.3.3) (Baggiolini *et al.* 1993). In addition to its important role in the microbicidal actions of neutrophils, NAD(P)H oxidase-derived $O_2^{\bullet-}$ also promotes transendothelial migration of these cells by inactivating NO, which inhibits the recruitment of immune cells through suppression of adhesion molecule activity (e.g. CD11/CD18) (Kubes *et al.* 1991). Indeed, Clark and colleagues (2002) have demonstrated that activated neutrophils accelerate NO disappearance from aerobic buffer *in vitro* in an NAD(P)H oxidase-dependent manner (Clark *et al.* 2002). Furthermore, leukocyte–endothelial cell interactions are upregulated in hypercholesterolemia via a mechanism involving $O_2^{\bullet-}$ generation by leukocyte NAD(P)H oxidase (Stokes *et al.* 2001). In this respect, NAD(P)H oxidase activity appears to play a critical role in the innate immune response.

In addition to their role in innate immunity, neutrophils are believed to contribute to the development of adaptive immunity, including delayed-type hypersensitivity reaction (DTH) (Figure 1.8) (Yamashiro *et al.* 2001). Although the precise mechanisms are still

being elucidated, it has been suggested that prolonged exposure of neutrophils to inflammatory cytokines (e.g. TNF-α) leads to phenotypic and functional changes in these cells, known as 'priming', some of which may be associated with a role in adaptive immunity (Yamashiro *et al.* 2001). For example, upon activation, inflammatory-primed neutrophils have been shown to synthesise and release an array of cytokines and chemokines, including IL-1β, IL-8, macrophage inflammatory peptide (MIP)-1α and MCP-1, which induce activation and chemotaxis of monocytes, dendritic cells and T/B cells to the site of infection; processes that are highly important in the transition from innate to adaptive immunity (Cassatella, 1999; Kasama *et al.* 2005; Scapini *et al.* 2000; Yamashiro *et al.* 2001).

1.6.2 Neutrophils and endothelial dysfunction

While the importance of neutrophils in the innate immune response is well documented, relatively little work has been carried out to investigate a role for these cells in the endothelial dysfunction commonly observed in inflammatory vascular disease. Indeed, given the importance of NO bioavailability in vascular homeostasis, it is entirely plausible that the rapid generation of $O_2^{\bullet-}$ by neutrophils may have a deleterious effects on endothelial function. With this in mind, various *in vitro* studies have examined vasoactivity in isolated vessels following the addition of neutrophils to the tissue baths. In this setting, activated neutrophils were shown to prevent or reverse endothelium-dependent relaxation in phenylephrine (PE)-precontracted vascular tissues, indicating a loss of endothelial function (Ohlstein & Nichols, 1989). Similarly, the addition of

activated neutrophils to rabbit aorta and cat coronary arteries *in vitro* elicited vasocontraction in an endothelium- and $O_2^{\bullet-}$-dependent manner, indicating that neutrophils may alter vascular tone via $O_2^{\bullet-}$-mediated inactivation of basally released NO (Ma *et al.* 1991; Ohlstein & Nichols, 1989). A recent study by Sugano and colleagues (2005) provided further insight into the mechanism behind these effects when neutrophils from hypercholesterolemic patients were found to readily adhere to cultured HUVECs *in vitro* and impair eNOS activity in these cells via the generation of $O_2^{\bullet-}$. Thus, it appears that activated neutrophils may impair endothelial function *in vitro* via the direct inactivation of NO and also by inhibiting eNOS activity in vascular cells. However, to our knowledge, no study has yet been carried out to explore the vasoactive effects of neutrophils *in vivo*, and as such the relevance of these findings under physiological conditions remains uncertain.

1.6.3 Neutrophil priming and vascular tone

As already mentioned, prolonged exposure to inflammatory cytokines can cause neutrophil priming. Neutrophil priming is be defined as the potentiation of neutrophil responses to biological activating agents, such as fMLP or the complement component C5a, following prior exposure to a 'priming agent' (Condliffe *et al.* 1998; Vercellotti *et al.* 1988). For example, neutrophil respiratory burst activity has been shown to be upregulated up to 20-fold and neutrophil integrin expression is also enhanced in primed cells (Condliffe *et al.* 1998). In addition, prolonged exposure to inflammatory stimuli also stimulates 5-LOX and COX-2 activity within neutrophils, promoting the release of AA

metabolites such as LTB_4 and TxA_2 following cell activation (Pouliot *et al.* 1998; Staňková *et al.* 1995).

LTB_4 is a potent promoter of neutrophil adhesion to the vessel wall, chemotaxis and degranulation (Ford-Hutchinson *et al.* 1980; Rola-Pleszczynski, 1985; Samuelsson *et al.* 1987). This lipid mediator also modulates many functions of other cell populations. For example it can stimulate IFN-γ production by T-cells and TNF-α production by monocytes/macrophages (Dubois *et al.* 1989; Horiguchi *et al.* 1989; Rola-Pleszczynski *et al.* 1986). Thus, the sustained production of LTB_4 by neutrophils is likely to potentiate the recruitment and activation of other immune cells. In addition, exogenous LTB_4 has recently been shown to promote endothelium-dependent vasoconstriction in the guinea pig aorta via the release of histamine and TxA_2 (Bäck *et al.* 2004). TxA_2 is a powerful constrictor of vascular smooth muscle and enhanced production of this molecule has been reported in several cardiovascular diseases, including unstable angina and hypertension (Matrougi *et al.* 1997; Narumiya *et al.* 1999; Noll & Luscher, 1998; Ungvari & Koller, 2000). Release of this mediator has also been shown to be enhanced in neutrophils following an increase in COX-2 mRNA levels (Pouliot *et al.* 1998). The reason for the lack of PGI_2 generation by these cells has not been determined, although it is possible that this due to the $ONOO^-$-mediated tyrosine nitration and concomitant inactivation of PGIS following $O_2^{\bullet-}$ release by neutrophil NAD(P)H oxidase (Zou *et al.* 1997).

A role for 5-LOX and COX-2 in the vasoactive effects of neutrophils has yet to be established, since previous studies have all used acutely activated cells, in which the

bioactivity of these enzymes is low. For example, Ma and colleagues (1991) found that neutrophil-induced vasoconstriction was unaffected by the addition of COX or LOX inhibitors when these cells were acutely activated with PMA or fMLP, indicating that LTB_4 and TxA_2 are not responsible for the observed vasoconstriction response. However, given that the bioactivities of 5-LOX and COX-2 are enhanced in neutrophils following prolonged exposure of these cells to inflammatory cytokines, LTB_4 and TxA_2 are more likely to play a role in the vasoactive effects of primed neutrophils. Furthermore, while the ability of acutely activated neutrophils to consume NO *in vitro* is entirely NAD(P)H oxidase-dependent, the enhanced expression of 5-LOX and COX-2 in primed cells may provide an addition mechanism by which these cells may alter NO bioavailability and, therefore, vascular tone (i.e. LOX- and COX-mediated catalytic NO consumption; see *sections 1.5.1.1 and 1.5.1.2*) (Clark *et al.* 2002). Therefore, in order to more effectively assess the potential for neutrophil 5-LOX and COX-2 to contribute to the regulation of vascular tone *in vivo*, the vasoactive effects of inflammatory-primed cells should be examined *in vitro*.

In addition to the upregulation of 5-LOX and COX-2 activity, neutrophil priming also results in the increased synthesis and release of an array of cytokines and chemokines (e.g. IL-8, MIP-1 and MCP-1), which promote activation and infiltration of monocytes into the inflammatory site. One of the most immediate responses of monocytes to activating stimuli is the production of $O_2^{\bullet-}$ via the NAD(P)H oxidase pathway (Cathcart, 2004). The NAD(P)H oxidase components in monocytes/macrophages are similar to those in neutrophils, although the enzyme appears to be regulated differently (Leusen *et*

al. 1996). For example, whereas neutrophils produce an immediate respiratory burst upon stimulation, peaking at 2–10 mins, $O_2^{\bullet-}$ production by monocytes increases more gradually and is still detectable after several hours (Cathcart *et al.* 1989; Dewald *et al.* 1984). Furthermore, after a sufficient recovery period, monocyte NAD(P)H oxidase may be restimulated; a process not observed in neutrophils. While the ability of activated monocytes to alter vascular tone has not been examined, these cells can consume NO *in vitro*, and macrophages have long been associated with the pathogenesis of atherosclerosis due to their accumulation in atherosclerotic lesions (Cathcart, 2004; Hancock & Jones, 1987). Thus, the recruitment of monocytes/macrophages following cytokine release by neutrophils may serve as a key step in the pathogenesis of inflammatory vascular disease. This underlines the importance of examining the ability of neutrophils to modulate vascular tone *in vivo*, as the vasoactive effects of these cells are likely to involve interactions with other immune cells and, as such, cannot be fully recreated *in vitro*.

1.6.4 Potential vasoactive effects of resting neutrophils

Currently, little is known about the ability of circulating neutrophils to alter vascular tone under healthy conditions. A study by Kerr and colleagues (1998) attempted to address this question by examining the effect of unactivated human neutrophils on isolated human umbilical veins. Interestingly they found that these cells caused an endothelium-independent vasoconstriction that was mediated by a soluble molecule released into the cell supernatant and inhibited by the leukotriene synthesis inhibitor BIRM-270. These

observations suggest that unstimulated circulating neutrophils may exert a vasoconstrictive effect on vascular smooth muscle via the constitutive release of a lipid mediator such as LTB_4 (Kerr *et al.* 1998). However, this is in marked constrast to the findings of other studies which showed that LTB_4 generation by unactivated or acutely activated neutrophils was low or undetectable without prior exposure to inflammatory stimuli (Dahinden *et al.* 1988; Di Persio *et al.* 1988; Zarini *et al.* 2006). As such, further investigation is required to more accurately gauge to vasoactive potential of circulating neutrophils under healthy conditions.

In summary, neutrophils play an important role in both innate and acquired immunity by rapidly infiltrating infected tissue, removing harmful microbes and recruiting other immune cells (e.g. monocytes). However, in addition to the beneficial role that neutrophils play, these cells have been associated with the loss of endothelial function and increased vascular tone commonly observed in inflammatory vascular disease. The mechanisms by which inflammatory-activated neutrophils may potentially alter vascular tone are outlined in Figure 1.10. These include the NAD(P)H oxidase-dependent generation of $O_2^{\bullet-}$ anions, which inactivate NO, leading to the loss of NO-mediated vasodilatory signalling, PGIS inactivation and upregulation of endothelial and leukocyte adhesion molecule expression. Furthermore, following prolonged exposure to inflammatory stimuli (e.g. TNF-α), neutrophils may release chemokines and chemoattractants, promoting the recruitment of other immune cells, such as monocytes/macrophages, which also release large quantities of $O_2^{\bullet-}$. Finally, primed neutrophils also express 5-LOX and COX-2, which have the potential to generate potent

vasoconstrictors (LTB_4 and TxA_2, respectively) as well as catalytically consuming NO as part of their enzyme cycle. However, despite the obvious potential for these cells to play a role in the altered vascular function commonly observed in many inflammatory vascular diseases, to date little work has been carried to test this hypothesis. With this in mind, the current study will focus on examining the role of neutrophils in the modulation of vascular tone, with particular interest given to the mechanisms by which these cells may exert their vasoactive effects *in vivo*.

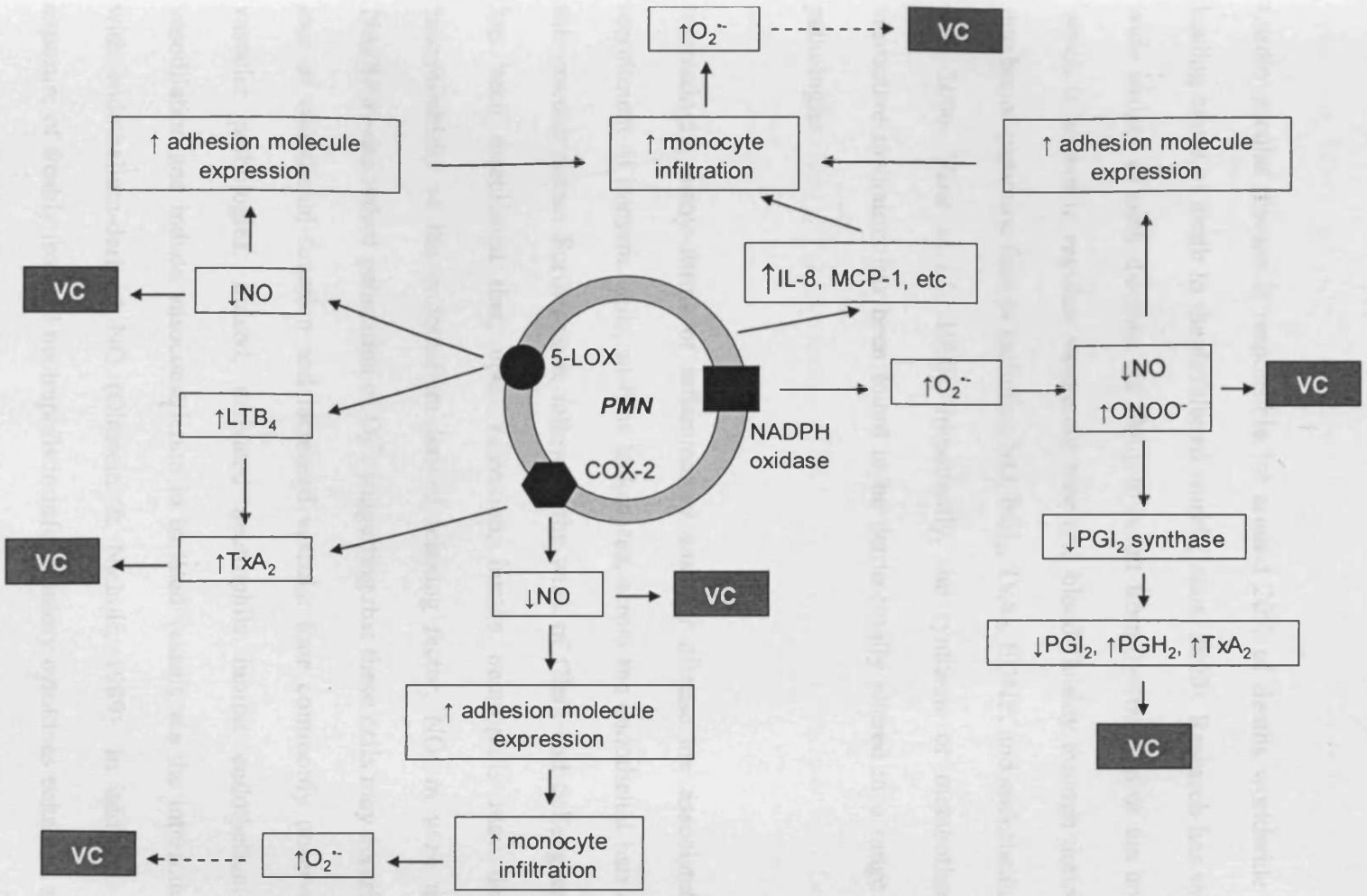

Figure 1.10 Schematic representation of the mechanisms by which activated neutrophils may potentially alter vascular tone

VC = vasoconstriction; ↑ = upregulated; ↓ = downregulated; ⟶ = directly leads to…; - - -▸ = indirectly leads to…

1.7 General aims

Cardiovascular disease is responsible for around 20% of deaths worldwide and is the leading cause of death in the developed world (Lusis, 2000). Research has shown that a wide variety of such diseases are likely to result from dysfunction of the endothelium, which is known to regulate vasomotor tone and blood fluidity through the release of a number of paracrine factors including NO, PGI_2, TxA_2, EDHF, and endothelin (Huang & Vita 2006; Vane *et al.*, 1990). Importantly, the synthesis or metabolism of these vasoactive mediators has been found to be detrimentally altered in a range of disease pathologies.

Interestingly, many forms of inflammatory vascular disease are associated with the recruitment of immune cells, such as leukocytes, across the endothelial barrier and into the vascular tissue. Furthermore, following the work of Clark and colleagues (2002), it has been established that, upon activation, human neutrophils may decrease the bioavailability of the endothelium-derived relaxing factor, NO, *in vitro* through the NAD(P)H-dependent generation of $O_2^{\bullet-}$, suggesting that these cells may contribute to the loss of endothelial function and increased vascular tone commonly observed in such vascular pathologies. Indeed, activated neutrophils inhibit endothelium-dependent vasodilation and induce vasoconstriction in isolated vessels via the interaction of ROS with endothelium-derived NO (Ohlstein & Nichols, 1989). In addition, prolonged exposure of freshly isolated neutrophils to inflammatory cytokines enhances a variety of neutrophil functions, including the release of vasoactive mediators and NAD(P)H

oxidase-dependent $O_2^{\bullet-}$ generation (Condliffe *et al.* 1998). However, to date the potential vasoactive effects of these cells have yet to be adequately explored, particularly with regard to relevance of these observations *in vivo*.

Herein, the aim of this study is to further examine a role for neutrophils in the regulation of vascular tone. Specifically, this thesis will focus on (1) the effect of inflammatory mediators on NO consumption by neutrophils *in vitro*, and (2) the effect of neutrophil depletion on vascular tone *in vivo*.

1.7.1 The effect of inflammatory mediators on NO consumption by neutrophils in vitro

Hypertensive and inflammatory vascular diseases, such as atherosclerosis, are associated with a reduction in NO bioavailability, partly due to the overproduction of $O_2^{\bullet-}$. Under inflammatory conditions, exposure of neutrophils to inflammatory cytokines (e.g. PAF, TNF-α, IL-8) potentiates neutrophil responses to activating agents such as fMLP; a phenomenon known as 'priming' (Condliffe *et al.* 1998). Of particular interest is the potentiation of neutrophil NAD(P)H oxidase, 5-LOX and COX-2 activity by various 'priming agents' *in vitro* and the potential outcome of this priming *in vivo*. To investigate whether priming enhances NO consumption, human neutrophils will be isolated and incubated with a range of priming agents. The ability of primed cells to regulate NO levels *in vitro* will be examined and compared to unprimed cells, initially in an attempt to better understand the ability of neutrophils to consume NO under inflammatory

conditions *in vitro*, before attempting to predict the implications of this NO uptake *in vivo*.

1.7.2 The effect of neutrophil depletion on vascular tone in vivo

Given the fact that activated neutrophils may alter NO bioavailability and generate vasoactive mediators *in vitro*, the importance of these cells in regulating vascular tone *in vivo* must be considered. Briefly, neutrophils will be depleted in healthy mice using monoclonal antibodies and the BP of these animals will be monitored over a period of 8 days to assess a potential role for these cells in vascular homeostasis. In addition, direct comparisons of vasoactivity in vessels from control and neutropenic mice will be carried out *ex vivo* to further examine the mechanisms by which neutrophils may modulate vascular smooth muscle tone.

It is hoped that the results of this study will provide an insight into the role of neutrophils in regulating vascular tone under normal and inflammatory conditions. Furthermore, identification of beneficial or detrimental changes in vascular function in the absence of neutrophils *in vivo* may better our understanding of the pathology of cardiovascular disease and therefore lead to the development of novel therapeutic strategies for the treatment of these conditions.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Unless stated otherwise, all chemicals used for the experiments described in this thesis were purchased from Sigma-Aldrich Company Ltd (Poole, Dorset, UK).

2.1.2 General buffers and solutions used

Krebs buffer

100 M NaCl, 50 mM HEPES, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $NaH_2PO_4.2H_20$, 1 mM $CaCl_2$, 2 mM D-glucose, pH 7.4.

Phosphate buffered saline (PBS)

137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4.

Citrate PBS

137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.4% (w/v) $Na_3C_6H_5O_7.2H_2O$, pH 7.4.

Dextran-Citrate-PBS

137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.8 % (w/v) $Na_3C_6H_5O_7.2H_2O$, 2% (w/v) 100,000–200,000 Da Dextran, pH 7.4.

0.2% saline

0.2% (w/v) NaCl, pH 7.4.

1.6% saline

1.6% (w/v) NaCl, pH 7.4.

Krebs-Henseleit buffer

120 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4.7H_20$, 24 mM $NaHCO_3$, 1.1 mM KH_2PO_4, 10 mM Glucose, 2.5 mM $CaCl_2.2H_20$, pH 7.4.

Nitric oxide

Anaerobic solutions of 1.9 mM nitric oxide (NO) were prepared by equilibrating NO gas in N_2-saturated deionised water (Beckman *et al.* 1996). NO_2 was eliminated by bubbling NO through 1 M NaOH.

SDS polyacrylamide stacking gel for western blotting

65 mM tris (hydromethyl)-methylamine, 0.1% (w/v) sodium dodecyl sulphate (SDS), 10% (w/v) acrylamide, 0.05% (w/v) ammonium persulphate, 0.1% (v/v) $N_1N_1N_1N_1$-tetramethylethylene diamine (TEMED).

10% SDS polyacrylamide running gel for western blotting

355 mM tris (hydromethyl)-methylamine, 0.1% (w/v) SDS, 10% (w/v) acrylamide, 0.1% (w/v) ammonium persulphate, 0.4% (v/v) TEMED.

Western running buffer

25 mM tris (hydromethyl)-methylamine, 190 mM glycine, 0.1% (w/v) SDS.

Western transfer buffer

25 mM tris (hydromethyl)-methylamine, 190 mM glycine, 20% (v/v) methanol.

Phosphate buffered saline with tween (PBS-T)

137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4, 0.1% tween 20.

Buffer A

10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% MP40.

Buffer C

20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA.

5X Reaction Buffer Stock

50 µl 1 M HEPES (pH 7.9), 250 µl 1 M KCl, 500 µl 100% glycerol, 90 µl dH_20.

5X Binding Buffer

89 µl reaction buffer stock, 10 µl 10 mg/ml acetylated BSA, 0.5 µl 1 M DTT, 1 µl 0.1 M phenylmethysulfonylfluoride (PMSF).

6X DNA Loading Buffer

0.03% xylene cyanol FF, 0.03% bromophenol blue, 60% glycerol in dH_20

2.2 Methods

2.2.1 Isolation of human neutrophils

Whole blood was obtained by venepuncture from the antecubital fossa of healthy volunteers who were free from non-steroidal anti-inflammatory drugs for at least fourteen days. Ethical permission was obtained from the Bro Taf Local Research Ethics Committee and written consent was obtained from all volunteers.

Human neutrophils were isolated by the method described by Segal & Peters (1976). Briefly, 20 ml of whole blood was drawn slowly and directly into an equal volume of Dextran-Citrate-PBS. Erythrocytes were allowed to sediment for 45–60 min, and the upper plasma layer removed and underlaid with lymphoprep (Nycomed Pharma, Oslo, Norway), using half the volume of lymphoprep to plasma. This was centrifuged at 800 g for 20 min at 4 °C. The supernatant was discarded and the pellet resuspended in 10 ml citrate-PBS and the transferred to a clean centrifuge tube. This was centrifuged at 400 g for 5 min at 4 °C. The supernatant was discarded and the red cells (RBCs) lysed by resuspending in 3 ml hypotonic saline solution (ice-cold 0.2% saline).

Isotonisity was restored after 45 s by addition of an equal volume of ice-cold 1.6% saline, and the solution centrifuged at 400 g for 5 min at 4 °C. This hypotonic shock procedure was repeated a further 1–2 times, each with an interval of just 30 s between the addition of 0.2% saline and 1.6% saline. The final pellet was resuspended in Krebs buffer and the neutrophil concentration determined by tryphan blue exclusion using a haemocytometer under an inverted microscope.

2.2.2 Assays

2.2.2.1 $O_2^{\bullet-}$ generation assay

$O_2^{\bullet-}$ generation by human neutrophils was assayed by the copper-zinc superoxide dismutase (CuZn-SOD)-inhibitable reduction of cytochrome *c* (cyt *c*). Cyt *c* reduction was monitored in a Uvikon 923 spectrophotometer (Bio-Tek Kontron Instruments) monitoring at 550 nm at 37 °C with stirring, using $\Delta\varepsilon = 21.1\ cm^{-1}\ mM^{-1}$ (Cross *et al.* 1982). 1 μM fMLP was added to 2 ml of Krebs buffers containing $0.5 \times 10^6\ ml^{-1}$ neutrophils and 50 μM cyt *c*. CuZn-SOD (Bio-Stat Diagnostic Systems, UK) (300 U ml^{-1}) was included as a negative control. The cuvette was quartz crystal and was coated with Sigmacote to prevent neutrophil activation.

In addition, neutrophils ($3.1 \times 10^4\ ml^{-1}$) were primed by incubation at 37 °C, with stirring, with either (95 nM) PAF for 5 min, TNF-α ($10\ ng.ml^{-1}$) for 10 min, or IL-8 (10 nM) for 10 min, before activation with fMLP.

2.2.2.2 NO consumption assay

NO was measured electrochemically using an NO electrode (Harvard AmiNo700 with inNO meter). In order to measure the background rate of NO loss, a bolus of NO (1.9 μM) was added with a Hamilton syringe to 0.5 ml Krebs buffer at 37 °C with stirring. Where NO loss was not linear, rates are given as first order rate constants (k_{obs}) calculated by determining the slope of ln[NO] *versus* time. For all k_{obs} determined, the square of the Pearson product moment correlation coefficient (r) of the slope of the replotted data was always greater than 0.9, confirming that the reaction followed first order kinetics. Where NO consumption was linear, the slope of the rate of 'NO disappearance was determined.

In order to measure the rate of NO consumption by neutrophils, once again a bolus of NO (1.9 μM) was added to 0.5 ml Krebs buffer containing neutrophils (0.5×10^6 ml^{-1}) at 37 °C with stirring. Once the NO peak had stabilised, neutrophils were activated with fMLP (1 μM), and the rate of NO disappearance monitored.

In order to measure the rate of NO consumption by primed neutrophils, cells (3.125×10^4 ml^{-1}) were primed by incubation at 37 °C, with stirring, with either PAF (95 nM) for 5 min, TNF-α (10 $ng.ml^{-1}$) for 10 min, or IL-8 (10 nM) for 10 min. After this period, a bolus of NO (1.9 μM) was added to the reaction vessel and, once the NO peak had stabilised, the neutrophils were activated with fMLP (1 μM), and the rate of NO disappearance monitored.

2.2.2.3 Protein concentration determination

Concentration of protein was determined by the method of Bradford (1976) using bovine serum albumin as standard.

2.2.2.4 Western blotting

Western blotting was performed as according to the method of Towbin and colleagues (1979). Briefly, mice were sacrificed and mouse blood was collected in a heparinised syringe by cardiac puncture and centrifuged at 10,000 g for 5 min at 4 °C to recover plasma. Samples were then assayed for protein concentration before being diluted into sample buffer. As a positive control for MPO, leukocytes (1.7 x 10^6 cells, equivalent to 1.5 μg protein) were prepared from mouse blood by hypotonic lysis of RBCs (1:10 blood:lysis buffer (1X RBC Lysis Buffer, eBiosciences, California, CA, USA)).

Samples were subjected to electrophoresis on 10% polyacrylamide gel in SDS electrode/running buffer at 170 V for 2 hrs. Proteins were then transferred from the wet gel to a nitrocellulose sheet at 100 V for 1 hr. Samples were probed with rabbit polyclonal anti-MPO antibody (Calbiochem, San Diego, CA, USA) (1:1000) and visualised using enhanced chemiluminescence autoradioagraphy (ECL) (Amersham, UK) after incubation with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:20,000).

2.2.2.5 Nitric oxide metabolite measurements

The NO metabolites nitrate and nitrite (NOx) were determined using the Griess Reaction (Schmidt & Kelm, 1996). Mouse blood was collected in a heparinised syringe by cardiac puncture and centrifuged at 10,000 g for 5 min at 4 °C to recover plasma, which was then centrifuged through a 10 kDa filter (Microcon; Millipore) at 10,000 g for 30 min at 4 °C. Filtrate was analysed for NOx by addition of sulphanilamide/HCl (1 mM, 0.6 mM, respectively) and *N*-(1-naphtyl)-ethylenediamine (NEDA, 1 mM), either with or without previous reduction using nitrate reductase (Aspergillus, Boehringer Ingelheim, Berkshire, UK) for 2 hours at 37°C. Absorbances were measured at 548 nm and compared with standard curves generated using sodium nitrate or nitrite.

2.2.2.6 Immunohistochemistry

The descending thoracic aorta was removed, cleaned of adipose tissue, and fixed in paraffin wax. 10 μm sections were taken using a microtome and methanol-fixed on glass slides, permeablised using 0.1% (w/v) Triton X-100/phosphate-buffered saline (PBS), and blocked using 1% (w/v) bovine serum albumin (BSA)/PBS. MPO or iNOS expression was visualised using either rabbit anti-MPO (1:200) or rabbit anti-iNOS (1:50; Calbiochem, Merck Biosciences Ltd, Nottingham, UK), with goat anti-rabbit IgG-Alexa 568 as secondary (1:100). Negative controls were equivalent concentrations of isotype control rabbit IgG antibody (Invitrogen, Paisley, UK). Imaging was performed on an Axiovert S100 TV inverted microscope connected to a Hamamatsu Orca I/ER 4742-95 camera (Hamamatsu Photonics UK Ltd,

Hertfordshire, UK) using standard analysis software (AQM-advance 6, Kinetic Imaging Ltd, Wirral, UK). Images were acquired using a X 20 air lens, with excitation at 568 nm and emission 560/40 nm. Post-acquisition processing used Adobe Photoshop, with average fluorescence intensity of specific stained regions (arbitrary units) of unprocessed images determined using AQM-advance. For each aorta, five separate sections were imaged and pixel intensity calculated at five separate areas of each section. Therefore, for each aorta 25 separate pixel intensity determinations were made.

2.2.2.7 *Limulus* amebocyte lysate test

Limulus amebocyte lysate (LAL) is an aqueous extract of blood cells from the horseshoe crab *Limulus polyphemus*. In the presence of endotoxin, factors in LAL are activated in a proteolytic cascade that results in the cleavage of a colourless artificial peptide substrate in Pyrochrome (Associates of Cape Cod, Inc, E. Falmouth, MA, USA). Proteolytic cleavage of the substrate liberates p-nitroaniline (pNA). The test is performed by adding a volume of Pyrochrome to a volume of specimen and incubating the reaction mixture at 37 °C; the greater the endotoxin concentration in the specimen, the faster pNA will be produced. In order to quantify endotoxin concentration in a sample, the pNA formed is reacted with nitrite in HCl and then with NEDA to form a diazotized magenta derivative that absorbs light at 540–550 nm.

All equipment used in this assay was endotoxin-free. Mouse blood was collected in a heparinised syringe by cardiac puncture and centrifuged at 10,000 g for 5 min at 4 °C to recover plasma. Plasma samples were subsequently diluted 1:10 in endotoxin-free

water (Associates of Cape Cod, Inc, E. Falmouth, MA, USA) and heated at 75 °C for 5 min in order to remove plasma inhibitors which may interfere with LAL proenzyme activation (e.g. α_1-globulin and α_1-lipoprotein esterase, IgM and IgG antibodies, and protease inhibitors, such as α_1-antitrypsin, α_2-macroglobulin and antithrombin III) (Harris *et al.* 1983). In addition, dilutions of endotoxin stock solution (0.5–0.016 $EU.ml^{-1}$) were prepared using control standard endotoxin (CSE, 0.2 EU) (Associates of Cape Cod, Inc, E. Falmouth, MA, USA) and endotoxin-free water in order to generate a standard curve.

The assay was carried out in a 96-well plate as indicated in the plate map below (Figure 2.1). The reaction mixture was incubated at 37 °C for 30 min before the reaction was stopped with 0.05 ml of NO_2^- in HCl. Samples were analysed for endotoxin by addition of ammonium sulphate and NEDA. Absorbances were measured at 548 nm and compared with standard curves generated with endotoxin stock dilutions.

2.2.2.8 Electrophoretic mobility shift assay (EMSA)

Preparation of nuclear extracts

Thoracic aortae were removed from Balb/c mice, cleaned of adipose tissue, and snap frozen in liquid nitrogen. For each sample, five snap frozen thoracic aortae were ground to a fine powder in liquid nitrogen using a pestle and mortar. Nuclear extracts were then prepared from the powder using a technique for the rapid extraction of nuclear proteins (Andrews & Faller, 1991). Briefly, the powder was resuspended in ice-cold Buffer A, containing 0.5 mM Dithiothreitol (DTT), 0.2 mM PMSF, 1 mM

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.25 EU Standard	0.125 EU Standard	0.06 EU Standard	0.015 EU Standard	0.0075 EU Standard							
B	Sample (control 1)	Inhibitor test (C1)	-ve control		Sample (C2)	Inhibitor test (C2)	-ve control		Sample (C3)	Inhibitor test (C3)	-ve control	
C												
D	0.25 EU Standard	0.125 EU Standard	0.06 EU Standard	0.015 EU Standard	0.0075 EU Standard							
E	Sample (depleted 1)	Inhibitor test (D1)	-ve control		Sample (D2)	Inhibitor test (D2)	-ve control		Sample (D3)	Inhibitor test (D3)	-ve control	
F	Sample (D4)	Inhibitor test (D4)	-ve control		Sample (D5)	Inhibitor test (D5)	-ve control					
G												
H												

Figure 2.1 Plate map for LAL test

A1–A5 and D1–D5 = standard curve. Well volume = 100 µl.

Inhibitor tests were carried out for each sample to test for the presence of plasma inhibitors (38 µl sample, 12 µl 0.5 $EU.ml^{-1}$ standard).

50 µl Pyrochrome working solution was added to each well to initiate the reaction.

sodium orthovanadate, 50 mM sodium fluoride (NaF) and proteinase inhibitors (diluted 1:1000, Sigma), and incubated on ice for 30 min. The nuclei were pelleted by centrifugation (17,000 g for 5 min) and the supernatant (cytosolic fraction) was removed. The nuclear pellet was resuspended in ice-cold Buffer C, containing 0.5 mM DTT, 0.2 mM PMSF, 1 mM sodium orthovanadate, 50 mM NaF, and proteinase inhibitors (diluted 1:1000, Sigma), and incubated on ice for 30 min to allow for high salt extraction. Cellular debris was removed by brief high-speed centrifugation (17,000 g for 5 min) and the resulting supernatants (nuclear extract) collected. Protein concentrations were determined using the Bradford method (see section 2.2.2.3).

Radiolabelling of double-stranded oligonucleotide probes

Oligonucleotide probes, containing either the SIE (signal transducer and activator of transcription (STAT)-inducing element) STAT-binding IFN-γ-activated site (GAS) elements (Figure 2.2A) or an interferon regulatory factor (IRF)-1-binding consensus sequence (Figure 2.2B), were annealed by heating reverse complementary oligonucleotide primers (1 $\mu g.ml^{-1}$) to 95 °C for 10 min, in the presence of 1 M NaCl and H_2O, and slowly cooling to room temperature overnight. The double-stranded probes were labelled with [α^{32}P]-dTTP (Amersham Pharmacia) using the Klenow fragment of DNA polymerase I and purified using probe quant micro columns (Amersham Pharmacia).

EMSA

For each binding reaction, 5 µg of nuclear extract was suspended in 7 µl of 5X binding buffer, an appropriate amount of water, 1 µl poly dIdC ($1mg.ml^{-1}$). After a 10 min incubation at room temperature, 2 µl of radiolabelled oligonucleotide probe was

A

SIE STAT (1)

5' – CGA CAT TTC CCG TAA ATC G – 3'

SIE STAT (2)

5' – CGA CGA TTT ACG GGA AAT G – 3'

B

IRF-1 (1)

5' – ATC GGC AGC GAA AAT GAA ATT GAG T – 3'

IRF-1 (2)

5' – ATA AGT CAA TTT CAT TTT CGC TTC C – 3'

Figure 2.2 Reverse complementary oligonucleotide primers for SIE STAT (*Panel A*) and IRF-1 (*Panel B*)

added to the mixture. After a further 20 min incubation at room temperature, the binding reaction was stopped by addition of 3.5 µl 6X DNA loading buffer. Reaction mixtures were analysed by electrophoresis on 6%,8 % or 10% polyacrylamide in 5X reaction buffer at 180 V for 2 hrs 30 min. Gels were then dried, and bands corresponding to complexes between transcription factors and the labelled probe were detected by autoradiography.

2.2.2.9 Urine analysis by gas chromatography-mass spectrometry (GC/MS)

Urine was collected in metabolic cages over a 24 hour period from control and neutropenic Balb/c mice (day 2½ to day 3½). Four mice were kept in each cage (three cages in total) at a constant temperature (20–22 °C), with free access to water and standard chow, and urine was collected at 12 hour intervals.

Following sample collection, GC/MS was used to detect and quantify the presence of the PGI_2 metabolite 2,3-dinor-6-keto prostaglandin $F1_\alpha$ (2,3-dinor-6-keto $PGF1_\alpha$) (this technique was kindly carried out by Dr Jason Morrow and Dr Stephanie Sanchez of Vanderbilt Medical Centre, Nashville, TN, USA) (Morrow & Minton, 1993).

2.2.3 Neutrophil depletion study

2.2.3.5 Neutrophil depletion

All animal experiments were performed in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act, 1986 (PPL 30/1934 & PPL

30/2252). 8–10 week old, male wild-type (WT) Balb/c mice, IFN-$\gamma^{-/-}$ Balb/c mice (Dalton *et al.* 1993), WT C57Bl/6 mice (Charles River, UK), and iNOS$^{-/-}$ C57Bl/6 mice were kept in constant temperature cages (20–22 °C) and given free access to water and standard chow (Afanasyeva *et al.* 2004; Chauhan *et al.* 2003; Dalton *et al.* 1993). iNOS$^{-/-}$ C57Bl/6 mice were a kind gift from Dr. A. J. Hobbs, Wolfson Institute for Biomedical Research, University College London, Gower Street, London. All experiments, except for iNOS knockouts and their controls were performed using male Balb/c mice.

Neutrophils were depleted using the antibody RB6-8C5, a rat anti-mouse IgG2b monoclonal antibody directed against Ly-6G, previously known as Gr-1, an antigen on the surface of mouse neutrophils (Ismail *et al.* 2003). Alternatively, mice received an equal dose of a control IgG antibody, GL113. The antibody was prepared for injection by dilution with sterile PBS under aseptic conditions.

To establish the optimum doses of RB6-8C5, mice were administered with varying amounts of the antibody by intraperitoneal (i.p.) injection and sacrificed at intervals. Mice were killed using schedule 1 methods and exanguinated by cardiac puncture using a heparinised syringe. RBCs were lysed by hypotonic lysis (1:10 blood:lysis buffer (1X RBC Lysis Buffer, eBiosciences, California, CA, USA)) for 4 min on ice, and isotonisity was restored by adding 20 ml PBS. The solution was then centrifuged at 120 g for 10 min at 4 °C and the supernatant discarded. The final pellet was resuspended in an appropriate volume of PBS, and the total white blood cell (WBC) concentration determined by haemocytometer counting and tryphan blue exclusion.

The cell concentration was adjusted to a maximum of 10^6 cells.ml^{-1} by adding PBS. 100 µl of the WBC solution (i.e. 10^5 cells) was cytospun onto glass slides at 100 g for 10 min (Shandon Cytospin® 4 Cytocentrifuge, Thermo Electron Corporation, Pittsburgh, PA, USA) and allowed to dry overnight. The next day, the slides were fixed with methanol and stained with eosin and azure solutions (Quick III stainkit™, Astra Diagnostics, New Jersey). When viewed under a microscope, neutrophils could be easily distinguished by their multi-lobed nuclei, monocytes by their large size and granular cytoplasm, and lymphocytes by their large, dense nuclei and clear cytoplasm. Numbers of monocytes, lymphocytes and neutrophils were then calculated by differential cell counting.

2.2.3.2 Blood pressure measurement

Systolic BP was measured in mice daily at the same time of day – for 3–5 days before neutrophil depletion (training) and for 8 days after depletion – by 'tail-cuff plethysmography' (Duo 18, World Precision Instruments, Florida). All BP measurements were carried out at 24–26 °C (Figure 2.3).

2.2.3.3 Infusion with Ang II

Control or neutrophil-depleted mice were anesthetised via inhalation of 2% isoflurane (98% oxygen). Osmotic minipumps (Alzet model 1002; Charles River UK Ltd, Kent, UK) containing Ang II (infusion rate 1.1 mg.kg^{-1}.day^{-1}) were implanted subcutaneously in the midscapular area. As a control, some animals underwent a 'sham' operation, in which no minipump was inserted. Systolic BP was monitored for

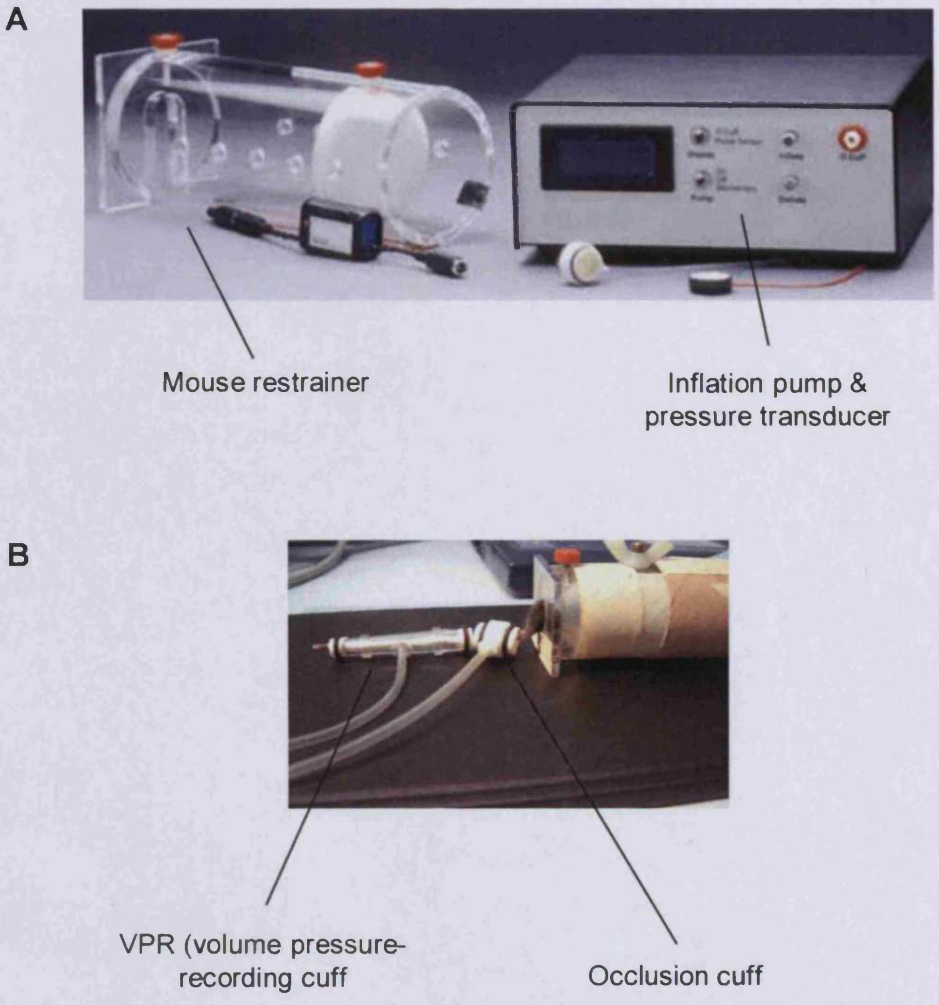

Figure 2.3. Non-invasive measurement of mouse blood pressure by tail-cuff plethysmography

at least 2 days before implantation (training) and 7 days after implantation via tail cuff plethysmography in conscious mice.

2.2.3.4 Assessment of Heart:Body Weight ratio

Following measurement of systolic BP on day 8, mice were sacrificed by asphyxiation and heart and body weights were recorded. From these values the heart:body weight ratio was calculated and used as a measure of cardiac hypertrophy. In addition, the descending thoracic aorta was removed, cleaned of adipose tissue, and fixed in paraffin wax.

2.2.3.5 Isometric tension functional studies

Isometric tension functional studies were carried out according to the method of Anning and colleagues (2005). Briefly, control or neutrophil-depleted mice were sacrificed by asphyxiation. The thoracic aorta was removed and placed in Krebs-Henseleit buffer. The aorta was dissected of adipose tissue, cut into rings (2–3 mm) and suspended on an isometric tension myograph (model 610; DMT, Aarhus, Denmark) containing Krebs buffer at 37°C and gassed at 5% CO_2/95% O_2. A resting tension of 3 mN was maintained, and changes in isometric tension were recorded via Myodaq software (DMT).

After a 60 min equilibration period, vessels were primed with 48 mM KCl before a concentration (0.1 μM) of PE producing ~75% contraction was added. Once responses stabilised, 1 μM ACh was added to assess endothelial integrity. Any rings

that did not maintain contraction to PE, or relaxed less than 50% of the PE-induced tone after addition of ACh (1 μM) were discarded. No differences in the number of rings discarded between wild-type and neutrophil-depleted vessels were observed.

Rings were washed for 30 min, after which a cumulative concentration-response curve to PE was constructed (1 nM–0.1 mM) to assess vasoconstrictor activity. Tissues were then washed for 60 min to restore basal tone. In order to assess endothelium-dependent relaxation, tissue were contracted to approximately 80% of the 0.1 mM PE-induced response; once a stable response to PE was observed, cumulative concentration response curves were constructed to ACh (1 nM–10 μM). After a 30 min wash, endothelium-independent relaxation was evaluation by pre-contracting aortic rings with 0.1 mM PE, before constructing concentration response curves to diethylamine NONOate (DEA-NONOate) (Cayman, 0.1 nM–0.1 mM). Responses were expressed as a percentage of either baseline tension (vasoconstriction) or contracted tension (vasodilation). Responses from patent rings of each animal (3–4 rings) were combined to produce an average value (*n*).

Experiments were repeated in the presence of various pharmacological inhibitors (e.g. L-nitroarginine-methyl ester (L-NAME) (300 μM, 15 min), 1400W (10 μM, 30 min), indomethacin (10 μM, 30 min), celecoxib (1 μM, 15 min) and SC-560 (1 μM or 100 nM, 30 min), with constriction and dilation curves being constructed as described above. PE, ACh, DEA-NONOate, L-NAME and 1400W solutions were prepared by dissolving in Krebs-Henseleit buffer; indomethacin, celecoxib and SC-560 solutions were prepared by dissolving in ethanol. Vehicle controls were carried out to confirm that ethanol did not cause not specific changes in vessel tone (Figure 2.4).

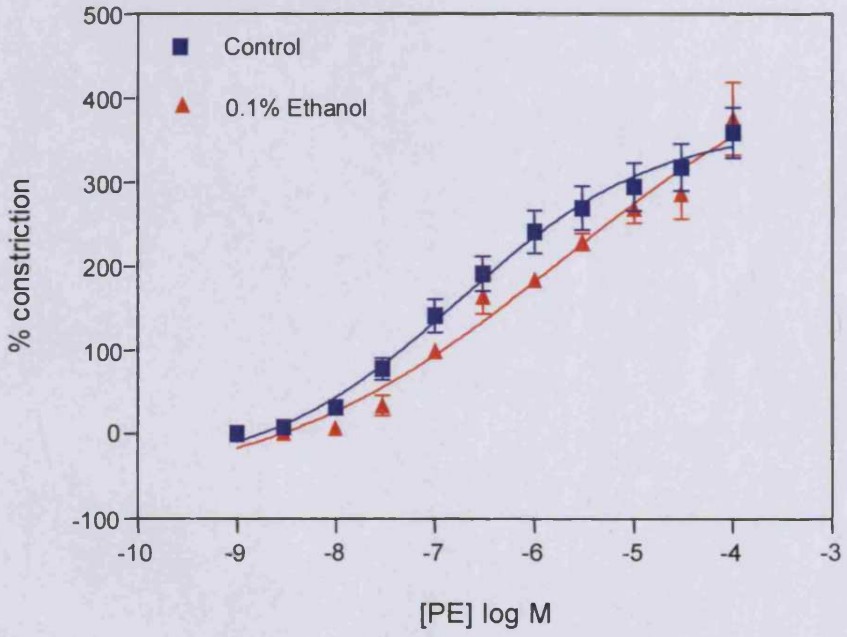

Figure 2.4 Ethanol does not affect vasoconstriction *in vitro*
PE-induced constriction responses in thoracic aortae from wild-type mice in the presence or absence of 0.1% ethanol (n ≥ 2, mean ± SEM). Aortic ring functional responses were determined as described in section 2.2.3.5 of *Materials and Methods*..

2.2.3.6 Inhibition of complement *in vivo*

BPs were measured in wild-type Balb/c mice administered RB6-8C5 with or without co-administration of the anti-complement C5 antibody BB5.1 (1 mg i.p, day 0 and day 2). BB5.1 was also administered alone as a control.

2.2.4 Cell culture

2.2.4.1 RB6-8C5 and GL113 cells

The RB6-8C5 and GL113 rat hybridomas were a kind gift from Dr Awen Gallimore, Department of Medical Biochemistry & Immunology, School of Medicine, Cardiff University. The RB6-8C5 cell line, originally established by Dr Robert Coffman (DNAX Research Institute of Molecular & Cell Biology, Palo Alto, CA, USA), secretes an IgG2b monoclonal antibody that specifically binds to and depletes mature mouse granulocytes (Chen *et al.* 2000). The GL113 antibody, anti-*Escherichia coli* β-galactosidase, is commonly used as a control IgG antibody in immunodepletion studies (Loughry *et al.* 2005; Suzuki *et al.* 1994).

Invitrogen Ltd (Paisley, Scotland, UK) supplied all cell culture reagents unless otherwise stated. Tissue culture hoods were treated with a solution of 70% (v/v) ethanol before use and regularly cleaned with biocidal ZF™ (Wak-Chemie Medical) or fumigated with vaporised-formaldehyde for several hours.

Frozen cells were thawed slowly at room temperature before being transferred into a falcon tube. Cells were washed twice with RPMI supplemented with 2% foetal calf serum (FCS), 2 mM L-glutamine, 50 $U.ml^{-1}$ penicillin G and 50 $\mu g.ml^{-1}$ streptomycin to remove any residual dimethyl suphoxide (DMSO), and then centrifuged for at 250 g for 5 min at 4 °C. Cells were resuspended in 5 ml of RPMI supplemented with 10% FCS, 1% minimum essential medium (MEM) non-essential amino acids (100X), 2 mM L-glutamine, 1 mM sodium pyruvate, 50 $U.ml^{-1}$ penicillin G and 50 $\mu g.ml^{-1}$ streptomycin and transferred into a T80 medium tissue culture flask. The volume was then made up to 20 ml.

Once cells had reached numbers of 2 x 10^7 $cells.ml^{-1}$, were centrifuged and re-suspended in RPMI supplemented with 10% low IgG FCS, 1% MEM non-essential amino acids (100X), 2 mM L-glutamine, 1 mM sodium pyruvate, 50 $U.ml^{-1}$ penicillin G and 50 $\mu g.ml^{-1}$ streptomycin ('complete media') and transferred to the cell compartment of an Integra CL1000 flask (Scientific Laboratory Supplies (SLS), Nottingham, UK). The nutrient compartment of the integra flask was filled with RPMI supplemented with 1% MEM non-essential amino acids (100X), 2 mM L-glutamine, 1 mM sodium pyruvate, 50 $U.ml^{-1}$ penicillin G and 50 $\mu g.ml^{-1}$ streptomycin ('nutrient media').

2.2.4.2 Purification of endotoxin free antibody

Twice-weekly, half of the liquid in the cell compartment of the integra flask was removed and replaced with fresh complete media. This cell solution was then centrifuged at 250 g for 5 min at 4 °C and the supernatant collected and stored at

–20°C. Once approximately 100 ml of supernatant had been collected, any cell debris was removed by centrifugation at 1000 g for 30 min at 4 °C and the supernatant passed through a 0.2 μm filter unit. The antibody was precipitated out of solution by incubating the supernatant overnight at 4 °C in an equal volume of 5 mM ammonium sulphate solution. The precipitated protein was then separated by centrifugation at 1000 g for 30 min at 4 °C and resuspended in 10 ml of endotoxin free water. This precipitation and centrifugation step was repeated and once again the precipitate was resuspended in 10 ml of endotoxin free water.

The antibody solution was concentrated by dialysis. This involved transferring the antibody solution into dialysis tubing (Serva Electrophoresis GmbH, Heidelberg, Germany) and placing it in 2 L of 1 X PBS with stirring for 3 days, replacing the PBS every 24 hours. After this period, the concentrated antibody solution was removed from the dialysis tubing and diluted 1:10 in PBS. Antibody content was assessed by measuring the optical density of the antibody solution at 280 nm, and purity was confirmed by running the solution on an SDS PAGE gel.

CHAPTER 3

CHARACTERISATION OF SUPEROXIDE GENERATION AND NITRIC OXIDE CONSUMPTION BY PRIMED HUMAN NEUTROPHILS

3.1 Introduction

Among the variety of signalling and mediatory roles that it plays, NO is known to regulate leukocyte function. More specifically, NO may restrict recruitment and attachment through suppression of adhesion molecule activity (e.g. CD11/CD18 in neutrophils) (Kubes *et al.* 1991). Neutrophils are by far the most abundant leukocytes (>70%) in the human immune system and play an essential role in infection and innate immunity, providing early defence against invading microorganisms (Parker *et al.* 2005). Thus, it is important that neutrophils are able to overcome the inhibitory effect of NO in order to carry out their activities *in vivo*.

As already mentioned in Section 1.6 of this thesis, neutrophils can utilise a number of potential mechanisms to inactivate NO. For example, neutrophils have long been known to generate and release a variety of oxygen radicals and other exocytosed granule products upon activation by agents such as phorbol 12-myristate 13-acetate (PMA), or chemotactic peptides such as fMLP (Carreras *et al.* 1994; Schmidt *et al.* 1989). In particular, activated neutrophils produce large amounts of $O_2^{\bullet-}$ (Curnette & Babior, 1974), which can rapidly react with NO to form $ONOO^-$, thus decreasing NO

bioavailability (Curnette & Babior, 1974; Nath & Powledge, 1997). Indeed, studies by Clark *et al.* (2002) and McBride & Brown (1997) have shown that fMLP or PMA-stimulated neutrophils can consume NO from aerobic buffer *in vitro*. Furthermore, a combination of SOD and DPI fully inhibited this process, indicating an absolute requirement for NAD(P)H oxidase in NO consumption by acutely activated human neutrophils *in vitro* (McBride & Brown, 1997; Clark *et al.* 2002). It has also been shown that NAD(P)H oxidase-knockout mice show decreased parasite- or hypercholerolaemia-induced leukocyte adhesion and migration, suggesting that leukocyte $O_2^{\bullet-}$ plays an essential role in these processes via the inactivation of NO *in vivo* (Murray & Nathan, 1999; Stokes *et al.* 2001).

Other potential pathways for NO consumption by neutrophils include LOX, MPO, and COX (Abu-Soud & Hazen, 2000; Eiserich *et al.* 2002; O'Donnell *et al.* 1999; O'Donnell *et al.* 2000). A role for these oxidases in neutrophil-mediated NO consumption *in vitro* was investigated by Clark and colleagues (2002). Despite constituting around 5% of neutrophil protein, inhibition of MPO activity with azide and aminotriazole (ATZ) was without effect on rates of NO consumption by activated neutrophils, suggesting that MPO is not responsible for NO consumption by intact human neutrophils (Abu-Soud & Hazen, 2000; Clark *et al.* 2002; Hazen *et al.* 1999). Furthermore, the addition of exogenous purified MPO to MPO-deficient cells did not result in accelerated rates of NO consumption by these cells (Clark *et al.* 2002). In contrast to MPO, COX expression by freshly isolated neutrophils was low, suggesting that this pathway does not consume NO in resting neutrophils (Maloney *et al.* 1998). Finally, while neutrophils constitutively express 5-LOX protein, its activation in response to fMLP was poor (Clark *et al.* 2002). Thus, Clark and colleagues concluded

that scavenging of NO by NAD(P)H oxidase-derived $O_2^{\bullet-}$ is the primary mechanism of NO consumption by neutrophils *in vitro*.

During inflammation *in vivo*, neutrophils are exposed to a series of inflammatory cytokines, such as TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF) and LPS, that can cause 'priming' (Vosbeck *et al.* 1990; Weisbart *et al.* 1987; Wewers *et al.* 1990). Priming can be defined as the potentiation of neutrophil responses to biological activating agents, such as fMLP or the complement component C5a, following prior exposure to a 'priming agent' (Condliffe *et al.* 1998; Vercellotti *et al.* 1988). For example, enhancement of the neutrophil respiratory burst in response to fMLP stimulation may be increased up to 20-fold by prior exposure of cells to a priming agent (Guthrie *et al.* 1984). In addition, neutrophil priming has been shown to augment leukocyte adhesion molecule expression and function, thereby promoting leukocyte recruitment to an inflamed focus, and to enhance agonist-induced degranulation (e.g. MPO and elastase release) and the generation of lipid mediators (e.g. AA, LTB_4 and PAF) (Condliffe *et al.* 1998; Doerfler *et al.* 1989; Doerfler *et al.* 1994; Fittschen *et al.* 1988).

A wide variety of substances, both physiological and pharmacological, have now been shown to act as priming agents (see Table 3.1). A key concept of priming is that these agents do not elicit the effector function(s) on their own and must be presented to the cell for a variable period before the cell is exposed to the activating stimulus. The mechanism underlying the priming of neutrophil respiratory burst activity is still the subject of great speculation. Indeed, the diversity of agents, and the differing preincubation times required to initiate maximal priming, raises the possibility that

there may be class- or even agent-specific variations in the signal transduction routes that lead to neutrophil priming. Furthermore, the variable potency displayed by different agents for priming also suggests that this process is not an all-or-nothing response. As a consequence of this uncertainty, a range of potential mechanisms for neutrophil priming has been proposed (for comprehensive review see Condliffe *et al.* 1998).

Priming agent	Time required to induce maximal priming
ATP	15 s
Substance P	1 min
Ionomycin	2 min
Inositol hexakisphosphate	2 min
PAF	5 min
TNF-α	10 min
IL-8	10 min
Orthovanadate	10 min
Influenza A virus	30 min
LPS	120 min
GM-CSF	120 min
Interferon-γ	120 min

Table 3.1 Variability of neutrophil priming agents

Adapted from Condliffe *et al.* 1998.

The physiological consequences of neutrophil priming have yet to be fully investigated, although this process has been shown to be critical for the induction of endothelial injury, both *in vitro* and *in vivo*. Indeed, Smedley *et al.* (1986) found that neutrophils stimulated with activated agents alone produced minimal damage to

endothelial cell monolayers, whereas prior incubation of cells with low concentrations of LPS, followed by stimulation with fMLP or C5a, resulted in extensive endothelial cell injury. Similarly, in a rabbit model, intravascular administration of LPS and fMLP together greatly enhanced neutrophil vascular sequestration within the lung, leading to lung damage; an effect not seen when either substance was used alone (Condliffe *et al.* 1998). Furthermore, Murohara and colleagues (1994) showed that PAF receptor antagonist, WEB-2170, caused marked attenuation of neutrophil-induced vasocontraction and endothelial dysfunction in isolated cat coronary artery rings *in vitro*. Thus, priming appears to be important in regulating the functional responsiveness of the neutrophils at an inflamed site, and may implicate neutrophils in the endothelial dysfunction commonly observed in the pathogenesis of conditions such as inflammatory vascular disease.

While the mechanisms responsible for the above observations have yet to be confirmed, the accelerated inactivation of NO by primed neutrophils is likely to play some role, particularly given that the enhancement of respiratory burst activity is viewed as the 'gold standard' priming response (Condliffe *et al.* 1998). However, as yet, the impact of neutrophil priming on NO bioavailability *in vitro* has not been fully evaluated, nor has the relative contribution of NO consuming pathways other than NAD(P)H oxidase. For example, in addition to enhancing respiratory burst activity, neutrophil priming has also been shown to promote the release of both MPO and AA into the extracellular space via degranulation (Daniels *et al.* 1992; Doerfler *et al.* 1994; Fittschen *et al.* 1988; Surette *et al.* 1998). As already described in section 1.5.1.1 of this thesis, MPO is a haem peroxidase enzyme capable of binding NO, and thus may serve as a potential mechanism for NO inactivation by primed neutrophils.

Similarly, the enhanced release of AA by these cells may facilitate LOX or COX activity within the neutrophil, both of which are capable of catalytically consuming NO. Indeed, the upregulation of LTB_4 release by primed neutrophils is indicative of enhanced 5-LOX activity in these cells (Bauldry *et al.* 1991; Doerfler *et al.* 1989; Surette *et al.* 1998; Zarini *et al.* 2006). Thus, a firmer appreciation of the capacity of primed neutrophils to consume NO *in vitro*, and the mechanism by which they may do so, is required if we are to elucidate a role for these cells in the complex pathogenesis of inflammatory vascular disease *in vivo*.

3.1.1 Aims

To date, the effect of priming on the consumption of NO by neutrophils is not known. Therefore the aim of this chapter was to investigate the potentiation of neutrophil activity by various 'priming agents' *in vitro* with regard to NO consumption, with particular interest given to the potential implications of neutrophil priming *in vivo*. This involved:

- The measurement of $O_2^{\bullet-}$ generation by unprimed and primed human neutrophils.

- The measurement of NO consumption by unprimed and primed human neutrophils.

- Examining the relationship between $O_2^{\bullet-}$ generation and NO consumption in unprimed and primed human neutrophils, and determining what proportion of this consumption was dependent on NAD(P)H oxidase-derived $O_2^{\bullet-}$ and whether other pathways also known to consume NO (e.g. MPO, COX, LOX) are involved.

3.2 Results

3.2.1 Characterisation of $O_2^{\bullet-}$ generation by unprimed and primed human neutrophils

$O_2^{\bullet-}$ generation by activated human neutrophils was measured by CuZn-SOD-inhibitable cyt *c* reduction. Resting, healthy human neutrophils do not reduce cyt *c*. Neutrophil activation with fMLP (1 μM) caused immediate and rapid reduction of cyt *c* that lasted for several minutes and this was completely inhibited by 300 $U.ml^{-1}$ CuZn-SOD, indicating that neutrophils were producing $O_2^{\bullet-}$ (Figure 3.1A). Similar results were obtained using PMA, although the duration and magnitude of activation was greater (Figure 3.1B).

Rates of fMLP-stimulated $O_2^{\bullet-}$ production were enhanced in neutrophils primed with 10 $ng.ml^{-1}$ TNF-α or 10 nM IL-8 for 10 min compared to cells exposed to fMLP alone (Figure 3.2 and Figure 3.3). Similarly, fMLP-stimulated $O_2^{\bullet-}$ production was significantly accelerated in cells primed with 95 nM PAF for 5 min compared to fMLP-stimulated $O_2^{\bullet-}$ production by unprimed cells (Figure 3.4). Quantification of $O_2^{\bullet-}$ generation rates in primed and unprimed cells showed an mean increase of 242.80 ± 72.21% in TNF-α-primed cells, 271.00 ± 71.45% in IL-8 primed cells and 212.00 ± 47.94% in PAF-primed cells (mean ± SEM, $n \geq 3$, Figure 3.5). These data are consistent with primed neutrophils having enhanced respiratory burst in response to stimulation by fMLP (Guthrie *et al.* 1984).

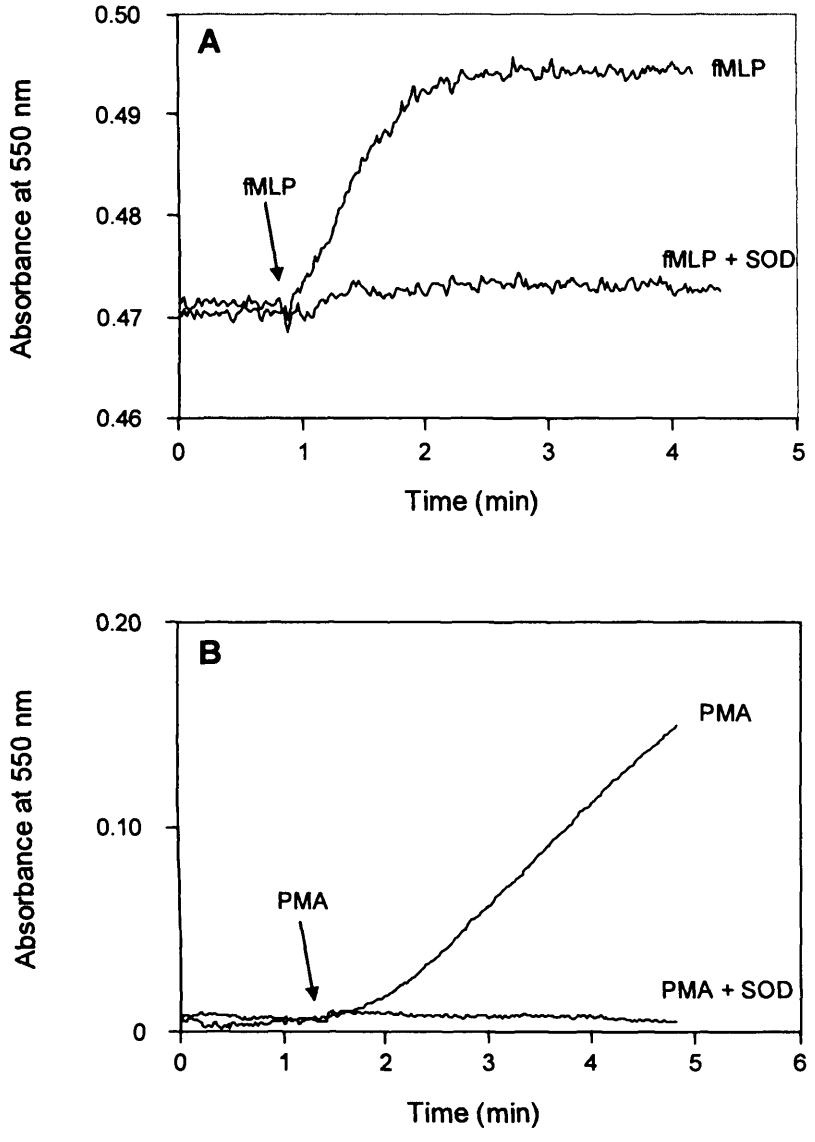

Figure 3.1 Activated human neutrophils reduce cyt *c*, consistent with $O_2^{\cdot-}$ generation

$O_2^{\cdot-}$ production by activated human neutrophils was measured by the CuZn-SOD-inhibitable reduction of cyt *c* at 550 nm. *Panel A.* Activation of human neutrophils by addition of 1 μM fMLP, as indicated by arrow, to 2 ml Krebs-Henseleit buffer containing cyt *c* (50 μM) and human neutrophils (1 x 10^6 cell.ml^{-1}) at 37 °C with stirring, in the presence or absence of 300 U.ml^{-1} CuZn-SOD. *Panel B.* Activation of human neutrophils by addition of 1 μg.ml^{-1} PMA, as indicated by arrow, to 2 ml Krebs-Henseleit buffer containing cyt *c* (50 μM) and human neutrophils (1 x 10^6 cell.ml^{-1}) at 37°C with stirring, in the presence or absence of 300 U.ml^{-1} CuZn-SOD. Results shown are of a representative experiment from a single donor.

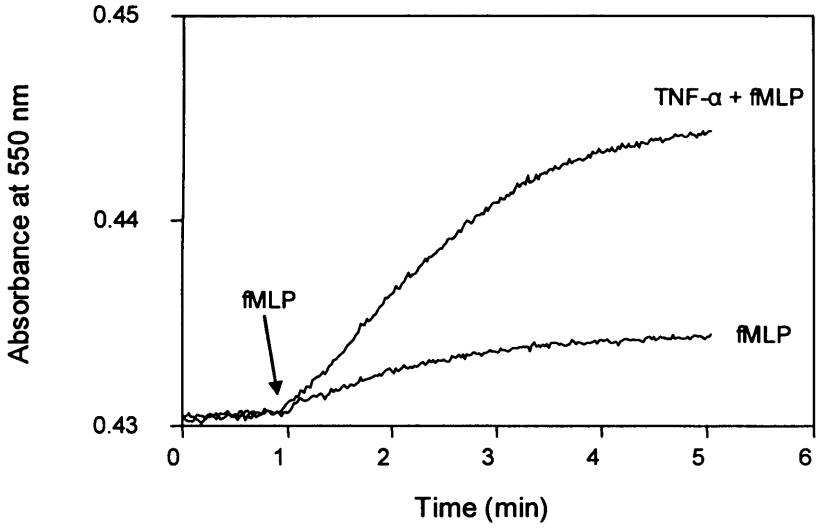

Figure 3.2 Neutrophils primed with TNF-α produce $O_2^{\cdot-}$ at a greater rate than control cells when activated

Activation of human neutrophils by addition of 1 μM fMLP, as indicated by arrow, to control cells and cells primed with 10 ng.ml-1 TNF-α for 10 min. fMLP (1μM) was added to 2 ml Krebs-Henseleit buffer containing cyt *c* (50 μM) and human neutrophils (3.1 x 10^4 cell.ml^{-1}) at 37 °C with stirring. $O_2^{\cdot-}$ production was measured by the CuZn-SOD-inhibitable reduction of cyt *c* at 550 nm.

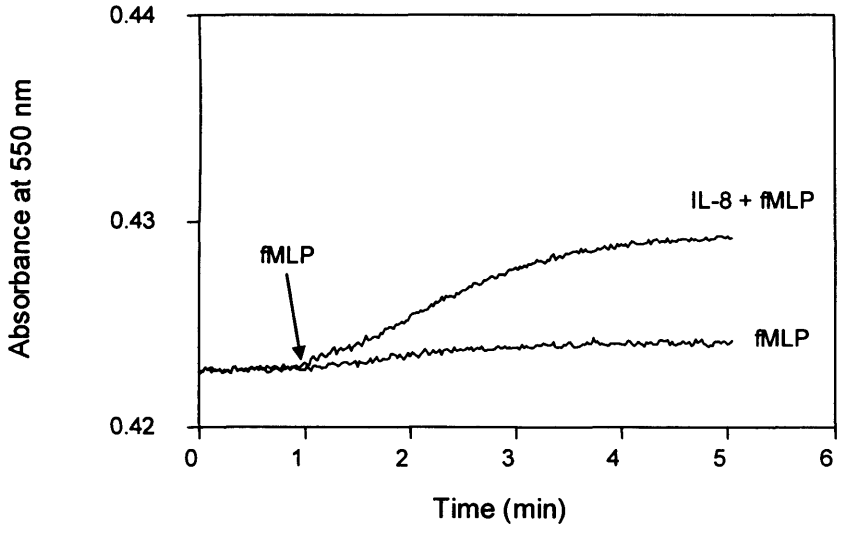

Figure 3.3 Neutrophils primed with IL-8 produce $O_2^{\bullet-}$ at a greater rate than control cells when activated

Activation of human neutrophils by addition of 1 μM fMLP, as indicated by arrow, to control cells and cells primed with 10 nM IL-8 for 10 min. fMLP (1μM) was added to 2 ml Krebs-Henseleit buffer containing cyt *c* (50 μM) and human neutrophils (3.1 x 10^4 cell.ml^{-1}) at 37 °C with stirring. $O_2^{\bullet-}$ production was measured by the CuZn-SOD-inhibitable reduction of cyt *c* at 550 nm.

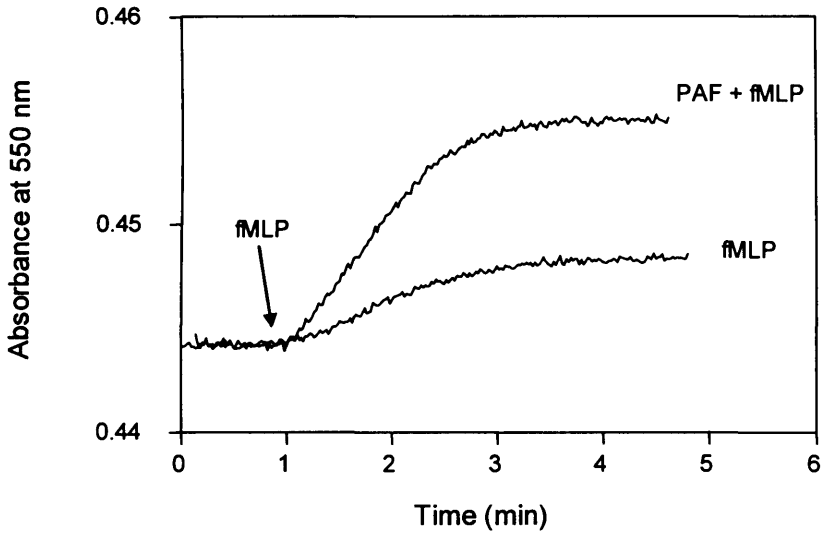

Figure 3.4 Neutrophils primed with PAF produce $O_2^{\bullet-}$ at a greater rate than control cells when activated

Activation of human neutrophils by addition of 1 μM fMLP, as indicated by arrow, to control cells and cells primed with 95 nM PAF for 5 min. fMLP (1μM) was added to 2 ml Krebs-Henseleit buffer containing cyt *c* (50 μM) and human neutrophils (3.1 x 10^4 cell.ml^{-1}) at 37 °C with stirring. $O_2^{\bullet-}$ production was measured by the CuZn-SOD-inhibitable reduction of cyt *c* at 550 nm.

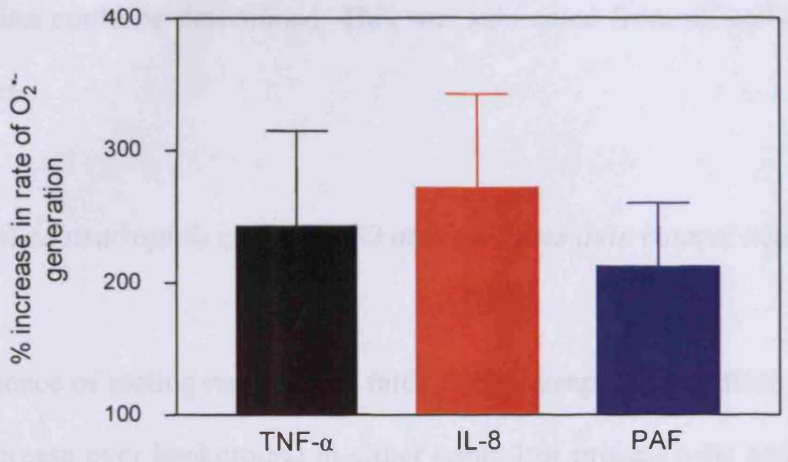

Figure 3.5 Neutrophils primed with 10 ng.ml^{-1} TNF-α, 10 nM IL-8 or 95 nM PAF generate $O_2^{\bullet-}$ at faster rates than unprimed cells

$O_2^{\bullet-}$ generation was measured as described in Figure 3.2. Results shown represent the mean increase in rates of $O_2^{\bullet-}$ generation by fMLP-activated cells from at least 3 donors, following incubation with either 10 ng.ml^{-1} TNF-α for 10 mins, 10 nM IL-8 for 10 mins or 95 nM PAF for 5 mins (Rates in unprimed cells = 100%; $n \geq 3$, mean ± SEM).

3.2.2 *Characterisation of NO loss in the electrode system*

NO (1.9 μM) decay in aerobic buffer at 37 °C in the electrode system followed first order kinetics with k_{obs} of 5.5 ± 0.5 x 10^{-3} s^{-1} (mean ± SD, n = 3) (Figure 3.6A (i)). While NO decay through reaction with O_2 is second order, NO decay in this system was primarily through diffusion into the gas phase and oxidation by the electrode (Clark *et al.* 2002). Using this k_{obs}, rates of background NO disappearance at any concentration could be determined. This was subtracted from all cell-dependent NO uptake rates.

3.2.3 *Primed neutrophils consume NO at faster rates than control neutrophils*

In the presence of resting neutrophils, rates of NO disappearance from aerobic buffer did not increase over background in either control or primed cells and still followed first order kinetics (e.g. for one representative donor, k_{obs} for NO decay = 5.4 ± 0.3 x 10^{-3} s^{-1} in the presence of resting unprimed cells; k_{obs} = 5.6 ± 0.8 x 10^{-3} s^{-1} in the presence of resting primed cells, mean ± SD) (Figure 3.6A (ii), B (ii)).

Initially a concentration of 0.5 x 10^6 cells.ml^{-1} were used in both $O_2^{\bullet-}$ generation and NO consumption assays, consistent with the method used by Clark *et al.* (2002). At this concentration, $O_2^{\bullet-}$ CuZn-SOD-inhibitable reduction of cytochrome C at 550 nm, and NO consumption was easily quantified to give an accurate representation of neutrophil respiratory burst activity (data not shown). However, upon priming and activation with fMLP, NO removal from the NO electrode chamber was so rapid that

neutrophil-mediated NO consumption could not be accurately determined. Therefore, the concentration of neutrophils used in subsequent $O_2^{\bullet-}$ generation and NO consumption assays was lowered until a suitable concentration of 3.1 x 10^4 cells.ml^{-1} was found. This gave easily measurable rates in both assays.

Upon activation with fMLP, neutrophils primed with 10 ng.ml^{-1} TNF-α or 10 nM IL-8 for 10 min showed accelerated NO consumption compared with unprimed cells (Figures 3.6 and 3.7). Similarly, priming of neutrophils with 95 nM PAF for 5 min caused an increase in the fMLP-stimulated rate of NO consumption compared to unprimed cells (Figures 3.8). Quantification of NO consumption rates in primed and unprimed cells showed an mean increase of 298.00 ± 63.56% in TNF-α-primed cells, 275.20 ± 18.81% in IL-8 primed cells and 237.90 ± 63.99% in PAF-primed cells (mean ± SEM, n ≥ 3, Figure 3.9). These data indicate that, upon stimulation with fMLP, primed human neutrophils consume NO from aerobic buffer at far greater rates than unprimed cells. This is consistent with the increase in $O_2^{\bullet-}$ generation observed in earlier experiments with primed and unprimed human neutrophils.

3.2.4 The rate of NO consumption by both unprimed and primed neutrophils is greater than the rate of $O_2^{\bullet-}$ production

By comparing rates of $O_2^{\bullet-}$ generation and NO consumption, it was observed that consumption of NO by both unprimed and primed neutrophils activated with fMLP

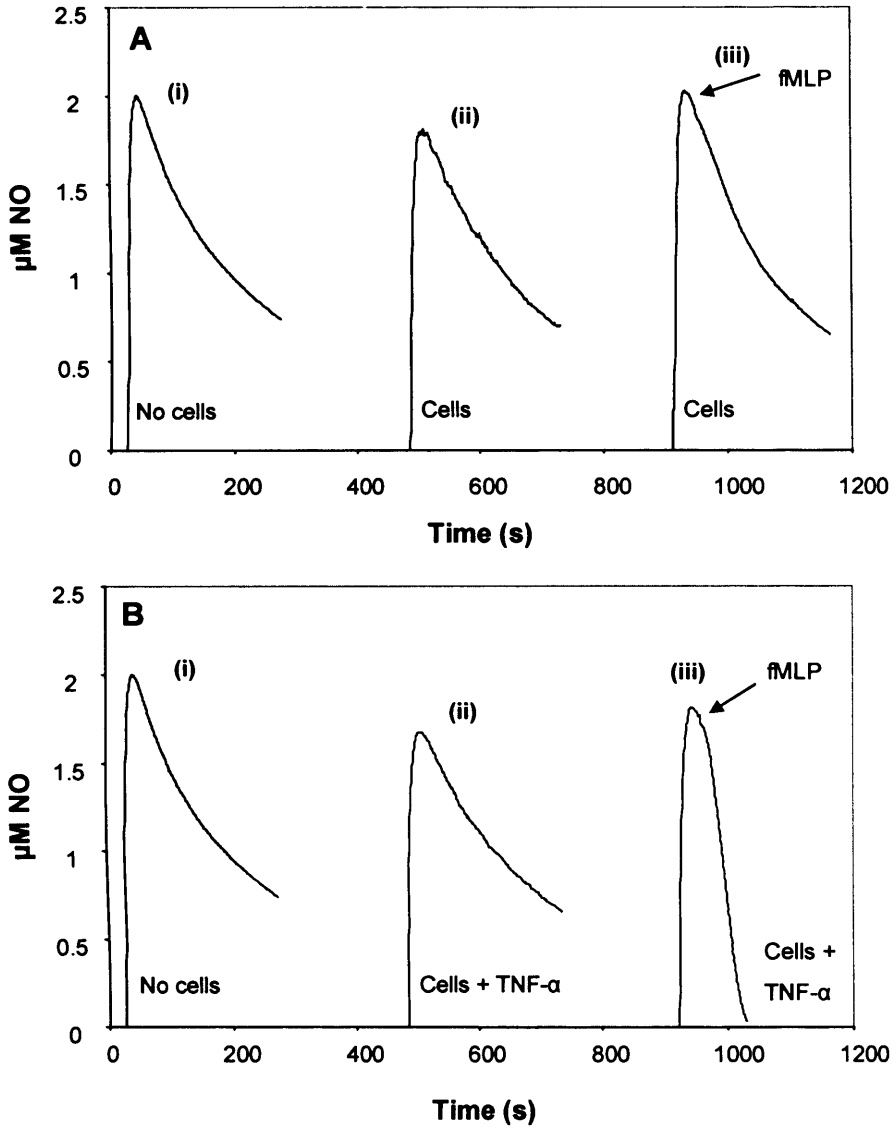

Figure 3.6 Control neutrophils and neutrophils primed with 10 ng.ml^{-1} TNF-α consume NO upon activation with fMLP

NO (1.9 µM) was added to 0.5 ml Krebs-Henseleit buffer containing human neutrophils (3.1 x 10^4 cells.ml^{-1}) in the chamber of the NO electrode at 37 °C with stirring. *Panel A. (i)* backgound NO loss, *(ii)* resting unprimed neutrophils, *(iii)* unprimed neutrophils activated with fMLP (1 µM), as indicated by arrow. *Panel B. (i)* background NO loss, *(ii)* resting neutrophils primed with 10 ng.ml^{-1} TNF-α for 10 min, *(iii)* primed neutrophils (10 ng.ml^{-1} TNF-α) activated with fMLP, as indicated by arrow. Results shown are of a representative experiment from a single donor for each panel.

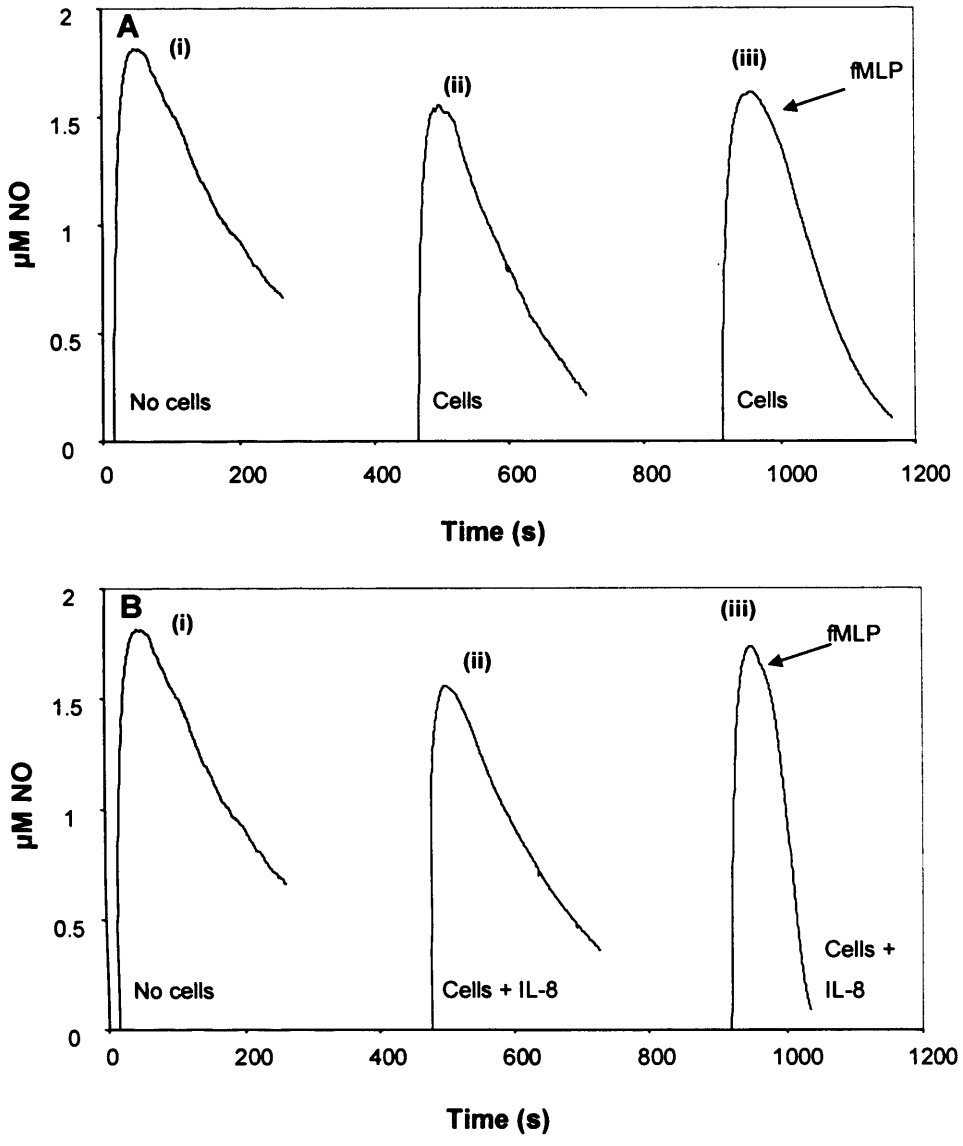

Figure 3.7 Control neutrophils and neutrophils primed with 10 nM IL-8 consume NO upon activation with fMLP

NO (1.9 µM) was added to 0.5 ml Krebs-Henseleit buffer containing human neutrophils (3.1 x 10^4 cells.ml^{-1}) in the chamber of the NO electrode at 37 °C with stirring. *Panel A. (i)* backgound NO loss, *(ii)* resting unprimed neutrophils, *(iii)* unprimed neutrophils activated with fMLP (1 µM), as indicated by arrow. *Panel B. (i)* background NO loss, *(ii)* resting neutrophils primed with 10 nM IL-8 for 10 min, *(iii)* primed neutrophils (10 mM IL-8) activated with fMLP, as indicated by arrow. Results shown are of a representative experiment from a single donor for each panel.

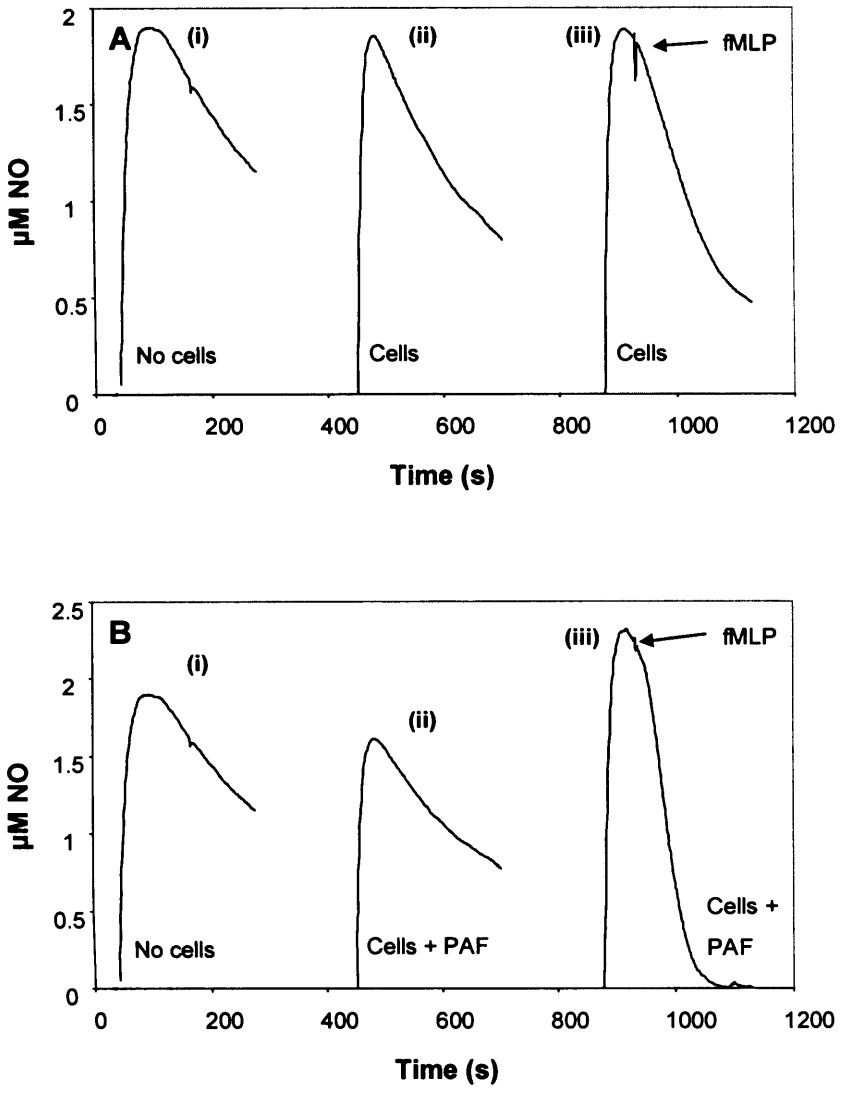

Figure 3.8 Control neutrophils and neutrophils primed with 95 nM PAF consume NO upon activation with fMLP

NO (1.9 µM) was added to 0.5 ml Krebs-Henseleit buffer containing human neutrophils (3.1 x 10^4 cells.ml^{-1}) in the chamber of the NO electrode at 37 °C with stirring. *Panel A. (i)* backgound NO loss, *(ii)* resting unprimed neutrophils, *(iii)* unprimed neutrophils activated with fMLP (1 µM), as indicated by arrow. *Panel B. (i)* background NO loss, *(ii)* resting neutrophils primed with 95 nM PAF for 5 min, *(iii)* primed neutrophils (95 nM PAF) activated with fMLP, as indicated by arrow. Results shown are of a representative experiment from a single donor for each panel.

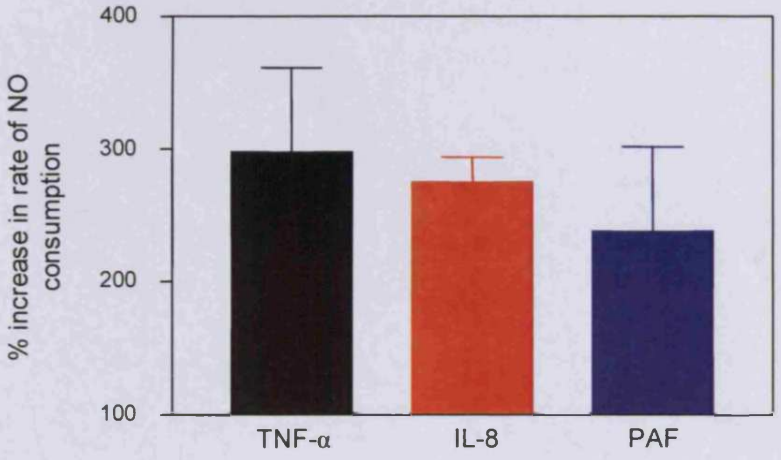

Figure 3.9 Neutrophils primed with 10 ng.ml^{-1} TNF-α, 10 nM IL-8 or 95 nM PAF consume NO at faster rates than unprimed cells

NO was measured as described in Figure 3.6. Results shown represent the mean increase in rates of NO consumption by fMLP-activated cells from at least 3 donors, following incubation with either 10 ng.ml^{-1} TNF-α for 10 mins, 10 nM IL-8 for 10 mins or 95 nM PAF for 5 mins (Rates in unprimed cells = 100%; $n \geq 3$, mean ± SEM).

was consistently faster than $O_2^{\bullet-}$ generation. For example, for a representative donor, there was a 2.07-fold difference between mean rates of $O_2^{\bullet-}$ generation and NO disappearance in the presence of unprimed cells, compared to a 2.65-fold difference between these rates in the presence of TNF-α-primed cells (Figure 3.10). Similar results were seen in experiments with IL-8; for a representative donor, there was a 1.81-fold difference between mean rates of $O_2^{\bullet-}$ generation and NO consumption in the presence of unprimed cells, compared to a 2.03-fold difference in the presence of IL-8 primed cells (Figure 3.11). Finally, for a reprensentative donor, there was a 2.25-fold difference between mean rates of $O_2^{\bullet-}$ generation and NO uptake in the presence of unprimed cells, compared to a 3.23-fold difference between these rates in the presence of PAF-primed cells (Figure 3.12). This is unexpected from the simple 1:1 termination between NO and $O_2^{\bullet-}$ and suggests a more complex mechanism of NO consumption than first predicted.

3.2.5 *Nitric oxide removal is prevented by blockade of $O_2^{\bullet-}$ generation by NAD(P)H oxidase*

Incubation of unprimed neutrophils with CuZn-SOD (3000 $U.ml^{-1}$) and DPI (10 μM) in combination completely blocked NO consumption by these cells following stimulation with fMLP. Similarly, CuZn-SOD (3000 $U.ml^{-1}$) and DPI (10 μM) also completely inhibited fMLP-stimulated NO consumption by neutrophils primed with TNF-α or IL-8 when applied in combination (Figures 3.13 and 3.14). This indicates that NO consumption by both unprimed and primed human neutrophils is entirely dependent on NAD(P)H oxidase-derived $O_2^{\bullet-}$.

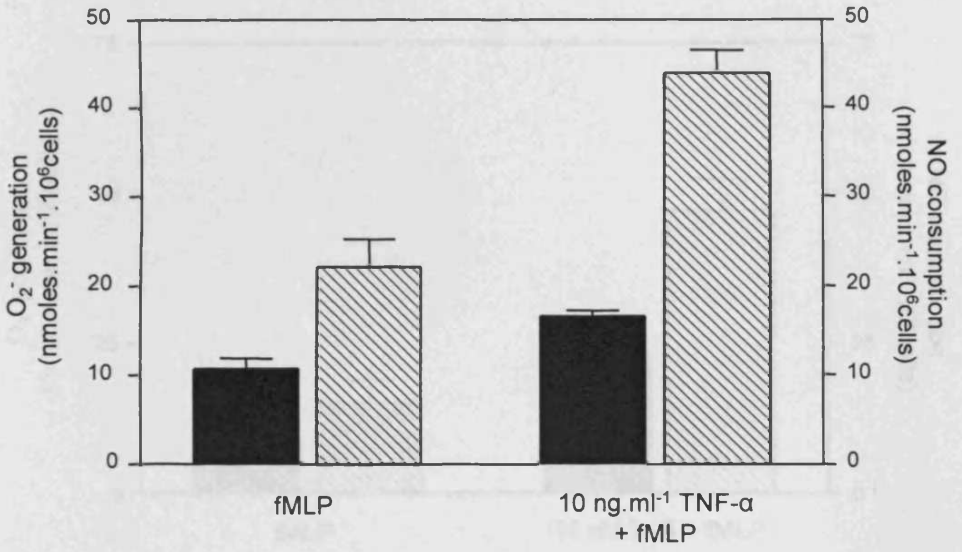

Figure 3.10 NO consumption by both control and TNF-α-primed human neutrophils is faster than $O_2^{\bullet-}$ production

Solid bar. $O_2^{\bullet-}$ production measured by Cu-Zn-SOD inhibitable reduction of cyt *c* at 550 nm. fMLP (1 μM) was added to 2 ml Krebs-Henseleit buffer containing control or TNF-α-primed human neutrophils (3.1 x 10^4 cells.ml^{-1}) and 50 μM cyt *c*, as described in section 2.2.2.1 of *Materials and Methods*. Results shown are of a representative experiment from a single donor.

Hatched bar. NO consumption was measured following fMLP (1 μM) addition to 0.5 ml Krebs-Henseleit buffer containing NO (1.9 μM) and control or TNF-α-primed human neutrophils (3.1 x 10^4 cells.ml^{-1}). Results shown are of a representative experiment from a single donor.

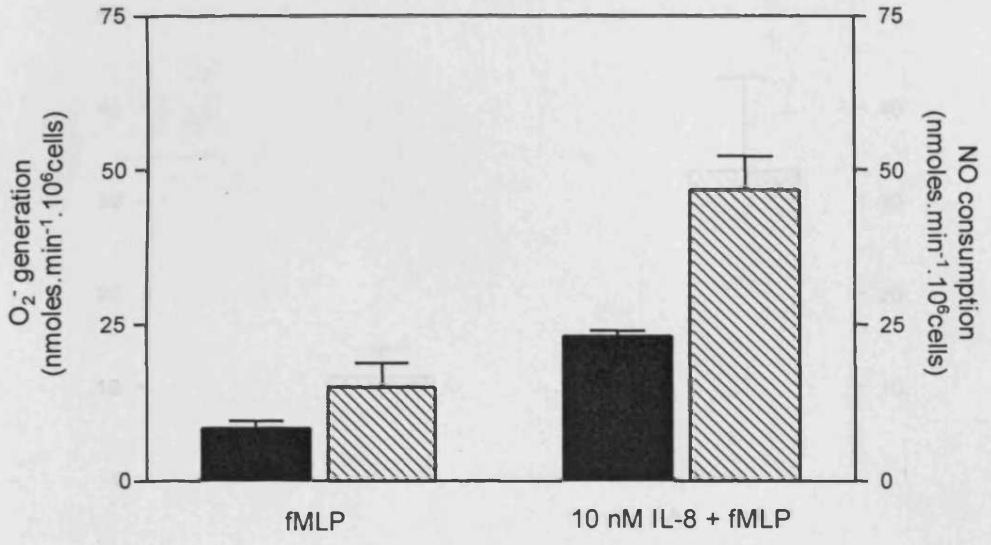

Figure 3.11 NO consumption by both control and IL-8-primed human neutrophils is faster than $O_2^{\cdot-}$ production

Solid bar. $O_2^{\cdot-}$ production measured by Cu-Zn-SOD inhibitable reduction of cyt *c* at 550 nm. fMLP (1 μM) was added to 2 ml Krebs-Henseleit buffer containing control or IL-8-primed human neutrophils (3.1 x 10^4 cells.ml^{-1}) and 50 μM cyt *c*, as described in section 2.2.2.1 of *Materials and Methods*. Results shown are of a representative experiment from a single donor.

Hatched bar. NO consumption was measured following fMLP (1 μM) addition to 0.5 ml Krebs-Henseleit buffer containing NO (1.9 μM) and control or IL-8-primed human neutrophils (3.1 x 10^4 cells.ml^{-1}). Results shown are of a representative experiment for a single donor.

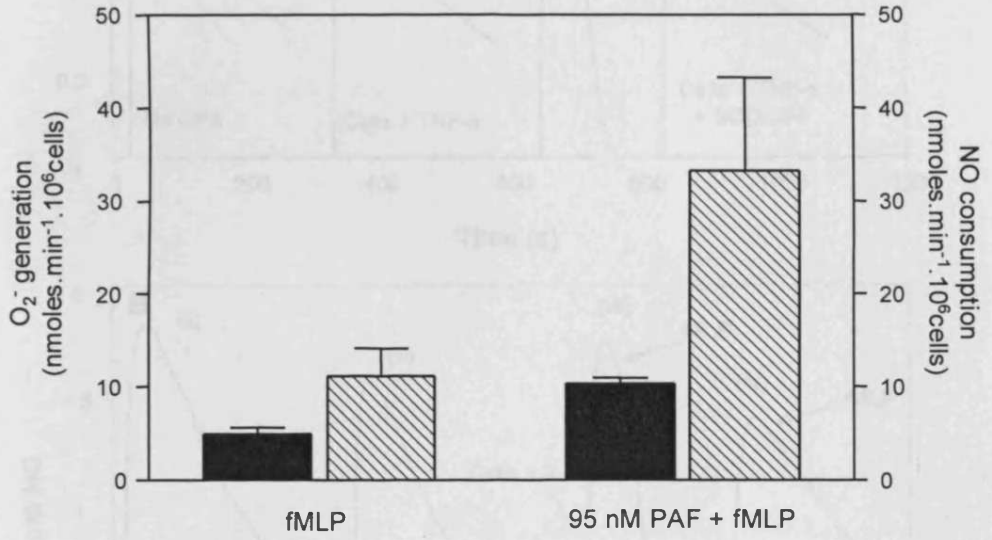

Figure 3.12 NO consumption by both control and PAF-primed human neutrophils is faster than $O_2^{\cdot-}$ production.

Solid bar. $O_2^{\cdot-}$ production measured by Cu-Zn-SOD inhibitable reduction of cyt *c* at 550 nm. fMLP (1 μM) was added to 2 ml Krebs-Henseleit buffer containing control or PAF-primed human neutrophils (3.1 x 10^4 cells.ml^{-1}) 50 μM cyt *c*, as described in section 2.2.2.1 of *Materials and Methods*. Results shown are of a representative experiments from a single donor.

Hatched bar. NO consumption was measured following fMLP (1 μM) addition to 0.5 ml Krebs-Henseleit buffer containing NO (1.9 μM) and control or PAF-primed human neutrophils (3.1 x 10^4 cells.ml^{-1}). Results shown are of a representative experiment from a single donor.

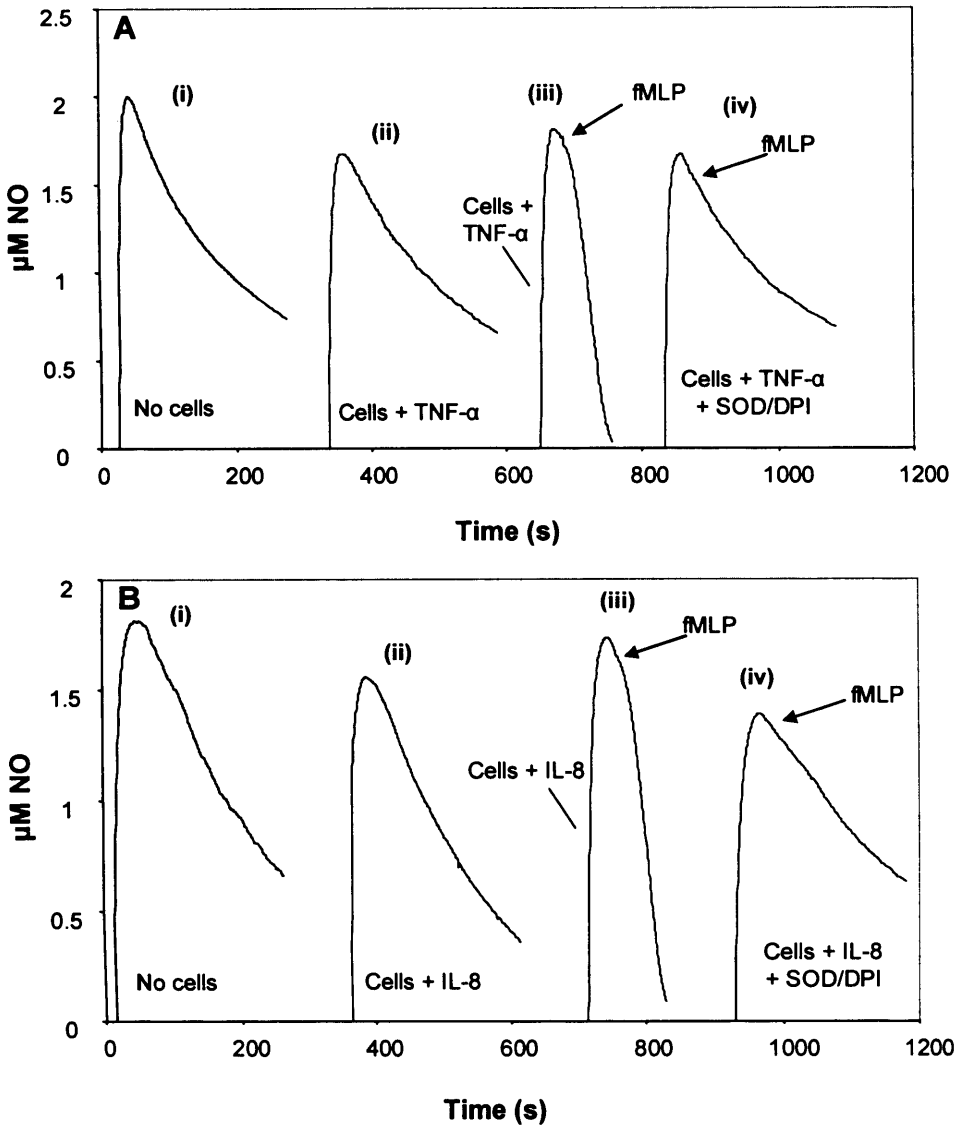

Figure 3.13 Neutrophils primed with TNF-α or IL-8 consume NO in an NAD(P)H oxidase-dependent manner upon activation with fMLP

NO (1.9 μM) was added to 0.5 ml Krebs-Henseleit buffer containing human neutrophils (3.1 x 10^4 cells.ml^{-1}) in the chamber of the NO electrode at 37°C with stirring. *Panel A. (i)* background NO loss, *(ii)* resting neutrophils primed with 10 ng.ml^{-1} TNF-α for 10 min, *(iii)* primed neutrophils (10 ng.ml^{-1} TNF-α) activated with fMLP, as indicated by arrow, *(iv)* primed neutrophils (10 ng.ml^{-1} TNF-α) activated with fMLP, as indicated, in the presence of CuZn-SOD (3000 U.ml^{-1}) and DPI (10 μM). *Panel B. (i)* background NO loss, *(ii)* resting neutrophils primed with 10 nM IL-8 for 10 min, *(iii)* primed neutrophils (10 mM IL-8) activated with fMLP, as indicated by arrow, *(iv)* primed neutrophils (10 nM IL-8) activated with fMLP, as indicated, in the presence of CuZn-SOD (3000 U.ml^{-1}) and DPI (10 μM). Results shown are of a representative experiment from a single donor for each panel, repeated with at least three different donors.

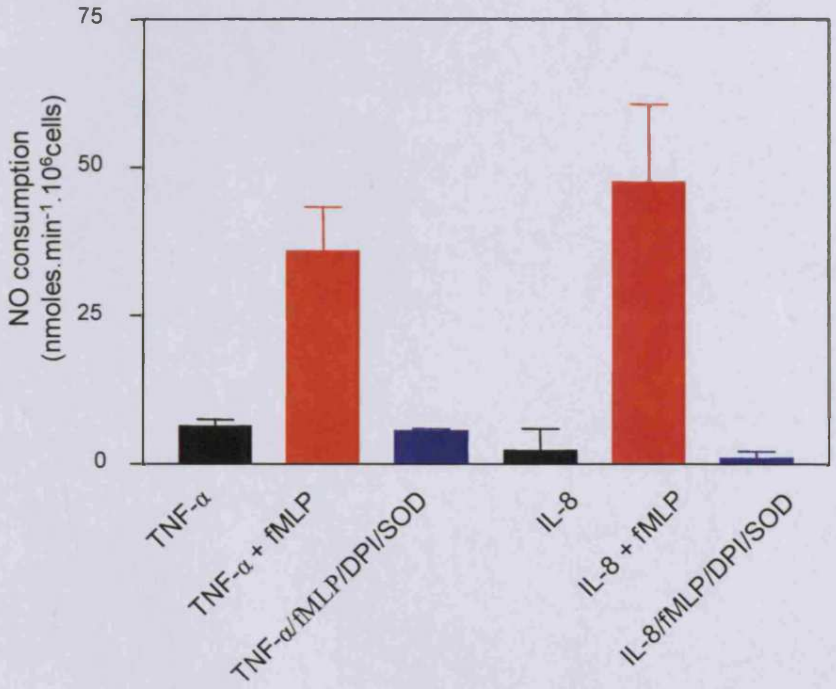

Figure 3.14 Neutrophils primed with TNF-α or IL-8 consume NO in an NAD(P)H oxidase-dependent manner upon activation with fMLP

NO uptake was measured as described in Figure 3.11. Results shown are of a representative experiment from a single donor, repeated with at least three donors ($n \geq 3$, mean ± SEM).

3.3 Discussion

The main finding of this chapter is that primed human neutrophils produce $O_2^{\bullet-}$ and consume NO at far faster rates than unprimed neutrophils upon activation with fMLP *in vitro*. Complete inhibition of $O_2^{\bullet-}$ production and NO removal with the NAD(P)H oxidase inhibitor DPI and $O_2^{\bullet-}$ scavenger CuZn-SOD indicate that NAD(P)H oxidase is required for these processes.

Previous studies have shown an enhancement of the respiratory burst response in primed human neutrophils compared to unprimed cells (Guthrie *et al.* 1984). Indeed, this was clearly demonstrated in this study using neutrophils primed with TNF-α, IL-8 or PAF prior to activation with fMLP, a cell surface receptor agonist (Figures 3.2–3.5). In light of the increased rate of $O_2^{\bullet-}$ generation by these primed cells, it was perhaps unsurprising that the rates of NO consumption by neutrophils primed with TNF-α, IL-8 or PAF were greater than those observed in unprimed cells (Figures 3.6–3.9). However, it is of note that the rates of NO consumption observed in this study were consistently faster than the rates of $O_2^{\bullet-}$ generation by these cells (Figures 3.10–3.12), suggesting the pathways other than NAD(P)H oxidase may also be responsible for the markedly accelerated rates of NO consumption by primed neutrophils.

In order to assess the relative contribution of NAD(P)H oxidase activity in the consumption of NO by primed neutrophils, a combination of the $O_2^{\bullet-}$ scavenger CuZn-SOD and the non-specific flavoenzyme inhibitor DPI was used, as previous work by Clark *et al.* (2002) showed that neither of these agents were completely effective in preventing NAD(P)H oxidase-dependent NO consumption in activated

human neutrophils when used alone. The inability of CuZn-SOD to completely inhibit NO consumption in previous studies is thought to be due the reversible nature of the SOD-catalysed reaction between $O_2^{\bullet-}$ and protons (H^+) yielding H_2O_2 (McBride *et al.*1999):

$$2\ O_2^{\bullet-} + 2\ H^+ \leftrightarrow H_2O_2 + O_2$$

In the presence of CuZn-SOD, $O_2^{\bullet-}$ generation leads to a rapid build up of H_2O_2, pushing the reaction in the opposite direction (i.e. to the left). The resulting $O_2^{\bullet-}$ reacts far more rapidly with NO than SOD and, hence, even when very high concentrations of CuZn-SOD are used (e.g. 3000 $U.ml^{-1}$) the SOD cannot compete with the rapid reaction of $O_2^{\bullet-}$ and NO, leading to NO consumption. Similarly, DPI alone does not fully inhibit NO consumption; inhibition of flavoenzymes by DPI occurs during flavin turnover when it reacts with a catalytic intermediate (reduced FAD), therefore some enzyme catalysis occurs before inhibition is complete (Figure 3.15) (O'Donnell *et al.* 1993). Nevertheless, the combination of DPI and CuZn-SOD has been shown to be fully effective in preventing NAD(P)H oxidase-dependent NO removal, as the small amount of $O_2^{\bullet-}$ produced in the presence of DPI is effectively removed by CuZn-SOD (Clark *et al.* 2002).

The use of CuZn-SOD/DPI in the current study resulted in the complete inhibition of NO consumption by primed neutrophils, suggesting that NAD(P)H oxidase activity is the primary mechanism responsible for NO consumption by these cells (Figures 3.13 & 3.14). This was unexpected, given that previous studies have shown the upregulation of LTB_4 and TxA_2 release by primed neutrophils, indicating increased 5-

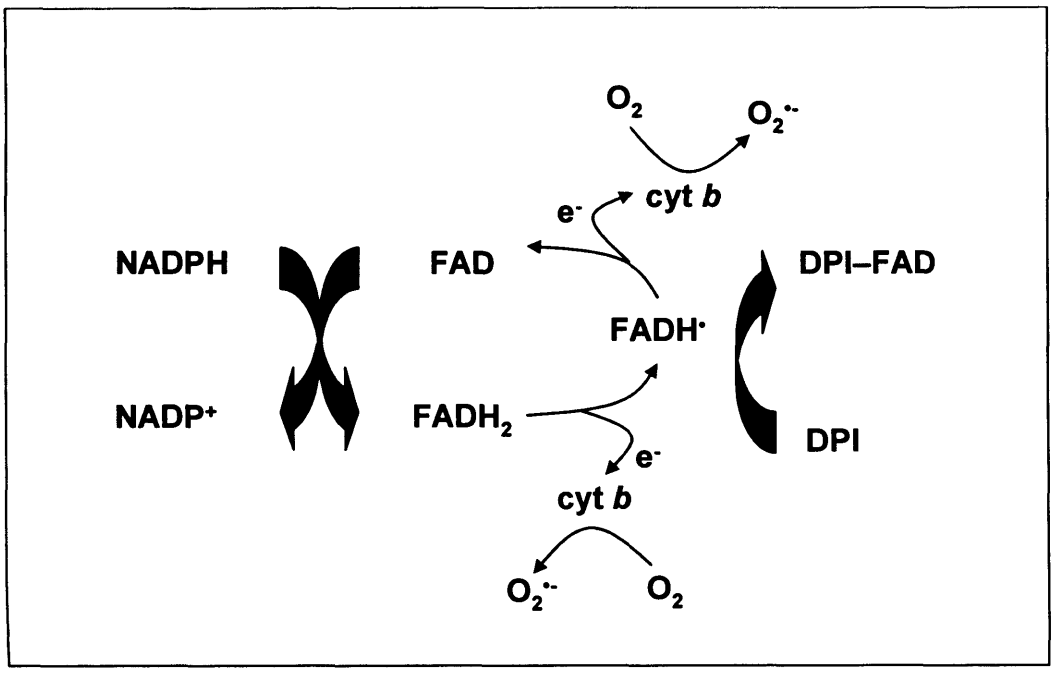

Figure 3.15 Mechanism of action of DPI

NAD(P)H oxidase is an enzyme complex with FAD and low potential cyt *b* as redox centres. Electrons are donated from NADPH to the cyt *b* via FAD, ultimately resulting in the reduction of O_2 to $O_2^{\bullet-}$. Inhibition of this flavoenzyme by DPI occurs during enzyme turnover when it reacts with a catalytic intermediate (reduced FAD). Therefore some enzyme catalysis occurs before inhibition is complete (Adapted from O'Donnell *et al.* 1993).

LOX and COX-2 activity – and, therefore, 5-LOX- and COX-2-dependent NO consumption – in these cells (Bauldry *et al.* 1991; Pouliot *et al.* 1998; Surette *et al.* 1998; Zarini *et al.* 2006). However, it may be that the high concentration of NO used in this study resulted in inhibition of COX-2 activity via NO binding to the central haem group of this peroxidase enzyme (Abu-Soud *et al.* 2001). Similarly, the lack of 5-LOX-dependent NO consumption by primed neutrophils may be due the activating stimulus used, since activation of this enzyme is poor in response to fMLP (Clark *et al.* 2002). Therefore, in order to better assess the contribution of 5-LOX to NO consumption by primed neutrophils, a different activating substance could be used (e.g. PMA or A23187).

The data presented here are consistent with the findings of Clark and colleagues (2002), who showed that NO consumption by fMLP-stimulated unprimed neutrophils was also mediated by NAD(P)H oxidase alone, despite a similar discrepancy between the observed rates of NO disappearance and $O_2^{\bullet-}$ generation. Indeed, in the current data, ratios of $O_2^{\bullet-}$ generation and NO consumption rates do not differ significantly between unprimed and primed cells (see Section 3.2.5), indicating that mechanisms of NO removal are likely to be similar. However, while these findings suggest that COX- and LOX-dependent mechanisms are unlikely to play a role in th NO consumption by either primed or unprimed neutrophils in this study, a role for other enzymes or reactions downstream of NAD(P)H oxidase cannot be ruled out.

The various enzymes and reactions that may occur downstream of $O_2^{\bullet-}$ production by NAD(P)H oxidase are outlined in Figure 3.16. These include MPO, which may use

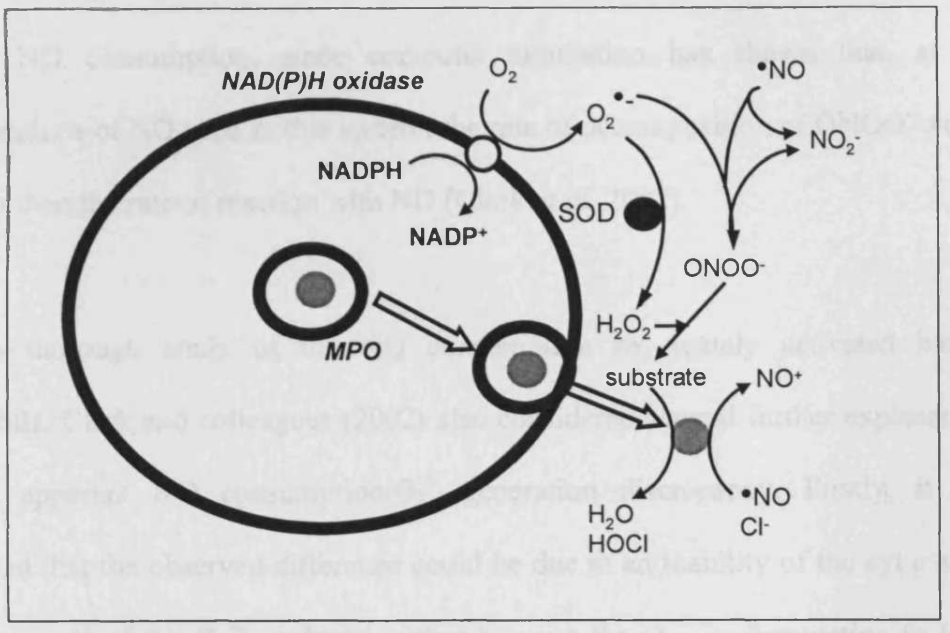

Figure 3.16 Potential routes of NO removal downstream of NAD(P)H oxidase in neutrophils

Abbreviations: myeloperoxidase (MPO); superoxide dismutase (SOD); molecular oxygen (O_2); superoxide ($O_2^{\bullet-}$); nitric oxide (NO); nitrosium ion (NO^+); nitrite (NO_2^-); peroxynitrite ($ONOO^-$); hydrogen peroxide (H_2O_2); hypochlorous acid (HOCl) (schematic designed by Dr Stephen R Clark).

NO as a substrate, and reaction of NO with $ONOO^-$ (Abu-Soud & Hazen, 2000); O'Donnell *et al.* 2000; Pfeiffer *et al.* 1997). However, despite the enhanced release of MPO by primed neutrophils, the observation in previous studies that rates of NO consumption by MPO-deficient cells were unaffected by addition of exogenous purified MPO makes a role for this pathway unlikely (Clark *et al.* 2002; Fittchen *et al.* 1988). Similarly, it is also doubtful that sequestration of NO by $ONOO^-$ plays a major role in NO consumption, since computer simulation has shown that, at the concentrations of NO used in this system, the rate of decomposition of $ONOO^-$ would be faster than the rate of reaction with NO (Clark *et al.* 2002).

In their thorough study of the NO consumption by acutely activated human neutrophils, Clark and colleagues (2002) also considered several further explanations for the apparent NO consumption/$O_2^{\bullet-}$ generation discrepancy. Firstly, it was postulated that the observed difference could be due to an inability of the cyt *c* assay to measure all of the $O_2^{\bullet-}$ produced, either because the $O_2^{\bullet-}$ is dismutating to H_2O_2 before it can react with cyt *c* or because a population of $O_2^{\bullet-}$ is produced in intracellular phagosomal compartments that are not accessible to cyt *c* but are accessible to lipophilic NO. However, kinetic simulation demonstrated that in the presence of 50 μM cyt *c*, $O_2^{\bullet-}$ preferentially reacts with cyt *c* and does not dismutate to H_2O_2 (Clark *et al.* 2002). Indeed, although rate constants for spontaneous $O_2^{\bullet-}$ dismutation and the reaction with $O_2^{\bullet-}$ with cyt *c* are similar, dismutation of $O_2^{\bullet-}$ is second order and will therefore be slow at low concentrations of $O_2^{\bullet-}$. Furthermore, it is unlikely that $O_2^{\bullet-}$ is produced in discreet intracellular compartments, since rates of $O_2^{\bullet-}$ generation were in agreement with rates of O_2 consumption.

It is important to note that, in contrast to the NO consumption assay, no NO is present in the system when $O_2^{\bullet-}$ generation is being measured. This is an important consideration, since NO may enhance NAD(P)H oxidase activity upon its addition to the aerobic buffer (e.g. by accelerating enzyme assembly). Nevertheless, when these assays were carried out in a cell-free reconstitution system with pre-assembled NAD(P)H oxidase the same trend towards NO consumption being significantly faster than $O_2^{\bullet-}$ generation was observed. Hence, these differences are not likely to be due to an effect of NO on the cellular processes that control activation and assembly of NAD(P)H oxidase (Clark *et al.* 2002).

Despite the apparent discrepancy between rates of $O_2^{\bullet-}$ generation and NO, it is clear that priming of human neutrophils with inflammatory stimuli potentiates $O_2^{\bullet-}$ generation by these cells, leading to increased rates of NO consumption from aerobic buffer *in vitro*. These findings have implications for the role of neutrophils in regulating NO bioavailability *in vivo* following inflammatory insult, specifically regarding regulation of vessel tone, smooth muscle proliferation, platelet aggregation and leukocyte adhesion (McBride & Brown, 1997). However, cells other than neutrophils are also subject to modulation by inflammatory cytokines. For example, NO production by macrophages has been shown to be upregulated in the presence of LPS, TNF-α, IL-1β and IFN-γ due to the enhanced iNOS expression in these cells (Szabo & Thiemermann, 1995; Wong & Billiar, 1995). Thus, under inflammatory conditions *in vivo*, accelerated $O_2^{\bullet-}$ production and NO synthesis by primed neutrophils and macrophages, respectively, may result to excessive formation of $ONOO^-$.

$ONOO^-$ is a potent oxidizing and nitrating agent that can attack a wide variety of biological molecules. For example, *in vitro* studies have demonstrated that $ONOO^-$ spontaneously reacts with protein tyrosine residues to yield the stable product 3-nitrotyrosine (Beckman *et al.* 1994). Elevated levels of this product have been implicated in a number of pathological conditions including heart disease, acute lung injury, Alzheimer's disease and atherosclerosis (Kooy *et al.* 1995; Kooy *et al.* 1997; Leeuwenburgh *et al.* 1997; Smith *et al.* 1997). Indeed, analysis of LDL isolated from human atherosclerotic lesions revealed a 90-fold elevation of the level of 3-nitrotyrosine compared with that of circulating LDL, indicating a causative role for $ONOO^-$ in the pathogenesis of atherosclerotic disease (Leeuwenburgh *et al.* 1997; White *et al.* 1994). Thus, in addition to the potential effects of enhanced NO consumption on vascular smooth muscle tone and leukocyte function, neutrophil priming may also have deterious effects on endothelial function through the accelerated generation of $ONOO^-$.

In summary, the current data indicate that priming of human neutrophils with inflammatory stimuli potentiates NAD(P)H oxidase activity in these cells, leading to increased rates of NO consumption from aerobic buffer *in vitro*. While the physiological impact of this rapid NO consumption still requires much investigation, it is speculated that the rapid generation of $O_2^{\bullet-}$ by primed neutrophils *in vivo* may induce endothelial dysfunction either through the rapid inactivation of NO, leading to increased vascular tone, or through excessive generation of cytotoxic $ONOO^-$.

In order to further investigate a role for primed neutrophils in the pathogenesis of inflammatory vascular disease it is important to examine these cells in a more

physiological setting. For example, intraperitoneal injection of IL-8 could be used to cause neutrophil priming *in vivo*. However, at the time of writing little information is available concerning the role of neutrophils in normal vascular homeostasis, particularly with regard to endothelial function and the modulation of vascular tone *in vivo*. Therefore, before the effects of neutrophil priming *in vivo* can be examined, it is important to establish baseline data for resting neutrophils. Previous studies have used specific *in vivo* immunodepletion techniques to examine the role of neutrophils in a number of processes, including resistance to *Listeria monocytogene* infection and wound healing (Conlan & North, 1994; Czuprynski *et al.* 1994; Dovi *et al.* 2003). With this in mind, subsequent chapters of this thesis will examine the role of these cells in the regulation of vascular tone *in vivo* using a specific model of neutropenia.

CHAPTER 4

A MODEL FOR NEUTROPHIL DEPLETION IN MICE AND EFFECTS OF NEUTROPENIA ON SYSTOLIC BLOOD PRESSURE

4.1 Introduction

Given the propensity of activated neutrophils to affect NO bioavailability *in vitro* via NADPH oxidase-dependent $O_2^{\bullet-}$ release, as described in the previous chapter, the importance of this effect *in vivo* must be considered. As well as enabling leukocyte-mediated defence against the infiltration of infectious agents, thereby comprising a fundamental component of the non-specific immune response, a neutrophil-induced decrease in NO bioavailability may alter local blood flow by increasing vascular smooth muscle tone. Indeed, there have been a variety of reports showing that the addition of activated neutrophils to isolated vascular tissue results in a change in the contractile tone of the vessel wall. In line with our findings *in vitro*, many of these studies attributed this neutrophil-induced vasoconstriction to the release of ROS that inactivate basally produced NO (Ma *et al.* 1991; Ohlstein & Nichols, 1989).

Interestingly, a study by Kerr and colleagues (1998) produced conflicting results regarding the nature of neutrophil-induced vasoconstriction. Unlike previous studies, they found that both unactivated and activated PMNs induced a cell number-dependent vasoconstriction and that the nature of the vasoconstriction response differed according

PMN activation state. The vasoconstrictor response for unactivated PMNs was endothelium-independent, was due to soluble factor(s) in the cell supernatant and was partially blocked by an inhibitor of leukotriene biosynthesis. In contrast, the response to activated PMNs was endothelium-dependent, was not due to a soluble factor and was linearly related to their activation state. The finding that unactivated neutrophils exerted effects on vascular smooth muscle tone would suggest that, even under basal conditions, neutrophils may play a role in the regulation of hemodynamics throughout the body. Furthermore, upon activation of neutrophils with inflammatory stimuli, changes in neutrophil-function may contribute to local endothelium-dependent modifications in blood flow and tissue perfusion. However, to our knowledge, no study has yet been carried out to explore the vasoactive effects of either resting or inflammatory-activated neutrophils *in vivo* and, as such, the relevance of these findings under physiological conditions remains uncertain.

To investigate neutrophil actions *in vivo*, an animal-model of neutropenia can be used. Early models, such as that used by Steinshamn *et al.* (1993) – in which cyclophosphamide was administered to mice – suffered from a lack of specificity, since cyclophosphamide also depletes lymphocytes at the concentrations used (LaGrange *et al.* 1974; Schwartz *et al.* 1976). More recent studies have induced neutropenia by injection of the monoclonal antibody (mAb) RB6-8C5. This is a rat anti-mouse IgG2b mAb directed against Ly-6G, previously known as Gr-1, a surface marker found on mature mouse granulocytes and the specificity of this mAb for neutrophils in mice has been confirmed by a range of cytofluorimetric, haematological and histological techniques

(Conlan & North, 1994; Czuprynski *et al.* 1994; Ismail *et al.* 2003). *In vivo* treatment of mice with RB6-8C5 has been shown to severely depress blood and spleen neutrophil counts for up to 5 days (Jensen *et al.* 1993), while repeated administration prolonged the period of neutropenia to 7–8 days (Vassiloyanakopoulos *et al.* 1998). Attempts at inducing longer-term neutropenia have been unsuccessful, with neutrophil numbers starting to return to normal after day 10 despite continued administration of RB6-8C5, a process concurrent with detection of IgG and IgM titres against RB6-8C5 being detected in the sera of these animals (Han & Cutler, 1997).

4.1.1 Aims

While the use of RB6-8C5 as a method for inducing neutropenia in various disease models is widespread, to date no baseline physiological measurements have been taken to compare control and neutropenic animals. Furthermore, while some investigation into the action of neutrophils in altering vascular smooth muscle tone *in vitro* has taken place, a similar role for neutrophils *in vivo* has not been investigated. Therefore, the aim of this chapter was to investigate a role for neutrophils in regulating vascular tone *in vivo* using a model of acute neutropenia induced by RB6-8C5. This involved:

- The characterisation and optimisation of RB6-8C5 as a method for inducing and maintaining neutropenia in Balb/c mice for 8 days.

- The non-invasive measurement of systolic BP in control and neutrophil-depleted Balb/c mice by tail-cuff plethysmography.

4.2 Results

4.2.1 RB6-8C5 specifically depletes neutrophils in mice

Typical basal cell counts for murine whole blood were approximately 1.1 ± 0.1 x 10^6 neutrophils.ml^{-1} (mean ± SEM, n = 3), 0.7 ± 0.1 x 10^6 monocytes.ml^{-1} (mean ± SEM, n = 3), and 1.9 ± 0.8 x 10^6 lymphocytes.ml^{-1} (mean ± SEM, n = 3) at baseline. A range of protocols for neutrophil depletion using RB6-8C5 were tested to determine the optimal dose (Table 4.1). In all cases, a total volume of 250 µl of RB6-8C5 in sterile PBS was administered by intraperitoneal injection.

	Protocol 1	**Protocol 2**	**Protocol 3**	**Protocol 4**	**Protocol 5**
Day 0	150 µg	300 µg	1 mg	150 µg	150µg
Day 1					
Day 2				300 µg	300 µg
Day 3					
Day 4					300 µg
Day 5					
Day 6					300 µg

Table 4.1 Trial dosage regimens for neutrophil depletion in Balb/c mice with RB6-8C5

Administration of 150 μg RB6-8C5, as in protocol 1, completely depleted neutrophils by day 1 of the study. However, by day 2 neutrophil levels had started to recover before returning to baseline levels by day 3 (Figure 4.1). Similarly, protocols 2 reduced neutrophil numbers to almost zero, but once again neutrophil numbers began to recover before the end of the testing period (Figure 4.2). Administration of a large dose of RB6-8C5, as in protocol 3, was successful in keeping neutrophils depleted for up to 6 days (Figure 4.3). In contrast, the administration of two smaller doses, as in protocol 4, failed to completely suppress neutrophil numbers on day 6 (Figure 4.4). Therefore, in order to prolong the period of neutropenia whilst using the minimum dose of antibody, all further experiments used three small doses of RB6-8C5, delivered on day 0, day 2, day 4 and day 6 (see Table 4.1, protocol 5). This caused neutrophils to remain completely depleted for the entire 6-day testing period (Figure 4.5).

1 day after injection with RB6-8C5 (see Table 4.1, protocol 5), neutrophil counts had decreased significantly ($0.04 \pm 0.03 \times 10^6$ cells.ml^{-1} *versus* $1.10 \pm 0.10 \times 10^6$ cells.ml^{-1}, day 1 *versus* day 0, mean ± SEM, $p < 0.001$, using one way ANOVA and Dunnett post hoc test analysis, $n = 3$, Figure 4.5) and remained depleted throughout the experiment. The number of monocytes did not change significantly throughout the experiment. Similarly, the total number of lymphocytes did not change significantly throughout the experiment. These data show that RB6-8C5 specifically depletes neutrophils without significantly affecting monocyte or lymphocyte numbers. Therefore, the protocol used is suitable for use in physiological experiments which require a murine model of neutropenia. Administration of an equal dose of a control antibody, GL113, did not result

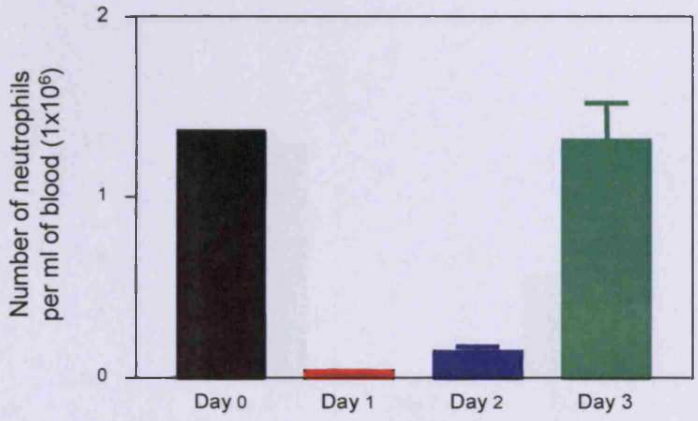

Figure 4.1 Neutrophil counts in mouse whole blood following administration of RB6-8C5 according to Protocol 1

150 µg RB6-8C5 was administered by intraperitoneal injection on day 0. Neutrophil numbers were determined by differential counting of murine whole blood samples, as described in section 2.2.3.1 of *Materials and Methods*, collected on day 0 (n = 1), day 1 (n = 2), day 2 (n = 2) and day 3 (n = 2). Briefly, white blood cells were isolated from whole mouse blood and the concentration adjusted to a maximum of 10^6 cells ml^{-1} by adding PBS. 100 µl of cells (i.e. 100,000 cells) were cytospun at 100 *g* for 10 mins and the neutrophils, monocytes and lymphocytes counted.

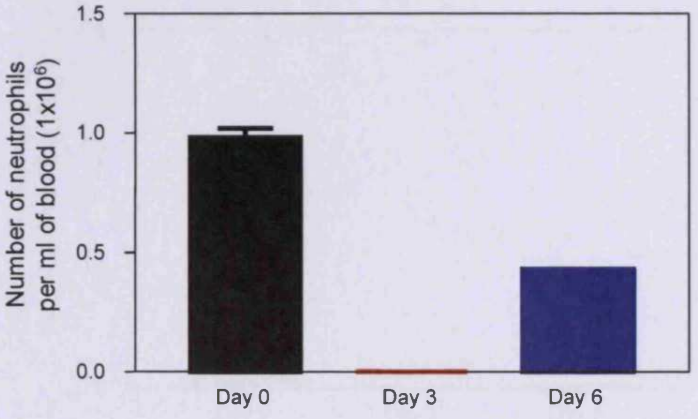

Figure 4.2 Neutrophil counts in mouse whole blood following administration of RB6-8C5 according to Protocol 2

300 µg RB6-8C5 was administered by intraperitoneal injection on day 0. Neutrophil numbers were determined by differential counting of murine whole blood samples, as described in section 2.2.3.1 of *Materials and Methods*, collected on day 0 (n = 2), day 3 (n = 1) and day 6 (n = 1). Briefly, white blood cells were isolated from whole mouse blood and the concentration adjusted to a maximum of 10^6 cells ml^{-1} by adding PBS. 100 µl of cells (i.e. 100,000 cells) were cytospun at 100 *g* for 10 mins and the neutrophils, monocytes and lymphocytes counted.

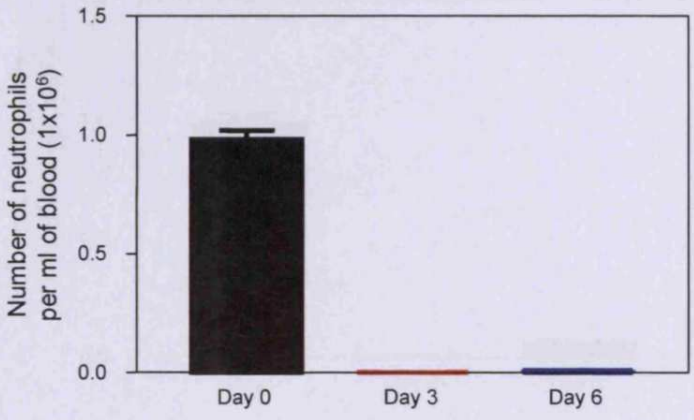

Figure 4.3 Neutrophil counts in mouse whole blood following administration of RB6-8C5 according to Protocol 3

1 mg RB6-8C5 was administered by intraperitoneal injection on day 0. Neutrophil numbers were determined by differential counting of murine whole blood samples, as described in section 2.2.3.1 of *Materials and Methods*, collected on day 0 (n = 2), day 3 (n = 2) and day 6 (n = 1). Briefly, white blood cells were isolated from whole mouse blood and the concentration adjusted to a maximum of 10^6 cells ml^{-1} by adding PBS. 100 µl of cells (i.e. 100,000 cells) were cytospun at 100 *g* for 10 mins and the neutrophils, monocytes and lymphocytes counted.

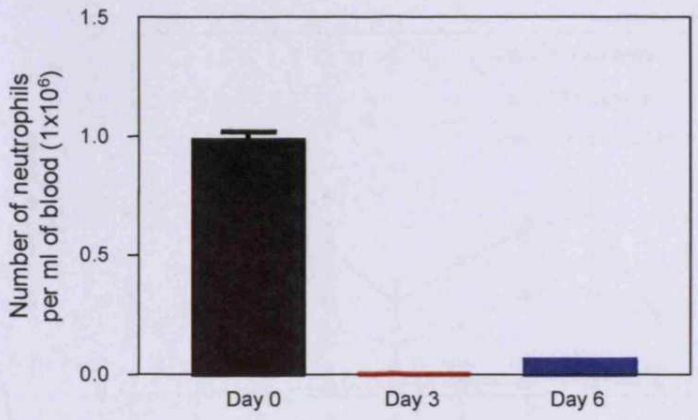

Figure 4.4 Neutrophil counts in mouse whole blood following administration of RB6-8C5 according to Protocol 4

150 µg RB6-8C5 was administered by intraperitoneal injection on day 0, with a further doses of 300 µg on day 2. Neutrophil numbers were determined by differential counting of murine whole blood samples, as described in section 2.2.3.1 of *Materials and Methods*, collected on day 0 (n = 2), day 3 (n = 2) and day 6 (n = 1). Briefly, white blood cells were isolated from whole mouse blood and the concentration adjusted to a maximum of 10^6 cells ml^{-1} by adding PBS. 100 µl of cells (i.e. 100,000 cells) were cytospun at 100 *g* for 10 mins and the neutrophils, monocytes and lymphocytes counted.

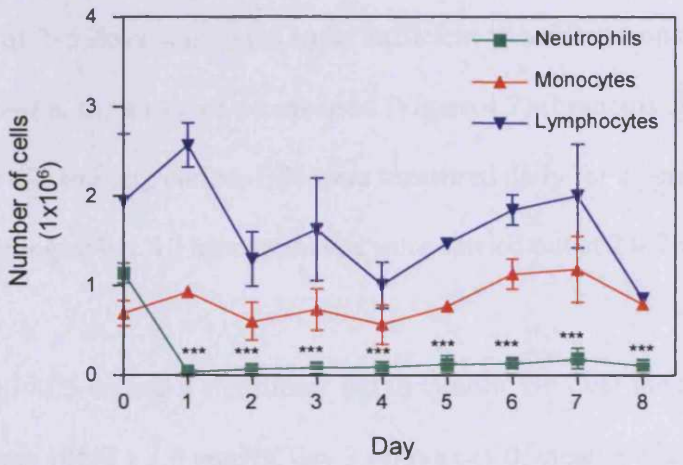

Figure 4.5 RB6-8C5 specifically depletes neutrophils in mice

8 week old, Balb/c mice administered 150 µg RB6-8C5 on day 0, and 300 µg on day 2, day 4 and day 6 by intraperitoneal injection. White blood cells were isolated from whole mouse blood, as described in section 2.2.3.1 of *Materials and Methods*, and the concentration adjusted to a maximum of 10^6 cells ml^{-1} by adding PBS. 100 µl of cells (i.e. 100,000 cells) were cytospun at 100 *g* for 10 mins and the neutrophils, monocytes and lymphocytes counted (n = 3, mean ± SEM). *** represents $p < 0.001$ *versus* day 0 using one way ANOVA and Dunnett post hoc test analysis.

in any significant change in circulating leukocyte numbers (Figure 4.6).

4.2.2 Depletion of murine neutrophils causes a significant fall in systolic BP

Initially, mice were subjected to a training period in order for them to become familiar with the procedure, thereby preventing artificially high BP measurements due to stress. A training period of 2–5 days was found to be sufficient to achieve consistent systolic BPs at a level expected in the strain of mouse used (Figure 4.7) (Francois *et al.* 2004; Yang *et al.* 2005). After this training period, BPs were measured daily for a period of 8 days using tail-cuff plethysmography. All measurements were carried out at 24–26°C.

Injection of RB6-8C5 caused a significant fall in systolic BP over the first 3 days (88.0 ± 3.5 mmHg, *versus* 104.0 ± 2.8 mmHg, day 3 *versus* day 0, mean ± SEM, $p < 0.001$, using one way ANOVA and Dunnett post hoc test analysis, $n \geq 15$). In contrast, i.p. administration of an equal dose of the control antibody (GL113) had no effect on BP. These data indicate that neutrophils may play a role in the regulation basal BP *in vivo*.

4.2.3 Neutropenia decreases PE-induced vasoconstriction ex vivo

To compare vasoactivity in healthy and neutropenic animals, myography was conducted on aortic rings from control mice and mice depleted of neutrophils for 3 days, since these animals exhibit the largest differences in systolic BP. Thoracic aortae from neutropenic mice exhibited a significant attenuation in PE-induced vasoconstriction compared to

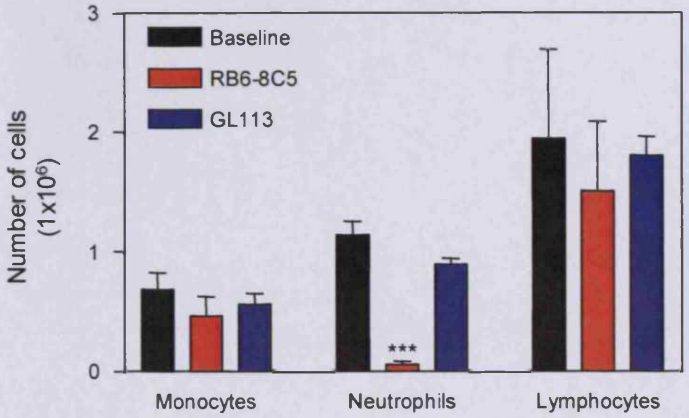

Figure 4.6 GL113 does not affect circulating leukocyte counts in mice

8 week old, Balb/c mice administered 150 µg GL113 on day 0, and 300 µg on day 2 by intraperitoneal injection. White blood cells were isolated from whole mouse blood on day 3, as described in section 2.2.3.1 of *Materials and Methods*, and the concentration adjusted to a maximum of 10^6 cells ml^{-1} by adding PBS. 100 µl of cells (i.e. 100,000 cells) were cytospun at 100 *g* for 10 mins and the neutrophils, monocytes and lymphocytes counted ($n \geq 3$, mean ± SEM). *** represents $p < 0.001$ *versus* baseline using one way ANOVA and Dunnett post hoc test analysis.

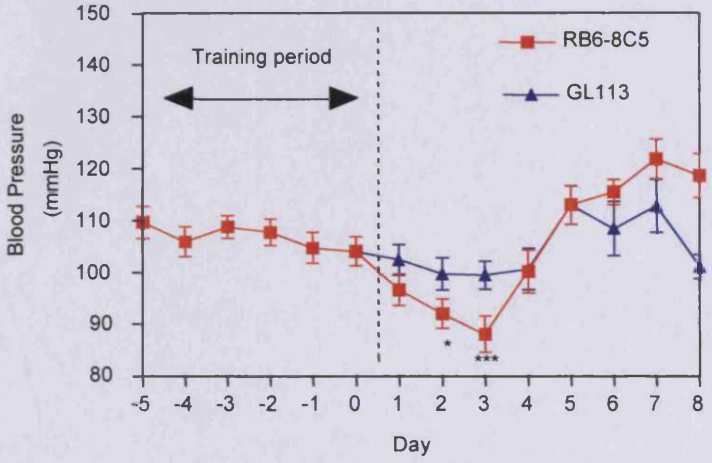

Figure 4.7 Neutrophil depletion causes a transient fall in systolic BP

BPs were measured daily by tail-cuff plethysmography under controlled conditions. Following a 5-day training period, mice randomized to receive either RB6-8C5 or an equal dose of GL113 (n = 3–20, mean ± SEM) by intraperitoneal injection. * represents $p < 0.05$ *versus* day 0, *** represents $p < 0.001$ *versus* day 0 using one way ANOVA and Dunnett post hoc analysis.

controls (205.91 ± 30.89% MAX *versus* 358.01 ± 30.42% MAX, neutropenic *versus* control, mean ± SEM, $p < 0.001$, using two way ANOVA, $n \geq 4$, Figure 4.8). In contrast, no differences in constriction responses to KCl were observed between groups, indicating that smooth muscle integrity is unaltered in neutropenia (138.40 ± 7.48 % MAX *versus* 119.30 ± 10.14 %, $n \geq 4$, Figure 4.9).

4.2.4 Neutrophil-depletion by RB6-8C5 does not cause neutrophil degranulation

The precise mechanism by which RB6-8C5 causes neutrophil depletion from the systemic circulation has yet to be fully determined. Potential mechanisms include phagocytosis by monocytes, neutrophil activation and apoptosis, and complement-mediated neutrophil clearance.

Antibody-mediated activation of neutrophils may result in the release of neutrophil intracellular contents into the systemic circulation, causing inflammatory changes such as monocyte activation or endothelial dysfunction. Therefore, to ensure that neutrophil activation and degranulation was not induced by RB6-8C5, plasma samples from control and day 1 mice were probed for the presence of MPO, which is commonly released following neutrophil activation *in vivo* (Baldus *et al.* 2001), using rabbit polyclonal anti-MPO antibody (Calbiochem, San Diego) (1:1000). No MPO was detected in either control or day 1 samples (Figure 4.10). Similarly, quantification of pixel intensity following immunohistochemistry of aortic sections from control and day 1 mice revealed no increase in MPO deposition in the vessel wall (Figure 4.11). These data indicate that

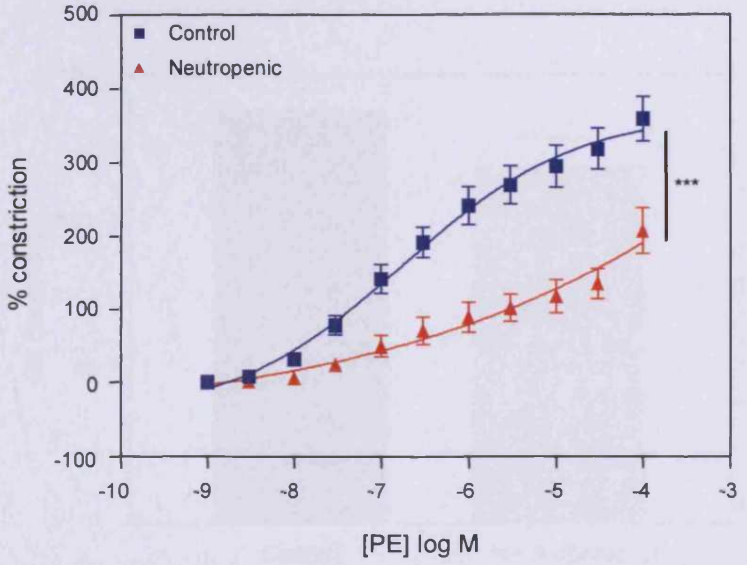

Figure 4.8 Neutropenia decreases vasoconstriction *ex vivo*

PE-induced constriction responses in thoracic aortae from control mice (n = 6, mean ± SEM) and mice depleted of neutrophils for 3 days (n = 4, mean ± SEM). Aortic ring functional responses were determined as described in section 2.2.3.5 of *Materials and Methods*. *** represents $p < 0.001$, neutropenic versus control using two way ANOVA.

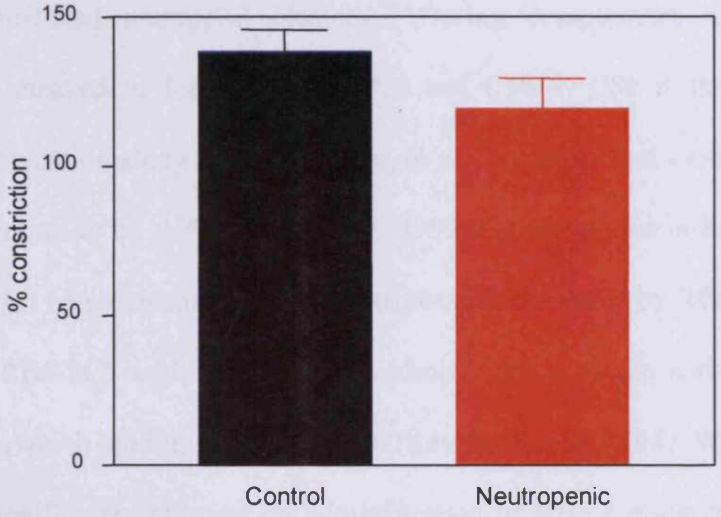

Figure 4.9 Neutropenia does not effect KCl-induced vasoconstriction *ex vivo*

KCl-induced constriction responses in thoracic aortae from control mice (n = 6, mean ± SEM) and mice depleted of neutrophils for 3 days (n = 4, mean ± SEM). Aortic ring functional responses were determined as described in section 2.2.3.5 of *Materials and Methods*.

RB6-8C5 does not cause neutrophil activation and degranulation when administered *in vivo.*

4.2.5 Complement C5 is not involved in neutrophil depletion by RB6-8C5

Another potential mechanism for the onset of RB6-8C5-induced neutropenia involves complement-mediated neutrophil clearance. During complement activation the C5 component is cleaved to form products C5a and C5b–9. C5a is thought to promote neutrophil margination along the vessel walls, as well as peripheral vasodilation (Drapeau *et al.* 1993; Frangi *et al.* 1994; Short *et al.* 1999). Therefore, in order to investigate a potential role for complement activation in neutrophil clearance by RB6-8C5, mice were administered RB6-8C5 with or without co-administration of the anti-C5/C5b antibody BB5.1 (1 mg), which inhibits C5a generation (Ravirajan *et al.* 2004). WBC counts on day 3 were not significantly affected by administration of BB5.1 alone (Figure 4.12). Co-administration of RB6-8C5 and BB5.1 resulted in significantly decreased neutrophil counts compared to baseline counts ($0.107 \pm 0.033 \times 10^6$ cells.ml^{-1} *versus* $1.1 \pm 0.1 \times 10^6$ cells.ml^{-1}, RB6-8C5 + BB5.1 *versus* control, mean ± SEM, $p < 0.001$, using one way ANOVA and Dunnett post hoc test analysis, $n = 3$). Neither monocyte nor lymphocyte counts were significantly affected by co-administration of RB6-8C5 and BB5.1 compared to controls. These data indicate that complement C5 is not involved in neutrophil depletion by RB6-8C5.

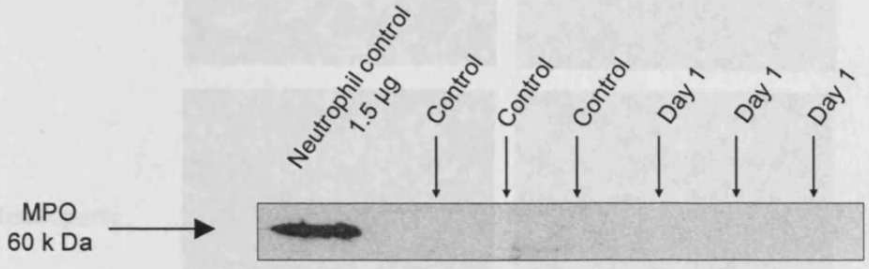

Figure 4.10 Neutrophil-depletion by RB6-8C5 does not cause vascular MPO release into plasma

Plasma from control and neutropenic mice (1 day post injection) were probed for MPO by Western Blot. As a positive control, leukocytes (1.7×10^6 cells, equivalent to 1.5 µg) were prepared from control blood by hypotonic lysis of RBCs. Samples were probed with rabbit polyclonal anti-MPO antibody (1:1000; Calbiochem, San Diego) and visualised using ECL (Amersham, UK) after incubation with a HRP-conjugated anti-rabbit secondary antibody (1:20,000).

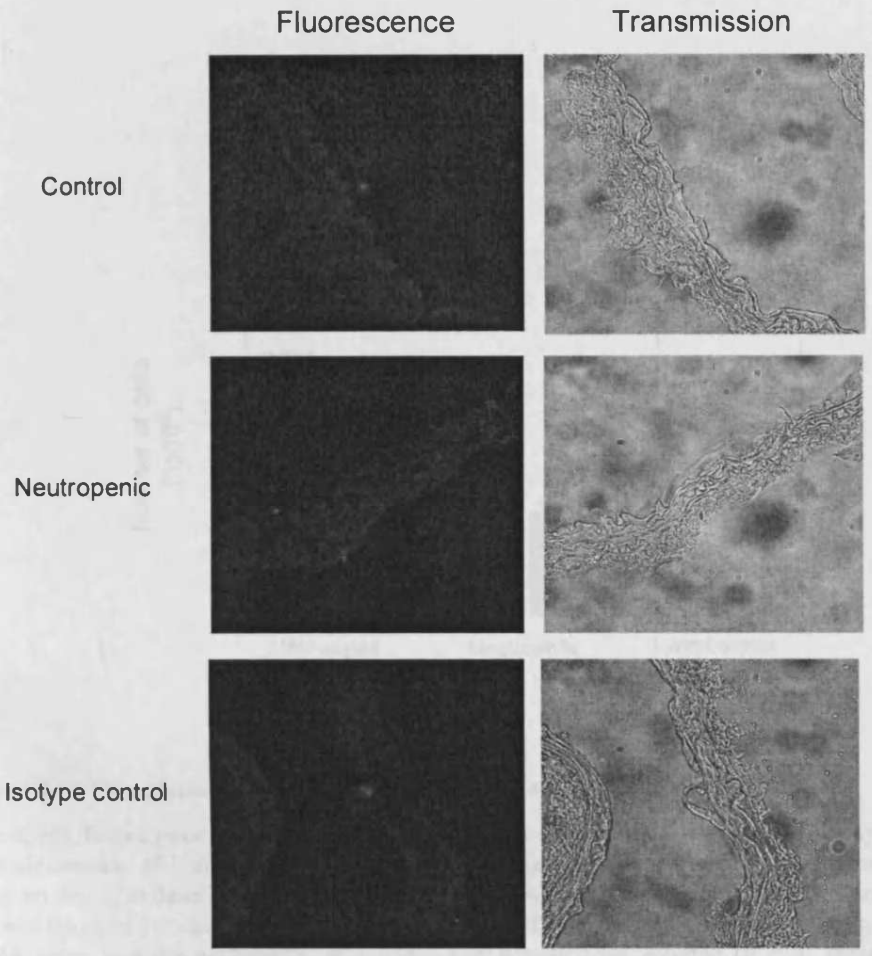

Figure 4.11 MPO deposition is not observed in aortae of neutrophil-depleted mice

Immunohistochemical detection of MPO in aortae from control and neutropenic mice (day 1). Aortic sections from control and neutropenic mice were sectioned and stained for MPO. Representative sections are shown for each condition. MPO was visualised using rabbit anti-MPO (1:200; Calbiochem), with goat anti-rabbit IgG-Alexa 568 as secondary (1:100). Negative controls used equivalent concentrations of isotype rabbit IgG antibody.

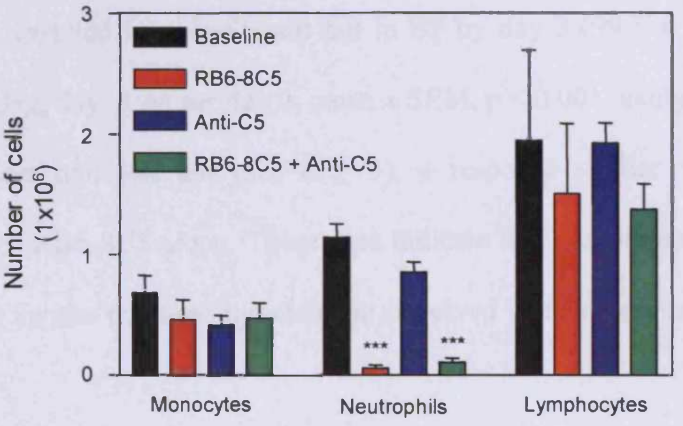

Figure 4.12 Complement C5 is not involved in neutrophil depletion by RB6-8C5

8 week old, Balb/c mice were administered 150 μg RB6-8C5 on day 0, and 300 μg on day 2, with or with coadministration of 1 mg BB5.1, by intraperitoneal injection. WBCs were isolated from whole mouse blood on day 3, as described in section 2.2.3.1 of *Materials and Methods*, and the concentration adjusted to a maximum of 10^6 cells ml^{-1} by adding PBS. 100 μl of cells (i.e. 100,000 cells) were cytospun at 100 *g* for 10 mins, and the neutrophils, monocytes and lymphocytes counted (n = 3, mean ± SEM). *** represents $p < 0.001$ *versus* Anti-C5 using one way ANOVA and Dunnett post hoc test analysis.

4.2.6 Complement C5 is not involved in the decrease in BP observed in RB6-8C5-induced neutropenia

Administration of BB5.1 alone (1 mg) had no effect on systolic BP, as measured by tail-cuff plethysmography on days 0 to 3 (114.1 ± 2.2 mmHg, *versus* 107.0 ± 3.0 mmHg, day 3 *versus* day 0, mean ± SEM, $n \geq 3$, Figure 4.13). In contrast, co-administration of RB6-8C5 and BB5.1 resulted in a significant fall in BP by day 3 (99.1 ± 5.1 mmHg, *versus* 107.0 ± 3.0 mmHg, day 3 *versus* day 0, mean ± SEM, $p < 0.001$, using one way ANOVA and Dunnett post hoc test analysis, $n \geq 3$), a response similar to that seen upon administration of RB6-8C5 alone. These data indicate that complement C5 activation is not responsible for the transient hypotension observed in neutropenia induced by RB6-8C5.

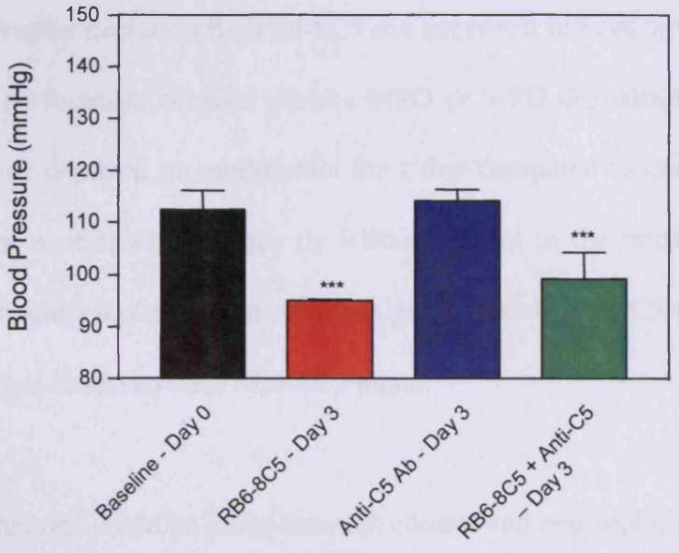

Figure 4.13 Complement C5 is not involved in the decrease in BP observed in RB6-8C5-induced neutropenia

Systolic BPs were measured daily by tail-cuff plethysmography under controlled conditions. Following a 3-day training period, mice were randomized to receive RB6-8C5 and/or BB5.1 ($n \geq 3$, mean ± SEM) by intraperitoneal injection. *** represents $p < 0.001$ *versus* day 0 using one way ANOVA and Dunnett post hoc analysis.

4.3 Discussion

The main finding of this chapter is that neutrophil depletion *in vivo* causes a transient fall in systolic BP and reduced vasoconstriction *ex vivo* in Balb/c mice. This effect was specific to the action of RB6-8C5, since administration of an equal dose of a control antibody, GL113, which does not deplete neutrophils, did not result in any fall in BP. Furthermore, neutrophil depletion by RB6-8C5 did not result in neutrophil activation and degranulation, as no increase in either plasma MPO or MPO deposition on vessel walls was evident in mice depleted on neutrophils for 1 day compared to controls. A role for complement C5 in neutrophil clearance by RB6-8C5, and in the transient hypotension observed in neutropenia, has also been ruled out given that the anti-C5 Ab BB5.1 had no effect on the changes observed with RB6-8C5 alone.

Before any comparisons could be made between control and neutrophil-depleted animals, the murine model of neutropenia first had to be optimized. Initial attempts to deplete neutrophils using RB6-8C6 were based on the dosage regimen used by Vassiloyanakopoulos *et al.* (1998), which was successful in inducing neutropenia in Balb/c mice. However, while Vassiloyanakopoulos and colleagues found a single dose of 150 μg RB6-8C5 to be sufficient for depleting neutrophils for up to 5 days, the same dose only depleted neutrophils for up to 1–2 days in this study, before neutrophil numbers returned to basal levels on day 3 (Figure 4.1). It is believed that these contrasting results are likely to be due to differences in antibody potency, perhaps due perhaps as a result of different storage or preparation methods. After further characterisation, a dosage regimen

consisting of an injection of 150 μg on day 0, and further injections of 300 μg on day 2, day 4 and day 6 was found to deplete neutrophils for up to 8 days (Figure 4.5), a period deemed sufficient to study the role of neutrophils in regulating vascular tone *in vivo*.

The Gr-1 antigen is expressed on both neutrophils and monocytes, but not lymphocytes (Dovi *et al.* 2003; Largasse & Weissman, 1996). Nevertheless, while Gr-1 expression increases on neutrophils as they differentiate from immature cells in the bone marrow to mature polymorphs in the blood and spleen, expression of Gr-1 in monocytes decreases as they move from the bone marrow into the general circulation (Largasse & Weissman, 1996). Hence, the low levels of Gr-1 expression in monocytes and the absence of Gr-1 in lymphocytes mean that these cells are unlikely to be depleted by RB6-8C5. Indeed, the specificity of RB6-8C5 for neutrophils has previously been demonstrated by Conlan & North (1994) in male CB6/F_1 mice using a range of cytofluorimetric, hemotological and histological techniques. However, a study by Han & Cutler (1997) using female Balb/cByJ mice produced conflicting results, indicating that while intravenous administration of low doses of RB6-8C5 (25 μg) produced a specific depletion of neutrophils, higher doses of this antibody (≥ 50 μg) resulted in a general leukopenia, as reflected by a decrease in total white blood cell count that was greater than could be accounted for by loss of neutrophils. Thus, before proceeding with the use of this model, it was important to examine the specificity of RB6-8C5 at the doses used in this study.

The results of this study demonstrate that RB6-8C5 can be used to specifically deplete neutrophils in Balb/c mice for a period of up to 8 days (Figure 4.5). 1 day after injection

of RB6-8C5 neutrophil counts had decreased significantly ($0.04 \pm 0.03 \times 10^6$ cells.ml^{-1} *versus* $1.14 \pm 0.11 \times 10^6$ cells.ml^{-1}, day 1 *versus* day 0, mean ± SEM, $p < 0.001$, using one way ANOVA and Dunnett post hoc test analysis, $n = 3$) and remained depleted throughout the experiment. The number of monocytes did not differ significantly from control levels at any point. Similarly, the total number of lymphocytes did not change significantly throughout the experiment compared to controls. Thus, at the doses used in this experiment, neutropenia was induced in Balb/c mice without affecting circulating monocyte or lymphocyte levels. Finally, the finding that administration of GL113 did not affect circulating leukocyte numbers confirms the suitability of this antibody as a control (Figure 4.6).

Following injection of RB6-8C5 on day 0, neutropenic mice demonstrated a significant fall in BP by day 2 (91.97 ± 2.83 mmHg *versus* 103.97 ± 2.77 mmHg, day 2 *versus* day 0, mean ± SEM, $p < 0.05$, using one way ANOVA with Dunnett post hoc analysis, $n \geq 15$, Figure 4.7) reaching a minimum at day 3. BPs then rose above basal levels by day 5 and remained raised for the rest of the experiment. In contrast, administration of a control antibody, GL113, did not result in any fall in BP between day 0 and day 3; although BPs were similarly elevated after day 4. It is thought that the observed elevation in systolic BP in both RB6-8C5 and GL113-treated animals after day 4 is a limitation of this model of neutropenia. Previous studies have reported the detection of IgG and IgM titers against RB6-8C5 in the sera of animals after 10 days of neutropenia, which prevented the extension of studies similar to this (Han & Cutler, 1997). It may be that immune processes related to these observations are responsible for the rise in systolic BP seen in

this study, particularly given the similar trends observed following administration of RB6-8C5 or GL113. Therefore, no conclusions regarding the role of neutrophils in regulating vascular tone *in vivo* can be drawn from these data after day 3 post-injection.

There are several potential mechanisms by which neutrophils may modulate vascular tone. For example, a role for a NAD(P)H oxidase-dependent $O_2^{\bullet-}$ generation in the regulation of basal BP was previously suggested by Wang *et al.* (2001), who showed that $gp91^{phox-/-}$ mice, which lack an essential subunit of NAD(P)H oxidase involved in $O_2^{\bullet-}$ production, exhibit significantly lower baseline BPs compared with wildtype animals of the same age and weight. However, this does not distinguish between NAD(P)H activity in leukocytes and vascular cells. Furthermore, as discussed in the previous chapter, the reaction between neutrophil-derived $O_2^{\bullet-}$ and NO is extremely rapid; therefore, according to this hypothesis, the complete disappearance of neutrophils by day 1 in this model should produce an increase in NO bioavailability in the vasculature within the same timeframe, resulting in prompt vasodilation and a concurrent decrease in systolic BP. In contrast, a much more gradual fall in BP is observed in this study, indicating that a simple reduction in $O_2^{\bullet-}$-mediated NO activation is not entirely responsible for the changes observed. As such, local upregulation of NOS expression exists as a potential mechanism for these changes. Alternatively, the observed change in vascular tone may be independent of any changes to NO bioavailability. For example, flow-dependent and agonist-induced vasodilation can also be regulated by PGI_2 or via the hyperpolarisation of vascular smooth muscle cells, attributed to the release of EDHF (Büssemaker *et al.* 2003; Hennen *et al.* 2001). A decrease in local vasoconstrictive stimuli may also explain the

hypotensive effect of neutropenia in this study, such as that described Kerr and colleagues (1998), who observed an endothelium-independent vasoconstriction in isolated human umbilical veins in the presence of unactivated neutrophils *in vitro*.

To further investigate a potential role for neutrophils in regulating vessel tone *in vivo*, isometric tension functional studies were carried out on thoracic aortae isolated from control and neutropenic (day 3) animals. While the examination of vascular responses in the aorta do necessarily not reflect those of the resistance vessels which regulate BP, such observations allow a useful insight into the changes in vascular cell signalling that may occur in coordination with altered disease states. Indeed, many investigators have made use of large vessels from a variety of species, including mouse, rat, rabbit, dog and even humans to examine changes in cell signalling pathways in a variety of disease models (Agewell *et al.* 2006; Bagi *et al.* 2005; Bueno *et al.* 2005; Flavahan *et al.* 1994; Jones *et al.* 2005). Mice depleted for 3 days were used, as the largest differences in systolic BP were observed on this day *in vivo* relative to baseline measurements. Interestingly, aortic rings taken from neutropenic animals exhibited a significantly attenuated PE-induced vasoconstriction response compared to controls (Figure 4.8), indicating that cell signalling pathways in aortae become altered following the onset on neutropenia. From these observations the exact nature of such cell signalling changes cannot be determined, although any doubts regarding the contractile function of aortae from neutropenic animals can be dismissed, since no differences in constriction responses to KCl were evident (Figure 4.9), providing evidence that smooth muscle integrity was unaltered in

neutropenia. Hence, further examination of vessel function in healthy and neutropenic mice will be the subject of subsequent chapters of this thesis.

While the specificity of RB6-8C5 for neutrophils is now well characterised, the precise mechanism by which these cells are cleared from the systemic circulation is still subject to question. In fact, only one published study that has attempted to address this question: using radiolabelled RB6-8C5, Thakur and colleagues (1996) monitored the distribution of this antibody following injection into Balb/c mice and found that the radioactivity become concentrated in the liver and spleen. Concurrently, a composite of photomicrographs of histological slices showed that the number of neutrophils in the liver and spleen had increased. Thus, it was concluded that neutrophils interacting with RB6-8C5 sequestered in the liver and spleen and did not return to the circulation.

Although it has yet to be investigated fully, there are three schools of thought regarding the mechanism by white neutrophils are cleared from the body following RB6-8C5 administration. The first involves neutrophil activation and apoptosis. The second possibility involves complement-mediated neutrophil depletion, either by neutrophil margination in the vessel wall or by complement-mediated cell lysis. The final theory involves antibody-mediated cell-mediated cytotoxicity, whereby binding of RB6-8C5 to Gr-1 on the surface of the neutrophil attracts liver macrophages or natual killer (NK) cells to bind via the FcR molecule on their cell surface, inititating clearance of the neutrophil by phagocytosis or antibody-dependent cell-mediated cytotoxicity, respectively.

Antibody-mediated activation of neutrophils may cause activation of the neutrophil, resulting in degranulation. The release of neutrophil intracellular contents into the systemic circulation can result in pro-inflammatory changes such as monocyte activation or endothelial dysfunction, leading to changes in vascular tone. Indeed, a recent study has shown that intravenous administration immunoglobulin preparations (IVIGs) often induce a long-lasting hypotension associated with macrophage-derived PAF (Bleeker *et al.* 2000). MPO is considered a good general index of the neutrophil activity in the vasculature, since previous studies have reported an increase in free MPO in the plasma of patients following neutrophil activation *in vivo* (Baldus *et al.* 2001; Biasucci *et al.* 1996). In addition, the intraluminal release of MPO is known to result in its binding to the vascular intima (Baldus *et al.* 2001; Malle *et al.* 2000). Therefore, in order to investigate the possibility that RB6-8C5 may be causing pro-inflammatory changes in its action against neutrophils, plasma samples from control and neutrophil-depleted animals (day 1 post injection) were assayed for the presence of MPO by Western blotting, and aortic sections from control and neutrophil-depleted animals (day 1 post injection) were subjected to immunohistochemical detection of MPO. No MPO was detected in either control or day 1 samples by Western blotting (Figure 4.10) and quantification of pixel intensity following immunohistochemistry of aortic sections from control and day 1 mice revealed no increase in MPO deposition in the vessel wall (Figure 4.11). Therefore these data indicate that RB6-8C5 does not cause intravascular degranulation of neutrophils is when administered *in vivo*.

A role for complement mediators in the clearance of neutrophils by RB6-8C5 has been suggested, since other antibodies of the IgG2b isotype have previously been reported to be complement-activating *in vitro* (Fust *et al.* 1980; Medgyesi *et al.* 1978). Furthermore, complement activation *in vivo*, following administration of streptokinase to patients after acute myocardial infarction, has been associated with a transient decrease in the number of circulating neutrophils (Frangi *et al.* 1994). Complement mediator and anaphylatoxin, C5a, is a well known chemotactic agent and receptors for C5a have been described on the surface of neutrophils and monocytes/macrophages. Therefore, it has been postulated that the apparent depletion of neutrophils is due to neutrophil adhesion and margination along vessel walls as a result of C5a receptor activation. Indeed, intravenous administration of C5a has been shown to produce an acute fall in circulating neutrophil count (Drapeau *et al.* 1993; Frangi *et al.* 1994; Short *et al.* 1999). However, given the high MPO content of these cells, such margination would be expected to result in enhanced MPO staining along the vessel wall, which did not occur in this study (Figure 4.11).

Complement activation following high doses of purified anaphylatoxin C5a or C5a receptor agonists produce transient hypotension in a variety of human and animal models (Lundberg *et al.* 1987, Short *et al.* 1999). It is thought that the fall in BP may be caused by peripheral vasodilation due to C5a-induced increases in vasodilatory COX products, such as PGI_2, since hypotension was largely abolished by pre-treatment with COX inhibitors, and increases in circulating 6-keto-$PGF_{1\alpha}$ (a metabolite of PGI_2) levels occur parallel to changes in BP (Lundberg *et al.* 1987; Short *et al.* 1999). While the induction of neutropenia and onset of hypotension following complement activation are

consistently shown to occur in parallel, prior induction of neutropenia in rabbits by nitrogen mustard was found to have no effect on the C5a-induced hypotensive response, and thus the two responses appear independent of each other (Lundberg *et al.* 1987). Tissue macrophages, dispersed in the connective structure of the vessel wall, are thought to be the source of the vasoactive prostanoids released, since C5a is a well documented chemoattractant and activator of these cells (Marceau *et al.* 1990). Furthermore, histochemical studies of macrophages in umbilical artery sections revealed a similar distribution to binding of labelled C5a in this tissue (Marceau *et al.* 1990; Short *et al.* 1999). Nevertheless, the observed changes in neutrophil numbers, BP and circulating COX metabolites following C5a administration were immediate (usually in 15 to 30 s) and recovered to baseline levels within 30 min. Therefore while these observations are interesting, the changes observed in this study occur over a much longer period than those previously reported and thus further investigation into a role for complement in RB6-8C5-induced neutropenia and hypotension was required.

In order to investigate a role for complement C5 in this model, WBC counts and BP measurements were made in mice administered RB6-8C5, with or without co-administration of the anti-C5 Ab BB5.1. When given alone, this antibody had no adverse effect on circulating leukocyte numbers or systolic BP measurements (Figure 4.12 and Figure 4.13). Furthermore, inhibition of complement C5 did not prevent the observed changes in WBC counts or BP previously seen with RB6-8C5 alone. Therefore, it appears that complement C5 is not responsible for neutrophil clearance or induction of hypotension following administration of RB6-8C5 *in vivo*. Of additional note is the

observation by Morgan that RB6-8C5 and GL113 caused an identical degree complement activation *in vitro* (Figure 4.14, unpublished observations). Given that GL113 was without effect on either circulating leukocyte counts or systolic BP when administered at an equal dose to that of RB6-8C5 in this model of neutropenia, it can be concluded that the importance of such *in vitro* observations is minimal in the *in vivo* setting.

Collectively, these data provide substantial evidence that the administration of RB6-8C5 to Balb/c mice results in a highly specific and controlled depletion of neutrophils from the systemic circulation, without resulting in complement activation or neutrophil degranulation. However, a role for antibody-mediated cell-mediated cytotoxicity, via the action of macrophages and/or NK cells, in the clearance of neutrophils by RB6-8C5 has yet to be investigated.

In summary, depletion of neutrophils results in a gradual alteration of vascular tone over 3 days that is specific to the action of RB6-8C5 on these cells. This change is likely to be the result of local changes in cell signalling pathways rather than a reduction in $O_2^{\bullet-}$-mediated NO inactivation, as previously predicted, since the time taken for systolic BPs to reach a minimum was much longer than would be expected if neutrophil NAD(P)H oxidase were involved. Furthermore, thoracic aortae isolated from neutropenic animals exhibited significantly attenuated vasoconstriction responses to PE compared to controls, providing further evidence for altered vasoregulatory mechanisms in the vasculature of neutropenic animals. Given that NO is thought to be the predominant vasoactive mediator in large conductance vessels under healthy conditions, changes in NO signalling

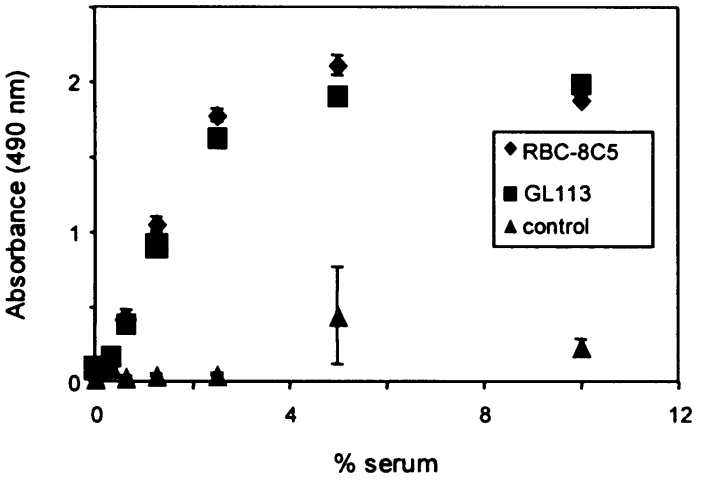

Figure 4.14 RB6-8C5 and GL113 activate complement to a comparable extent *in vitro*

Activation of mouse complement by 0.05 mg antibodies was determined by measuring C3 deposition from mouse serum using an ELISA (n = 3, mean +/- SEM). To determine mouse complement-activating capacity in vitro, plates (96-well, Nunclon Maxisorb) were coated with a Fab fragment of a polyclonal anti-rat IgG (made in-house) at 10μg/ml in carbonate buffer pH 8.0, blocked in PBS containing 0.1% tween-20 (PBS-T) and 1 % BSA, and incubated with mAb RB6-8C5 or the control mAb GL113 at 0.05mg/ml (a dose shown to saturate the capture antibody in preliminary titrations) in PBS-T. Unbound antibody was removed by washing four times in PBS-T. The wells were blotted dry and dilutions of mouse serum in veronal-buffered saline (CFD, Oxoid) added to the wells in triplicate. Plates were incubated for 60 min at 37 °C, washed in PBS-T and incubated with biotinylated rabbit anti-human C3 IgG (10μg/ml in PBS-T; made in-house, known cross-reactivity with mouse C3) for 30 min at 4 °C. After washing, plates were incubated with avidin-peroxidase (BioRad 1:1000 in PBS-T), washed and developed using OPD substrate. Absorbance values were read at 490nm in a Dynex ELISA plate reader (Morgan, unpublished observations).

pathways, such as eNOS upregulation, are the most likely explanation for the changes described above (Shimokawa *et al.* 1996). However, COX-mediated signalling pathways are also known to influence vascular tone, particularly under pathophysiological conditions, so a role for COX-derived vasoactive agents (e.g. PGI_2) in these observations cannot be discounted (Davidge, 2001). With this in mind, the nature of the mechanisms involved in the regulation of vascular tone in the presence and absence of neutrophils will be the subject of intensive investigation in subsequent chapters of this study.

CHAPTER 5

CHARACTERISATION OF NO SIGNALLING PATHWAYS IN THE VASCULATURE OF NEUTROPENIC MICE

5.1 Introduction

In the previous chapter, the induction of neutropenia following injection with RB6-8C5 was shown to cause a transient decrease in systolic BP, which reached significance at day 2 post-injection and a minimum at day 3. The gradual nature of this fall in BP may be indicative of changes in local cell signalling pathways regulating vascular tone rather than a simple decrease in $O_2^{\bullet-}$-mediated NO inactivation, which would result in a more dramatic fall in BP upon neutrophil disappearance between day 0 and day 1. In addition, thoracic aortae isolated from animals depleted of neutrophils for 3 days exhibited significantly attenuated vasoconstriction responses to the α_1-adenoreceptor agonist PE compared to control vessels, providing further evidence for altered vasoregulatory mechanisms in the vasculature of neutropenic animals.

It is well established that NO is the predominant mediator regulating vascular smooth muscle tone in the aorta (Shimokawa *et al.* 1996). However, it is currently not known what effect the onset of neutropenia has on NOS-derived cell signalling in Balb/c mice.

5.1.1 Aims

With this in mind, the aim of this chapter was to characterise local and systemic NO signalling pathways in healthy and neutropenic animals both *in vivo* and in isolated vessel preparations *ex vivo*. This involved:

- The characterisation of NO signalling pathways in isolated aortic sections from healthy and neutropenic animals using various pharmacological inhibitors.

- Measurement of plasma concentrations of the NO breakdown products nitrate and nitrite in plasma samples from control and neutropenic mice (day 3 of neutropenia) using the Griess Reaction.

5.2 Results

5.2.1 Aortae from neutropenic mice exhibit enhanced NO signalling ex vivo *compared to controls*

To compare vasoactivity in healthy and neutropenic animals, myography was conducted on aortic rings from control mice and mice depleted of neutrophils for 3 days, since these animals exhibit the largest differences in systolic BP. Incubation of control aortic rings with the non-specific NOS inhibitor L-NAME (300 μM, 15 min) caused a small but non-significant increase in the maximal vasoconstriction response to PE (434.86 ± 51.89% MAX *versus* 358.01 ± 30.42% MAX, control + L-NAME *versus* control, mean ± SEM, n ≥ 4, Figure 5.1). In contrast, aortae from neutrophil-depleted animals exhibited a significantly enhanced vasoconstriction response to PE in the presence of L-NAME so that it was normalized to control levels (374.38 ± 25.76% MAX *versus* 205.91 ± 30.89% MAX, neutropenic ± L-NAME *versus* neutropenic, mean ± SEM, $p < 0.001$, using two way ANOVA, n ≥ 4, Figure 5.1). These data indicate an increase in NOS-derived NO signalling in aortae of neutropenic mice.

In the absence of pharmacological inhibitors, ACh-induced endothelium-dependent relaxation did not differ between aortae from neutropenic mice and controls, suggesting that eNOS bioactivity is not altered in these tissues upon induction of neutropenia (Figure 5.2). Furthermore, L-NAME caused complete attenuation of endothelium-dependent relaxation in vessels from both control and neutropenic mice,

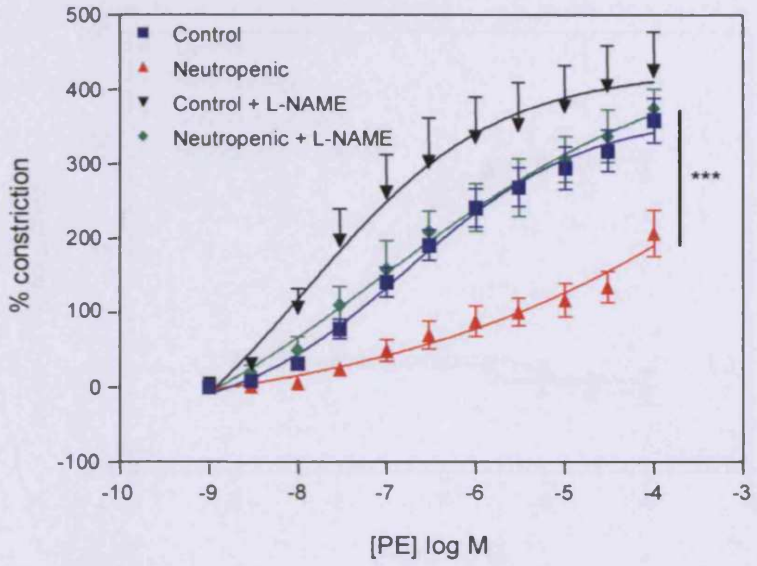

Figure 5.1 Thoracic aortae from neutrophil-depleted mice exhibit enhanced NO signalling compared to controls

PE-induced constriction responses in thoracic aortae from control mice (n = 6, mean ± SEM) and mice depleted of neutrophils for 3 days (n = 4, mean ± SEM) in the presence or absence of L-NAME (300 μM) (n = 4, mean ± SEM). Aortic ring functional responses were determined as described in section 2.2.3.5 in *Materials and Methods*. *** represents $p < 0.001$, neutropenic + L-NAME *versus* neutropenic using two way ANOVA.

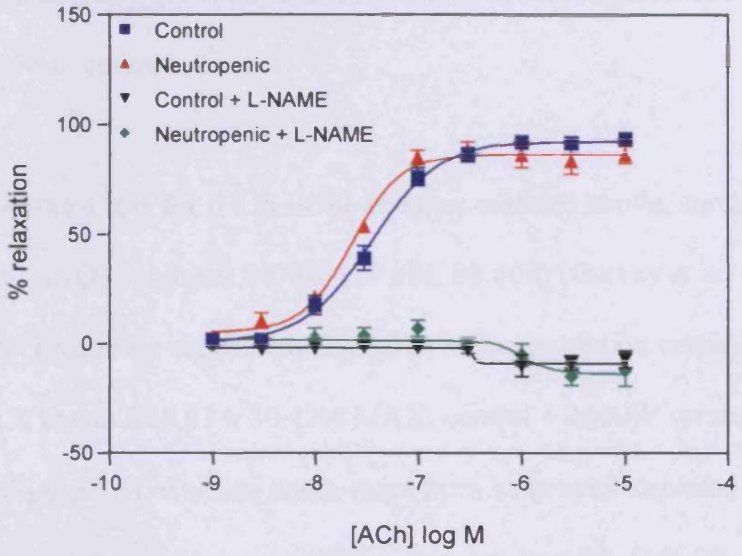

Figure 5.2 Neutropenia does not affect eNOS-derived NO signalling

ACh-relaxation dose-response curves in thoracic aortae from control mice (n = 6, mean ± SEM) and mice depleted of neutrophils for 3 days (n = 4, mean ± SEM) in the presence or absence of L-NAME (300 μM) (n = 4, mean ± SEM). Aortic ring functional responses were determined as described in section 2.2.3.5 of *Materials and Methods*.

demonstrating the NO-dependent nature of this response (Figure 5.2). Similarly, endothelium-independent relaxation responses to DEA-NONOate were also unchanged in neutropenia, in the presence or absence of L-NAME, indicating no change in smooth muscle sensitivity to NO in neutropenic animals (Figure 5.3).

5.2.2 Thoracic aortae from neutrophil-depleted mice exhibit enhanced iNOS bioactivity compared to controls

In order to examine a role for iNOS in the changes outlined above, aortae were incubated with the specific iNOS inhibitor 1400W (10 μM, 30 min) (Garvey *et al.* 1997). In control vessels, 1400W caused no significant change in vasoconstriction response to PE (337.76 ± 36.09% MAX *versus* 358.01 ± 30.42% MAX, control + 1400W *versus* control, mean ± SEM, $n \geq 4$, Figure 5.4), whereas aortic rings from neutrophil-depleted mice exhibited a significant enhancement of vasoconstriction response to PE (366.20 ± 27.55% MAX *versus* 205.91 ± 30.89% MAX, neutropenic + 1400W *versus* neutropenic, mean ± SEM, $p < 0.001$, using two way ANOVA, $n \geq 4$, Figure 5.4), once again being normalized to control levels. These data suggest that an alteration in iNOS bioactivity is responsible for the altered PE-induced vasoconstriction response observed in aortae from neutropenic mice. However, no effect of 1400W on endothelium-dependent or -independent relaxation was observed, indicating that endothelial function and vascular smooth muscle sensitivity is to NO unchanged in neutropenia (Figure 5.5). These findings were confirmed by immunohistochemical detection of iNOS, which showed a clear increase in iNOS in segment from neutropenic animals (Figure 5.7).

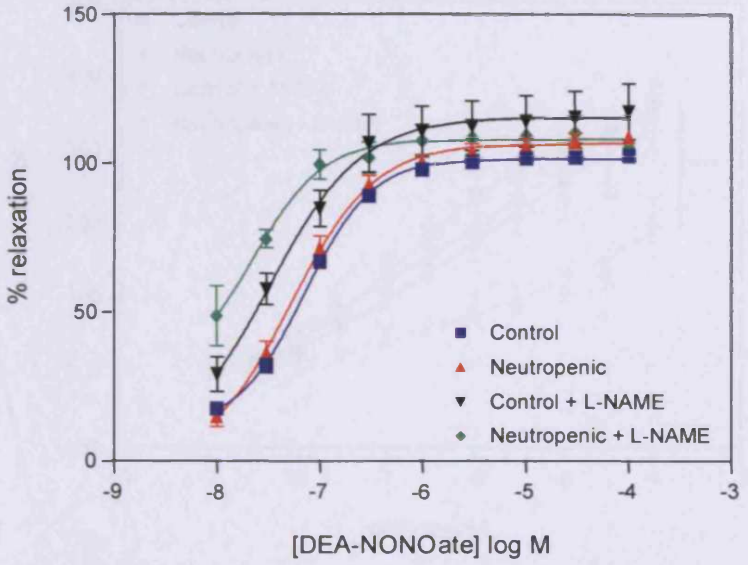

Figure 5.3 Neutropenia does not affect endothelium-independent vasodilation

Dose-response curves to DEA-NONOate in thoracic aortae from control mice (n = 6, mean ± SEM) and mice depleted of neutrophils for 3 days (n = 4, mean ± SEM) in the presence or absence of L-NAME (300 μM) (n = 4, mean ± SEM). Aortic ring functional responses were determined as described in section 2.2.3.5 of *Materials and Methods*.

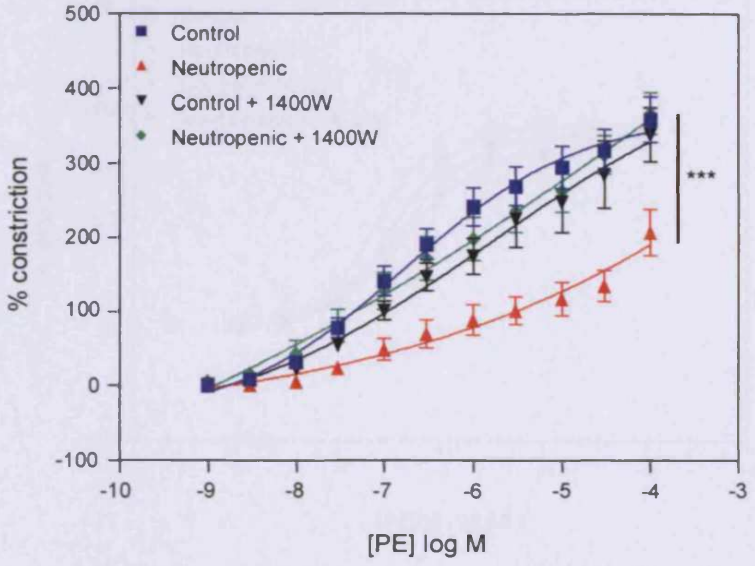

Figure 5.4 Thoracic aortae from neutrophil-depleted mice exhibit enhanced iNOS bioactivity compared to controls

PE-induced constriction responses in thoracic aortae from control mice (n = 6, mean ± SEM) and mice depleted of neutrophils for 3 days (n = 4, mean ± SEM) in the presence or absence of 1400W (10 μM) (n = 4, mean ± SEM). Aortic ring functional responses were determined as described in section 2.2.3.5 of *Materials and Methods*. *** represents $p < 0.001$, neutropenic + 1400W versus neutropenic using two way ANOVA.

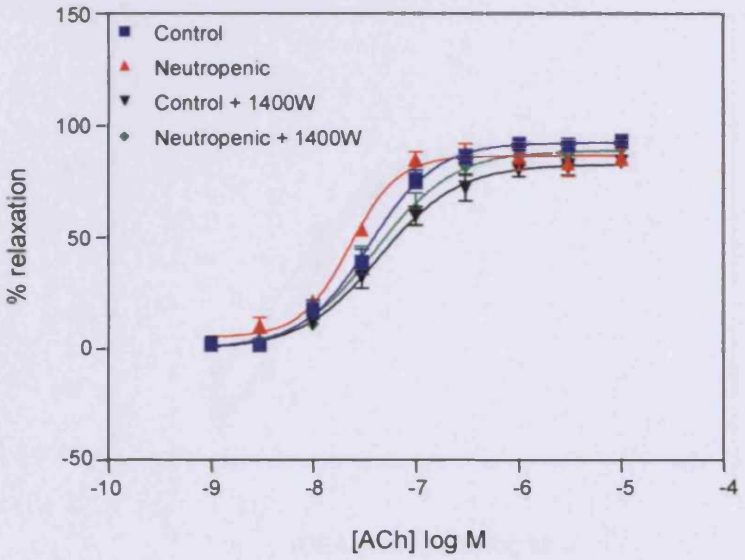

Figure 5.5 Effect of 1400W on endothelial function in isolated thoracic aortae from healthy and neutropenic mice

ACh-relaxation dose-response curves in thoracic aortae from control mice (n = 6, mean ± SEM) and mice depleted of neutrophils for 3 days (n = 4, mean ± SEM) in the presence or absence of 1400W (10 μM) (n = 4, mean ± SEM). Aortic ring functional responses were determined as described in section 2.2.3.5 of *Materials and Methods*.

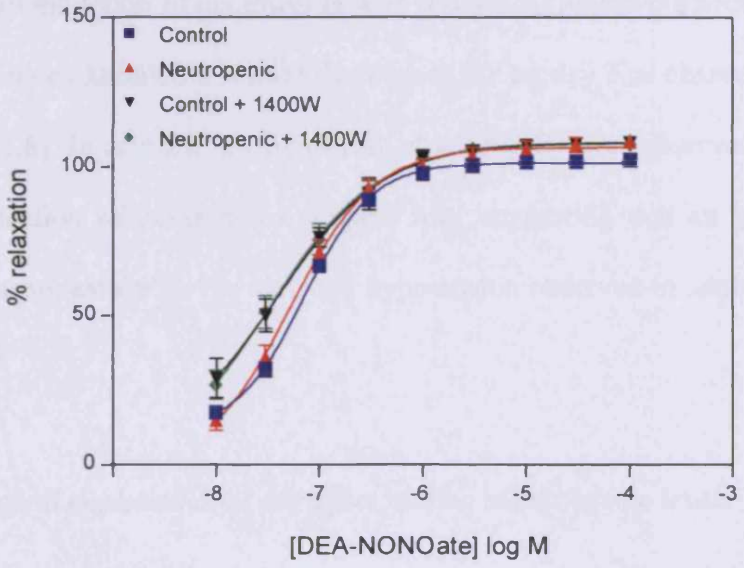

Figure 5.6 Neutropenia does not affect endothelium-independent vasodilation

Dose-response curves to DEA-NONOate in thoracic aortae from control mice (n = 6, mean ± SEM) and mice depleted of neutrophils for 3 days (n = 4, mean ± SEM) in the presence or absence of 1400W (10 μM) (n = 4, mean ± SEM). Aortic ring functional responses were determined as described in section 2.2.3.5 of *Materials and Methods*.

5.2.3 Neutropenia does not alter vascular tone in iNOS$^{-/-}$ mice

To examine the functional relevance of the increased iNOS bioactivity in RB6-8C5-induced neutropenia, BP measurements were taken in WT and iNOS$^{-/-}$ C57Bl/6 mice, with or without induction of neutropenia with RB6-8C5. Following neutrophil depletion, WT C57Bl/6 mice exhibited a similar decrease in BP by day 3 as observed in WT Balb/c mice (Figure 5.8). In contrast, no significant change in BP was observed in iNOS$^{-/-}$ mice following induction of neutropenia (Figure 5.9), suggesting that an increase in iNOS bioactivity is responsible for the transient hypotension observed in neutropenic Balb/c or C57Bl/6 mice.

5.2.4 Neutrophil depletion does not affect plasma nitrate/nitrite levels

In order to determine the extent iNOS upregulation in neutropenia, concentrations of the NO breakdown products nitrite and nitrate were assayed in plasma samples from control and neutropenic mice (day 3 of neutropenia) using the Griess Reaction. No significant differences were observed between the concentrations of either plasma nitrite (1.31 ± 0.05 μM *versus* 1.30 ± 0.02 μM, day 3 *versus* day 0, mean ± SEM) or total nitrate/nitrite (2.22 ± 0.08 μM *versus* 2.30 ± 0.10 μM, day 3 *versus* day 0, mean ± SEM) in samples taken from animals depleted of neutrophils for 3 days compared to controls (Figure 5.10). These data suggest that the observed increase in iNOS expression may be restricted to vasculature tissue.

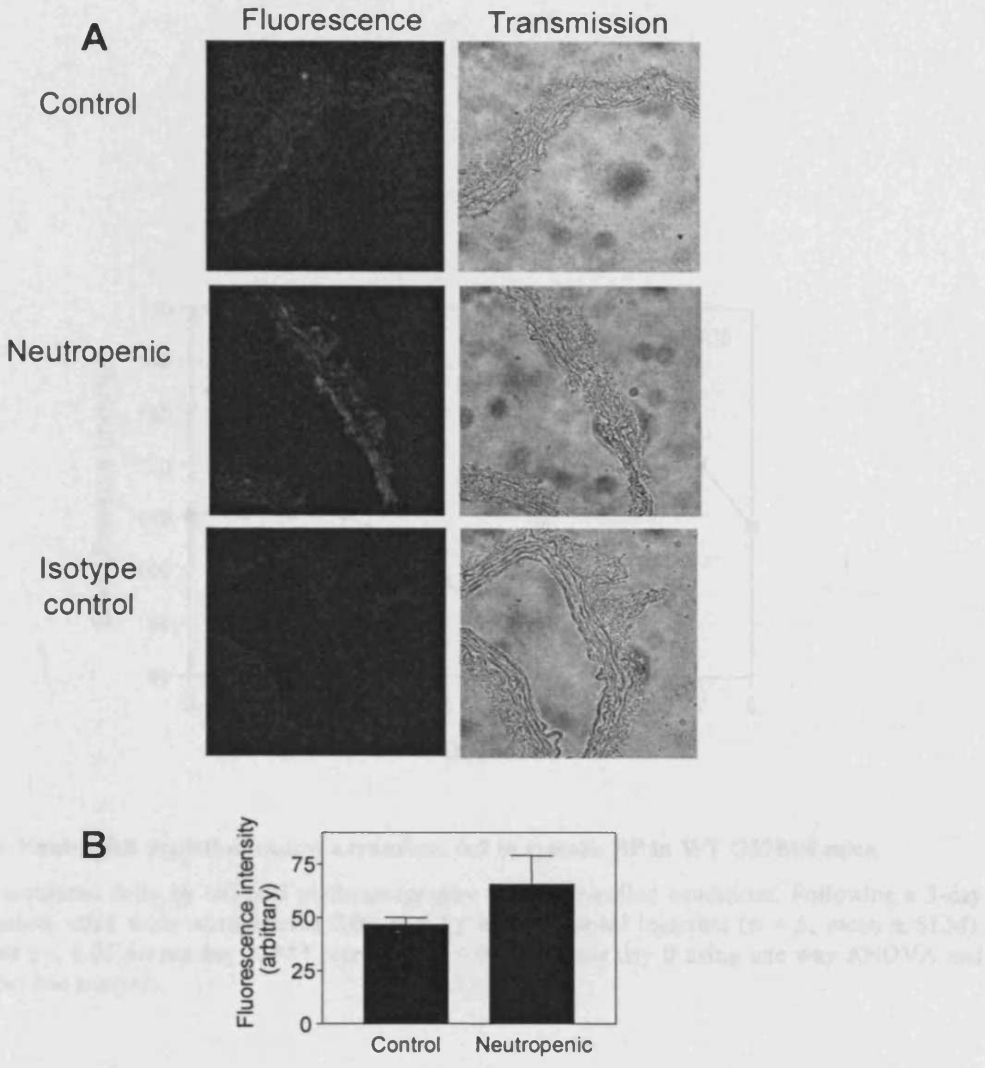

Figure 5.7 Neutropenic mice exhibit elevated aortic iNOS expression *in vivo*

Immunohistological detection of iNOS in aortae from control and neutropenic mice (day 3). *Panel A.* Representative sections are shown for each condition. *Panel B.* For each aorta, five separate sections were imaged and pixel intensity calculated at five separate areas of each section (n = 3). iNOS was visualised using rabbit anti-iNOS (Calbiochem), with goat anti-rabbit IgG-Alexa 568 as secondary. Negative controls used equivalent concentrations of isotype rabbit IgG antibody.

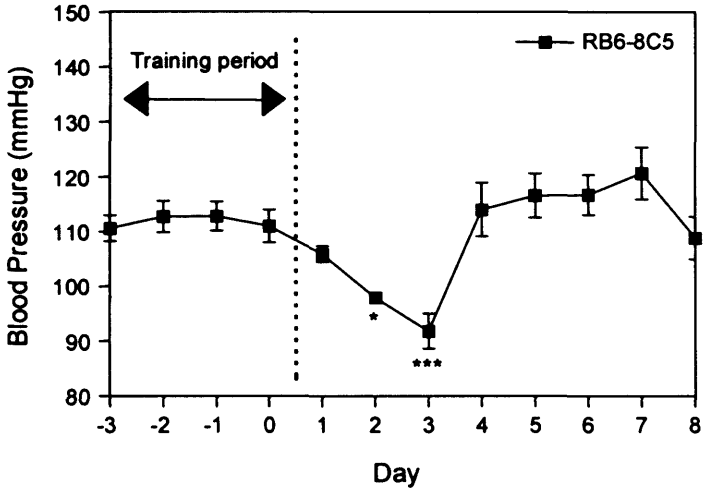

Figure 5.8 Neutrophil depletion causes a transient fall in systolic BP in WT C57Bl/6 mice

BPs were measured daily by tail-cuff plethysmography under controlled conditions. Following a 3-day training period, mice were administered RB6-8C5 by intraperitoneal injection (n = 5, mean ± SEM). * represents $p < 0.05$ *versus* day 0, *** represents $p < 0.001$ *versus* day 0 using one way ANOVA and Dunnett post hoc analysis.

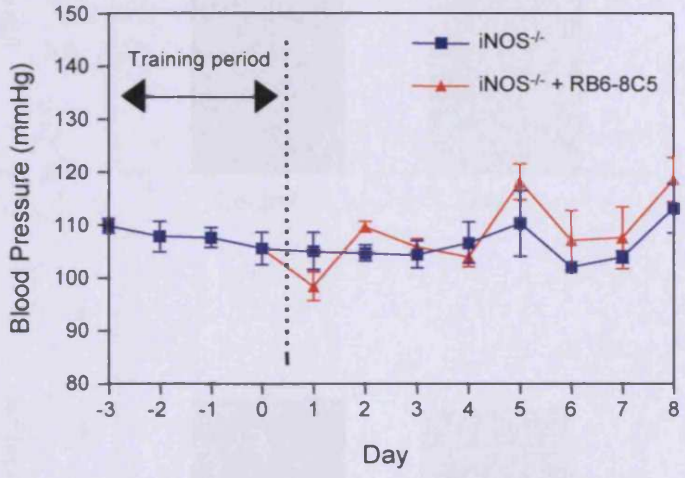

Figure 5.9 Neutrophil depletion does not alter basal blood pressure in iNOS$^{-/-}$ C57Bl/6 mice

BPs were measured daily by tail-cuff plethysmography under controlled conditions. Following a 3-day training period, mice were randomized to receive either RB6-8C5 by intraperitoneal injection or left untreated ($n \geq 4$, mean ± SEM).

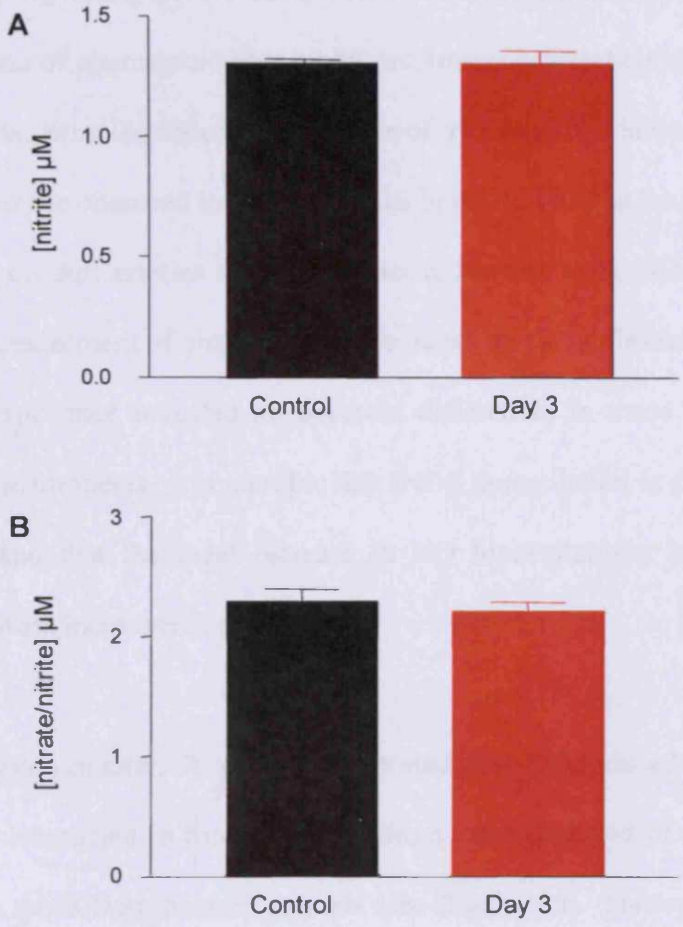

Figure 5.10 Neutrophil depletion does not affect plasma nitrite/nitrate levels

Neutrophil-depleted mice exhibit comparable plasma nitrite/nitrate levels *in vivo*. Plasma nitrite (*A*) and total nitrate/nitrite (*B*) was measured in control and neutropenic mice using the Griess Reaction.

5.3 Discussion

The main finding of this chapter is that thoracic aortae from neutropenic mice exhibit enhanced NO signalling *ex vivo* compared to vessels from healthy animals. Furthermore, through the use of pharmacological inhibitors, immunohistochemistry and gene knockout mice, iNOS has been identified as the source of increased NO in the aortae of neutropenic mice. Whether the observed increase in iNOS bioavailability is limited to the vasculature, or even just conduit arteries such as the aorta, has yet to be fully explored. However, given that measurement of nitrite and nitrate/nitrite levels in plasma samples from control and neutropenic mice revealed no apparent differences in these NO metabolites upon induction or neutropenia, it is possible that iNOS upregulation is at least restricted to the vasculature and that the local increase in NO bioavailability is not sufficient to be reflected in gross increases in plasma NO_x.

In the previous chapter, it was demonstrated that PE-induced vasoconstriction was significantly attenuated in thoracic aortae from mice depleted of neutrophils for 3 days compared to those from healthy animals (see Figure 4.8). Through the use of the non-specific NOS inhibitor L-NAME, we have determined that this altered vasoactivity observed in neutropenia is due to enhanced NO signalling, since PE-induced vasoconstriction was normalized in vessels from neutropenic animals in the presence of this inhibitor (Figure 5.1). The contribution of eNOS-derived NO signalling pathways in these changes was examined by comparing ACh-induced endothelium-dependent relaxation responses in vessels from neutropenic mice and controls, since previous

studies have shown that, following gene transfer, increased eNOS expression in rabbit carotid arteries resulted in significantly enhanced ACh-dependent relaxation in these vessels (Kullo *et al.* 1997; Ooboshi *et al.* 1997). However, in the present study, no differences were observed between endothelium-dependent relaxation responses in vessels from healthy and neutropenic mice, suggesting that eNOS bioactivity is unchanged in vessels from neutropenic animals (Figure 5.2). Furthermore, the complete inhibition of ACh-induced relaxation in aortae from both healthy and neutropenic mice following incubation with L-NAME confirms that endothelium-dependent relaxation is mediated primarily by NO in these tissues rather than by any other vasodilatory mechanism (e.g. PGI_2) (Figure 5.2).

To confirm to that the decreased vascular tone observed in neutropenia is not due to due to sensitization of the vascular smooth muscle to NO, endothelium-independent relaxation responses were examined using the NO donor DEA-NONOate. Comparison of DEA-NONOate concentration-response curves in aortae from healthy and neutropenic mice revealed no differences between these tissues in the presence or absence of L-NAME (Figures 5.3). Thus, smooth muscle sensitivity to NO is unaltered in neutropenia.

In the presence of the specific iNOS inhibitor 1400W, vessels isolated from healthy mice exhibited no change in vasoconstriction response to PE, indicating that iNOS expression in these tissues is negligible or absent (Figure 5.4). In contrast, incubation with 1400W caused vasoconstriction responses to be normalized to control levels in vessels from neutropenic mice, indicating that iNOS bioactivity is upregulated in these tissues. This

was confirmed by immunohistological detection of iNOS in aortic ring sections taken from healthy and neutropenic mice (day 3), which showed a clear increase in iNOS expression following the induction of neutropenia (Figure 5.7).

Inhibition of iNOS with 1400W had no effect on endothelium-dependent relaxation in aortae from either healthy or neutropenic mice (Figure 5.5). This is in contrast to the findings of Chauhan and colleagues (2003) who showed iNOS-associated endothelial dysfunction in the aortae and mesenteric arteries of LPS-treated WT C57Bl/6 mice. Indeed, it is believed that 'high-output' iNOS-derived NO presents a negative feedback mechanism to inhibit the expression and activity of eNOS, possibly by interacting with the haem prosthetic group of NOS and thereby interfering with electron transport and substrate oxygenation within the enzyme (Buga *et al.* 1993; Chauhan *et al.* 2003; Connelly *et al.* 2005). However, the levels of iNOS activity achieved following LPS treatment are likely to far exceed those occurring in the current model, especially since LPS-induced sepsis is often associated with easily measurable increases in plasma levels of NO_2^- and NO_3^- (Gomez-Jimenez *et al.* 1995). Indeed, no such increase was observed in this study, suggesting that iNOS-derived NO bioavailability in neutropenia is insufficient to significantly alter eNOS bioactivity (Figure 5.10).

To determine whether the upregulation of iNOS in the thoracic aorta is responsible for the transient hypotension observed during neutropenia, BP measurements were taken in $iNOS^{-/-}$ mice following administration of RB6-8C5. Due to the fact that all preceding observations regarding neutrophil-depletion in this study had been carried out in Balb/c

mice, direct comparison with iNOS$^{-/-}$ C57Bl/6 mice was not possible. Therefore, initial characterization of RB6-8C5-induced neutropenia in WT C57Bl/6 mice was required. As shown in Figure 5.8, administration of RB6-8C5 in these WT mice resulted in a similar fall in BP by day 3 as observed in Balb/c mice, thus providing a suitable platform for comparison with observations in C57Bl/6 iNOS$^{-/-}$ mice. Importantly, iNOS$^{-/-}$ mice showed no change in BP following induction of neutropenia (Figure 5.9), confirming the importance of iNOS upregulation in the physiological changes observed in neutropenic mice.

A limitation of the current data is that, although increased iNOS bioactivity is evident in the thoracic aortae of these animals, the extent of iNOS expression or activity elsewhere in the vasculature has not been examined. Given the importance of iNOS upregulation in the transient hypotension observed in this model, it is likely that expression of this enzyme is increased in resistance vessels such as those found in the mesentery in addition to conduit vessels. Any increase in NO signalling in these tissues could be quantified using cGMP accumulation as a measure of NOS activity. Alternatively, specific immunohistological detection of iNOS would allow visualization and quantification of enzyme expression in these tissues. In addition to cells within the vasculature, iNOS expression has previously been observed in macrophages, hepatocytes, kidney cells, chondrocytes, cardiac myocytes, pancreatic islets, and fibroblasts (Morris & Billar, 1994), providing the possibility that a more systemic increase in iNOS bioactivity may occur than characterized thus far. Nevertheless, while this cannot be ruled out, the fact

that plasma concentrations of NO metabolites were not found to be altered in this study suggests that iNOS upregulation in neutropenic mice is limited to specific tissues.

Together these data provide substantial evidence for altered iNOS bioactivity in the vasculature of mice rendered neutropenic by administration of RB6-8C5. These changes are, at least in part, responsible for the transient hypotension and defective vasoconstriction response observed in this experimental model. The mechanisms responsible for this increase in vascular iNOS bioactivity have yet to be examined, although it appears that neutrophils may play an important role in regulating iNOS expression in the vasculature of healthy mice. With this in mind, the next chapter of this thesis will examine the specific cell signalling pathways which lead to iNOS expression, and the extent to which these are affected by RB6-8C5-induced neutropenia.

CHAPTER 6

CHARACTERISATION OF MECHANISMS OF iNOS UPREGULATION IN NEUTROPENIC MICE

6.1 Introduction

The previous two chapters have shown that immunodepletion of neutrophils in mice results in onset of hypotension *in vivo* and defective vasoconstriction *ex vivo* due, at least in part, to the upregulation of iNOS bioactivity in the thoracic aortae for these animals. As such, these data suggest that neutrophils play a role in the regulation of iNOS expression under basal conditions. In order to investigate how this occurs, it is important the gain a better understanding of the cell signalling pathways involved in the observed iNOS upregulation.

Traditionally iNOS expression is associated with a generalised or localised inflammatory response resulting from infection or tissue injury (Morris & Billar, 1994). Accordingly, the enzyme is induced by stimulatory cytokines such as IFN-γ, IL-1β, IL-6, and TNF-α and oxidative stress (Kleinert *et al.* 2003). For example, the induction of iNOS protein by LPS is associated with increased TNF-α and IL-1 release following activation of toll-like receptor 4 (Abbas & Lichtman, 2003). In contrast to constitutive NOS isoforms, iNOS bioactivity is primarily regulated at the level of protein expression level by transcriptional and post-transcriptional mechanisms (Morris & Billar, 1994). Indeed, induction of iNOS

expression by inflammatory mediators has been shown to involve a variety of different cell signalling pathways, which lead to transcription factor/iNOS gene binding, depending on the species and cell type involved. In the case of murine cells, the promoter region of the iNOS gene contains several binding sites for nuclear factor κB (NF-κB) and for Jun/Fos heterodimers (collectively known as activating protein (AP)-1), as well as for transcription factors such as CCAAT-enhancer box binding protein (C/EBP), STAT1, and IRF-1 (Hecker *et al.* 1999). Despite the marked species and tissue heterogeneity observed regarding the specific regulation of iNOS expression by inflammatory compounds, in general it seems that iNOS regulatory pathways can be separated into those stimulated by IFN-γ, which involve activation or inhibition of the janus kinase (JAK)/STAT and the IRF-1 pathways, or those stimulated by other inflammatory stimuli, such as the NF-κB pathway (Figure 6.1) (Kleinert *et al.* 2004).

Exposure of cells to IFN-γ results in dimerization of the IFN-γ receptor and activation of cytoplasmatic JAKs that tyrosine-phosphorylate themselves, the IFN-γ receptor and the latent cytoplasmatic STAT transcription factors (Kleinert *et al.* 2004; Schindler & Darnell, 1995). After tyrosine-phosphorylation, the STATs dimerize, translocate to the nucleus and activate STAT-dependent gene expression (Kleinert *et al.* 2004; Schindler & Darnell, 1995). Both iNOS and IRF-1 promoters contain a binding site for STAT1α, and therefore this transcription factor is likely to be essential in the stimulation of iNOS induction, either directly by binding the iNOS promoter at the IFN-γ-activated site (GAS), or indirectly by inducing IRF-1 acitivity (Dell'Albani *et al.* 2001; Samardizic *et al.* 2001) (Figure 6.1). The importance of this activity is demonstrated by the findings of

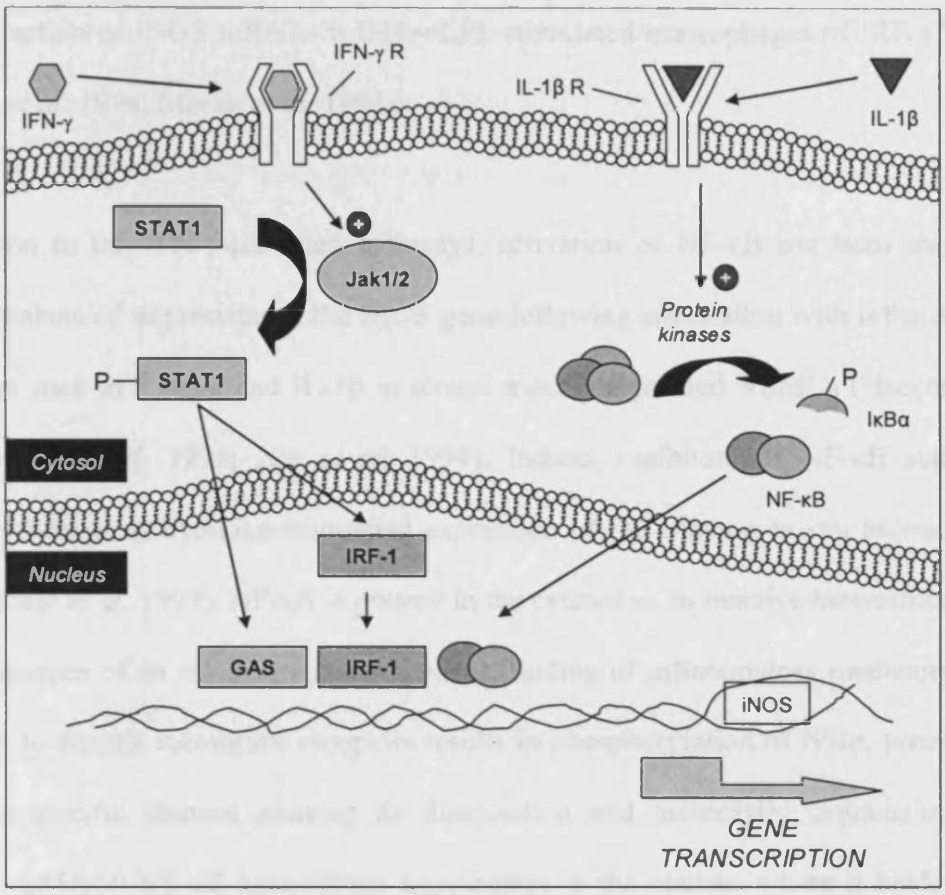

Figure 6.1 Simplified schematic of iNOS induction by IFN-γ and IL-1β pathways in murine cells

Abbreviations: interferon-γ (IFN-γ); IFN-γ receptor (IFN-γ R); janus kinase 1/2 (Jak1/2); signal transducer and activator of transcription 1 (STAT1); interferon regulatory factor 1 (IRF-1); IFN-γ-activated site (GAS); interleukin-1β (IL-1β); IL-1β receptor (IL-1β R); nuclear factor κB (NF-κB); inducible nitric oxide synthase (iNOS). *(Adapted from Bach* et al. *1997; Hecker* et al. *1999; Miljkovic & Trajkovic, 2004).*

Gao and colleagues (1997), who reported that binding of STAT1α to the GAS of the murine iNOS promoter is absolutely required for optimal induction of the iNOS gene in macrophages following IFN-γ and LPS coadministration. Furthermore, the essential role for IRF-1 in IFN-γ-induced iNOS expression has been demonstrated by the absence of NO production or iNOS mRNA in IFN-γ/LPS-stimulated macrophages of IRF-$1^{-/-}$ mice (Kamijo *et al.* 1994; Martin *et al.* 1994).

In addition to the IFN-γ-activated pathways, activation of NF-κB has been shown to mediate enhanced expression of the iNOS gene following stimulation with inflammatory mediators such as TNF-α and IL-1β in mouse macrophages and VSMCs (Hecker *et al.* 1999; Spink *et al.* 1995; Xie *et al.* 1994). Indeed, inhibitors of NF-κB activation effectively suppress cytokine-stimulated expression of the enzyme *in situ* as well as *in vivo* (Hecker *et al.* 1999). NF-κB is present in the cytosol as an inactive heterotrimer, due to the presence of an inhibitory subunit IκBα. Binding of inflammatory mediators, such as IL-1β, to plasma membrane receptors results in phosphorylation of IκBα, presumably via IκBα-specific kinases, causing its dissociation and proteolytic degradation. The activated p65/p50 NF-κB heterodimer translocates to the nucleus where it binds to the corresponding response element in the iNOS promoter (Hecker *et al.* 1999) (Figure 6.1).

Despite evidence for inflammatory mediators activating specific signalling pathways that lead to transcription factor/iNOS promoter binding, these pathways are not mutually exclusive. Indeed, IRF-1 has been shown to facilitate the binding of other transcriptional factors, such as NF-κB, and thus appears to be important in coordinating the effects of a

number of cytokines to achieve regulated gene activation (Spink & Evans, 1997). Furthermore, mutation of the enhancer-linked κB element of the iNOS promoter was shown to significantly reduce responsiveness of the enhancer in mouse macrophages not just to LPS, but to IFN-γ as well, in spite of the fact that NF-κB itself is not activated by IFN-γ (Gao *et al.* 1997). Taken together, these observations suggest that transcription factors interact with the iNOS enhancer in a synergistic fashion, such that the simultaneous binding of all transcription factors leads to optimal iNOS gene expression.

6.1.1 Aims

The induction of neutropenia via RB6-8C5 administration has been associated with the upregulation of vascular iNOS bioactivity. As such, it appears that neutrophils may regulate iNOS expression in the vasculature of healthy animals. To gain a better understanding of the mechanisms by which neutrophils may exert these effects, the mechanisms regulating the induction of iNOS expression were characterised in healthy and neutropenic animals. This involved:

- The non-invasive measurement of systolic BP in control and neutrophil-depleted IFN-$\gamma^{-/-}$ Balb/c mice by tail-cuff plethysmography.

- The characterisation of vasoconstriction, endothelium-dependent and -independent responses of isolated aortic sections from control and neutropenic (day 3) IFN-$\gamma^{-/-}$ Balb/c mice.

- Measurement of STAT1α and IRF-1 induction in nuclear extracts of thoracic aortae from control and neutropenic mice (day 3 of neutropenia) by EMSA.

- Measurement of plasma LPS concentrations in samples from control and neutropenic mice (day 3 of neutropenia) using the LAL test.

6.2 Results

6.2.1 Systolic BP is unaffected by neutropenia in IFN-γ$^{-/-}$ mice

To examine the mechanisms by which neutrophils regulate iNOS expression, systolic BP was measured by in healthy and neutropenic IFN-γ$^{-/-}$ Balb/c mice, which lack one of the pathways that induce iNOS. Following neutrophil-depletion on day 0, IFN-γ$^{-/-}$ mice did not exhibit any change in BP by day 3 (100.55 ± 2.51 mmHg *versus* 99.5 ± 2.06 mmHg, day 3 *versus* day 0, mean ± SEM, n ≥ 6, Figure 6.2) or at any time thereafter. These data indicate that IFN-γ is essential for the transient hypotension observed in RB6-8C5-induced neutropenia in WT mice.

6.2.2 Neutropenia does not affect vasoactivity in IFN-γ$^{-/-}$ mice

In order to compare vasoactivity in healthy and neutropenic IFN-γ$^{-/-}$ Balb/c mice, myography was conducted on aortic rings from these animals. No differences were observed between PE-induced constriction responses of thoracic aortae isolated from healthy animals and those removed on day 3 of neutropenia (404.58 ± 26.79% MAX *versus* 339.77 ± 22.13% MAX, neutropenic *versus* control, mean ± SEM, Figure 6.3). Endothelium-dependent and -independent relaxation responses were also unchanged in vessels from neutropenic IFN-γ$^{-/-}$ mice (Figures 6.4 & 6.5). Therefore, these data suggest that IFN-γ is essential for the upregulation of iNOS in thoracic aortae of neutropenic WT Balb/c mice.

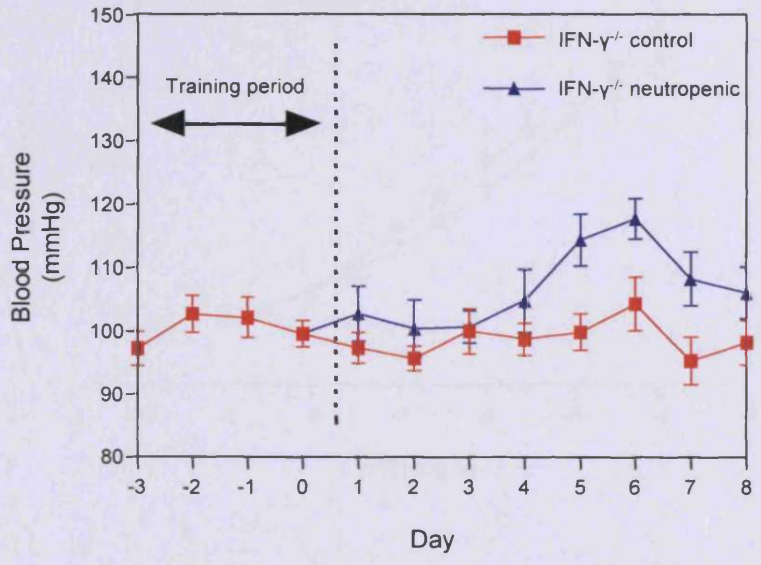

Figure 6.2 Neutropenia does not affect BP in IFN-$\gamma^{-/-}$ mice

BPs were measured daily by tail-cuff plethysmography under controlled conditions. Following a 3-day training period, mice were randomized to receive either RB6-8C5 by intraperitoneal injection or left untreated ($n \geq 6$, mean ± SEM).

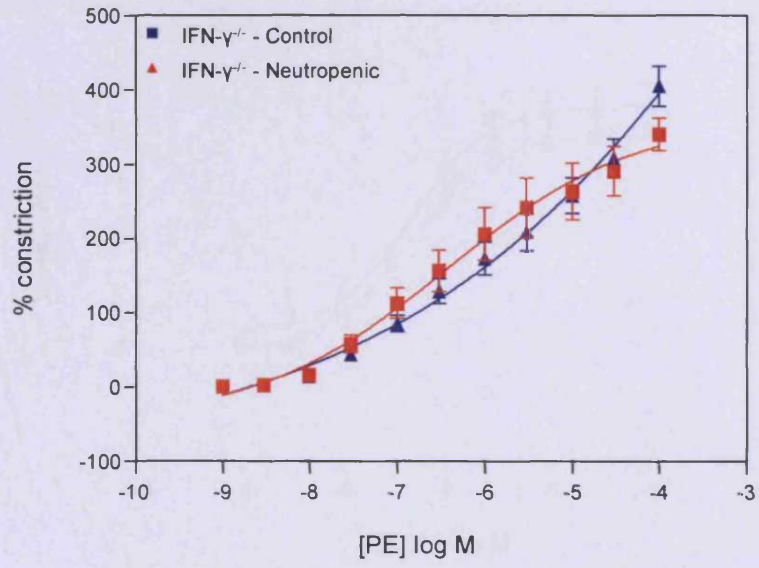

Figure 6.3 Neutropenia does not affect PE-induced vasoconstriction in IFN-$\gamma^{-/-}$ mice

PE-induced dose-responses in thoracic aortae from control IFN-$\gamma^{-/-}$ mice and IFN-$\gamma^{-/-}$ mice depleted of neutrophils for 3 days (n = 6, mean ± SEM). Aortic ring functional responses were determined as described in section 2.2.3.5 of *Materials and Methods*.

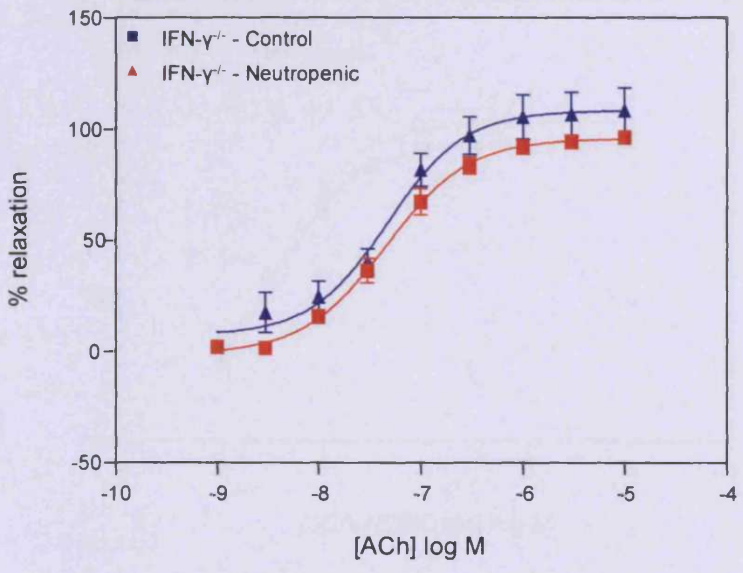

Figure 6.4 Neutropenia does not affect eNOS-derived NO signalling in IFN-$\gamma^{-/-}$ mice

ACh-induced relaxation responses in thoracic aortae from control IFN-$\gamma^{-/-}$ mice and IFN-$\gamma^{-/-}$ mice depleted of neutrophils for 3 days (n = 6, mean ± SEM). Aortic ring functional responses were determined as described in section 2.2.3.5 of *Materials and Methods*.

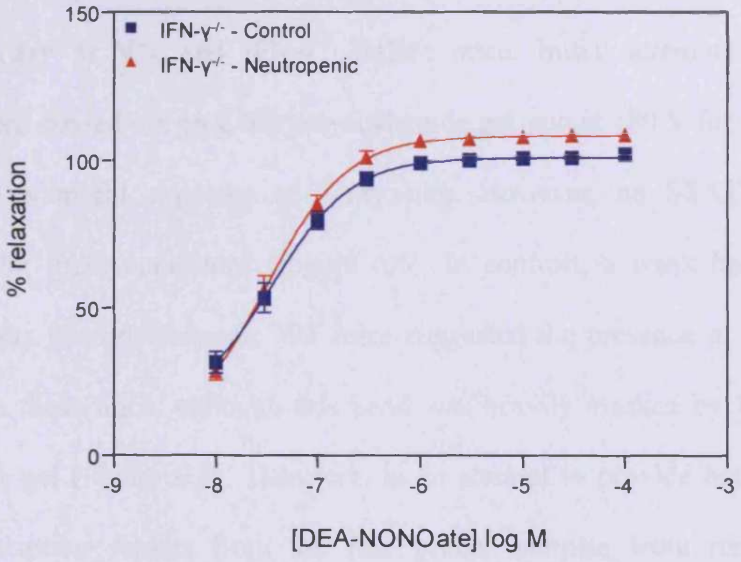

Figure 6.5 Neutropenia does not affect endothelium-independent vasodilation in IFN-$\gamma^{-/-}$ mice

Dose-response curves to DEA-NONOate in thoracic aortae from control IFN-$\gamma^{-/-}$ mice and IFN-$\gamma^{-/-}$ mice depleted of neutrophils for 3 days (n = 6, mean ± SEM). Aortic ring functional responses were determined as described in section 2.2.3.5 of *Materials and Methods*.

6.2.3 Examination of STAT1α and IRF-1 expression in aortae from healthy and neutropenic mice

With the use of double-stranded oligonucleotide probes containing a complementary site for either STAT1α or IRF-1, EMSAs were conducted to assess activity of these transcription factors in nuclear extracts prepared from the thoracic aortae of healthy and neutropenic (day 3) WT and IFN-$\gamma^{-/-}$ Balb/c mice. Initial attempts to detect these molecules were carried out on a 6% polyacylamide gel, run at 180 V for 2 hours 30 mins, followed by overnight exposure to X-ray film. However, no STAT1α activity was observed under these conditions (Figure 6.6). In contrast, a weak band for IRF-1 in nuclear extracts from neutropenic WT mice suggested the presence of this molecule in samples from these mice, although this band was heavily masked by free probe at the bottom of the gel (Figure 6.6). Therefore, in an attempt to provide better separation of bound transcription factors from the free probe, samples were run on either 8% (STAT1α) or 10% (IRF-1) polyacrylamide gels at 180 V for 2 hours 30 mins. However, despite exposure of these gels to X-ray film for 6 hrs, 24 hrs or 4 days, no STAT1α activity was evident in the nuclear extracts tested (Figure 6.7). Similarly, despite the apparent presence of weak band for IRF-1 in nuclear extracts from neutropenic WT mice, the binding of this transcription factor to the probe appears minimal (Figure 6.8). The reason for this is unclear, although it may reflect an insufficient concentration of STAT1α or IRF-1 in each sample, such that levels of these proteins are below the limit of detection.

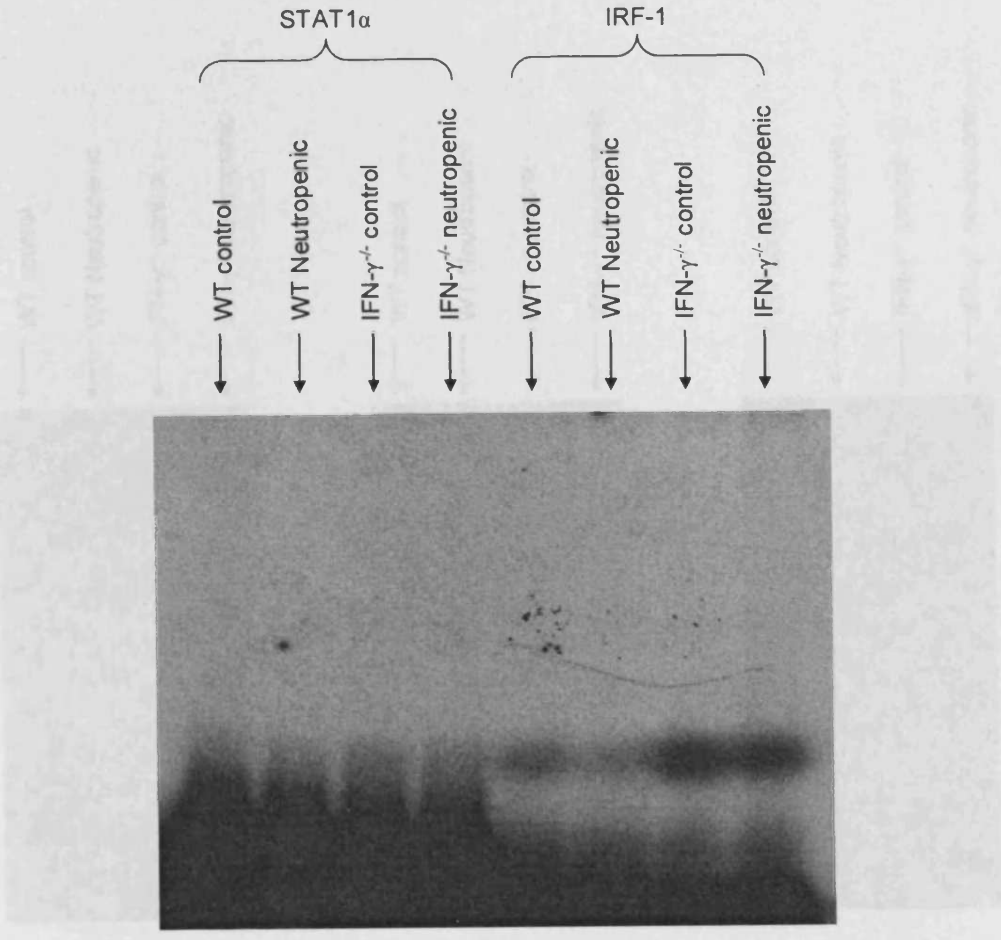

Figure 6.6 Analysis of STAT1α and IRF-1 activity by EMSA

With the use of double-stranded oligonucleotide probes containing a complementary site for either STAT1α or IRF-1, EMSAs were performed on nuclear extracts derived from thoracic aortae of healthy and neutropenic (day 3) WT and IFN-$\gamma^{-/-}$ Balb/c mice. The gel shown is a 6% polyacrylamide gel, run at 180 V for 2 hours 30 mins, followed by overnight exposure to X-ray film.

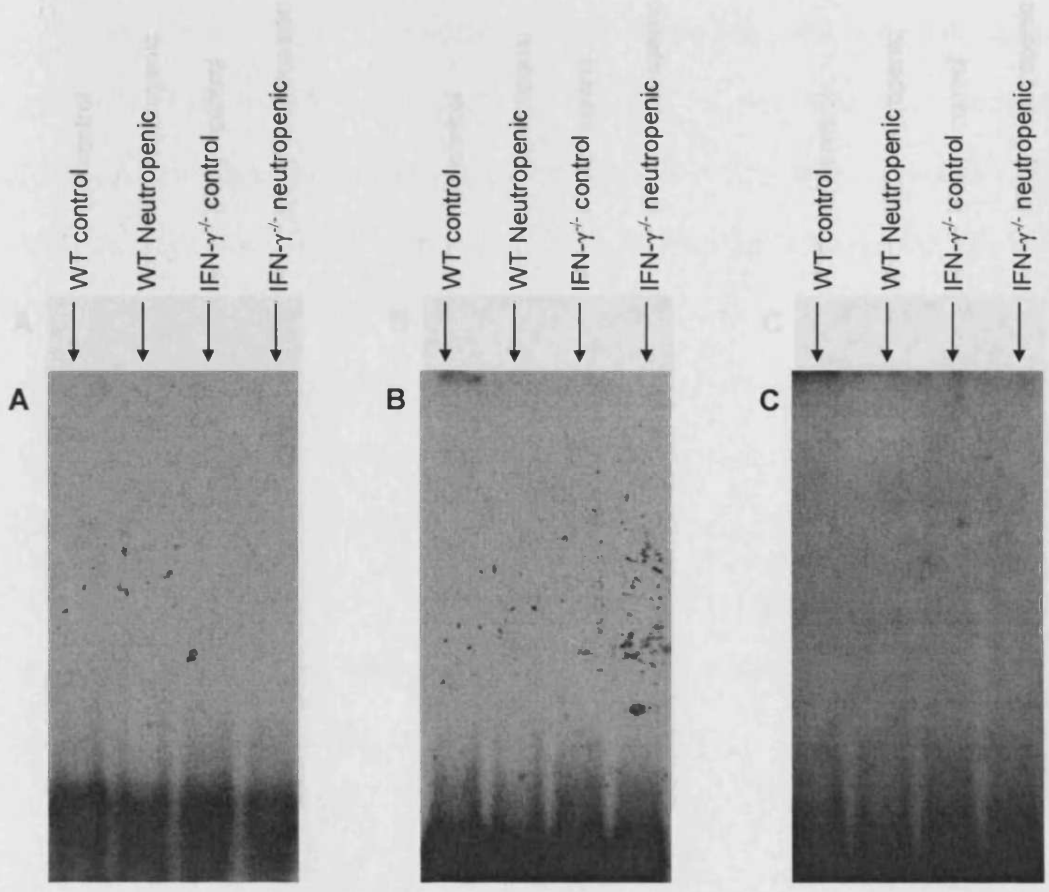

Figure 6.7 Analysis of STAT1α activity by EMSA

With the use of double-stranded oligonucleotide probes containing a complementary site for STAT1α, EMSAs were performed on nuclear extracts derived from thoracic aortae of healthy and neutropenic (day 3) WT and IFN-$\gamma^{-/-}$ Balb/c mice. The gel shown is a 8% polyacrylamide gel, run at 180 V for 2 hours 30 mins, followed by exposure to X-ray film for 6 hours (*panel A*), 24 hours (*panel B*), or 4 days (*panel C*).

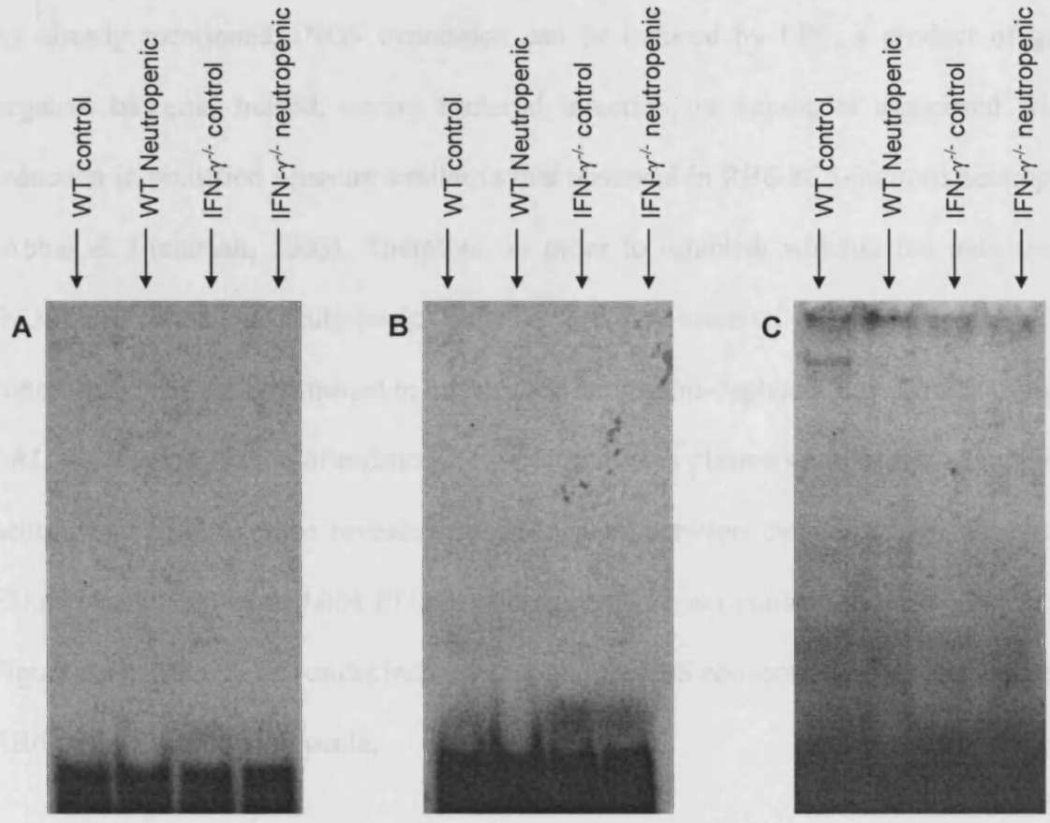

Figure 6.8 Analysis of IRF-1 activity by EMSA

With the use of double-stranded oligonucleotide probes containing a complementary site for IRF-1, EMSAs were performed on nuclear extracts derived from thoracic aortae of healthy and neutropenic (day 3) WT and IFN-$\gamma^{-/-}$ Balb/c mice. The gel shown is a 10% polyacrylamide gel, run at 180 V for 2 hours 30 mins, followed by exposure to X-ray film for 6 hours (*panel A*), 24 hours (*panel B*), or 4 days (*panel C*).

6.2.4 Plasma concentrations of endotoxin (LPS) are not changed in RB6-8C5-induced neutropenia

As already mentioned, iNOS expression can be induced by LPS, a product of gram-negative bacteria. Indeed, severe bacterial infection, or sepsis, is associated with a reduction in perfusion pressure similar to that observed in RB6-8C5-induced neutropenia (Abbas & Lichtman, 2003). Therefore, in order to establish whether the induction of iNOS expression in neutropenic mice is due to bacterial infection, plasma LPS concentrations were determined in healthy and neutrophil-depleted (day 3) mice using the LAL test. Quantification of endotoxin concentrations in plasma samples from healthy and neutropenic (day 3) mice revealed no differences between the groups (0.044 ± 0.003 $EU.ml^{-1}$ *versus* 0.052 ± 0.004 $EU.ml^{-1}$, neutropenic *versus* control, mean ± SEM, n = 5, Figure 6.9). Thus, these results indicate that plasma LPS concentrations are unchanged in RB6-8C5-induced neutropenia.

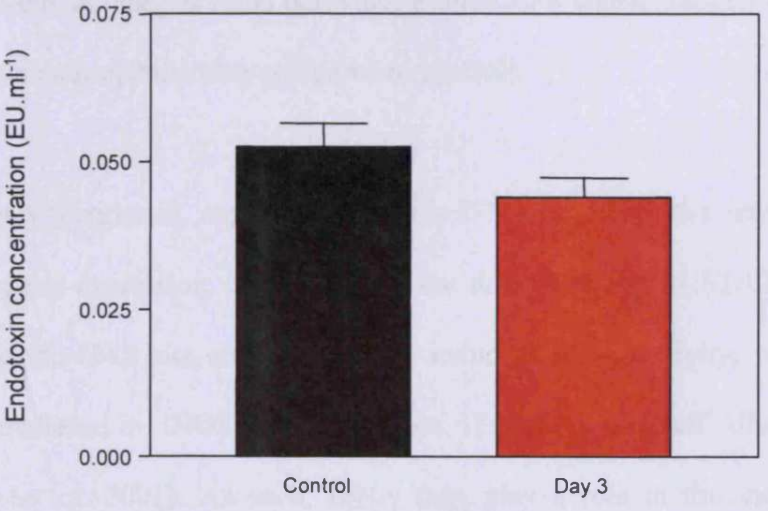

Figure 6.9 Plasma concentration of LPS is not changed in RB6-8C5-induced neutropenia

Plasma endotoxin (LPS) concentration was determined in healthy and neutrophil-depleted (day 3) mice using the LAL test, as described in section 2.2.2.7 of *Materials and Methods* (mean ± SEM, n = 5).

6.3 Discussion

The main finding of this chapter is that IFN-γ is absolutely required for the increased iNOS bioactivity observed in RB6-8C5-induced neutropenia. This is demonstrated by the resistance of IFN-$\gamma^{-/-}$ mice to changes in vascular tone *in vivo* and *ex vivo* following the induction of neutropenia. Furthermore, a role for endotoxemia in the induction of iNOS in WT neutropenic mice is ruled out, since plasma LPS concentrations were found to be unchanged in neutropenic mice compared to controls.

As previously mentioned, exposure of cells to IFN-γ results in the activation of STAT-dependent gene expression, either through the direct binding of STAT1α to the iNOS promoter at the GAS site, or indirectly by inducing IRF-1 activity, which itself is an essential mediator in iNOS gene induction (Figure 6.1) (Dell'Albani *et al.* 2001; Samardizic *et al.* 2001). As such, IFN-γ may play a role in the induction of iNOS expression in RB6-8C5-induced neutropenia. This was examined using mice IFN-$\gamma^{-/-}$ mice. Interestingly, these mice were found to be resistant to the transient hypotension observed in WT mice following RB6-8C5 administration, and PE-induced constriction responses were similar in healthy and neutropenic mice (Figures 6.2 & 6.3). These responses are similar to those already described in iNOS$^{-/-}$ mice *in vivo* or following pharmacological inhibition of iNOS *ex vivo*, respectively (see Chapter 5). Thus, it is reasonable to conclude that IFN-γ signalling is an essential component of the mechanism for iNOS induction in neutropenic animals.

In order to further explore a role for IFN-γ signalling in the upregulation of iNOS in RB6-8C5-induced neutropenia, EMSAs were carried out on nuclear extracts from thoracic aortae of healthy and neutropenic (day 3) WT and IFN-$\gamma^{-/-}$ Balb/c mice, using DNA probes for STAT1α and IRF-1. The EMSA technique is based on the observation that protein-DNA complexes migrate more slowly than free DNA molecules when subjected to non-denaturing polyacrylamide or agarose gel electrophoresis (Tokudome *et al.* 2004); thus binding of the DNA probe containing complementary sequences for STAT1α to this transcription factor will result in retardation of the protein-DNA complex on the gel, while remaining free DNA probe will migrate freely. In this instance, a major advantage of the use of EMSA over other DNA probing techniques (e.g. filter binding assay, footprint analysis) is that the source of the DNA-binding protein may be a crude nuclear or whole cell extract rather than a purified protein preparation, thereby allowing qualitative identification of sequence-specific DNA-binding protein, such as transcription factors, in tissue samples (Fried, 1989; Fried & Crothers, 1981; Fried & Crowthers, 1984; Garner & Revzin, 1981). In this study, DNA probes were radiolabelled with ^{32}P, allowing easy detection by exposure to X-ray film, although alternatively DNA can also be labelled with a biotinylated or hapten-labelled dNPT, then probed and detected using an appropriately sensitive fluorescent or chemiluminescent substrate (Maclean *et al.* 2004; Tokudome *et al.* 2004). In addition to the labelled DNA probe, non-specific competitor DNA (poly dIdC, 1 mg/ml) was included in the binding reaction to minimise the binding of non-specific proteins to the labelled target DNA.

Attempts to detect the presence of STAT1α activity were unsuccessful in all samples from healthy or neutropenic (day 3) WT and IFN-γ Balb/c mice (Figures 6.6 & 6.7). Initial attempts to detect IRF-1 activity in these tissues showed promise, revealing the presence of a strong set of bands, particularly in samples from IFN-$\gamma^{-/-}$ animals (Figure 6.6). However these bands are unlikely to represent IRF-1 activity, since this IFN-inducible transcription factor is likely to be absent in IFN-$\gamma^{-/-}$ mice. Rather, these bands are likely to represent IRF-2 activity, a transcription factor that binds similar DNA motifs to IRF-1 and yet acts as a repressor of IFN-stimulated genes and IRF-1 itself (Paludan *et al.* 1999; Sharf *et al.* 1997). Indeed, a recent study by Teng and colleagues (2002) revealed the presence of similar bands following the detection of IRF-1 in nuclear extracts from cultured rat aortic smooth muscle cells (RASMC) by EMSA. Further attempts to detect IRF-1 activity were equally unsuccessful in all aortae from healthy or neutropenic (day 3) animals (Figure 6.8).

Nevertheless, while STAT1α and IRF-1 appear to be absent in these tissues following 3 days of neutropenia, these molecules may play a role in iNOS induction at an earlier time-point during neutropenia. Indeed, Teng and colleagues (2002) showed that STAT1 and IRF-1 levels in RASMC were increased following just 24 hours treatment with IFN-γ. Furthermore, although the amounts of nuclear extract used in this study are consistent with the methods of Teng *et al.* (2002) (i.e. 5 μg), the current method differs in that nuclear extracts were prepared from whole tissue samples rather than RASMC. As such, the nuclear extracts used in this study will have contained nuclear proteins from cells other than smooth muscle cells, such as adventitial cells and endothelial cells, in

which iNOS expression may not be upregulated. Thus, the lack of STAT1α or IRF-1 binding in the current study may be due to levels of these proteins being below the limit of detection. With this in mind, it is proposed that by using nuclear extracts derived from the thoracic aortae of mice subjected to just 1 or 2 days of neutropenia and by increasing the amount of protein added to each binding reaction, future experiments will demonstrate increased STAT1α and IRF-1 activity in vessels from neutropenic mice compared to controls.

The reduced vascular resistance and increased iNOS bioactivity described in RB6-8C5-induced neutropenia are similar in nature to the symptoms observed in clinical and experimental models of bacterial sepsis (Chauhan *et al.* 2003; Gomez-Jimenez *et al.* 1995). Indeed, the importance of iNOS upregulation in the pathophysiology of this condition has been demonstrated in animal models of bacterial sepsis, in which $iNOS^{-/-}$ mice were protected against contractile hyporeactivitiy of both conduit and resistance arteries induced by administration of LPS, a component of the cell wall of gram-negative bacterial (Chauhan *et al.* 2003). Given the protective role of neutrophils in the immune response to infection, and the similarity of the symptoms of RB6-8C5-induced neutropenia and bacterial sepsis, it is possible that the increased iNOS bioactivity observed in the absence of neutrophils is due to bacterial infection, leading to increased levels of LPS in bloodstream of RB6-8C5-treated mice. Therefore, in order to establish a potential role for LPS in the induction of iNOS expression in neutropenic mice, plasma LPS concentrations were determined in healthy and neutrophil-depleted (day 3) mice using the LAL test.

Quantification of endotoxin concentrations in plasma samples from healthy and neutropenic (day 3) mice revealed no differences between these groups (0.044 ± 0.003 $EU.ml^{-1}$ *versus* 0.052 ± 0.004 $EU.ml^{-1}$, neutropenic *versus* control, mean ± SEM, n = 5, Figure 6.9). Although no measures of endotoxin levels in healthy mice were available, the concentrations measured in this sutdy were in line with those described previously in samples from healthy human volunteers (0.05 ± 0.022 $EU.ml^{-1}$, Nadhazi *et al.* 2002) and well below endotoxin concentrations measured in patients with sepsis (> 0.3 $EU.ml^{-1}$, Bengsch *et al.* 2005). These results indicate that plasma LPS concentrations are unchanged in RB6-8C5-induced neutropenia, thereby ruling out endotoxemia as a cause for the upregulation of iNOS observed in this model.

In conclusion, the data described in this chapter indicate that the IFN-γ signalling is required for the upregulation of iNOS in RB6-8C5-induced neutropenia in Balb/c mice. Thus, while further work is required to fully characterise the signalling pathways induced following the onset of neutropenia, the current data suggest that IFN-γ signalling is likely to be upregulated in neutropenic mice.

The mechanism underlying this increase IFN-γ signalling has yet to be investigated, although it is likely to involve an increase in inflammatory cytokine release by monocytes/macrophages and other immune cells. Such an increase in macrophage IFN-γ release in the absence of neutrophils, may indicate an anti-inflammatory role for neutrophils under basal conditions. The mechanism by which neutrophils may exert this effect is currently unknown, although previous studies have shown that neutrophils may

modulate the actions of other immune cells under certain conditions. For example, Daley and colleagues (2005) have recently postulated that neutrophils may suppress TNF-α and IL-6 release by macrophages in a model of acute sterile inflammation through the release of an unidentified soluble mediator. Similarly, neutrophils have been reported to influence CD4+ Th cell differentiation following infection with *Leishmania major*, promoting the immunosuppressive Th2 response and inhibiting the inflammatory Th1 response (Tacchini-Cottier *et al.* 2000). Although the precise mechanism(s) behind these effects are currently unknown, it has been speculated that neutrophils may act via the secretion of IL-10 and TGF-β (Tacchini-Cottier *et al.* 2000). This is important, as following differentiation, Th2 cells are known to produce IL-4, IL-10 and IL-13 (Yu *et al.* 1998); anti-inflammatory cytokines which suppress various pathways leading to iNOS expression (Ruetten & Thiemermann, 1997). Indeed, IL-4 and IL-10 have been shown to inhibit the production of IFN-γ, IL-1β and TNF-α by LPS-stimulated macrophages, and also to enhance the expression of an endogenous IL-1 receptor antagonist (IL-1ra) (Fiorentino *et al.* 1991; Hart *et al.* 1989; Moore *et al.* 1993; Vannier *et al.* 1992).

However, it is also possible that the observed increase in IFN-γ signalling may simply be a by-product of antibody-mediated neutrophil clearance (i.e. IFN-γ release by phagocytosing macrophages). This could be further investigated by examining the behaviour of isolated monocytes/macrophages in the presence or absence of RB6-8C5-coated synthetic beads. If the pro-inflammatory changes observed in RB6-8C5-induced neutropenia are neutrophil-independent, phagocytosis of RB6-8C5-coated beads would be expected to result in an increased IFN-γ concentration in the cell supernatant, as

measured by enzyme-linked immunosorbant assay (ELISA). In contrast, if IFN-γ levels remained unchanged in the presence of RB6-8C5-coated beads, this would confirm that the vasoactive changes observed in RB6-8C5-treated mice are neutrophil-dependent.

CHAPTER 7

CHARACTERISATION OF COX SIGNALLING PATHWAYS IN THE VASCULATURE OF NEUTROPENIC MICE

7.1 Introduction

While NO is widely regarded as the predominant mediator of vascular tone in conduit vessels under healthy conditions, COX-derived vasoactive agents also play a role under physiological and pathophysiological conditions. Indeed, COX situated in the endothelium or vascular smooth muscle cells, is known to produce both vasorelaxant compounds (e.g. PGI_2), or vasoconstrictors (e.g. PGH_2), depending on stimuli (Davidge, 2001).

Under normal physiological conditions, COX-mediated cell signalling usually promotes vasodilation through the action of eicosanoids such as PGI_2. However, in vascular pathologies an imbalance may occur whereby COX-derived vasoconstrictors become more prominent, leading to impaired vascular function (Davidge, 2001). For example, a recent study by Alvarez and colleagues (2005) examined changes in hypertension by comparing PE-induced vasoconstriction responses of aortic sections taken from spontaneously hypertensive rats (SHR) with those from normotensive Wistar-Kyoto rats (WKY). Aortae from SHR exhibited an increased PE-induced constriction response

compared to controls, which was significantly abrogated by incubation with either dexamethasone, which represses COX-2 mRNA expression, or the selective COX-2 antagonist NS 398, suggesting a greater participation of vasoconstrictor prostanoids (e.g. PGH_2) derived from the inducible COX-2 enzyme in these hypertensive animals compared to control WKY (Alvarez *et al.* 2005).

COX may also influence vascular tone by affecting NO bioavailability either directly or indirectly. For example, COX-1 removes NO *in vitro* through peroxide-dependent catalytic NO consumption, thus providing a novel mechanism by which COX-1 may alter BP and contribute to vascular disease in addition to generation of vasoactive prostanoids and thromboxanes (O'Donnell *et al.* 2000). Alternatively, COX may alter NO bioavailability indirectly by acting as a source of $O_2^{\bullet-}$ anions through its ability to cooxidise substances such as NAD(P)H upon activation (Kukreja *et al.* 1986; Wolin, 2000).

The nature and extent to which COX influences vascular tone is, in part, a factor of the expression and activity of this enzyme. COX-1 is typically expressed in platelets, although some studies suggest that this enzyme is constitutively expressed throughout the vasculature and that its expression can be upregulated by shear stress (Doroudi *et al.* 2000; Wang *et al.* 1993). In contrast, while COX-2 is present in the endothelium due to continuous stimulation of shear stress response elements, expression of this enzyme can be upregulated by inflammatory cytokines (IL-1α, IL-1β), cholesterol, lipoproteins, and hypoxia (Davidge, 2001; Doroudi *et al.* 2000; Guan *et al.* 1998; Toratani *et al.* 1999).

Oxidative stress can also cause changes to COX-derived cell signalling since ROS are known to inactivate PGIS, the enzyme responsible for the formation of PGI_2, leading to accumulation of the vasoconstrictors TxA_2/PGH_2 (Zou *et al.* 2002). Alternatively, ROS or lipid hydroperoxides may activate COX directly, once again resulting in increased levels of vasoconstrictive mediators and a potential change in vascular tone (Ling *et al.* 2005).

Interestingly, NO has been reported to increase COX activity in a variety of cell types, including vascular endothelial cells, although no direct effect of NO on COX activity in isolated enzyme preparations has been demonstrated (Davidge, 1995; Davidge, 2001). Similarly, other studies have shown that NO can either increase or decrease COX activity depending on cell activation state and/or COX isoform (Onodera *et al.* 2000). These findings may be relevant to the altered vascular function seen in the current murine model of neutropenia, particularly given the increased iNOS bioactivity observed in the thoracic aortae of neutropenic mice.

7.1.1 Aims

COX plays an important role in the regulation of vascular tone through the synthesis of vasoactive mediators, and possibly by influencing NO bioavailability. Expression and activity of COX is known to be influenced by both NO and ROS, suggesting that COX-derived cell signalling may be altered according to disease state. Therefore, in light of the enhanced iNOS bioactivity observed in the thoracic aortae of neutropenic mice, the effect of RB6-8C5-induced neutropenia on COX-derived cell signalling pathways was also investigated. This involved:

- Quantification of COX metabolites in urine samples collected from healthy and neutropenic mice, using GC/MS (this technique was kindly carried out by Dr Jason Morrow and Dr Stephanie Sanchez of Vanderbilt Medical Centre, Nashville, TN, USA).

- The characterisation of COX-derived signalling pathways in isolated aortic sections from healthy and neutropenic animals using various pharmacological inhibitors.

7.2 Results

7.2.1 Neutropenic mice exhibit increased levels of 2,3-dinor-6-keto $PGF_{1\alpha}$ in urine

In order to examine the effect of RB6-8C5-induced neutropenia on COX-derived signalling pathways *in vivo*, GC/MS was used to detect and quantify the presence of the PGI_2 metabolite 2,3-dinor-6-keto $PGF_{1\alpha}$ in mouse urine collected over a 24-hour period from healthy and neutropenic (day 2½ to day 3½) mice. Levels of this metabolite were significantly greater in urine from neutropenic animals (7.30 ± 0.94 $ng.mgCr^{-1}$ *versus* 4.19 ± 1.04 $ng.mgCr^{-1}$, neutropenic *versus* control, mean ± SEM, $p < 0.05$, using one-tailed student t test, $n \geq 3$, Figure 7.1) indicating an increase in systemic PGI_2 synthesis in neutropenia.

7.2.2 Aortae from neutropenic mice exhibit decreased COX bioactivity compared to controls

In order to investigate the potential source of the systemic increase in PGI_2 described above, and the importance of COX-derived cell signalling in the regulation vascular tone regulation in aortic rings from healthy and neutropenic animals, myography was conducted on aortae from healthy and neutropenic (day 3) mice in the presence or absence of various pharmacological inhibitors. Incubation of control aortic rings with the non-specific COX inhibitor indomethacin (10 μM, 30 min) caused a significant reduction in the maximal vasoconstriction response to PE (222.45 ± 25.18% MAX *versus* 358.01 ±

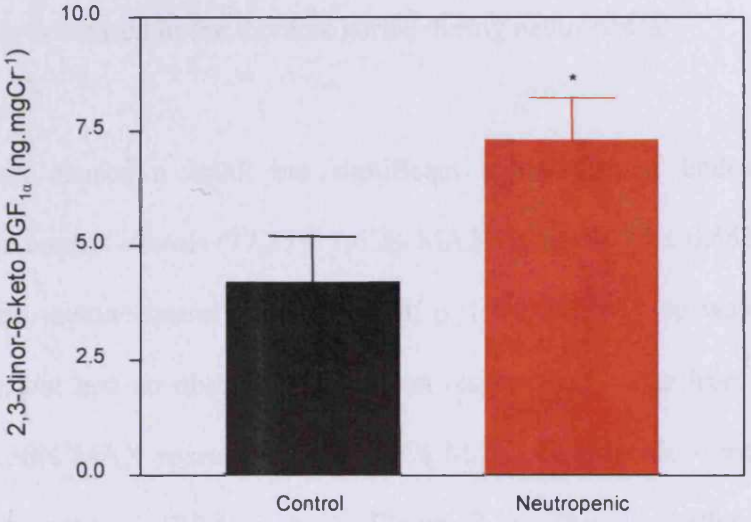

Figure 7.1 Mice exhibit an increase in 2,3-dinor-6-keto $PGF_{1\alpha}$ in urine during neutropenia

Concentration of 2,3-dinor-6-keto $PGF_{1\alpha}$ in urine of healthy and neutropenic animals ($n \geq 3$, mean ± SEM), detected by GC/MS. * represents $p < 0.05$, using one-tailed student t-test.

30.42% MAX, control + indomethacin *versus* control, mean ± SEM, $p < 0.05$, using two way ANOVA, $n \geq 4$, Figure 7.2). These data suggest that COX promotes vasoconstriction in healthy mice, perhaps through the release of PGH_2. In contrast, indomethacin did not significantly alter the vasoconstriction response to PE in aortae taken from neutrophil-depleted animals (185.33 ± 11.32% MAX *versus* 205.91 ± 3.89% MAX, neutropenic + indomethacin *versus* neutropenic, mean ± SEM, $n \geq 4$, Figure 7.2), suggesting that COX bioactivity is decreased in the thoracic aortae during neutropenia.

Indomethacin caused a small but significant attenuation of endothelium-dependent relaxation in control vessels (77.35 ± 1.63% MAX *versus* 93.25 ± 0.56% MAX, control + indomethacin *versus* control, mean + SEM, $p < 0.05$, using two way ANOVA, $n \geq 4$, Figure 7.3), but had no observable effect on responses of rings from neutropenic mice (99.37 ± 0.56% MAX *versus* 85.81 ± 3.88% MAX, neutropenic + indomethacin *versus* neutropenic, mean ± SEM, $n \geq 4$, Figure 7.3). This is further demonstrated in representative experimental traces of ACh-induced relaxation responses in the presence or absence of indomethacin (Figure 7.4). Once again, these data indicate a decrease COX-signalling in neutropenia, although in controls COX appears to promote vasodilation rather than vasoconstriction. However, given that preconstruction with PE was significantly attenuated in aortic rings from neutropenic mice (Figure 7.4), the reliability of this relaxation data is questionable. Furthermore, this contradicts the action of COX observed in PE-induced vasoconstriction above, and may indicate that the nature of COX-derived signalling pathways is dependent on the assay used.

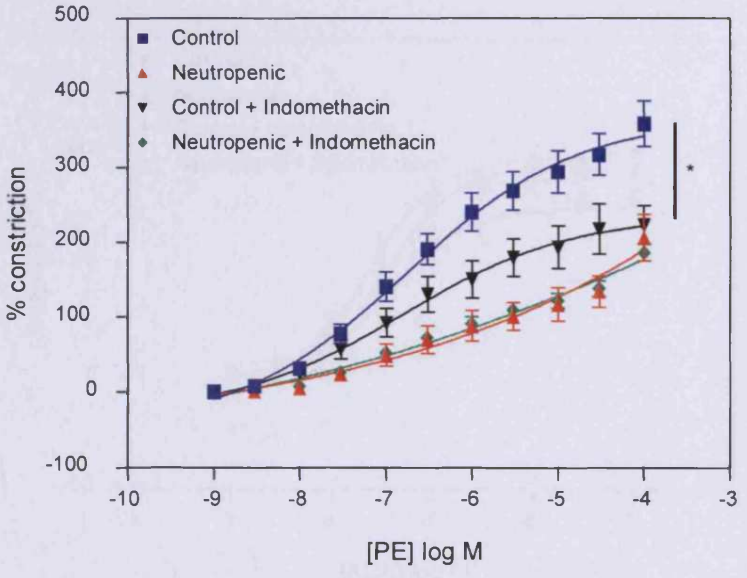

Figure 7.2 Thoracic aortae from neutrophil-depleted mice exhibit decreased COX bioactivity compared to controls

PE-induced constriction responses in thoracic aortae from control mice (n = 6, mean ± SEM) and mice depleted of neutrophils for 3 days (n = 4, mean ± SEM) in the presence or absence of indomethacin (10 μM) (n = 4, mean ± SEM). Aortic ring functional responses were determined as described in section 2.2.3.5 of *Materials and Methods*. * represents $p < 0.05$, control + indomethacin *versus* control using two way ANOVA.

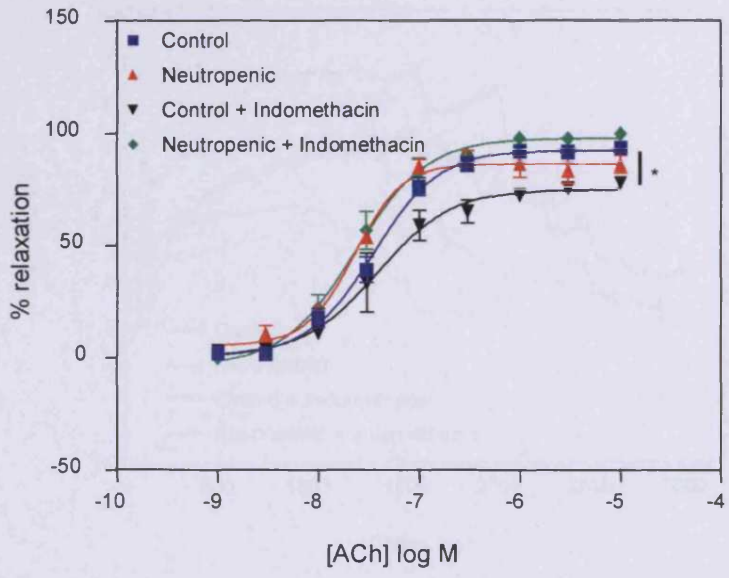

Figure 7.3 Effect of indomethacin on ACh-induced endothelium-dependent relaxation in aortae from healthy and neutropenic animals

ACh-relaxation dose-response curves in thoracic aortae from control mice (n = 6, mean ± SEM) and mice depleted of neutrophils for 3 days (n = 4, mean ± SEM) in the presence or absence of indomethacin (10 μM) (n = 4, mean ± SEM). Aortic ring functional responses were determined as described in section 2.2.3.5 of *Materials and Methods*. * represents $p < 0.05$, neutropenic + indomethacin *versus* neutropenic using two way ANOVA.

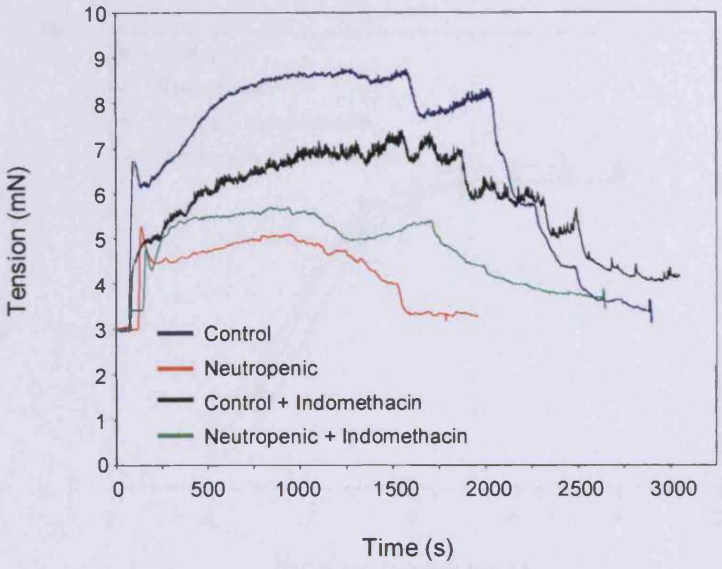

Figure 7.4 Representative experimental traces of endothelium-dependent relaxation in aortic rings from control and neutropenic mice in the presence or absence of indomethacin

Representative ACh-relaxation dose-response curves of thoracic aortae from control mice and mice depleted of neutrophils for 3 days in the presence or absence of indomethacin (10 μM). Aortic ring functional responses were determined as described in section 2.2.3.5 of *Materials and Methods*.

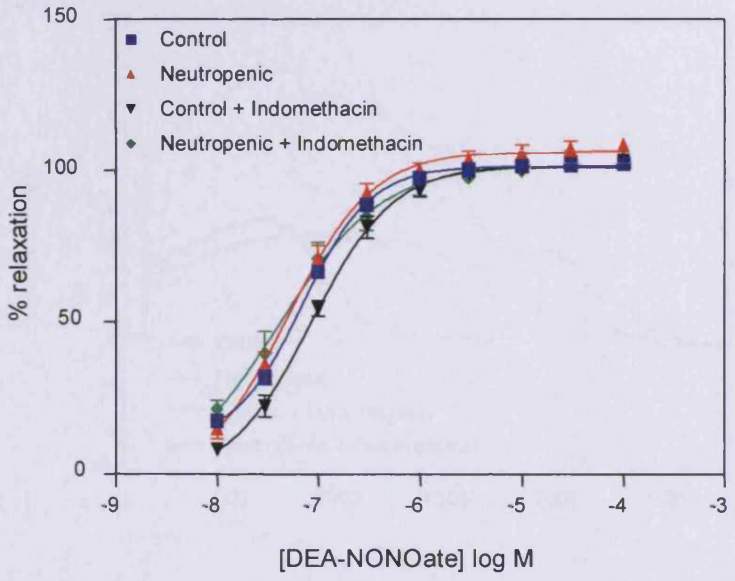

Figure 7.5 Neutropenia does not affect endothelium-independent vasodilation

Dose-response curves to DEA-NONOate in thoracic aortae from control mice (n = 6, mean ± SEM) and mice depleted of neutrophils for 3 days (n = 4, mean ± SEM) in the presence or absence of indomethacin (10 μM) (n = 4, mean ± SEM). Aortic ring functional responses were determined as described in section 2.2.3.5 of *Materials and Methods*.

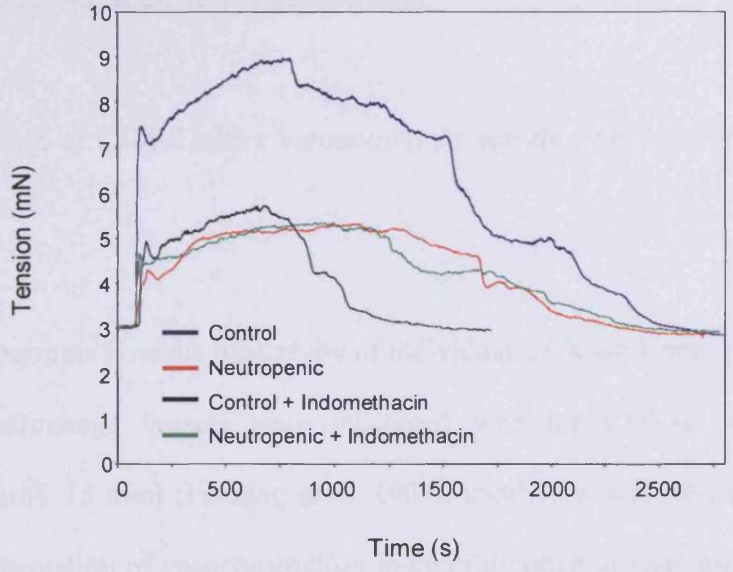

Figure 7.6 Representative experimental traces of endothelium-independent relaxation in aortic rings from control and neutropenic mice in the presence or absence of indomethacin

Representative DEA-NONOate-relaxation dose-response curves of thoracic aortae from control mice and mice depleted of neutrophils for 3 days in the presence or absence of indomethacin (10 μM). Aortic ring functional responses were determined as described in section 2.2.3.5 of *Materials and Methods*.

Finally, no differences in endothelium-independent relaxation to DEA-NONOate were evident following incubation with indomethacin in either group (Figure 7.5 and Figure 7.6), indicating that COX bioactivity has no effect on sGC/cGMP signalling pathways in either condition. However, given that preconstruction with PE was significantly attenuated in aortic rings from neutropenic mice (Figure 7.4 and Figure 7.6), the reliability of this relaxation data is questionable.

7.2.3 Inhibition of COX-2 alters vasoactivity in vessels from control and neutropenic mice

In order to determine how the bioactivity of individual COX isoforms was affected by the onset of neutropenia, vessels were incubated with the COX-2 selective inhibitor celecoxib (1 μM, 15 min) (Penning *et al.* 1997). Incubation with this inhibitor caused a significant attenuation of vasoconstriction in control aortae in response to PE (185.57 ± 25.92% MAX *versus* 358.01 ± 30.42% MAX, control + celecoxib *versus* control, mean ± SEM, $n \geq 3$, $p < 0.001$, Figure 7.7), and aortic rings from neutrophil-depleted animals demonstrated a similar trend (67.91 ± 21.83% MAX *versus* 205.91 ± 30.89% MAX, neutropenic + celecoxib *versus* neutropenic, mean ± SEM, $p < 0.01$, $n \geq 3$, Figure 7.7). These data suggest that COX-2 promotes vasoconstriction in the thoracic aortae of both control and neutropenic mice.

No effect of celecoxib on endothelium-dependent relaxation was observed in either group (healthy and neutropenic) (Figure 7.8), suggesting that COX-2 does not play a role in this

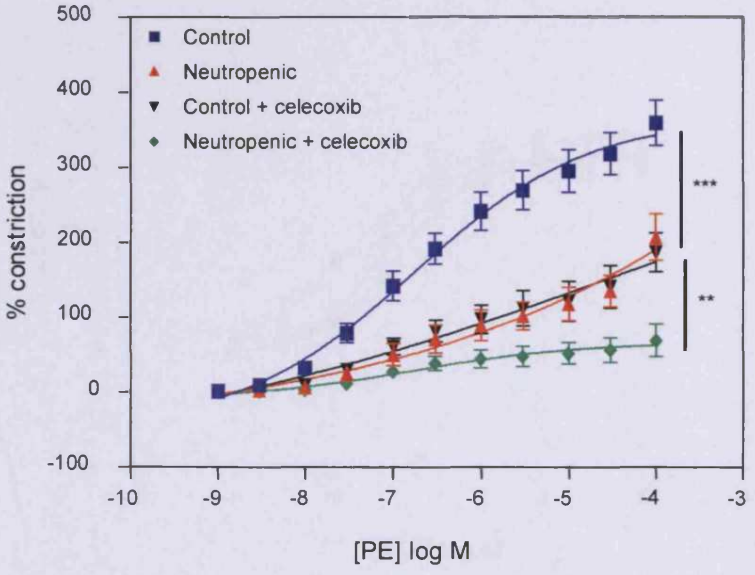

Figure 7.7 Effect of celecoxib on PE-induced constriction responses in vessels from healthy and neutropenic animals

Phenylephrine-induced constriction responses in thoracic aortae from control mice (n = 6, mean ± SEM) and mice depleted of neutrophils for 3 days (n = 4, mean ± SEM) in the presence or absence of celecoxib (1 μM) (n ≥ 3, mean ± SEM). Aortic ring functional responses were determined as described in section 2.2.3.5 of *Materials and Methods*. *** represents $p < 0.001$, control + celecoxib *versus* control using two way ANOVA; ** represents $p < 0.01$, neutropenic + celecoxib *versus* neutropenic using two way ANOVA.

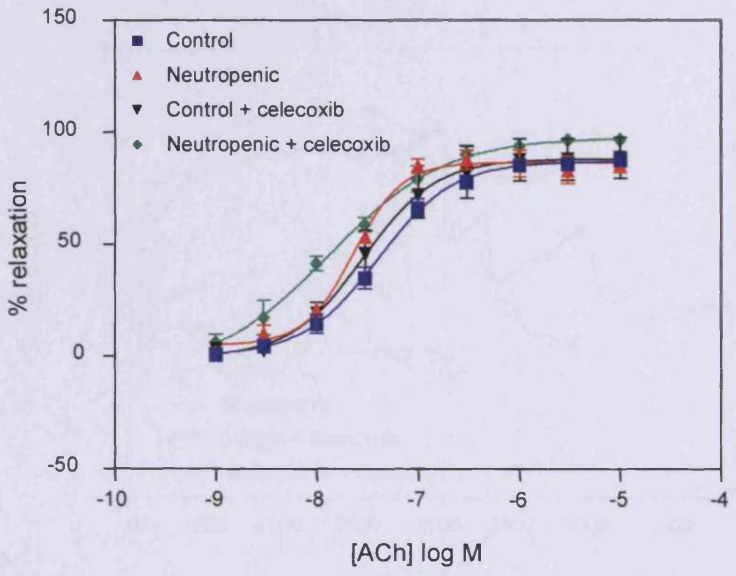

Figure 7.8 Effect of celecoxib on ACh-induced relaxation responses in vessels from healthy and neutropenic animals

ACh-relaxation dose-response curves in thoracic aortae from control mice (n = 6, mean ± SEM) and mice depleted of neutrophils for 3 days (n = 4, mean ± SEM) in the presence or absence of celecoxib (1 μM) (n ≥ 3, mean ± SEM). Aortic ring functional responses were determined as described in section 2.2.3.5 of *Materials and Methods*.

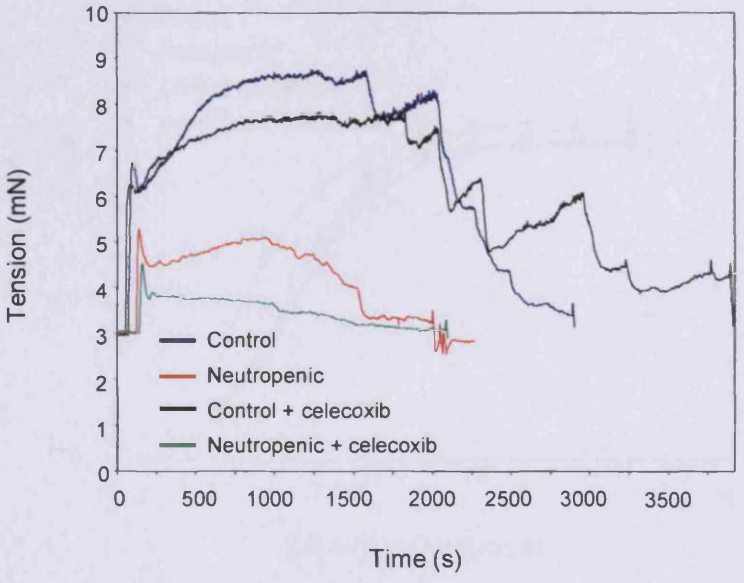

Figure 7.9 Representative experimental traces of endothelium-dependent relaxation in aortic rings from control and neutropenic mice in the presence or absence of celecoxib

Representative ACh-relaxation dose-response curves of thoracic aortae from control mice and mice depleted of neutrophils for 3 days in the presence or absence of celecoxib (1 μM). Aortic ring functional responses were determined as described in section 2.2.3.5 of *Materials and Methods*.

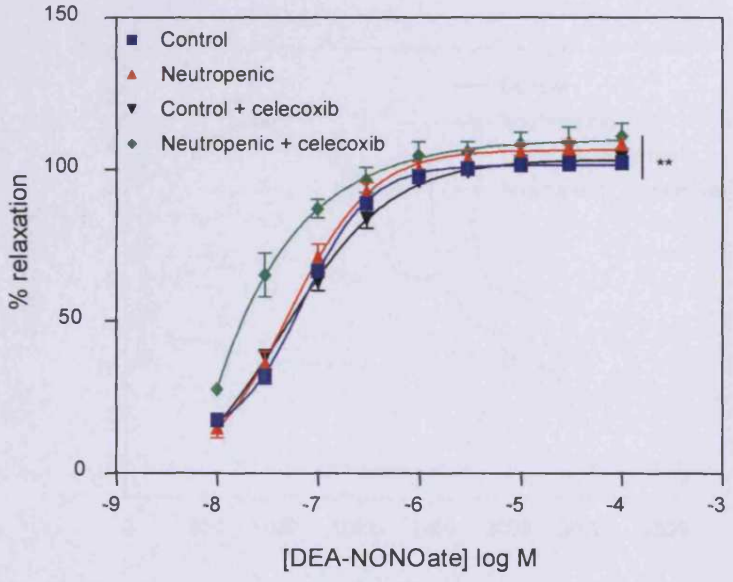

Figure 7.10 Effect of celecoxib on endothelial-independent relaxation in vessels from healthy and neutropenic animals

Dose-response curves to DEA-NONOate in thoracic aortae from control mice (n = 6, mean ± SEM) and mice depleted of neutrophils for 3 days (n = 4, mean ± SEM) in the presence or absence of celecoxib (1 μM) (n ≥ 3, mean ± SEM). Aortic ring functional responses were determined as described in section 2.2.3.5 of *Materials and Methods*. ** represents $p < 0.01$, neutropenic + celecoxib *versus* control + celecoxib using two way ANOVA.

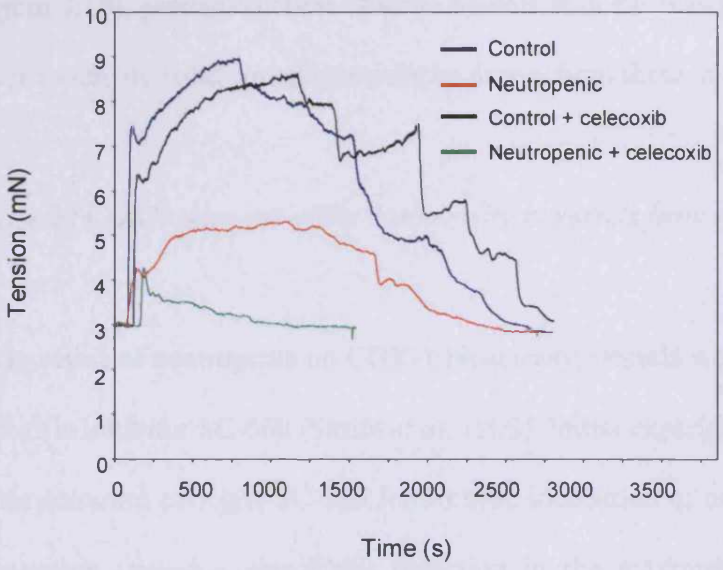

Figure 7.11 Representative experimental traces of endothelium-independent relaxation in aortic rings from control and neutropenic mice in the presence or absence of celecoxib

Representative DEA-NONO-relaxation dose-response curves of thoracic aortae from control mice and mice depleted of neutrophils for 3 days in the presence or absence of celecoxib (1 μM). Aortic ring functional responses were determined as described in section 2.2.3.5 of *Materials and Methods*.

response. However, given that preconstruction with PE was significantly attenuated in aortic rings from neutropenic mice in the presence of celecoxib (Figure 7.9), the reliability of this endothelium-dependent relaxation data is questionable. Similarly, while vessels from neutropenic animals appeared to show a leftward shift in endothelium-independent vasodilation response to DEA-NONOate following incubation with celecoxib (Figure 7.10), preconstruction of these vessels with PE was severely inhibited (Figure 7.11). As such, no solid conclusions can be drawn from these data.

7.2.4 Inhibition of COX-1 does not affect vasoactivity in vessels from neutropenic mice

To determine to effect of neutropenia on COX-1 bioactivity, vessels were incubated with the COX-1-specific inhibitor SC-560 (Smith *et al.* 1998). Initial experiments were carried out using a concentration of 1 μM SC-560 for 30 min. Incubation of control aortic rings at this concentration caused a significant reduction in the maximal vasoconstriction response to PE (178.37 ± 30.94% MAX *versus* 358.01 ± 30.42% MAX, control + 1 μM SC-560 *versus* control, mean ± SEM, $p < 0.001$, using two way ANOVA, $n \geq 4$, Figure 7.12). In contrast, 1 μM SC-560 did not significantly alter the vasoconstriction response to PE in aortae taken from neutrophil-depleted animals (192.42 ± 38.35% MAX *versus* 205.91 ± 3.89% MAX, neutropenic + 1 μM SC-560 *versus* neutropenic, mean ± SEM, $n \geq 4$, Figure 7.12). This was unexpected, given that the responses observed with indomethacin and celecoxib, described above, seemed to predict a vasodilatory role for COX-1 products in the vasculature of healthy and neutropenic mice.

The IC_{50} values of SC-560 when tested on recombinant enzymes were found to be 9 nM for COX-1 and 6.3 μM for COX-2 (Smith *et al.* 1998). Therefore, while a dose of 1 μM should retain selectivity for COX-1, a second, lower dose of 100 nM SC-560 was also used to ensure that the effects of this inhibitor were not due to non-specific inhibition of COX-2. Incubation of control aortic rings at this concentration also caused a significant reduction in the maximal vasoconstriction response to PE (216.16 ± 14.40% MAX *versus* 358.01 ± 30.42% MAX, control + 100 nM SC-560 *versus* control, mean ± SEM, $p < 0.001$, using two way ANOVA, $n \geq 4$, Figure 7.13). Furthermore, 100 nM SC-560 did not significantly alter the vasoconstriction response to PE in aortae taken from neutrophil-depleted animals (235.86 ± 12.99% MAX *versus* 205.91 ± 3.89% MAX, neutropenic + 100 nM SC-560 *versus* neutropenic, mean ± SEM, $n \geq 4$, Figure 7.13). These data suggest that COX-1 bioactivity is decreased or absent in aortae from neutropenic mice, and that COX-1 promotes vasoconstriction in control vessels undergoing stimulation with PE.

No effect on endothelium-dependent relaxation was observed with either concentration of SC-560 in vessels from healthy or neutropenic mice (Figures 7.14, Figure 7.15 and Figure 7.16), suggesting that COX-1 bioactivity is not a contributing factor to endothelial function in these animals.

In the presence of 1 μM SC-560, vessels from both control and neutropenic animals showed a significant leftward shift in endothelium-independent vasodilation response to DEA-NONOate relative to untreated tissue (Figure 7.17 and Figure 7.18). This trend was

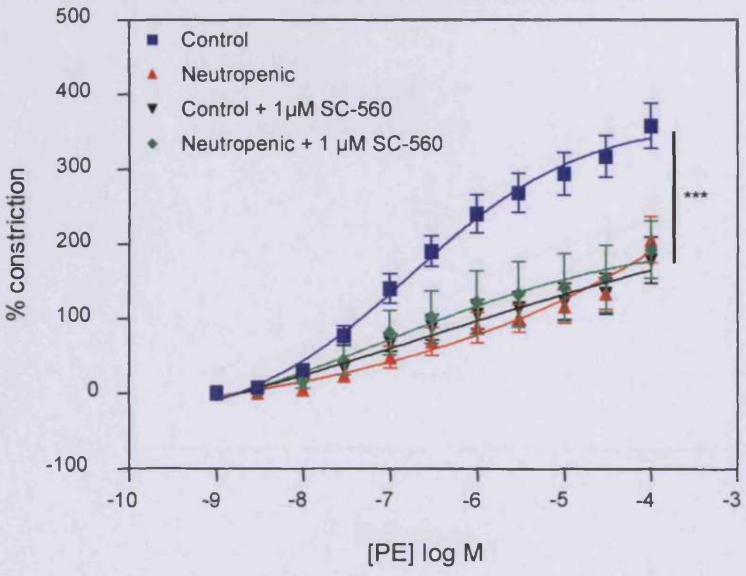

Figure 7.12 Effect of 1 μM SC-560 on PE-induced constriction responses in vessels from healthy and neutropenic animals

PE-induced constriction responses in thoracic aortae from control mice (n = 6, mean ± SEM) and mice depleted of neutrophils for 3 days (n = 4, mean ± SEM) in the presence or absence of SC-560 (1 μM) (n = 4, mean ± SEM). Aortic ring functional responses were determined as described in section 2.2.3.5 of *Materials and Methods*. *** represents $p < 0.001$, control + SC-560 *versus* control using two way ANOVA.

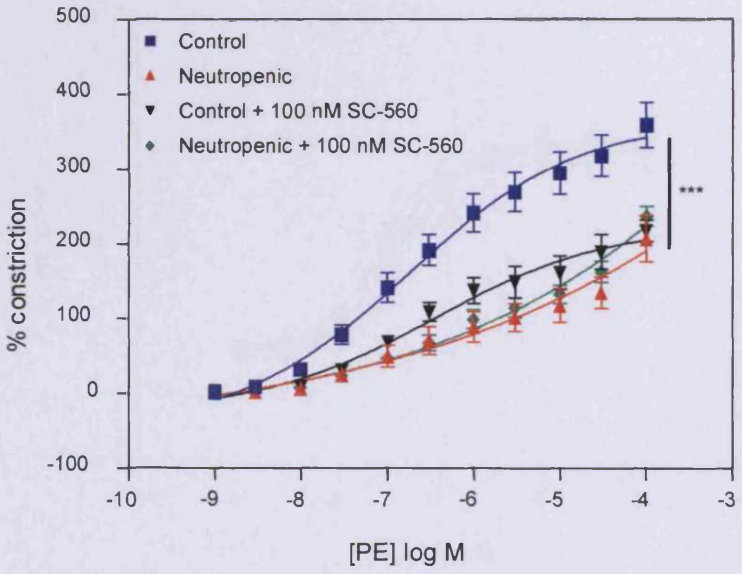

Figure 7.13 Effect of 100 nM SC-560 on PE-induced constriction responses in vessels from healthy and neutropenic animals

PE-induced constriction responses in thoracic aortae from control mice (n = 6, mean ± SEM) and mice depleted of neutrophils for 3 days (n = 4, mean ± SEM) in the presence or absence of SC-560 (100 nM) (n = 4, mean ± SEM). Aortic ring functional responses were determined as described in section 2.2.3.5 of *Materials and Methods*. *** represents $p < 0.001$, control + SC-560 *versus* control using two way ANOVA.

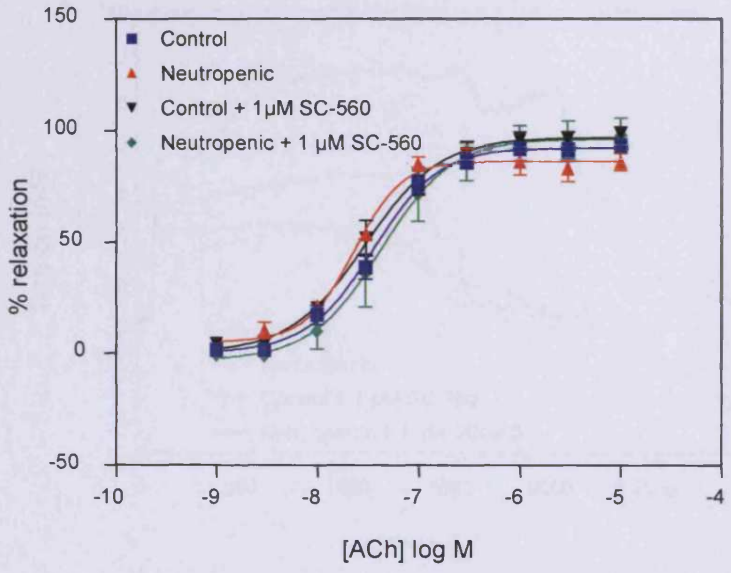

Figure 7.14 Effect of 1 μM SC-560 on ACh-induced relaxation responses in vessels from healthy and neutropenic animals

ACh-relaxation dose-response curves in thoracic aortae from control mice (n = 6, mean ± SEM) and mice depleted of neutrophils for 3 days (n = 4, mean ± SEM) in the presence or absence of SC-560 (1 μM) (n = 4, mean ± SEM). Aortic ring functional responses were determined as described in section 2.2.3.5 of *Materials and Methods*.

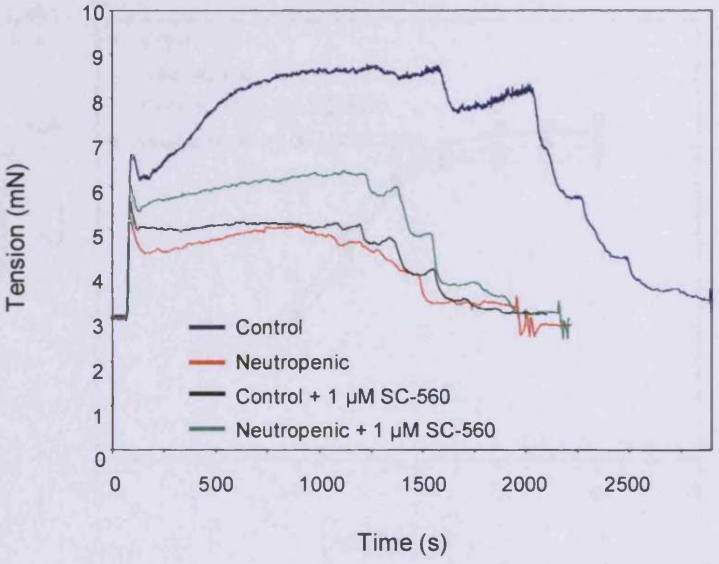

Figure 7.15 Representative experimental traces of endothelium-dependent relaxation in aortic rings from control and neutropenic mice in the presence or absence of SC-560

Representative ACh-relaxation dose-response curves of thoracic aortae from control mice and mice depleted of neutrophils for 3 days in the presence or absence of SC-560 (1 μM). Aortic ring functional responses were determined as described in section 2.2.3.5 of *Materials and Methods*.

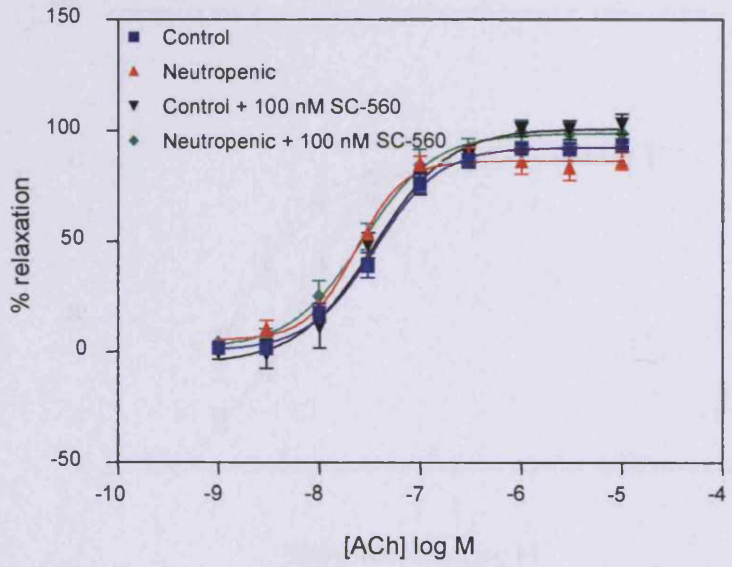

Figure 7.16 Effect of 100 nM SC-560 on ACh-induced relaxation responses in vessels from healthy and neutropenic animals

ACh-relaxation dose-response curves in thoracic aortae from control mice (n = 6, mean ± SEM) and mice depleted of neutrophils for 3 days (n = 4, mean ± SEM) in the presence or absence of SC-560 (100 nM) (n = 4, mean ± SEM). Aortic ring functional responses were determined as described in section 2.2.3.5 of *Materials and Methods*.

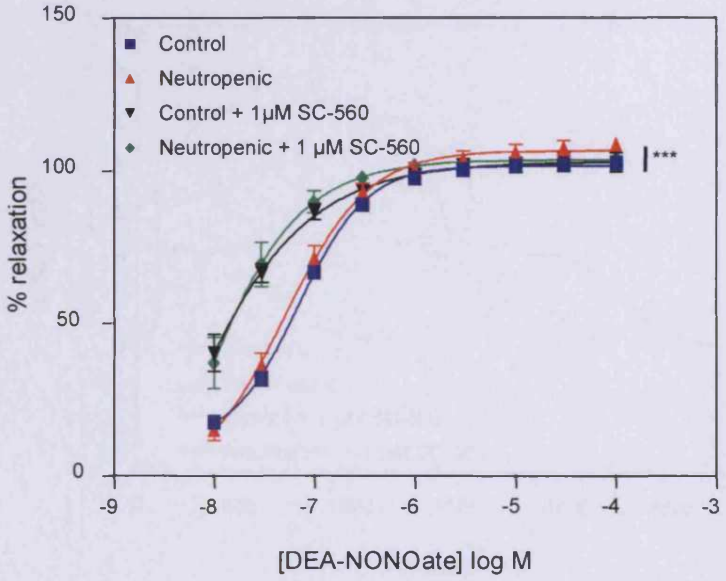

Figure 7.17 Neutropenia does not affect endothelium-independent vasodilation

Dose-response curves to DEA-NONOate in thoracic aortae from control mice (n = 6, mean ± SEM) and mice depleted of neutrophils for 3 days (n = 4, mean ± SEM) in the presence or absence of SC-560 (1 µM) (n = 3, mean ± SEM). Aortic ring functional responses were determined as described in section 2.2.3.5 of *Materials and Methods*. *** represents $p < 0.001$, neutropenic + SC-560 *versus* neutropenic, and control + SC-560 *versus* control using two way ANOVA.

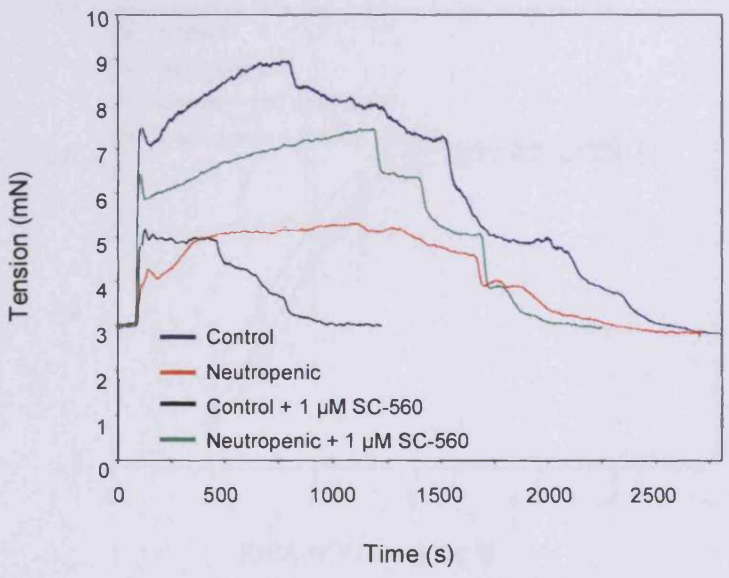

Figure 7.18 Representative experimental traces of endothelium-independent relaxation in aortic rings from control and neutropenic mice in the presence or absence of SC-560

Representative DEA-NONO-relaxation dose-response curves of thoracic aortae from control mice and mice depleted of neutrophils for 3 days in the presence or absence of SC-560 (1 µM). Aortic ring functional responses were determined as described in section 2.2.3.5 of *Materials and Methods*.

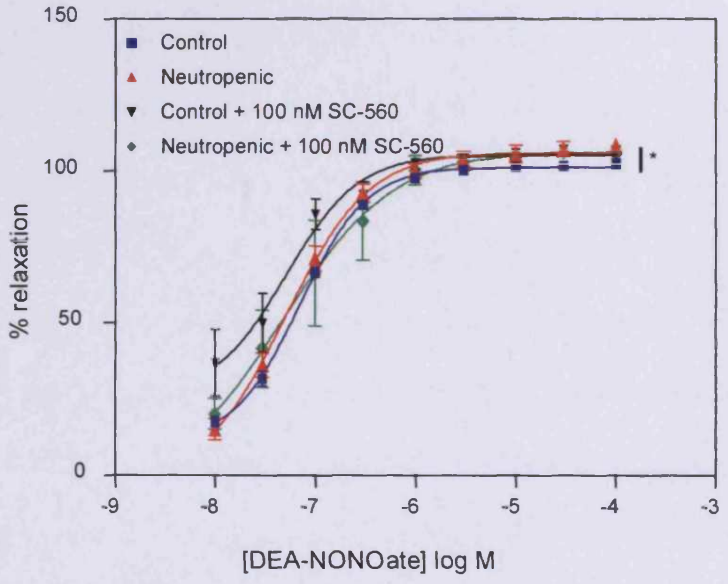

Figure 7.19 Neutropenia does not affect endothelium-independent vasodilation

Dose-response curves to DEA-NONOate in thoracic aortae from control mice (n = 6, mean ± SEM) and mice depleted of neutrophils for 3 days (n = 4, mean ± SEM) in the presence or absence of SC-560 (100 nM) (n = 3, mean ± SEM). Aortic ring functional responses were determined as described in section 2.2.3.5 of *Materials and Methods*. * represents $p < 0.05$, control + SC-560 *versus* control using two way ANOVA.

not seen at the lower dose of SC-560 (Figure 7.19), perhaps indicating some non-specific action of this inhibitor on COX-2 at the higher concentration. However, given that preconstruction with PE was significantly attenuated in aortic rings from neutropenic mice (Figure 7.15 and Figure 7.18), the reliability of this relaxation data is questionable.

7.3 Discussion

The main finding of this chapter is that PGI_2 synthesis is increased in neutropenia, as detected by measurement of the stable metabolite 2,3-dinor-6-keto-$PGF_{1\alpha}$ in urine samples collected from healthy and neutropenic mice (Figure 7.1). PGI_2 is the major COX product of the macrovascular endothelium, and physical or chemical stimulation of the vasculature has been shown to increase production of this eicosanoid in humans (Braden *et al.* 1991; Clarke *et al.* 1991; McAdam *et al.* 1999). While the source of this increased PGI_2 production was not determined here, a recent study by Anning and colleagues (2006) showed that oral administration of celecoxib to mice *in vivo* (400 $mg.kg^{-1}.day^{-1}$) reduced urinary excretion of 2,3-dinor-6-keto-$PGF_{1\alpha}$ by 71%, suggesting that COX-2 is the primary source of PGI_2 in mice. Indeed, given the increased iNOS bioactivity already described in this model, and the fact that COX-2 expression is induced by many of the same stimuli as iNOS (e.g. IL-1β), it is likely COX-2 bioactivity is potentiated in neutropenic mice.

In an effort to investigate this possibility further, the role of COX-1 and -2-signalling pathways were characterised in thoracic aortae isolated from healthy and neutropenic mice (day 3). However, the *ex vivo* data described in this chapter should be interpreted with caution, since the nature of COX signalling in isolated thoracic aortae appears to be dependent upon the assay used, such that COX-inhibition can have either a pro-constrictive or vasodilatory effect in the same vessel (Figures 7.2 & 7.3). Furthermore, many of the control data contradict the findings of others. For example, in the presence of

indomethacin, ACh-induced endothelium-dependent relaxation was significantly attenuated in vessels from control animals (Figure 7.3), suggesting that COX may play a role in determining endothelial function under healthy conditions. This is in marked contrast to the findings of Anning *et al.* (2006), who found indomethacin to be without effect on endothelium-dependent relaxation in vessels from C57Bl/6 mice (Anning *et al.* 2006). Similarly, the significant attenuation of PE-induced vasoconstriction observed in control vessels in the presence in of celecoxib (Figure 7.7) is in marked contrast to the findings of other investigators, who found celecoxib to be without effect on PE-induced constriction in vessels from healthy animals (Anning *et al.* 2006; Zhou *et al.* 2006). The reasons behind these differences are unknown, although previous studies were conducted using vessels from C57Bl/6 mice rather than the Balb/c mice used in the current study. As such, there may be differences between these two strains with regards to immune and vascular responses.

Nevertheless, from the current data it appears that aortae from neutropenic animals exhibit a decreased level of COX-1 signalling, since SC-560 was without effect of PE-induced vasoconstriction in these vessels (Figures 7.12 & 7.13). While the reason for this apparent downregulation of COX-1 bioactivity is currently unknown, characterisation of the promoter region of the human COX-1 gene by Wang and colleagues (1993) has revealed a shear stress responsive element. Indeed, shear stress has been shown to induce COX-1 expression in HUVECs (Doroudi *et al.* 2000). Therefore, it is proposed that the downregulation of COX-1 bioactivity observed in this model of neutropenia is a result of

the iNOS-induced transient hypotension already described, since the level of shear stress of the aortae of neutropenic animals is likely to be similarly reduced.

The effect of neutropenia on COX-2 bioactivity in the vasculature is less clear-cut, as celecoxib caused a similar decrease in PE-induced vasoconstriction in both control vessels and those from neutropenic mice (Figure 7.7). Importantly, these data indicate that COX-2 promotes vasoconstriction in both healthy and neutropenic animals. Unfortunately, a major limitation of the current study is that reductions in PE-induced preconstriction do not allow reliable interpretation of endothelium-dependent and -independent relaxation responses in vessels from neutropenic mice. As such, no solid conclusions can be made regarding the role of COX in endothelium-dependent and -independent relaxation in vessels from neutropenic mice. Nevertheless, it is of note that PE-induced vasoconstriction is almost completely absent in vessels from neutropenic mice in the presence of celecoxib, reiterating the possibility that COX-2 may play a vasoconstrictive role in the vasculature of neutropenic mice (Figure 7.9 and Figure 7.11). As already mentioned, given the increase in vascular iNOS bioactivity described in this model, and the fact that COX-2 expression is induced by many of the same stimuli which promote iNOS upregulation (e.g. IL-1β), an increase in COX-2 expression in neutropenia is highly plausible. This could be further investigated through immunohistological detection of COX-2 expression in vessels from healthy and neutropenic mice. However, the apparent pro-constrictive effect of COX-2-signalling in the thoracic aorta is at odds with the systemic increase in PGI_2 synthesis in neutropenic mice, which is vasodilatory in nature. Therefore, the source of this increased PGI_2 production in neutropenic mice needs

to be determined. This could be done by examining the effect of oral celecoxib on 2,3-dinor-6-keto-$PGF_{1\alpha}$ excretion in neutropenic mice; a return to baseline levels would indicate that COX-2 is solely the responsible for the systemic increase in PGI_2 synthesis in neutropenia, while a smaller decrease would suggest that COX-1 is also involved. In addition, the role of COX-2 in regulating vascular tone *in vivo* could be further evaluated by examining the effect of oral celecoxib administration on the transient hypotension observed in this model; a blunting of this transient hypotension would indicate that COX-2 promotes vasodilation of resistance vessels, while a further decrease in BP would support the pro-constrictive nature of COX-2-signalling already observed in isolated aortae.

In summary, the data described in this chapter indicate that COX signalling is altered in RB6-8C5-induced neutropenia. Specifically, neutropenic mice exhibit a systemic increase in PGI_2 synthesis in Balb/c mice, although the source of this PGI_2 production is currently unknown. Nevertheless, *ex vivo* studies indicate that COX-1 bioactivity is downregulated in the thoracic aortae of neutropenic mice, perhaps due to the lower shear stress experienced following iNOS-upregulation. Thus, further investigation into the role of COX-2 in neutropenic mice is required. The current *ex vivo* data indicate that COX-2 exerts a pro-constrictive effect in thoracic aortae of neutropenic Balb/c mice. However, it remains to be determined whether vascular COX-2 expression is upregulated in neutropenia, and whether inhibition of this enzyme normalises PGI_2 levels. Once this is known, further investigation into the vasoactive effects of COX-2 can be carried out, with a view to gaining a better understanding of how neutrophils may regulate vascular tone.

CHAPTER 8

CHARACTERISING THE ROLE OF NEUTROPHILS IN ANGIOTENSIN II-INDUCED HYPERTENSION AND CARDIAC HYPERTROPHY

8.1 Introduction

Ang II is a multifunctional hormone known to exert a variety of effects on the cardiovascular system, including vasoconstriction, smooth muscle proliferation, stimulation of protooncogene expression, modulation of myocardial hypertrophy, and induction of ventricular remodelling after myocardial infarction (Lee *et al.* 1993). Ang II can also increase mononuclear cell and platelet adhesion to the intima, thus enhancing local endothelial inflammation and injury (Paragh *et al.* 2002). Furthermore, a range of pathological states, including certain forms of hypertension, cardiac hypertrophy, congestive heart failure, atherosclerosis and nitrate tolerance are associated with elevated plasma renin activity and circulating levels of Ang II (Delbosc *et al.* 2002; Paragh *et al.* 2002; Rajagopalan *et al.* 1996).

The effects of Ang II on vascular tone are thought to be predominantly mediated via interaction with the vascular smooth muscle AT_1 receptor, causing vasoconstriction (Rajagopalan *et al.* 1996). More specifically, Griendling *et al.* (1994) have described a signalling mechanism in which Ang II increases production of $O_2^{\bullet-}$ anions via membrane-bound NADH- and NADPH-driven oxidases in cultured VSMCs in a

dose- and time-dependent fashion. As such, infusion of Ang II has been shown to induce hypertension in rats and impair vascular relaxations to ACh, the calcium ionophore A23187, and nitroglycerin, demonstrating that Ang II can exert this effect *in vivo* (Landmesser *et al.* 2002; Rajagopalan *et al.* 1996). The prevention of these effects by pre-treatment with SOD indicates that the Ang II-induced increase in $O_2^{\bullet-}$ production by VSMCs may contribute to alterations in endothelium-dependent vascular relaxation and responses to exogenous nitrovasodilators in the intact vessel (Rajagopalan *et al.* 1996).

Interestingly, Ang II has been shown to cause the release of neutrophil chemoattractant factors from cultures of human and bovine arterial endothelial cells *in vitro* (Farber *et al.* 1990). Furthermore, AT_1 receptors have been found to be expressed on the surface of neutrophils, with activation of these receptors resulting in enhanced adhesion molecule expression on the neutrophil cell surface (Ito *et al.* 2001). As such, it has been proposed that the effects of Ang II on neutrophils may contribute to inflammation at the vessel wall level, by favouring their cooperation with endothelial and vascular cells during the initiation of the inflammatory process (El Bekay *et al.* 2003). Indeed, previous studies have shown that inhibition of ACE attenuates postischemic adhesion of neutrophils in isolated perfused hearts, and ameliorates endothelial injury via inhibition of neutrophil-endothelial cell interaction (Guba *et al.* 2000; Kupatt *et al.* 1996).

Despite the observations described above, a direct role for neutrophils in Ang II-induced hypertension has not been fully investigated. Indeed, while some studies have shown that Ang II stimulates NAD(P)H oxidase-dependent $O_2^{\bullet-}$ generation by

neutrophils *in vitro*, the relevance of this action *in vivo* is currently unknown (Paragh *et al.* 2002). Therefore, given that oxidative stress is known to be increased in Ang II-induced hypertension, the involvement of $O_2^{\bullet-}$ generated by neutrophil NAD(P)H oxidase in this phenomenon requires further investigation.

8.1.1 Aims

Activation of human neutrophils, with or without priming by inflammatory mediators, is known to cause rapid $O_2^{\bullet-}$ generation by these cells *in vitro*, with a resultant decrease in local NO bioavailability. In addition, Ang II has been shown to induce hypertension and cardiac hypertrophy *in vivo*, via a mechanism involving increased ROS production within the vasculature (Rajagopalan *et al.* 1996). However, to date the role of neutrophils in these conditions has yet to be fully evaluated. Therefore, the aim of this chapter was to investigate the effect of RB6-8C5-induced neutropenia on Ang II-induced hypertension and cardiac hypertrophy *in vivo*. This involved:

- The non-invasive measurement of BP following infusion of Ang II via subcutaneous implantation of osmotic minipumps in healthy and neutropenic mice.

- The assessment of cardiac hypertrophy by comparison of heart:body weight ratios of healthy and neutropenic mice following Ang II infusion.

8.2 Results

8.2.1 Depletion of neutrophils with RB6-8C5 prevents Ang II-induced hypertension

In order to examine a potential role for neutrophils in Ang II-induced hypertension *in vivo*, systolic BP was measured in control and neutropenic mice following Ang II treatment. In control mice, subcutaneous infusion of Ang II caused significant increases in systolic BP (128.47 ± 4.50 mmHg on day 4, *versus* 111.33 ± 2.38 mmHg on day 0, mean ± SEM, $p < 0.001$, using one way ANOVA and Dunnett post hoc analysis, $n \geq 7$, Figure 8.1), whereas no such increase was observed in neutropenic mice. Rather, systolic BP was found to be significantly decreased even in the presence of Ang II infusion (98.45 ± 4.54 mmHg on day 3, *versus* 111.33 ± 2.38 mmHg on day 0, mean ± SEM, $p < 0.05$, using one way ANOVA and Dunnett post hoc analysis, $n \geq 7$). Indeed, the overall BP responses of neutrophil-replete and neutropenic mice during Ang II infusion were found to be significantly different over 7 days of measurement ($p < 0.001$ using two way ANOVA, $n \geq 4$, mean ± SEM). In contrast, no differences were found between BP responses of neutropenic mice in the presence or absence of Ang II infusion (Figure 8.2). These data indicate that depletion of neutrophils by RB6-8C5 prevents the onset of Ang II-induced hypertension.

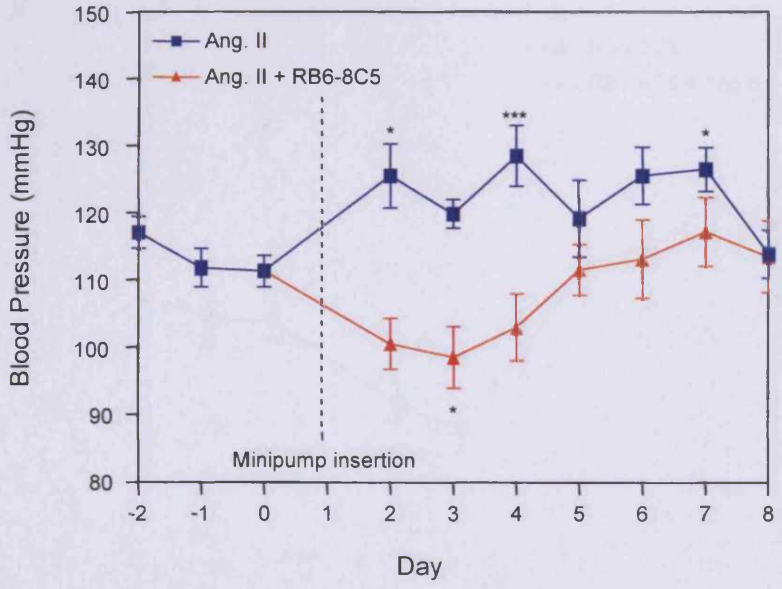

Figure 8.1 Neutrophil depletion with RB6-8C5 prevents induction of hypertension by Ang II infusion

BPs were measured daily by tail-cuff plethysmography under controlled conditions. Following training, mice were randomized to receive either RB6-8C5 by intraperitoneal injection or left untreated. Healthy or neutropenic mice were then infused with Ang. II (1.1 $mg.kg^{-1}.day^{-1}$), by insertion of an osmotic minipump 1 day following induction of neutropenia, in order to induce hypertension ($n \geq 4$, mean ± SEM). * represents $p < 0.05$ *versus* day 0, *** represents $p < 0.001$ *versus* day 0, using one way ANOVA and Dunnett post hoc analysis.

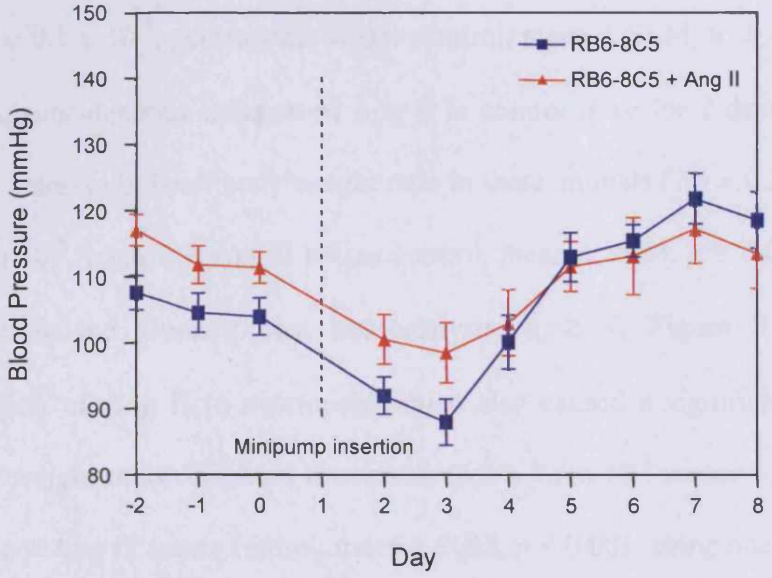

Figure 8.2 Ang II infusion does not prevent the decrease in BP observed in RB6-8C5-induced neutropenia

This figure allows comparison of BPs of neutropenic mice infused with Ang II (1.1 $mg.kg^{-1}.day^{-1}$) or left untreated ($n \geq 3$, mean ± SEM). Baseline values differ due to the fact that different groups of mice were observed in each experiment. BPs were measured daily by tail-cuff plethysmography under controlled conditions.

8.2.2 *Neutrophil depletion does not prevent cardiac hypertrophy following Ang II infusion*

Heart and body weights were measured in control mice, and mice depleted of neutrophils for 8 days, with or without subcutaneous infusion with Ang II (1.1 $mg.kg^{-1}.day^{-1}$) for 7 days. Depletion of neutrophils in the absence of Ang II infusion did not result in any change in heart:body weight ratio ($4.6 \pm 0.1 \times 10^{-3}$ *versus* $4.7 \pm 0.1 \times 10^{-3}$, neutropenic *versus* control, mean ± SEM, $n \geq 4$, Figure 8.3). In contrast, subcutaneous infusion of Ang II in control mice for 7 days resulted in a significant increase in heart:body weight ratio in these animals ($7.0 \pm 0.2 \times 10^{-3}$ *versus* $4.7 \pm 0.1 \times 10^{-3}$, control + Ang.II *versus* control, mean ± SEM, $p = 0.001$, using one way ANOVA and Dunnett post hoc analysis, $n \geq 4$, Figure 8.3). Similarly, administration of Ang II to neutropenic mice also caused a significant increase in heart:body weight ratio compared to controls ($6.2 \pm 0.5 \times 10^{-3}$ *versus* $4.7 \pm 0.1 \times 10^{-3}$, neutropenic + Ang II *versus* control, mean ± SEM, $p < 0.001$, using one way ANOVA and Dunnett post hoc analysis, $n \geq 5$, Figure 8.3). No significant differences were observed between heart:body weight ratios of healthy or neutropenic mice infused with Ang II ($6.2 \pm 0.5 \times 10^{-3}$ *versus* $7.0 \pm 0.2 \times 10^{-3}$, neutropenic + Ang II *versus* control + Ang II, mean ± SEM, $n \geq 4$, Figure 8.3). These data indicate that induction of neutropenia has no effect of heart:body weight ratio in the absence of Ang II. Furthermore, neutrophil depletion does not prevent the development of cardiac hypertrophy following subcutaneous Ang II infusion.

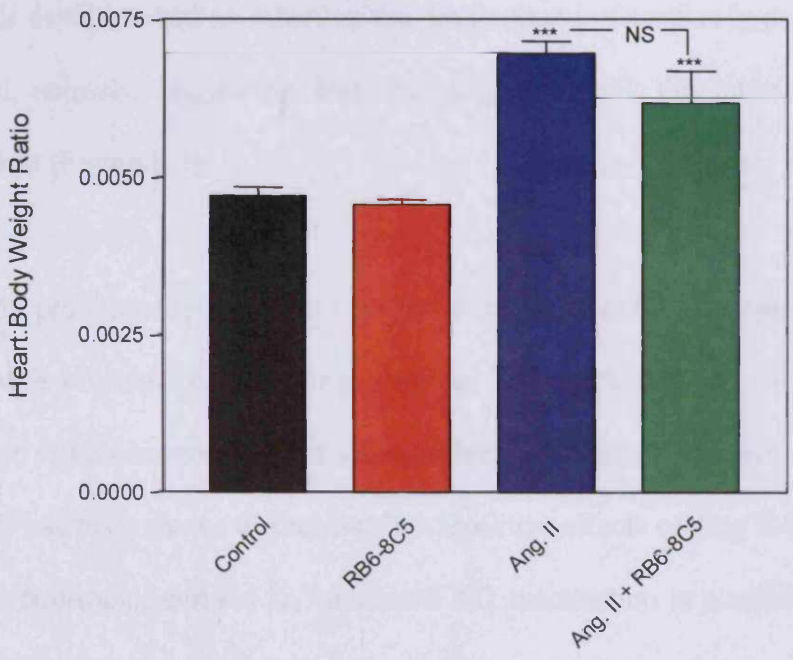

Figure 8.3 Neutrophil depletion does not prevent cardiac hypertrophy following Ang II infusion

Heart and body weight was recorded in healthy and neutropenic mice (day 8) in the presence or absence of Ang II infusion (7 days, 1.1 $mg.kg^{-1}.day^{-1}$, $n \geq 4$, mean ± SEM). NS = not significant, using an unpaired *t* test. *** represents $p < 0.001$ *versus* control, using one way ANOVA and Dunnett post hoc analysis.

8.3 Discussion

The main finding of this chapter is that neutrophil depletion with RB6-8C5 appears to prevent the onset of hypertension in Balb/c mice following subcutaneous infusion with Ang II (Figure 8.1). Furthermore, RB6-8C5-induced neutropenia resulted in a significant fall in systolic BP even in the presence of Ang II, once again highlighting the potential importance of neutrophils in maintaining vascular tone. In contrast, neutrophil depletion had no effect on the development of cardiac hypertrophy in Ang II-infused animals, suggesting that the progression of this state is neutrophil-independent (Figure 8.3).

This study provides the first direct evidence that neutrophils may play a role in the pathogenesis of Ang II-induced hypertension. The mechanism by which neutrophils exert these effects requires further characterisation, although given that pre-treatment with SOD has been shown to prevent the vasoactive effects of Ang II infusion in rats, a role for neutrophil-derived $O_2^{\bullet-}$-mediated NO inactivation is possible (Rajagopalan *et al.* 1996). Nevertheless, previous studies have proposed that Ang II-induced leukocyte-endothelial cell interaction are partly mediated through the production of ROS (Alvarez & Sanz, 2001), so the protective effect of SOD may be due to reduced neutrophil infiltration into the vascular tissue in addition to preserving NO bioavailability. As such, a role for other neutrophil functions, such as inflammatory cytokine release, in the pathogenesis of Ang II-induced hypertension cannot be ruled out.

In addition, it remains to be determined whether the resistance of neutropenic mice to Ang II-induced hypertension is related to the upregulation of iNOS already described in neutropenic mice in the absence of Ang II-infusion (see chapters 5 and 6). Indeed, the similarity between BP responses of untreated and Ang II-infused neutropenic mice suggests that an increase in iNOS bioactivity in the vasculature of these animals may act to counteract the impaired endothelial function consistently observed in Ang II-infused animals (Figure 8.2). Furthermore, NO is known to downregulate AT_1 receptor expression in VSMCs at the transcriptional level, thereby providing further protection against Ang II-mediated vasoconstriction (Ichiki *et al.* 1998; Watanabe *et al.* 2005; Yan *et al.* 2003). Therefore, immunohistochemical detection of iNOS in aortic sections taken from control and neutropenic mice following Ang II infusion is required in order to evaluate the role of iNOS in the above observations. In addition, vasoactivity of isolated aortae from these animals could be examined using myography and various pharmacological inhibitors, as described in the earlier chapters of this thesis.

Ang II-induced hypertrophy of VSMCs is thought to be associated with both $O_2^{\bullet-}$ and H_2O_2 (El Bekay *et al.* 2003; Griendling *et al.* 1994). Indeed, it has been shown that H_2O_2, one immediate product of $O_2^{\bullet-}$ metabolism, stimulates mitogeneses in VSMCs (Rao & Berk, 1992). Furthermore, the activities of both protein tyrosine kinase and protein tyrosine phosphatase, pathways intimately involved in the growth response of many cell types, are regulated by ROS (Larsson & Cerutti, 1988; Pumiglia *et al.* 1992). However, the specific growth-related pathways regulated by H_2O_2 remain to be determined in VSMCs (Griendling *et al.* 1994). In this study, immunodepletion of neutrophils prior to Ang II infusion had no effect of the development of cardiac

hypertrophy in Balb/c mice (Figure 8.2), suggesting that neutrophils do not play a role in cell growth. Indeed, given the prolonged activation of vascular NAD(P)H oxidase, and predominantly intracellular generation of $O_2^{\bullet-}$ by this enzyme in response to Ang II, it appears that vascular NAD(P)H oxidase is responsible for the development of cardiac hypertrophy in Ang II-treated mice rather than the faster acting phagocytic NADPH oxidase found in neutrophils, which releases $O_2^{\bullet-}$ into the extracellular space (Bauldry *et al.* 1992; Griendling *et al.* 1994; Watson *et al.* 1991). As such, these data are in line with previous reports that Ang II can induce medial thickening and increase vascular cross-sectional area independently of BP elevation (Griffin *et al.* 1991; Schiffers *et al.* 1993; Wang *et al.* 2001).

In summary, the data presented in this chapter provide the first direct evidence that neutrophils may play a role in the onset of Ang II-induced hypertension *in vivo*. However, further investigation is required to examine the specific mechanisms involved in these changes, particularly with regard to changes in iNOS expression. Nevertheless, the current data provide evidence that neutrophils may play an important role in the pathogenesis of vascular pathologies related to elevated circulating Ang II levels (e.g. atherosclerosis). In contrast, immunodepletion of neutrophils did not appear to prevent the development of cardiac hypertrophy in Ang II-treated animals, indicating that neutrophils are unlikely to play a role in the progression of this condition.

CHAPTER 9

GENERAL DISCUSSION

The experiments in this thesis set out to examine a role for neutrophils in the regulation of vascular tone. Specifically, initial experiments aimed to assess the capacity of inflammatory-primed neutrophils to consume NO *in vitro*, and investigate the mechanisms by which they do so, in order to better understand how these cells may contribute to the pathogenesis of inflammatory vascular disease *in vivo*. Subsequent experiments then focussed on elucidating a role for neutrophils in regulating vascular tone *in vivo* using immunodepletion techniques. Importantly, this is the first study to explore the vasoactive effects of neutrophils *in vivo*.

In the first part of this thesis, it was demonstrated that inflammatory-priming of neutrophils with TNF-α, IL-8 or PAF potentiates NAD(P)H oxidase activity in these cells, resulting in increased rates of $O_2^{\bullet-}$ generation upon activation with fMLP *in vitro*. Furthermore, activation of primed neutrophils led to significantly accelerated rates of NO disappearance from aerobic buffer *in vitro* in an NAD(P)H oxidase-dependent manner.

Neutrophil priming is likely to occur *in vivo* following exaggerated inflammatory mediator release, such as that observed in septic shock, haemorrhagic shock, acute respiratory distress syndrome, rheumatoid arthritis and atherosclerosis (Ayala *et al.* 2002; Condliffe *et al.* 1998). Indeed, neutrophils derived from the peripheral circulation of mice

exposed to haemorrhagic shock exhibit increased respiratory burst capacity, consistent with *in vivo* priming, and primed neutrophils have been identified in the peripheral blood of patients after blunt trauma, in acute respiratory distress syndrome, and following bacterial and fungal infections (Ayala *et al.* 2002; Bass *et al.* 1986; Chollet-Martin *et al.* 1992; Krause *et al.* 1988). Given the importance of NO in regulating vessel tone, smooth muscle proliferation, platelet aggregation and leukocyte adhesion *in vivo*, the rapid consumption of NO by primed neutrophils is likely to have a detrimental effect on vascular homeostasis *in vivo*. For example, a decrease in NO bioavailability will result in the upregulation of adhesion molecule expression in the surface of endothelial cells and leukocytes (e.g. CD11/CD18 in neutrophils), such that NO consumption by primed neutrophils may serve a key step in the infiltration of immune cells, such as neutrophils and monocytes, into inflamed vascular tissue (Kubes *et al.* 1991). The rapid inactivation of vasodilatory NO by primed neutrophils may also have a pro-constrictive effect *in vivo*, since the non-specific inhibition of basal NO release by the endothelium results in endothelium-dependent vasoconstriction *in vivo* and *in vitro* (Manning *et al.* 1993; Rees *et al.* 1990). Finally, the $O_2^{\bullet-}$-mediated inactivation of NO is likely to result in excessive formation of $ONOO^-$, a potent oxidizing agent that has been implicated in the pathogenesis of atherosclerosis (Leeuwenburgh *et al.* 1997; White *et al.* 1994). Thus, neutrophil priming may also have a deleterious effect on endothelial function *in vivo* through the excessive generation of cytotoxic $ONOO^-$.

While these data clearly demonstrate enhanced NO clearance by primed neutrophils, a direct vasoactive effect of these cells could not be studied in this *in vitro* system.

Therefore, future studies could aim to demonstrate the vasoactive effects of primed neutrophils *in vitro* using isolated vessel segments. Similar experiments have already been carried out using unprimed neutrophils, which were shown to elicit an endothelium-dependent vasoconstriction in rabbit aortae and cat coronary arteries through the $O_2^{\bullet-}$-mediated inactivation of basally released NO (Ma *et al.* 1999; Ohlstein & Nichols, 1989). In light of the significantly enhanced neutrophil-mediated NO clearance observed in the current study, addition of primed neutrophils to isolated tissue segments would be expected to result in even greater vasoconstriction. In addition, due to the fact that PGIS is inactivated under conditions of oxidative stress, excessive $O_2^{\bullet-}$ generation by neutrophil NAD(P)H oxidase is likely to inhibit endothelial PGI_2 synthesis *in vivo,* further promoting vasoconstriction (Zou *et al.* 2002). Finally, given that neutrophil priming has been shown to enhance 5-LOX and COX-2 bioactivity *in vitro*, leading to release of the pro-constrictive mediators LTB_4 and TxA_2 upon neutrophil activation, primed neutrophils may also have vasoactive effects *in vivo* that are independent of changes in NO bioavailability (Pouliot *et al.* 1998; Staňková *et al.* 1995). The contribution of these mediators to the vasoactive effects of primed neutrophils could be examined by isolating supernatants from preparations of primed neutrophils following acute activation, and adding them to tissue baths containing isolated vessel segments. An increase in vessel tone would confirm that LTB_4 and TxA_2 are synthesised and released by primed neutrophils in sufficient quantities to contribute to the vasoactive effect of these cells *in vitro*.

However, while much can be learned from examining neutrophil activity *in vitro*, the interaction of these cells with the endothelium and other immune cells *in vivo* provides an additional layer of complexity. For example, exposure of cultured endothelial cells to shear stress modulates their ability to recruit neutrophils in responses to TNF-α, due to downregulation of E-selectin and IL-8 expression, such that recruitment of leukocytes in inflamed tissue is generally concentrated in postcapillary venules where shear stress is low (Sheikh *et al.* 2003). Furthermore, neutrophil priming results in the increased synthesis and release of an array of cytokines and chemokines (e.g. IL-8, MIP-1 and MCP-1), which promote the adhesion and transendothelial migration of monocytes *in vivo*. Like neutrophils, acute activation of monocytes results in $O_2^{\bullet-}$ generation *in vitro*, suggesting that these cells may also consume NO (Hancock & Jones, 1987). While the potential for mononuclear cells to alter vascular tone has yet to be examined, accumulation of macrophages has long been associated with the pathogenesis of atherosclerosis due to their abundance in atherosclerotic lesions (Cathcart, 2004); as such, the recruitment of monocytes/macrophages following cytokine release by primed neutrophils may be a key factor in vasoactive effects of neutrophils *in vivo*. Therefore, in order to properly investigate a role for neutrophils in the modulation of vascular tone, it is important to examine these cells in a more physiological setting (e.g. *in vivo*).

With this in mind, subsequent chapters of this thesis aimed to elucidate a role for neutrophils in regulating vascular tone *in vivo* using immunodepletion techniques. Briefly, neutropenia was induced in 8–10 week old Balb/c mice using the mAb RB6-8C5, which depleted neutrophils for up to 8 days. The main finding of this study was that,

following the onset of RB6-8C5-induced neutropenia, mice exhibited a gradual fall in systolic BP over 3 days, and PE-induced vasoconstriction was significantly attenuated in thoracic aortae isolated from neutropenic animals compared to controls. These effects were due, at least in-part, to the IFN-γ-dependent upregulation of iNOS bioactivity in the thoracic aortae of neutropenic mice. In addition, immunodepletion of neutrophils resulted in an increase in PGI_2 synthesis, suggesting the upregulation of COX-2 bioactivity in neutropenic mice, although this has yet to be explored fully.

In light of these findings, it appears that under physiological conditions (i.e. in the absence of inflammatory stimuli) neutrophils may modulate vascular tone by negatively regulating the bioactivity of pro-inflammatory enzymes such as iNOS and COX-2, which promote the onset of hypotension. To our knowledge, this is the first study to demonstrate this vasoactive effect of neutrophils *in vivo*. In addition, these data suggest that neutrophils may play an anti-inflammatory role *in vivo* under basal conditions. However, while some studies have been carried out to investigate the pathways underlying RB6-8C5-mediated neutrophil depletion, a definitive mechanism for neutrophil clearance has yet to be elucidated. In the absence of such a mechanism, no definitive conclusions regarding the role of neutrophils in regulating immune function can be drawn. Indeed, it is possible that the observed increase in IFN-γ signalling in RB6-8C5-treated mice may simply be a by-product of antibody-mediated neutrophil clearance by macrophages. This could be further investigated by examining the behaviour of isolated monocytes/macrophages in the presence and absence of RB6-8C5-coated synthetic beads. If the pro-inflammatory changes observed in RB6-8C5-induced neutropenia are

neutrophil-independent, phagocytosis of RB6-8C5-coated beads would be expected to result in an increased IFN-γ concentration in the cell supernatant, as measured by ELISA. In contrast, if IFN-γ levels remained unchanged in the presence of RB6-8C5-coated beads, this would confirm that the vasoactive changes observed in RB6-8C5-treated mice are neutrophil-dependent.

With this in mind, the primary challenge for future work is to further characterise the nature of the changes observed in RB6-8C5-induced neutropenia with a view to elucidating the mechanism by which these neutrophils may exert this effect. Previous studies have already alluded to an anti-inflammatory role for neutrophils under certain conditions. For example, in Balb/c mice, neutrophils have been reported to influence CD4+ Th cell differentiation following infections with *Leishmania major*, promoting the immunosuppressive Th2 response and inhibiting the inflammatory Th1 response (Tacchini-Cottier *et al.* 2000). Furthermore, in a recent study by Daley and colleagues (2005), neutrophils were found to inhibit macrophage recruitment and the release of proinflammatory cytokines (e.g. TNF-α, IL-6) and $O_2^{\bullet-}$ by these cells in a sterile wound model via the release of an unidentified soluble mediator (Daley *et al.* 2005). Whether or not macrophages play a role in the upregulation of iNOS and COX-2 bioactivity observed in the current model of neutropenia has yet to be determined, although some macrophage-derived cytokines (e.g. TNF-α) are known to induce iNOS expression in VSMCs *in vitro* (Hecker *et al.* 1999; Kleinert *et al.* 2003). As such, an *in vivo* model of monocyte depletion would provide a useful tool for determining the role of these cells in cardiovascular function.

Clearly, the soluble mediator described by Daley and colleagues (2005) requires identification, although preliminary studies have ruled out a role for PGE_2 and adenosine in the modulation of macrophage phenotype *in vitro* (Daley *et al.* 2005). Potential candidates could include IL-10 and TGF-β; anti-inflammatory cytokines known to be secreted by neutrophils and also to promote immunosuppressive Th2 cell differentiation (Tacchini-Cottier *et al.* 2000). Indeed, in a sterile wound model, local TGF-β concentrations were found to be significantly lower in wound fluid from neutropenic mice compared to controls (Daley *et al.* 2005). IL-10 is known to have a variety of anti-inflammatory roles, as exemplified by the exaggerated inflammatory responses exhibited by IL-$10^{-/-}$ mice in various disease models (Berg *et al.* 1995a; Berg *et al.* 1995b; Kuhn *et al.* 1993). Furthermore, several studies of the protective effects of IL-10 directly or indirectly implicate iNOS as a pathogenic mediator in these IL-$10^{-/-}$ mice (Gunnett *et al.* 1999; Mallat *et al.* 1999; Raisanen-Sokolowski *et al.* 1997; Yang *et al.* 2000). In the case of TGF-β, addition of this growth factor to cultured rat astrocytes or RAW 264.7 cells (a murine macrophage-like cell line) prevented iNOS expression in response to exogenous LPS (Kozuka *et al.* 2007; Takaki *et al.* 2006). Similarly, the absence of this signalling molecule, as in TGF-$\beta^{-/-}$ mice, has been associated with upregulation of iNOS bioactivity and IFN-γ signalling *in vivo* (Kulkarni *et al.* 1993; McCartney-Francis & Wahl, 2002; Shull *et al.* 1992; Vodovotz *et al.* 1996).

Following differentiation, Th2 cells produce a variety of anti-inflammatory cytokines (e.g. IL-4, IL-10 and IL-13), which suppress iNOS and/or COX-2 expression (Dworski &

Sheller, 1997; Mertz *et al.* 1994; Ruetten & Thiemermann, 1997). For example, addition of IL-4 suppressed both LPS- and cytokine-induced iNOS expression in cultured rat astrocytes without affecting mRNA levels (Kozuka *et al.* 2007). In addition, IL-4 and IL-10 also inhibit the production of IFN-γ, IL-1β and TNF-α by macrophages and T-cells (Fiorentino *et al.* 1991). With this in mind, further studies should examine a role for both IL-10 and TGF-β in the vascular effects observed in RB6-8C5-induced neutropenia. Specifically, systolic BP could be measured in Balb/c mice injected with anti-IL-10 and/or anti-TGF-β antibodies (JES-2A5 and 1D11, respectively) (Benigni *et al.* 2003; Guilbault *et al.* 2002). A similar fall in BP to that observed in the current study would suggest that these molecules may play a role in the neutrophil-mediated anti-inflammatory effect. A role for neutrophils in the JES-2A5- or 1D11-mediated hypotension could be confirmed by administering RB6-8C5 and JES-2A5 or 1D11 together; if the transient fall in BP was greater than that seen with JES-2A5 or 1D11 alone, this would indicate that neutrophils exert their immunosuppressive effects, at least in part, through another mechanism. However, if no additional effect is observed following neutrophil depletion, this would suggest that the neutrophil-mediated anti-inflammatory effects observed in this study are IL-10 or TGF-β-dependent.

However, it remains to be seen whether neutrophils can synthesise and secrete IL-10 and TGF-β in sufficient quantities *in vivo* to account for the increase in iNOS bioactivity observed in the current model of neutropenia. Furthermore, the precise stimulus responsible for the synthesis and release of these molecules requires clarification. For example, while previous studies have shown that neutrophils secrete IL-10 and TGF-β *in*

vitro in the presence of yeast and PMA, respectively, the current study was carried out *in vivo* under basal conditions (Fava *et al.* 1991; Romani *et al.* 1997). Therefore, closer examination of neutrophil phenotype in healthy mice *in vivo* is required before these questions can be answered. Recent studies have examined the expression of cell surface receptors by isolated human neutrophils using flow cytometry, and RT-PCR can be used to investigate the expression of intracellular signalling molecules at the mRNA level (Ratthé *et al.* 2007). In addition, IL-10 release by human peripheral blood monocytes has been measured by ELISA (Davidson *et al.* 2007). As such, the current data provide an excellent platform for further investigation into the phenotype of isolated murine neutrophils under physiological and pathophysiological conditions.

While it is clear the exposure of neutrophils to inflammatory cytokines causes these cells to undergo phenotypic and functional changes, which facilitate the removal of injurious agents from infected tissue, neutrophil phenotype can also be altered in the presence of Ang II. Specifically, stimulation of AT_1 receptors on the neutrophil cell surface results in enhanced adhesion molecule expression by these cells, and it has been proposed that the effects of Ang II on neutrophils may contribute to inflammation at the vessel wall level by favouring their cooperation with endothelial and vascular cells during the initiation of the inflammatory process (El Bekay *et al.* 2003; Ito *et al.* 2001). However, little is currently known of the functional consequence of these changes *in vivo*, particularly with regard to the regulation of vascular tone. With this in mind, the final chapter of this thesis aimed to investigate the effect of RB6-8C5-induced neutropenia on Ang II-induced hypertension *in vivo*.

Briefly, neutrophil depletion prevented the onset of hypertension following subcutaneous infusion with Ang II, providing the first evidence that neutrophils may be directly involved in the development of hypertension associated with this octapeptide *in vivo*. While further investigation is required to characterise the role of neutrophils in Ang II-induced hypertension, the finding that pre-treatment with SOD prevents the vasoactive effect of Ang II in rats suggests that NAD(P)H oxidase-dependent $O_2^{\bullet-}$ generation may be contributing factor to the progression of the hypertensive state (Rajagopalan *et al.* 1996). These data provide yet another example of how changes in neutrophil phenotype may invoke significant changes in vascular physiology. However, it remains to be determined whether the resistance of neutropenic mice to Ang II-induced hypertension is related to the upregulation of iNOS already described in neutropenic mice in the absence of Ang II-infusion. Indeed, the similarity between BP responses of untreated and Ang II-infused neutropenic mice suggests that an increase in iNOS bioactivity in the vasculature of these animals may act to counteract the impaired endothelial function consistently observed in Ang II-infused animals. Nevertheless, the possibility of a direct link between neutrophils and Ang II-induced hypertension suggests that these cells may play a role in a range of pathological states associated with elevated plasma Ang II concentrations *in vivo*, including atherosclerosis, congestive heart failure and nitrate tolerance, as well as other forms of hypertension (Delbosc *et al.* 2002; Paragh *et al.* 2002; Rajagopalan *et al.* 1996).

In conclusion, the data described in this thesis highlight the diversity of neutrophil activities *in vivo* and *in vitro* and how changes in neutrophil phenotype may impact upon

vascular physiology. For example, in an inflammatory setting, neutrophils act to rapidly consume NO via the release of $O_2^{\bullet-}$, an effect that may be related to their role in Ang II-mediated hypertension. In contrast, under basal conditions, neutrophils may modulate vascular tone primarily by altering the bioactivity of enzymes such as NOS and COX. In light of these findings, it may be possible to identify a role for these cells in different pathological conditions using specific disease models (e.g. Ang II-mediated hypertension). As such, while much work has yet to be done, the current data may provide a platform for novel therapeutic strategies that focus on modulating neutrophil phenotype in order to exert more widespread effects throughout the vascular system.

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