Reactive oxygen species in human blood vessels: sources of superoxide production and effects of angiotensin II

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This being a thesis submitted for the degree of Doctor of Philosophy in the Faculty of Medicine of the University of Glasgow

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1.1 Synopsis of thesis

The purpose of this thesis was to investigate the enzymatic sources of, and stimuli for, reactive oxygen species (ROS) production in human vascular cells and blood vessels. In particular, the possibility that vasoactive hormones, such as angiotensin II (Ang II), might stimulate vascular ROS production was also explored.

Internal mammary arteries (IMA) and saphenous veins (SV) were collected at the time of coronary artery bypass surgery. Initial validation studies demonstrated that lucigenin-enhanced chemiluminescence was a sensitive and specific method for quantification of superoxide ($\cdot O_2^-$) concentrations in these blood vessels. The enzymatic sources of ROS generation were NAD(P)H oxidase, xanthine oxidase and in some, but not all, patients, nitric oxide synthase. Superoxide production was greater in IMA than in SV, whereas the amount of superoxide dismutase protein was quantitatively similar in these blood vessels.

In subsequent studies, treatment of IMA with pharmacological concentrations of Ang II (1 micromolar, 1 nanomolar) for 1 and 4 hours was associated with an increase in $\cdot O_2^-$ production. Treatment of IMA with picomolar, or physiological, concentrations of Ang II tended to increase $\cdot O_2^-$ production. Further studies demonstrated that this was an Ang type 1 (AT_1) receptor-dependent, NAD(P)H oxidase-mediated pathway. Furthermore, inhibition of the AT_2 receptor did not prevent Ang II-stimulated increase in $\cdot O_2^-$ production in IMA, suggesting that this receptor does not contribute to $\cdot O_2^-$ generation in human arteries.

Immunodetection studies for the NAD(P)H oxidase phox subunits were performed in IMA. Using monoclonal antibodies and antisera, p22phox, gp91 phox (or a homologue), p67phox and p47 phox protein subunits were identified within the endothelium, vascular smooth muscle cell layer and adventitia. Xanthine oxidase was identified in the endothelium and adventitial layers of IMA. In this chapter, studies of the effects of Ang II on the abundance of cDNA transcripts of the subunits of NAD(P)H oxidase are reported. In subsequent molecular studies, it was demonstrated that treatment of human vascular cells and intact IMA with 1 micromole of Ang II for 4 hours led to an increase in the abundance of cDNA transcripts of p22phox, which

was attenuated by co-treatment with either actinomycin D, an inhibitor of gene transcription, or losartan, an AT_1 receptor antagonist.

In other studies, the possiblity that Ang II-stimulated $\cdot O_2^-$ production might contribute to vascular tone in human subcutaneous resistance arteries (SRA) was investigated. Isometric tension studies failed to demonstrate any positive effect of Ang II on the contractile response of SRA to norepinephrine. One reason for this may be the relatively minor contribution of nitric oxide to the vasorelaxant response of these arteries.

An investigation of the effect, if any, of demographic characteristics, risk factors for atherosclerosis, and individual drug therapies, on vascular $\cdot O_2^-$ production in the IMA of 79 patients was also performed. Multivariate analyses demonstrated that increasing age was weakly associated with increased vascular $\cdot O_2^-$ production whereas treatment with an angiotensin converting enzyme inhibitor or AT_1 receptor antagonist was independently associated with reduced vascular $\cdot O_2^-$ concentrations.

In summary, $\cdot O_2^-$ production is greater in IMA than in SV. Several enzymes capable of generating ROS are distributed throughout the wall of these arteries. NAD(P)H oxidase is a major source of $\cdot O_2^-$ generation. The activity of this enzyme is enhanced by Ang II, through a mechanism which involves and increase in gene transcription and protein synthesis. This may be clinically important as inhibitors of the renin-aldosterone-angiotensin system may reduce vascular $\cdot O_2^-$ production.

1.2 Declaration

I declare that this thesis has been composed by myself and is a record of work performed by myself. It has not been previously submitted for any other degree. The work described in this thesis was carried out under the supervision of Professor AF Dominiczak and Professor JJV McMurray in the Department of Medicine and Therapeutics at the Western Infirmary, Glasgow.

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Colin Berry

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1.6 List of Publications

Full publications containing work undertaken for this thesis:

Original papers:

Berry C, Hamilton CA, Brosnan MJ, Magill F.G, Berg G, McMurray JJV, and Dominiczak AF (2000a): An investigation into the sources of superoxide production in human blood vessels: Angiotensin II increases superoxide production in human internal mammary arteries. *Circulation* **101,** 2206-2212.

Berry C, Anderson N, Kirk A, Dominiczak AF, and McMurray JJV (2001b): Renin-angiotensin system inhibition is associated with reduced free radical concentrations in arteries of patients with coronary heart disease. *Heart* **86,** 217-220.

Brosnan MJ, Berry C, Hamilton CA, Alexander MY, Berg G, McMurray JJV, Dominiczak AF (2001). Distribution of NAD(P)H oxidase subunits in human arteries and mechanisms of activation by angiotensin II. (Submitted to *Hypertension*).

Reviews

Berry C, Touyz R, Dominiczak A, Webb RC, and Johns DG (2001): Angiotensin receptors: signaling, pathophysiology and novel interactions with ceramide. *American Journal of Physiology* 281:H2337 – H2365.

Berry C, Brosnan J, Hamilton C, Fennell J, and Dominiczak AF (2001): Oxidative stress and vascular damage in hypertension. *Current Opinion in Nephrology and Hypertension* **10,** 247-255.

Berry C and Clark AL (2000): Catabolism in chronic heart failure. *European Heart Journal* **21,** 521-532.

Oral Presentations to Learned Societies containing work undertaken in for this thesis:

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Experimental Medicine.

F Magill, MJ Brosnan, F Magill, Hamilton CA, **Berry C**, Dominiczak AF (1999). Quantification of superoxide dismuatse proteins in human internal mammary arteries and saphenous veins. *Scottish Society of Experimental Medicine.*

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Poster Presentations

Berry C, Alexander MY, Brosnan MJ, Hamilton CA, Dominiczak AF McMurray JJV (2001). Free radical producion in human blood vessels. *Clin Sci,* M21.

Brett M, Berry C, Hamilton CA, Pathi V, McMurray J, and Dominiczak AF (2001): Does endothelin stimulate free radical production in human blood vessels? *Seventh International Conference on Endothelin*

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1.7 List of Abbreviations

Act D	Actinomycin D
ACE-I	Angiotensin converting enzyme inhibitor
ACh	Acetylcholine
ALP	Allopurinol
Ang II	Angiotensin II
ARA	Angiotensin receptor antagonist
AT	Angiotensin receptor
CL	Chemiluminescence
DETCA	Diethyldithiocarbamate
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonulcease
DPI	Diphenyleniodonium
ED	Endothelial denudation
EDRF	Endothelium – derived relaxant factor
ELISA	Enzyme – linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
FADH	reduced flavin adenine dinucleotide

GAPDH	Glyceraldeyde – 3- phosphate deydrogenase
HMG-CoA	3-Hydroxy-3-methylglutaryl coenzyme A reductase.
HUVEC	Human umbilical vein endothelial cell
IMA	Internal mammary artery
iNOS	Inducible nitric oxide synthase
JAK	Janus kinase
Kb	Kilobase
Kd	Kilodalton
L-NAME	N^{ω}-Nitro-L-arginine methyl ester
LVEF	Left ventricular ejection fraction
mRNA	messenger ribonucleic acid
NAD(P)H	reduced nicotinamide adenine dinucleotide phosphate
NE	Norepinephrine
·NO	Nitric oxide
NOS	Nitric oxide synthase
NYHA	New York Heart Association
$\cdot O_2^-$	Superoxide
PCR	Polymerase chain reaction

PE	Phenylephrine
Phox	phosphate oxidase
RAAS	Renin angiotensin aldosterone system
RNA	Ribonucleic acid
RT	Reverse transcriptase
RTPCR	Reverse transcription polymerase chain reaction
SHR	Spontaneously hypertensive rat
SHRSP	Spontaneously hypertensive stroke – prone rat
SH3	SRC Homology 3
SOD	Superoxide dismutase
SRA	Subcutaneous resistance artery
STAT	Signal transducers and activators of transcription
SV	Saphenous vein
TBARS	Thiobarbituric acid-reactive substances
VSMC	Vascular smooth muscle cell
XOR	Xanthine oxidoreductase

1 Introduction

Reactive oxygen species (ROS) are being increasingly recognised as pleiotropic chemicals which participate in multiple metabolic reactions. Cell growth and behaviour can be modulated by ROS, which have a variety of differing biological effects. Increased vascular ROS production can result in reduced bioavailable nitric oxide with pathophysiological effects such as impaired endothelium-dependent relaxation, which are features of disease states such as hypertension and coronary heart disease.

The purpose of this thesis is to investigate the enzymatic sources of and stimuli for ROS production in human vascular cells and blood vessels. In particular, the possibility that vasoactive hormones, such as angiotensin II (Ang II), might stimulate vascular ROS production will also be explored.

1.1 Reactive oxygen species: basic biochemistry

ROS are oxygen-containing chemicals, which may have a single unpaired electron. A free radical is defined as any chemical species capable of independent existence that contains one or more unpaired electrons (Halliwell and Gutteridge 1988). However, not all ROS are free radicals. For example, hydrogen peroxide and singlet oxygen are ROS, however neither of these chemicals have single unpaired electrons, and are therefore not free radicals.

Free radicals are formed by the loss or gain of a single electron. The characteristic properties of free radicals include:

A chemical species with a single unpaired electron

Chemically unstable and usually highly reactive

Low chemical specificity

Autocatalytic: diverse reaction products produced

Generated in vitro and in vivo

In the ground state, molecular oxygen contains two unpaired electrons in parallel states

of spin, which contrasts with other stable organic molecules which typically have paired electrons in anti-parallel spin states. Oxygen (O_2) is therefore an electron acceptor. Together with its high midpoint redox potential (which leads to maximal energy conservation in redox reactions) and its solubility in aqueous solutions, oxygen is an ideal candidate for the transfer of electrons during oxidative phosphorylation (Figure 1.1) (Bunn and Poyton 1996; Fridovich 2001).

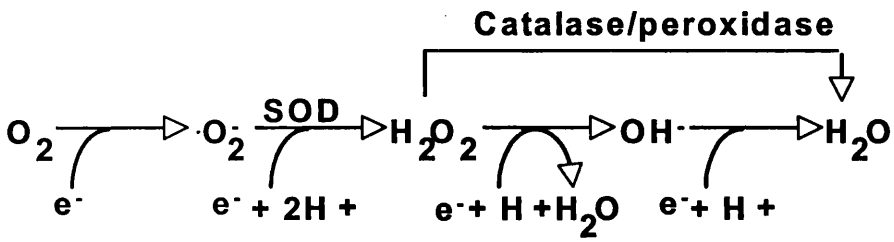


Figure 1.1 Steps in the 4 electron reduction of molecular oxygen to water by mitochondrial oxidase enzymes.

The reduction of oxygen to water in the mitochondria is, however, frequently incomplete, resulting in the generation of reactive oxygen intermediates, such as $\cdot O_2^-$ and the hydroxyl radical ($\cdot$OH). ROS are, therefore, a heterogeneous group of reactive, intermediate chemical species. Other examples of chemicals which have similar *in vivo* activity include the reactive nitrogen species, such as nitric oxide ($\cdot$NO) and the sulphur-derived thiyl radicals. Interestingly, $\cdot$NO is a very stable radical, and may exist as a free entity *in vivo*.

In vivo, ROS have important physiological effects. The reaction of $\cdot$NO with $\cdot O_2^-$ *in vivo* is a function of both the kinetics of this reaction and the relative tissue concentrations of these ROS. Ordinarily, endogenous SOD activity modulates $\cdot O_2^-$ availability (Gupte *et al* 1999), such that concentrations of $\cdot O_2^-$ are within the picomolar range, and reaction with $\cdot$NO is minimised (Figure 1.2).

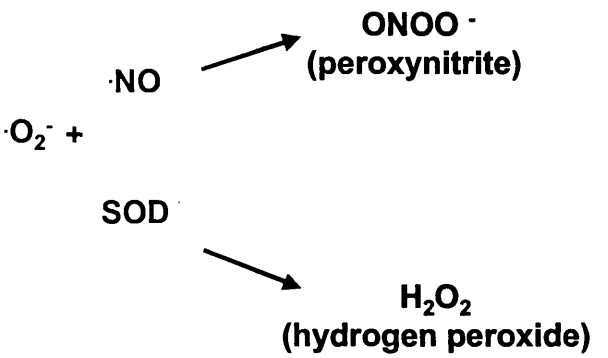


Figure 1.2 Schematic diagram representing the inactivation of superoxide ($\cdot O_2^-$) by either nitric oxide ($\cdot NO$) or superoxide dismutase (SOD).

If either $\cdot NO$ or $\cdot O_2^-$ concentrations become elevated to within the nanomolar range then these two radicals may react to form peroxynitrite (Table 1.1) (Fridovich 2001; Wolin 2000).

Table 1.1 Oxidative reactions

Oxidative Reaction	Enzyme	Rate constant ($M^{-1}s^{-1}$)
O_2 + electron $\rightarrow \cdot O_2^-$	oxidase	8×10^4
$\cdot O_2^- + \cdot O_2^- \rightarrow H_2O_2 + O_2$	SOD	2×10^9
$\cdot O_2^- + \cdot NO \rightarrow ONOO^-$	-	7×10^9

Superoxide may also be metabolised to secondary ROS. Superoxide is dismutated to hydrogen peroxide (H_2O_2), which in turn, forms the hydroxyl radical ($\cdot OH$) and hydrogen peroxide anion (OH^-) via the Fenton reaction:

$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH + OH^{\cdot}$

The Fenton reaction may occur at biologically important rates. In the liver, micromolar concentrations of hydrogen peroxide and divalent iron may lead to the generation of 4.58 x 10^{13} hydroxyl radical per second, roughly equivalent to 500 radicals per hepatocyte per second (Halliwell and Gutteridge 1988). Superoxide is readily converted into the hydroperoxyl radical ($^{\cdot}HO_2^-$), which is a much more reactive oxidant (Fridovich 2001).

1.2 Methods of measurement of vascular superoxide concentrations

Given the pleiotropic biological effects of ROS, there has been considerable interest in the development of methods for their measurement. These include *in vitro* methods based on chemiluminescence, cytochrome C reduction, electron spin resonance and fluorescence. These methods differ widely in their specificity and sensitivity for individual ROS, their utility for laboratory and clinical research, including ease of use and cost.

1.2.1 Chemiluminescence

Chemiluminescence may be defined as the light emission which accompanies the generation of reaction products, such as ROS. There is therefore a background light emission by cells, and in particular by activated phagocytes, which is most likely due to the conversion of chemical energy present in ROS to photons of light, occurring on the metabolism of ROS to less active forms (Halliwell and Gutteridge 1988). This light emission may be enhanced by the addition of a luminophore, a compound which emits light under certain conditions of chemical excitation, thereby releasing energy resulting a return to ground state.

Luminophores, such as firefly luciferin, are naturally occurring compounds. Lucigenin, or bis-*N*-methylacridinum nitrate, and luminol, 5-amino-2,3-dihydro-1,4-phtalazinedione, are luminophores which have been used to measure as $^{\cdot}O_2^-$ and $^{\cdot}OH$, respectively. Lucigenin was first described as a tool to measure as $^{\cdot}O_2^-$ by Gyllenhammar (1987), previously, as $^{\cdot}O_2^-$ was measured by the cytochrome *C* reduction method. Luminol is a non-specific luminophore as it reacts with a variety of different ROS (Halliwell and Gutteridge 1988). Alternatively, lucigenin, has been found to

luminesce under conditions attributed to $\cdot O_2^-$ generation (Gyllenhammar 1987). For example, Gyllenhammar (1987) reported that in cell-free conditions, the lucigenin-enhanced chemiluminescence signal associated with the xanthine (20 μmol/L) -xanthine oxidase (0.03 U) reaction was completely inhibited by the addition of treatments to specifically remove $\cdot O_2^-$. This was done both by the addition of superoxide dismutase (SOD) which catalyses the dismutation of $\cdot O_2^-$ to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), and by the addition of 4,5-dihydroxy-1,3-benzene-disulphonic acid salt (Tiron) which scavenges $\cdot O_2^-$. Furthermore, the addition of catalase, an enzyme which converts H_2O_2 to H_2O and O_2, had no effect on this signal, suggesting that neither H_2O_2 nor $\cdot OH$, an intermediate by-product of this reaction, do not contribute to xanthine/xanthine oxidase – induced lucigenin-enhanced chemiluminescence.

Lucigenin reacts with superoxide to form a dioxetane intermediate which decays by a light emitting process (Spasojevic *et al* 1999; Spasojevic *et al* 2000; VasquezVivar *et al* 1997). This can be represented by the following steps:

1) $LC^{2+} + e^- \rightarrow \cdot LC^+$ Formation of lucigenin radical by univalent reduction

2) $\cdot LC^+ + \cdot O_2^- \rightarrow$ dioxetane Formation of dioxetane intermediate

3) Dioxetane decomposition into two molecules of *N*-methylacridone, one of which is electrically excited.

4) Emission of a photon from the excited acridone state with return to ground state.

Lucigenin itself has been reported to generate $\cdot O_2^-$ because of redox cycling at higher concentrations (Liochev and Fridovich 1997; Liochev and Fridovich 1998; Vasquez-Vivar *et al* 2000; Skatchkov *et al* 1999; VasquezVivar *et al* 1997). The reason for this is the propensity for the $\cdot LC^+$ radical to undergo auto-oxidation. Lucigenin may therefore be responsible for $\cdot O_2^-$ generation and this has been confirmed by *in vitro* cyclic voltametry studies in aqueous solutions (Spasojevic *et al* 2000).

As a result of these observations, other workers have undertaken studies to confirm

whether or not lucigenin-enhanced chemiluminescence is a valid method for quantification of $\cdot O_2^-$ generation in biological systems (Li *et al* 1998a; Skatchkov *et al* 1999). In studies by Li *et al* (1998a) three methods were used to investigate the specificity of lucigenin-enhanced chemiluminescence for the detection of $\cdot O_2^-$ generation and its relationship with lucigenin concentration. Their studies of $\cdot O_2^-$ generation in cultured monocytes included sensitive measurement of lucigenin-enhanced chemiluminescence using a Berthold LB9505 luminometer, SOD-inhibitable ferricytochrome C reduction at 550 nm, O_2 consumption by O_2 polarography, and $\cdot O_2^-$ production by 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) spin trapping. They demonstrated that low concentrations of lucigenin (i.e. <50 μmol/L) were associated with constant, reproducible measurements of $\cdot O_2^-$ concentrations, however, lucigenin concentrations at, or above 50 μmol/L, were associated with incremental increases in signal, or $\cdot O_2^-$ concentrations. This was also reflected by increments in O_2 consumption and DEPMPO spin trapping. These data suggest that in an aqueous environment, lucigenin is itself reduced when present in higher concentrations, leading to an enhanced chemiluminescence signal. These effects can be explained by the ability of lucigenin to undergo redox cycling when present in sufficiently high concentrations. When the effect of addition of either xanthine/xanthine oxidase, at concentrations of 4μg/ml and 0.5 mmol/L, respectively, or lipoamide dehydrogenase NADH, at concentrations of 10 μg/ml and 0.5 mmol/L, respectively, to these cells was assessed, a lucigenin concentration-effect was observed only with the NADH system. The authors concluded that the availability of the lucigenin cation radical, and therefore the potential for redox cycling, was dependent on both the concentration of lucigenin and the experimental condition. Furthermore, the lucigenin concentration at which redox cycling does *not* occur should therefore be determined for any particular experimental condition.

1.2.2 Electron spin resonance spectroscopy

Electron spin resonance (ESR) spectroscopy is an electromagnetic technique which can be used to detect and quantify free radical activity (Halliwell and Gutteridge 1988). An unpaired electron has a spin of either +1/2 or –1/2, and therefore behaves as a small magnet. If it is exposed to an external magnetic field, the electron will align itself in opposition to that field, in either a parallel or anti-parallel alignment. If an appropriate electromagnetic field is applied, this energy will be absorbed by the free radical, causing a shift in the single electron's energy state. This absorbance can be measured by an ESR

spectrometer. In fact, the spectrometer actually measures the derivative of absorbance, that is the rate of change of absorbance:

$$\Delta E = g\beta H$$

where ΔE is the difference in energy between the two energy levels of the electron, H is the applied magnetic field, β a constant known as the Bohr magneton, and g is the 'splitting factor' which for a free electron is a constant of 2.00232. Absorption spectra have typical patterns according to the chemical species that is being detected. These spectra are known as 'hyperfine structures'.

ESR spectroscopy is a highly sensitive technique for the measurement of free radicals. It may be used to detect free radicals at concentrations as low as 1×10^{-10} mol/L. One limiting factor for the use of this method is that many free radicals are highly labile, and may not exist for long enough to be reliably measured by this technique. A variety of methods have been developed in order to overcome this problem, including flow systems, low-temperature solid matrix systems, and spin trapping. This latter approach causes the labile radical to react with a spin-trap compound to form a radical which has a longer half-life. An ideal 'spin-trap' is a compound which will react specifically with the radical of interest, to form another radical which is more stable, and has a characteristic ESR spectrum. DEMPO is a spin trap which reacts with both $^{\cdot}OH$ and $^{\cdot}O_2^-$ to form products with different ESR spectra. In this case, ethanol can be used to specifically quench $^{\cdot}OH$ radicals, therefore eliminating this radical's ESR spectra to leave that of $^{\cdot}O_2^-$. ESR spectroscopy may be used to measure $^{\cdot}O_2^-$ concentrations in biological systems in a highly specific manner (Li *et al* 1998a).

The use of this technique, however, is limited by a number of factors (Halliwell and Gutteridge 1988). Cellular reducing agents, such as vitamin C, may reduce the spin-trap compound to give an 'ESR-silent' species. ESR spectroscopy is also very expensive because of the type of equipment and reagents that are required. Furthermore, this equipment may occupy a considerable area of laboratory space. Additionally, such experiments are also very time-consuming to perform.

1.2.3 Cytochrome C reduction

Superoxide production can also be assessed by the cytochrome *C* reduction method. In this case, the generation of $^{\cdot}O_2^-$ is measured indirectly by the reduction of

ferricytochrome *C* at 550 nm. Non - $\cdot O_2^-$ dependent reduction of cytochrome *C* can be corrected for by deducting all activity not inhibited by the addition of SOD. However, the integrative nature of this method makes the study of dynamic response patterns difficult. In order to overcome this problem, chemiluminescence techniques, which have wider applicability and higher sensitivity, have received greater attention.

1.2.4 Oxidative fluorescent microtopography

Superoxide concentrations may also be measured in biological systems by fluorescent techniques. Fluorescent dyes, which are membrane permeable, can be used in conjunction with image analysis technology to measure both intra and extracellular ROS concentrations. Oxidative fluorescent microtopography is a technique which allows the *in situ* localisation of $\cdot O_2^-$ in intact blood vessels (Carter *et al* 1994; Miller *et al* 1998). Hydroethidine (HEt) is a dye, which is oxidised in the presence of $\cdot O_2^-$ to ethidium bromide (Et). The dye is topically applied to sections (approximately 50mm width) of blood vessels and the fluorescence can then be assessed microscopically after exciting at 585nm wavelength. The intensity and localisation of the oxidised HEt, which reflects $\cdot O_2^-$ production, can then be observed. This method of analysis is affected by fluctuations in both temperature and pH, and the fluorescent signal decays with time. Quantification of ROS concentrations using fluorescent techniques is, therefore, only semi-quantative, however, these techniques can yield useful qualitative information on, for example, the location and distribution of ROS production in a blood vessel wall.

1.3 Sources of vascular superoxide production

Superoxide may be produced by a variety of enzyme reactions *in vivo*. In experimental animals, the main enzymatic sources of $\cdot O_2^-$ production within the vascular wall are nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase (Bouloumie *et al* 1997; Mohazzabh *et al* 1994; Pagano *et al* 1995) xanthine oxidase (Mugge *et al* 1994 ; Ohara *et al* 1993), and nitric oxide synthase (NOS) enzyme (Holland *et al* 1990; Kerr *et al* 1999; McIntyre *et al* 1997). The cellular sources of vascular $\cdot O_2^-$ production in animals are the endothelium (Brandes *et al* 1997; Kerr *et al* 1999) (Mohazzabh *et al* 1994), vascular smooth muscle cells (VSMC) (Miller *et al* 1998; Mohazzab and Wolin 1994) and fibroblasts within the adventitia (Wang *et al* 1999). The cellular and enzymatic sources of ROS production in human blood vessels have not been fully explored.

1.3.1 NADP(H) Oxidase

NAD(P)H oxidase is ubiquitous throughout eukaryotic cells, but a plasma membrane-bound form has been demonstrated to be responsible for transmembrane electron transport (Morre and Brightman 1991). This enzyme catalyses the transfer of electrons from reduced pyridine nucleotides (NADH and NADPH) to molecular oxygen (O_2), which results in the formation of $\cdot O_2^-$. Other characteristic features of this enzyme include resistance to cyanide, which distinguishes it from mitochondrial oxidases, and activation by ligands, such as hormones (Morre and Brightman 1991). In teleological terms, phagocyte NAD(P)H oxidase is host-protective. The importance of vascular NAD(P)H oxidase in relation to cardiovascular physiology is not yet clear.

1.3.1.1 Physical chemistry of NAD(P)H oxidase

The enzyme was initially isolated from and studied in neutrophils. The enzyme is formed by the aggregation of a minimum of 5 proteins (Figure 1.3): a plasma membrane cytochrome b558 which is composed of a glycoprotein 91 kilodalton (Kd) phosphate oxidase (gp91phox) and a 22 Kd (p22) phox, two cytosolic proteins, p47phox and p67phox and a cytosolic G protein (either rac1 or rac2).

These four cytosolic proteins physically associate with the cytochrome to form the membrane-bound NAD(P)H oxidase (Babior 1999; Cross and Jones 1991). The protein-protein interactions are made possible by the presence of Src homology 3 (SH3) domains and polyproline motifs. These are small protein loci known to mediate interactions between proline-rich proteins (Fuchs *et al* 1996; Ren *et al* 1993) The mechanisms involved in the activation of NAD(P)H oxidase by metabolites of arachidonic acid were investigated by Sumimoto *et al* (1994). Both p47phox and p67phox contain SH3 domains (Leto *et al* 1990). In these studies, the mechanisms of assembly of the phox subunits were investigated by treating phagocytes with arachidonic acid (AA), in the presence and absence of a p47phox - SH3 domain fusion protein (anti-p47-SH3 antibody). Binding studies of p47 phox to either a monoclonal antibody directed to this protein or to p67phox, were also performed. These investigations demonstrated that p47phox SH3 fusion protein only bound to native p47phox in the presence of AA. In the absence of AA, p47phox SH3 fusion protein only bound to native phox if the phagocytes had been pre-treated with a p47phox binding protein tagged with SH3. Taken together, these observations suggest that treatment with AA induces a change in conformation of the p47phox protein, therefore exposing its

SH3 domain (i.e. p47phox-SH3). Further studies demonstrated that p47phox only bound to p67phox in the presence of arachidonic acid. This suggests that arachidonic acid may have similar effects on p67phox.

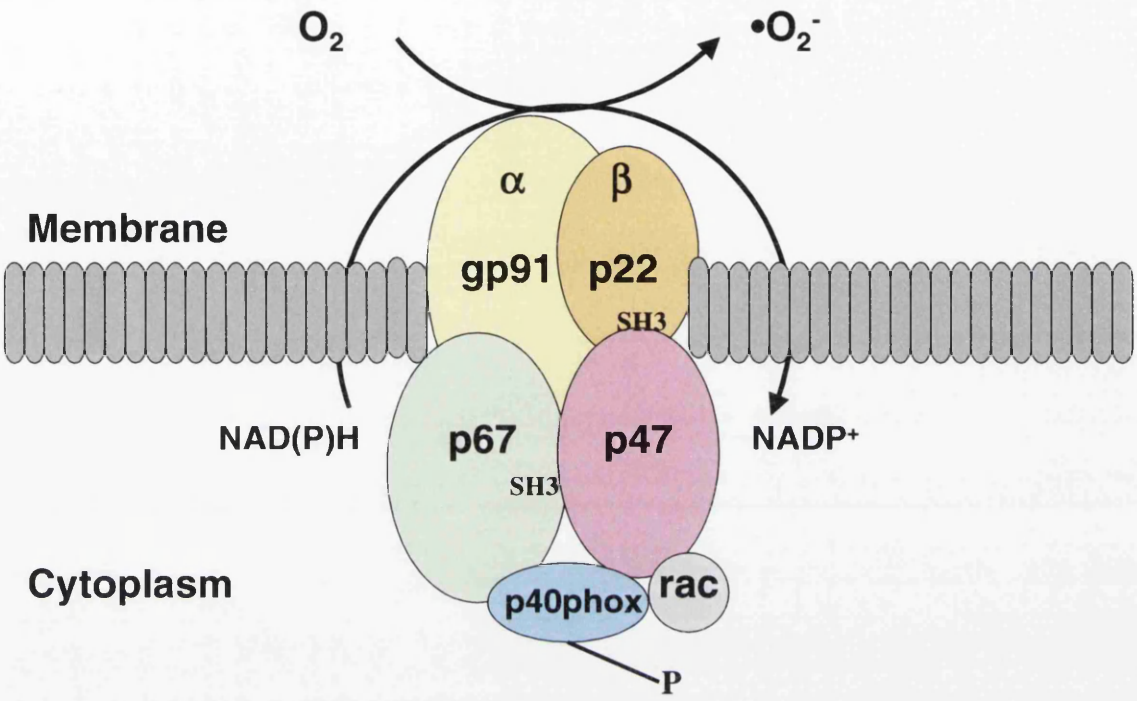


Figure 1.3 Vascular NAD(P)H oxidase, which is constituted by the aggregation of the cytoplasmic and membrane subunits. P47phox (phosphate oxidase) and p67phox, along with the small GTPase, rac-1, are cytoplasmic subunits. P40phox, another cytoplasmic subunit, facilitates the assembly of these proteins. Gp91phox and p22phox, which together form cytochrome b_{558}, constitute the locus of electron transfer.

In other studies, p47-SH3 was demonstrated to bind to the cytoplasmic domain of p22phox (expressed as a fusion protein), this being prevented by a single amino acid substitution (Gln for Pro-156) in p22phox. This amino acid substitution occurs in some patients with chronic granulomatous disease who have an inactive membrane cytochrome b558 (Dinauer *et al* 1991), illustrating the importance of this interaction for phox assembly and NAD(P)H oxidase activation. By contrast, gp91phox does not have a binding site for p47-phox (Dinauer *et al* 1987).

In addition, a sixth protein, p40phox, may also physically associate with NAD(P)H oxidase (Wientjes *et al* 1993). P40phox is a cytosolic protein which is bound to p67phox and p47phox (Rinckel *et al* 1999). Phosphorylation of p40phox, which is

subject to regulation by GTPases (Rinckel *et al* 1999), facilitates translocation of this protein, along with p47phox and p67phox, to the cell membrane, thus resulting in the formation of NAD(P)H oxidase. The formation of this enzyme occurs as a result of tyrosine kinase-mediated phosphorylation of its cytosolic protein subunits (Fuchs *et al* 1997). Interestingly, topological studies by (Fuchs *et al* 1996) led to the hypothesis that p40phox may also serve to prevent *spontaneous* interaction of these subunits.

More recently, it has been established that approximately 90% structural homology exists between the cytochrome b558 subunits (gp91phox and p22phox) of rat neutrophil and endothelial cell NAD(P)H oxidases (Fukui *et al* 1995; Bayraktutan *et al* 2000). In rat endothelial cells, however, these subunits have a predominatly subcellular location being within the endoplasmic reticulum rather than in the plasma membrane, as is the case in neutrophils (Bayraktutan *et al* 2000). At a tissue level, however, differences in the distribution of gp91phox within the vascular wall have been demonstrated. This protein can be detected at the mRNA and protein level in endothelial and adventitial cells, but gp91phox is undetectable using these techniques in rat VSMC (Gorlach *et al* 2000; Suh *et al* 1999). Alternatively, VSMC express a protein, nox1 (for NAD(P)H oxidase –1; or non-phagocytic oxidase) which has 56% homology with gp91phox. This homologous protein may also be coupled with p22phox to form the membrane cytochrome b558 subunit, and support electron transport, in these cells (Lassegue *et al* 1999; Suh *et al* 1999). There are now several homologues (nox1, nox4 and gp91phox) described in the nox family (Lambeth *et al* 2000). These data pertain to studies undertaken in rat VSMC and the existence, and abundance, of nox family members in human vascular cell types is not known.

The activated form of plasma membrane NAD(P)H oxidase is responsible for the univalent reduction of O_2 to $\cdot O_2^-$ (Cross and Jones 1991; Morre and Brightman 1991). Electron transport by NAD(P)H oxidase occurs on the membrane-bound cytochrome b558 complex, which transfers a single electron from NADH or NADPH to molecular O_2:

$$NAD(P)H + 2O_2 \rightarrow NAD(P)^+ + H^+ + 2\cdot O_2^-$$

As the cytochrome b558 is a transmembrane protein, it may accept electrons from both intracellular and extracellular reducing equivalents. Superoxide production is maximal in conditions of both high oxygen tension and NAD(P)H concentration, such that oxygen consumption by this pathway will increase. The redox potential of this reaction

is low (E_m = -245mV), such that this cytochrome has a greater propensity than any other cytochrome to reduce molecular oxygen to $\cdot O_2^-$ (Cross and Jones 1991). NAD(P)H reducing equivalents are generated by the pentose phosphate pathway. The extracellular release of electrons depolarises the cell membrane, which is counterbalanced by the concomitant release of H^+ ions. This enzyme therefore generates superoxide in a low-output constitutive manner, in contrast to the high-output bursts of phagocytes.

1.3.1.2 Biochemistry of NAD(P)H oxidase

What is the role of membrane-bound NAD(P)H oxidase in the generation of $\cdot O_2^-$? Studies of NAD(P)H oxidase activity have used a number of pharmacological antagonists to characterise the biological effects of this enzyme. For example, $\cdot O_2^-$ generation by endothelial cell NAD(P)H oxidase is inhibited by quinone analogs, such as Coenzyme Q, flavoprotein inhibitors, such as quinacrine and diphenyleneiodonium (DPI), and cytosolic subunit assembly inhibitors, such as apocynin (Holland *et al* 2000).

In neutrophils, $\cdot O_2^-$ is released into the phagocytic vacuole where it has a bactericidal effect. In non-phagocytic cells, NAD(P)H oxidase serves as a disposal mechanism for intracellular electrons. Vascular NAD(P)H oxidase-dependent $\cdot O_2^-$ production may also have a protective role. This enzyme may also serve as an oxygen-sensor in the vasculature (Bunn and Poyton 1996; Goligorsky 2000). In this case, vascular tone and blood flow may be modulated by alteration in NAD(P)H oxidase-dependent $\cdot O_2^-$ production (and $\cdot$NO removal), according to variation in oxygen tension in both blood and the vessel wall. The concept of NAD(P)H oxidase as an oxygen sensor has attracted some controversy (Bunn and Poyton 1996). For example, the neuronal stimulus to ventilation during hypoxia occurs through an inhibition of $\cdot O_2^-$-sensitive K^+ channels in the cells of the carotid body where NAD(P)H oxidase is present in abundance (Bunn and Poyton 1996). During *in vitro* studies, inhibition of NAD(P)H oxidase by treatment of these cells with the flavin-dependent enzyme inhibitor, DPI, reduced the hypoxia-induced discharge of these cells, implicating this enzyme as an oxygen sensor (Cross and Jones 1991). One argument against this hypothesis include the fact that mice lacking the gp91phox subunit of NAD(P)H oxidase have both appropriate K^+ channel inhibition and pulmonary artery vasoconstriction to hypoxia (Archer *et al* 2000). Furthermore, that patients with chronic granulomatous disease, an inherited disorder which results in the deletion of one of the protein subunits of NAD(P)H oxidase, are normoxaemic and have normal respiration (Bunn and Poyton 1996) also mitigates against NAD(P)H oxidase acting as an oxygen sensor.

The phox subunits seem to play different roles in NAD(P)H oxidase activation. Cross *et al* (1999; 2000) studied the potential roles of the cytosolic proteins for NAD(P)H oxidase activity. They used the SOD-inhibitable cytochrome *c* reduction assay in order to measure $\cdot O_2^-$ production by purified neutrophil membranes (i.e. containing cytochrome b558 (p22phox and gp91phox) and neutrophil cytosol in a cell-free system. The relative contribution of individual cytosolic protein subunits of NAD(P)H oxidase was assessed by the addition of recombinant phox proteins to the neutrophil membranes. Addition of either p47phox or p67phox proteins to the reaction mixture resulted in an increase in $\cdot O_2^-$ production, whereas this was not observed when p40phox recombinant protein was added. In this case, the addition of p40phox was associated with a very small rise in $\cdot O_2^-$ production, but only in the presence of p47phox. Further experiments confirmed that p40phox promoted $\cdot O_2^-$ production by p47phox. The phosphorylation and binding of p40phox may facilitate the conformational changes necessary for p47phox binding and activation of NAD(P)H oxidase. This thesis is supported by *in vitro* observations by Fuchs *et al* (1997) who demonstrated that in promyelocytes, p40phox was phosphorylated even in a resting state, and that on activation, additional phosphorylation of this protein occurred, which correlated with $\cdot O_2^-$ production by these cells. Furthermore, after $\cdot O_2^-$ production by NAD(P)H oxidase had declined, p40phox was observed to be dephosphorylated.

The importance of membrane and cytosolic proteins for $\cdot O_2^-$ production by NAD(P)H oxidase has been investigated in a number of physiological studies of vascular phenotypes such as cell hypertrophy or $\cdot NO$-regulated myogenic tone in resistance arteries. Genetic manipulation, by either transfection of antisense p22phox DNA in rats (UshioFukai *et al* 1996), immunodepletion of p67phox in rabbits (Pagano *et al* 1997a), or knockout of gp91phox in mice (Gorlach *et al* 2000), have all demonstrated these subunits to be functionally important for both NAD(P)H oxidase and Ang II-dependent $\cdot O_2^-$ production (UshioFukai *et al* 1996), (Pagano *et al* 1997a). It would appear, therefore, that these subunits are crucial elements for NAD(P)H oxidase-dependent ROS generation. The functional importance of these and other components of this enzyme, such as p47phox, rac 1 and nox1, in human blood vessels remain to be explored.

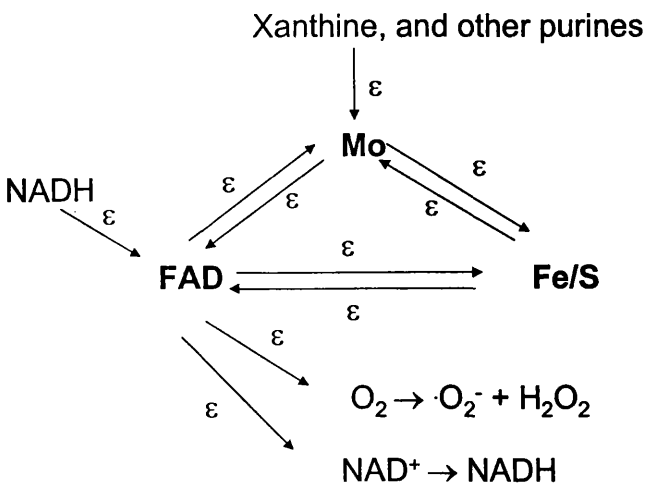
NAD(P)H oxidase is ligand-sensitive, such that $\cdot O_2^-$ production by this enzyme may be affected by a variety of hormones, cytokines and lipid metabolites (Cross and Jones 1991; DeKeulenaer *et al* 1998a). This enzyme is therefore activated by physiological and pathophysiological stimuli and, as such, $\cdot O_2^-$ production may serve as a second

messenger response. Furthermore, vascular NAD(P)H oxidase activity can be inhibited by treatment with glucocorticoids, the mechanism for this effect being a steroid-induced down-regulation of p22phox transcription (Marumo *et al* 1998). Disease states, such as endotoxaemia, have been associated with pro-oxidant induced vascular damage (Brandes *et al* 1999). This suggests that the therapeutic effect of treatments such as glucocorticoids may be in part mediated through inhibition of pro-oxidant enzymes.

1.3.2 Xanthine oxidase

1.3.2.1 Physical chemistry

Xanthine oxidoreductase (XOR) is a molybdenum-containing enzyme which may exist in one of two interconvertible forms, xanthine dehydrogenase (XDH) or xanthine oxidase (XO). XOR is primarily involved in purine catabolism, whereas XDH, which is the predominant form, preferentially reduces NAD^+ and does not generate ROS (Harrison 2000). By contrast, XDH may be converted to XO by Ca^{2+} - calmodulin dependent proteolysis. This reaction also occurs *in vivo* in the context of ischaemia-reperfusion. In this case, XO catalyses the conversion of hypoxanthine to xanthine, resulting in the reduction of molecular oxygen to $\cdot O_2^-$. Moreover, Sanders *et al.* (Sanders *et al* 1997) recently demonstrated that XOR has NADH oxidase activity as it may oxidise NADH, leading to increased production of $\cdot O_2^-$ (Figure 1.4).



Mo - molybdenum; Fe/S - Iron/sulphur; FAD - Flavin adenine dinucleotide; NADH - Reduced nicotinamide adenine dinucleotide; O_2 - oxygen; $\cdot O_2^-$ - Superoxide; H_2O_2 - Hydrogen peroxide.

Figure 1.4 Schematic representation of electron transfer catalysed by XOR. This

enzyme may use both NADH, xanthine and other purines as reducing substrates. Allopurinol, an inhibitor of XOR and uric acid production, inhibits $\cdot O_2^-$ generation at the molybdenum site, but does not prevent flavin-dependent $\cdot O_2^-$ generation.

1.3.2.2 Biochemistry of xanthine oxidase

XOR protein is widely distributed in a variety of cell types. In humans, it is recognised to be abundant in mammary, hepatic, and intestinal epithelial cells. XOR protein is also present in cardiovascular tissues and has been identified in the heart (Abadeh *et al* 1992) and in cultured vascular endothelial cells (Rouquette *et al* 1998). Using immunofluorescent techniques with confocal microscopy imaging, Adachi *et al* (1993) identified this protein both within the cytosol and on the external aspect of these cells. This suggests that these cells can synthesise XOR protein (Rouquette *et al* 1998). It is postulated that the XOR protein present on the external aspect of the cell surface may arise either as a result of secretion from the cytosol or by binding of circulating protein in the blood (Harrison 2000).

XOR enzyme activity in human plasma has been measured in healthy subjects (Yamamoto *et al* 1996), in hepatic (Ramboer and Piessins 1972) and rheumatic disease (Miesel and Zuber 1993), and in ischaemia-reperfusion (Friedl *et al* 1991; Tan *et al* 1995). This activity may be relevant to the pathophysiology of these diseases, given the potential of XOR for ROS generation. Interestingly, antibodies to XOR, rather than the protein itself, have also been measured in the blood of healthy human subjects (Benboubetra *et al* 1997). Benboubetra *et al.* (Benboubetra *et al* 1997) used an enzyme-linked immunosorbent assay (ELISA) to measure anti-human XOR antibodies in the sera of 258 subjects. They found that anti-human XOR IgM was 36.7±25.1 μg/mL and anti-human XOR IgG was 4.2±2.6 μg/mL representing 3% and 0.04% of total serum IgM and IgG respectively. Anti-human XOR antibody concentrations were significantly lower in women under the age of 50 years, however the reasons for this were not clear. Further studies demonstrated that XOR activity could be inhibited by treatment with anti-XOR antibody and that XOR in these sera samples was predominatly present in the form of immune complexes formed by association with these antibodies. The teleological function of circulating anti-XOR may be, therefore, to bind and neutralise XOR in the blood. This thesis is supported by the fact that the majority of blood XOR is bound to anti-XOR antibody, forming immune complexes, and that plasma activity of this enzyme is low (Yamamoto *et al* 1996).

In other studies, plasma concentrations of anti-human XOR were found to be increased in patients with an acute myocardial infarction (Benboubetra *et al* 1990; Harrison *et al* 1990). This may represent an adaptive response, however, the blood concentrations of XOR protein in cardiovascular and other disease states are not known. Moreover, it has been hypothesised, that the plasma activity of XOR may become biologically important in disease states where the plasma concentrations of this protein exceed those of the antibody binding proteins (Benboubetra *et al* 1990; Harrison *et al* 1990).

The XOR protein has been shown to bind to cell surface heparin-like glycosoaminoglycans. Studies by Adachi *et al* (1993) using cultured porcine aortic endothelial cells, column chromatography and antisera to human XOR, demonstrated that heparin may also bind XOR within a physiological pH range. The association between XOR protein and heparin could be inhibited by co-treatment with protamine, a heparin binding protein (Adachi *et al* 1993). In other studies by the same group, injection of intravenous heparin into healthy human subject was associated with a rapid increase in plasma concentrations of XOR.

That XOR is present within, and on the surface of, vascular cells suggests that ROS generated by this protein may be involved in cell signalling (Harrison 2000). XOR may also have an important role in the tissue damage which arises after ischaemia-reperfusion (McCord 1985). Adenosine triphosphate, which accumulates in ischaemia, undergoes anaerobic catabolism, which leads to the accumulation of hypoxanthine. In the hyperoxic conditions of reperfusion, XOR may then convert hypoxanthine to uric acid and $\cdot O_2^-$. This then may be one important pathway for excessive ROS production in ischaemic vasculature (McCord 1985).

By contrast, urate is an anti-oxidant. Urate suppresses ROS-induced vascular damage by, for example, reaction with peroxynitrite (a product of the reaction of $\cdot NO$ with $\cdot O_2^-$). This generates a variety of metabolites, some of which are nitrovasodilators, and therefore prevents protein nitration by peroxynitrite (Reiter *et al* 2000). Urate is also an anti-oxidant in human plasma (Ames *et al* 1981)

The production of XOR is quantitatively most abundant in the liver and intestine (Harrison 2000). XOR is, however, implicated in ROS-induced injury within the cardiovascular system (de Jong *et al* 2000; Dowell *et al* 1993; Gimpel *et al* 1995; Harrison *et al* 1990; Hori *et al* 1991; Nielsen *et al* 1994; Weinbroum *et al* 1995; Yokoyama *et al* 1990). This then raises the question as to the origin of XOR in the

cardiovascular system. Is XOR synthesised within cardiac and vascular tissues, where it may then be responsible for ROS generation, or alternatively, is XOR generated within hepatic and intestinal tissues, from which it may then be released into the circulation and bind to cardiovascular cells? Neilson *et al* (1996) investigated this question in a rabbit model of hepatic ischaemia and intestinal reperfusion. In this model, hepatic ischaemia was induced by occlusion of the supra-hepatic descending aorta for 40 minutes by use of a balloon catheter, followed by a 2-hour period of reperfusion. Venous blood samples were obtained at baseline and during reperfusion in the intervention group. Plasma XOR activity increased during reperfusion compared with baseline (729 +/- 140 mu U/ml, mean +/- standard error of the mean vs. 132 +/- 18 mu U/mL; $p < 0.001$). This was associated with increased plasma concentrations of hepatic enzymes such as aspartate aminotransferase, alanine transferase, and lactate dehydrogenase. In further studies undertaken to control for any possible effect of the surgical procedure (but without causing hepatic ischaemia), suprarenal-infrahepatic occlusion had no effect on plasma concentrations of alanine transferase.

In further studies, these investigators explored the possibility that increased release of hepatic and intestinal XOR could be associated with distant lung injury (Nielsen *et al* 1994). Pretreatment with tungstate, an inhibitor of XOR, reduced XOR activity and ameliorated liver and intestinal injury. Lung injury, manifested by increased bronchoalveolar lavage (BAL) protein concentration, BAL lactate dehydrogenase (LDH) activity and increased lung oedema, was associated with liver injury and circulating XOR activity. XOR inactivation significantly decreased BAL protein concentration, BAL LDH activity, and lung oedema. This suggests that remote lung injury may be modulated by hepatic injury leading to an increased production of circulating XOR.

Recently, XOR gene expression was compared between mice and humans (Xu *et al* 2000), in order to determine the basis for the low XOR activity in humans relative to non-primate mammalian species. The expression of XOR in human hepatocytes and vascular cells was found to be markedly lower than those of mice. Studies of both transcription rates and core promoter activity of this gene in the human cells demonstrated that the human XOR gene contains both repressor and activator binding regions which regulate core promoter activity, which may explain the low expression of this protein in human cells.

The pathways leading to XOR protein synthesis within human vascular cells and tissues

are poorly understood. Data on the presence or absence, and possible functional importance of XOR protein in human blood vessels, are lacking. As yet, the concentrations of XOR in the blood of healthy humans and patients with cardiovascular disease are unknown. The putative role of XOR in the pathophysiology of cardiovascular disease makes investigation of these questions important.

1.3.3 Nitric oxide synthase

Nitric oxide exists as three distinct redox forms, nitroxyl anion (NO^-), the nitrosonium cation (NO^+), and the nitric oxide free radical ($^{\cdot}NO$) (Nelli *et al* 2000). Although, NO^- is a potent relaxant of vascular and non vascular smooth muscle, it appears to do so indirectly, through conversion by copper-containing enzymes to $^{\cdot}NO$ (Nelli *et al* 2000). Although nitric oxide synthase (NOS) is a major source of $^{\cdot}NO$, paradoxically, this enzyme may also generate the $^{\cdot}NO$ – scavenger, $^{\cdot}O_2^-$. Neuronal (Xia *et al* 1996), inducible and endothelial NOS (Xia *et al* 1998) are all capable of $^{\cdot}O_2^-$ production. NOS will switch to generate $^{\cdot}O_2^-$ rather than $^{\cdot}NO$ in conditions where L-arginine and/or tetrahydrobiopterin, both of which are essential for $^{\cdot}NO$ synthesis, are deficient (Heinzel *et al* 1992; Pou *et al* 1992). As is the case for $^{\cdot}NO$ synthesis, $^{\cdot}O_2^-$ production by NOS is a function of intracellular free Ca^{2+}-dependent regulation of calmodulin kinase (Xia *et al* 1998). The versatility of ROS production by NOS is implicated in a number of vascular disease states, such as hypercholesterolaemia and heart failure, whereby a functional deficiency of L-arginine has been reported (Hirooka *et al* 1994; Stroes *et al* 1997). This suggests, therefore, that NOS may itself be dysfunctional and contribute to the pathophysiology of vascular damage.

1.4 Endogenous systems for superoxide removal

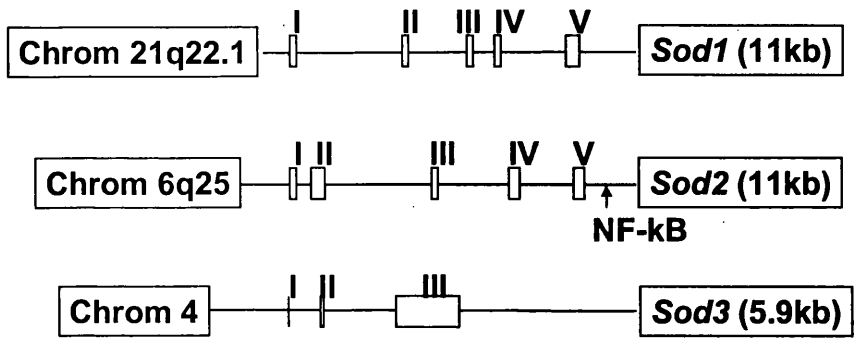
Superoxide may also be removed by endogenous scavenging enzymes, such as SOD (McCord and Fridovich 1969), catalase, peroxidase and by low molecular weight anti-oxidants such as vitamins C & E.

1.4.1 Endogenous enzymatic scavenging systems

SOD catalyses the dismutation of two $^{\cdot}O_2^-$ radicals to one molecule of H_2O_2 and one molecule of molecular O_2:

$$2\ {}^{\cdot}O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Three mammalian SOD proteins exist (Figure 1.5); copper/zinc SOD (Cu/Zn SOD) (McCord and Fridovich 1969), manganese SOD (Mn SOD), these being the intracellular isoenzymes, and extracellular SOD (EC-SOD) (Marklund 1982). These proteins are coded by three different genes.



NF-kB - Nuclear factor kappa B; SOD - superoxide dismutase; kb - kilobase
Chrom - chromosome; SOD1- Cu/Zn SOD; SOD2 - MnSOD; SOD3 - EC-SOD

Figure 1.5 The chromosomes which contain the genes for the three isoforms of superoxide dismutase (SOD) protein: copper/zinc SOD (Cu/Zn SOD), manganese SOD (Mn SOD), these being the intracellular isoenzymes, and extracellular SOD (EC-SOD) proteins.

Mn-SOD and Cu/Zn SOD are located within the mitochondria and cytosol respectively, and are major vascular isoenzymes which are important for both scavenging vascular $\cdot O_2^-$ and enhancing the bioavailability of endothelial $\cdot NO$ (Li *et al* 1995). EC-SOD has a high molecular weight (135,000), is composed of four non-covalently bound subunits each containing a copper atom. It is hydrophobic, and binds readily with proteoglycans, such as heparin (Sandstrom *et al* 1992), which suggests an affinity of this isoenzyme for cell membranes (Marklund 1982). The rate constant of the EC-SOD / $\cdot O_2^-$ dismutation reaction was originally determined to be 1×10^{-9} Mol/s (Marklund 1982). EC-SOD and cytosolic, or Cu-Zn, SOD are inhibited by diethyldithiocarbamate (DETCA), a copper chelating agent (Heikkila *et al* 1976; Mian and Martin 1995), which may be used in pharmacological studies to inhibit SOD activity.

Catalases are haem-containing enzymes which convert H_2O_2 to O_2 and water.

Peroxidases are again mostly haem-containing enzymes which utilise a variety of reducing substrates, such as NADH, to convert H_2O_2 to water (Fridovich 2001).

1.4.2 Endogenous non-enzymatic scavenging systems

Glutathione, or GSH, has a sulphydryl group, which enables it to scavenge hydroxyl and singlet oxygen radicals (Halliwell and Gutteridge 1988). Glutathione is abundant within cells, and is therefore an important intracellular anti-oxidant

$$2\ GSH + oxidant \leftrightarrow GSSG + reduced\ oxidant$$

An excess of GSSG, and consequently an imbalance in the GSH/GSSG ratio, occurs in situations of excess ROS activity. This is associated with abnormal cell metabolism, such as impaired protein synthesis (Halliwell and Gutteridge 1988). Excess activity of enzymes which metabolise glutathione, such as glutathione peroxidase (which oxidises glutathione), are therefore important in determining intra-cellular anti-oxidant activities (Mugge *et al* 1991a). In addition, thiyl radicals are formed when glutathione scavenges hydroxyl radicals, or when glutathione is oxidised by peroxidases. Thiyl radicals are, however, less reactive than hydroxyl radicals.

Uric acid is one other endogenously produced anti-oxidant. Uric acid is the end-product of purine metabolism and is present in human extracellular fluids, such as plasma, at concentrations of 0.25 – 0.4 mmol/L. Humans, unlike some other species, do not have urate oxidase, which appears to have been 'lost' during our evolutionary development. Consequently, urate is excreted in the urine. The abundance of uric acid in the human body may be advantageous, as it is a powerful scavenger of hydroxyl and peroxyl radicals, and singlet oxygen. Like GSH, however, the by-products of these reactions are themselves, weak radicals. Uric acid has important extracellular anti-oxidant activity, and in particular, inhibits lipid peroxidation by binding iron and copper ions into forms incapable of generating free radicals.

In vivo, ascorbic acid (vitamin C) and alpha-tocopherol (vitamin E) have ROS scavenging activities (Halliwell and Gutteridge 1988). Ascorbic acid is a water-soluble, reducing agent. It is present in human plasma at concentrations of 50 – 200 μmol/L and has a second order reaction rate with $\cdot O_2^-$ of 2.7 x 10^5 $M^{-1}s^{-1}$ at a physiological pH of 7.4. Dehydroascorbate is produced as a result of this reaction, which ultimately breaks down to form oxalic and threonic acids. Vitamin C is an important scavenger of ROS,

such as $\cdot O_2^-$ and $\cdot OH$, *in vivo*. This activity is concentration dependent. Furthermore, vitamin C is oxidised in the presence of transition metals, such as Fe^{3+} and Cu^{2+}, forming H_2O_2 and $\cdot OH$ as by-products of these reactions. Vitamin C is ubiquitous throughout the human body and, for example, this vitamin has important anti-oxidant activity in the eye. It is present in high concentrations in the lens, cornea and aqueous humour of day-living mammals (1 – 2 mmol/L), whereas in nocturnal animals, such as cats and rodents, vitamin C is much less abundant. Vitamin C is also an important anti-oxidant in cardiovascular tissues. Low plasma concentrations of vitamin C, and therefore inadequate endogenous anti-oxidant activity, have been reported to predict an increased risk of cardiovascular morbidity (Vita *et al* 1998).

By contrast, vitamin E is lipid soluble and reacts very slowly with $\cdot O_2^-$ in aqueous solutions. Nevertheless, vitamin E, which is present in various blood lipoproteins, does have important *in vivo* anti-oxidant activity by preventing lipid peroxidation. Other important endogenous anti-oxidants include certain extracellular proteins (Halliwell and Gutteridge 1988). These include albumin, and the acute phase proteins, such as caeruloplasmin and haptoglobin. By contrast, although haemoglobin has high affinity for $\cdot NO$ and $\cdot O_2^-$, this protein may also be a source of ROS *in vivo*. The haem product of this oxidation reaction, methaemoglobin, is unable to bind $\cdot O_2^-$. Methaemoglobin represents approximately 3% of total haemoglobin in red blood cells. The $\cdot O_2^-$ produced during this reaction must be removed by endogenous scavenging systems in these cells, such as Cu/Zn SOD, catalase and glutathione.

$$\text{Haem} - Fe^{2+} - O_2 \rightarrow \cdot O_2^- + \text{haem} - Fe^{3+}$$

$$\text{SOD} \searrow$$

$$H_2O_2 \qquad \cancel{O_2} + H_2O$$

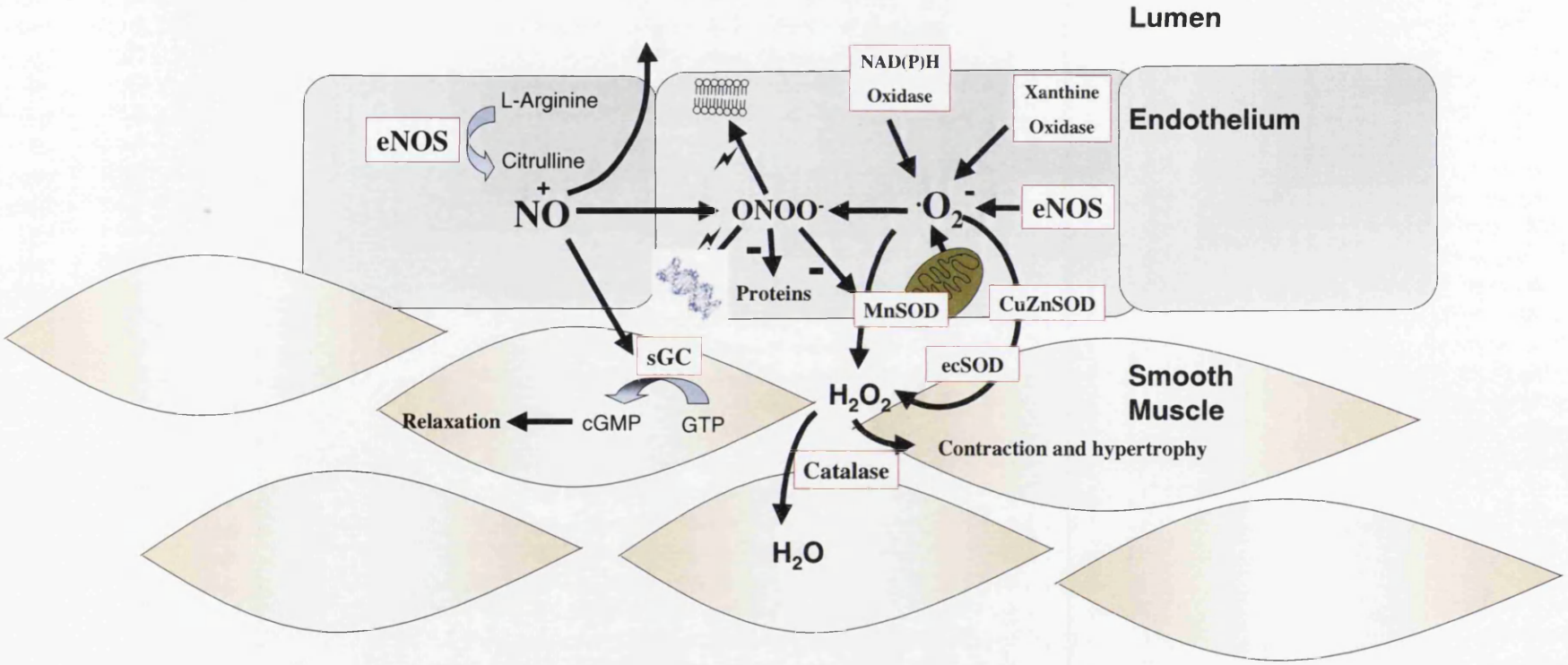
Catalase
or
Glutathione

1.5 Physiological effects of ROS activity

1.5.1 Regulation of vascular tone and blood flow: the regulation of bioavailable nitric oxide by superoxide and other ROS

Blood vessel tone is regulated by a variety of humoral and endothelium-derived relaxant

factors [Figure 1.6] (Mombouli and Vanhoutte 1995), the most important of which is ·NO (Moncada and Higgs 1993). ·NO is generated by three isoforms of the NOS. ·NO rapidly decomposes *in vivo*, having a half life of approximately 1-6 seconds (Brovkovych *et al* 1999). NO is metabolised by either interaction with other ROS, such as $\cdot O_2^-$ (Gryglewski *et al* 1986), or through catalysis by transition metals to nitrite and nitrate (Vallance and Collier 1994). A major physiological effect of ·NO is its interaction with the haem moiety of soluble guanylate cyclase leading to the activation of this enzyme, an increase in cyclic GMP, and a reduction in intracellular calcium concentrations. This in turn leads to relaxation of vascular smooth muscle cells (VSMC) (Waldman and Murad 1988).



·NO – nitric oxide; $ONOO^{-}$ peroxytnitrite; $\cdot O_2^-$ - superoxide; eNOS – endothelial nitric oxide synthase; NAD(P)H oxidase – reduced nicotinamide adenine dinucleotide oxidase; H_2O_2 – hydrogen peroxide; cGMP – cyclic guanosine monophosphate; GTP – guanosine triphosphate; sGC – soluble guanylate cyclase; MnSOD – manganese superoxide dismutase; Cu/ZnSOD – CopperZinc superoxide dismuatase; EC-SOD – extracellular superoxide dismutase; H_2O –water.

Figure 1.6 Schematic diagram summarising ROS activity and reduction in bioavailable ·NO in the blood vessel wall.

Vallance *et al* (1989) investigated the role of ·NO in the regulation of blood flow in healthy humans *in vivo.*, using strain-gauge plethysmography. This is a technique which can be used to measure forearm blood flow using a mercury-filled, silastic strain-gauge placed on the widest part of the forearm, and connected to a plethysmograph calibrated to measure percent change in volume (Greenfield *et al* 1963; Hokanson *et al* 1975). For each measurement, a cuff is placed on the upper forearm and inflated to a supravenous pressure (e.g. 40 mmHg) to occlude venous outflow, and a wrist cuff is inflated to supra-systolic pressures to exclude hand circulation. Serial measurements of arterial flow can be taken and averaged during any one measurement period, and expressed as mL per minute per 100 ml of forearm volume. The brachial artery (non-dominant arm) can be cannulated for the purposes of pharmacological studies. One advantage of local infusion of vasoactive compounds is that they do not attain concentrations within the systemic circulation to cause any important effect.

Studies by Vallance *et al* (1989) of forearm blood flow in healthy subjects demonstrated that intrabrachial artery infusion of an inhibitor of NOS, N^G monomethyl-L-arginine (L-NMMA), was associated with a reduction in basal and agonist (ACh)-augmented blood flow. This inhibition could be reversed by co-infusion of L-arginine. These observations demonstrated that ·NO is continuously synthesised from arginine in human blood vessels *in vivo,* where it has an important role in the regulation of basal and agonist-stimulated blood flow (Vallance *et al* 1989).

The inactivation of endothelium-derived relaxant factor (EDRF), subsequently determined to be ·NO (Palmer *et al* 1987), by ROS is a key factor in the regulation of vascular tone (Vallance and Collier 1994). Gryglewski *et al* (1986) developed an *in vitro* model which allowed for the differentiation between the effects of different substances on the release, action and stability of EDRF. In these studies, a chromatographic column of microbeads coated in 7-14 day-old cultured porcine aortic endothelial cells was perfused with physiological buffer solution maintained at 37°C. This buffer contained indomethacin to inhibit the production of prostaglandins in these cells. The column effluent was then used to superfuse three strips of dendothelialised, rabbit thoracic aorta. There was a predetermined temporal delay of 1 – 3s between perfusion of the column and the vascular strips. In this study, superfusion of nitroglycerin (20 – 200 nM), an endothelium-independent nitrovasodilator, and bradykinin (20 nM), was shown to relax all of the aortic strips (Gryglewski *et al* 1986), whereas co-infusion haemoglobin (a scavenger of EDRF/·NO) attenuated these relaxations. Reduction in effluent $\cdot O_2^-$ concentrations, by superfusion of the column with

SOD (5 – 30 U/ml), induced vasorelaxation. Furthermore, co-infusion of bradykinin with SOD markedly enhanced the vasorelaxant response previously observed with bradykinin, whereas, by contrast, co-infusion of bradykinin with catalase had no additional vasorelaxant effect. These data were the first to support the concept that $\cdot O_2^-$ destroys EDRF/$\cdot$NO in intact blood vessels.

1.5.1.1 Endogenous regulation of vascular superoxide concentrations and $\cdot$NO bioavailability: role of superoxide dismutase

Omar *et al* (1991) subsequently investigated the possibility that vasodilator responses might be regulated by the activity of endogenous SOD. Isometric tension studies of endothelium-dependent and -independent nitrovasodilator responses were performed in calf coronary arteries preconstricted to potassium. They found that both ACh- and nitroglycerin-dependent, but not endothelium-derived prostaglandin-mediated, vasorelaxation was abolished by inhibition of SOD through pretreatment of these arteries with 10 mmol/L of DETCA. This effect was abolished when the arteries in bath were exposed to severe hypoxia (pO_2 = 10 mmHg) or co-treatment with 300 nmol/L of SOD. In other studies, endothelial and smooth muscle cells were cultured from these blood vessels and $\cdot O_2^-$ concentrations in these cells were determined using lucigenin-enhanced chemiluminescence. Treatment of both cell types with DETCA resulted in increased $\cdot O_2^-$ concentrations in both cell types, this being inhibited by co-treatment with SOD. Taken together, these observations indicate that agonist-dependent regulation of vascular tone may be influenced by the activity of endogenous SOD, which modulates bioavailable concentrations of $\cdot O_2^-$ and $\cdot$NO. In addition, Omar *et al.* (1991) demonstrated that nitrovasodilator responses were inhibited by DETCA, which suggests that guanylate cyclase may be directly inhibited by $\cdot O_2^-$. These investigators also demonstrated that vascular smooth muscle cell guanylate cyclase activity is regulated by $\cdot O_2^-$.

The role of endogenous SOD in the regulation of bioavailable $\cdot$NO was also investigated by Mian and Martin (Mian and Martin 1995). In isometric tension studies in rat aortae, pre-treatment of aortic rings with SOD (1 – 300 u/ml) induced a concentration-dependent relaxation of phenylephrine (PE)-induced increase in vascular tone, which was inhibited by co-treatment with N^G-nitro-$_L$-arginine ($_L$-NOARG, 30μM), an inhibitor of NOS. By contrast, SOD had no such effect in dendothelialised rings. In subsequent studies, pre-treatment of aortic rings with 0.1 mmol/L of DETCA (a lower concentration than in those other studies described above (Omar *et al* 1991)) augmented

PE-induced, but inhibited L-NMMA-induced, tone. By contrast, this concentration of DETCA only slightly attenuated ACh-induced vasorelaxation. These effects were reversed by treatment with exogenous SOD (10 – 300 U/ml). These observations suggest that tonic production of endothelium-derived ·NO is protected from destruction by $\cdot O_2^-$ as a result of the activity of endogenous Cu/Zn SOD. Alternatively, the lack of effect of DETCA on agonist-induced vasorelaxation suggests that endogenous Cu/Zn SOD has a bimodal $\cdot O_2^-$ scavenging action, predominately limited to protecting basal ·NO synthesis.

1.5.2 Studies of SOD activity in human blood vessels

SOD protein content and activity has been quantified in human blood vessels obtained at autopsy, and compared with those of various other mammals. Using spectrophotometric and chromatographic techniques, Stralin *et al* (1995) demonstrated that EC-SOD was most abundant in human aortae and left anterior descending coronary artery (LAD) and less so saphenous veins (SV). By contrast, although CuZnSOD was most abundant in aortae, this isoform was more abundant in SV than in LAD. Furthermore, the amount and activity of these SODs was greater in human aortae than in cow, pig, dog, cat, rabbit, rat or mouse aortae. Immunostaining studies of EC-SOD in human blood vessels and vascular cells demonstrated this enzyme to be present throughout the blood vessel walls and evident in cultured VSMC and fibroblasts. This study my be criticised on the basis that human tissue was obtained up to 48 hours after the individuals died, and the causes of death were not detailed. Furthermore, Western blotting, which is the most accurate method of protein quantification, was not used. Nevertheless, these observations do suggest that SOD is abundant and functional within human blood vessels, and more so compared with lower mammals.

Isometric tension studies in human conduit blood vessels performed in our laboratory, using the response to treatment with SOD as an indirect assessment of $\cdot O_2^-$ production, demonstrated that increased $\cdot O_2^-$ production may contribute to impaired endothelium dependent vasorelaxation (Hamilton *et al* 1997).

1.5.2.1 ·NO metabolism: bioactivity of peroxynitrite

On the other hand, diffusible ·NO may be inactivated by $\cdot O_2^-$ (Rubanyi and Vanhoutte 1986a) to form peroxynitrite (Beckman 1993), which occurs readily and has a rate constant of 10^{10} mmol/L/s^{-1}(Fridovich 2001; Reiter *et al* 2000). This reaction is the

major path for ·NO metabolism at physiological pH (Reiter *et al* 2000), and it therefore out-competes that of SOD for $\cdot O_2^-$, which has a rate constant of 2 x10^9 $M^{-1}s^{-1}$ (Koppenol 1998). Dismutation of $\cdot O_2^-$ will therefore occur in situations where $\cdot O_2^-$, achieves micromolar concentrations, such as in ischaemia reperfusion (Vinten-Johansen 2000). Rate limiting steps for the formation of peroxynitrite include the diffusion rates of ·NO and $\cdot O_2^-$, and the availability of molecular oxygen. *Cis*-peroxynitrite is preferentially formed from ·NO and $\cdot O_2^-$, which is most likely due to the greater stability of the N-O bond in the *cis* conformation of this molecule (Reiter *et al* 2000).

Peroxynitrite is a highly reactive, and damaging intermediate. It has an important regulatory effect on vascular smooth muscle cell contractility and blood vessel tone (Rubanyi and Vanhoutte 1986b). It also has mild oxidative properties (Radi *et al* 1991), and is both negatively inotropic and a vasodilator (Ku *et al* 1992). Peroxynitrite may also oxidise lipoproteins, and oxidised lipoproteins can in turn inhibit endothelial G_i-protein and function (Liao 1994). Peroxyntrite readily oxidises tyrosine, to form nitrotyrosine (Reiter *et al* 2000), which can be measured in blood and tissues (Vinten-Johansen 2000). Spectrophotometric studies undertaken by Beckman's group using a stop-flow closed gas tonometer system, which allows for injection of reagents with consecutive spectrophotometric measurements, determined that peroxynitrite formed at a physiological pH causes tyrosine, and therefore protein, nitration (Reiter *et al* 2000). Chronic production of peroxynitrite can lead to the subsequent nitration of vascular proteins, such as Mn-SOD (MacMillanCrow *et al* 1996), which may contribute to the development of atherosclerosis (White *et al* 1994).

Peroxynitrite may have other harmful effects. Recent studies in isolated, working rat hearts treated with interleukin-1β, interferon-γ, and tumour necrosis factor-α, or vehicle, demonstrated that these cytokines caused marked impairment of myocardial contractile function, compared with controls (Ferdinandy *et al* 2000). This was associated with enhanced myocardial activity of XOR and NAD(P)H oxidase, and increased myocardial concentrations of $\cdot O_2^-$. Furthermore, the perfusate obtained from these hearts had higher concentrations of nitrotyrosine and dinitrotyrosine, which are metabolites of peroxynitrite. The production of these metabolites was associated with the development of myocardial dysfunction. All of these effects were attenuated by treatment with the NOS inhibitor, N^G –nitro-L-arginine, the $\cdot O_2^-$ scavenger, Tiron, or a catalyst of peroxynitrite decomposition, 5,10,15,20-tetrakis-[4-sulfonatophenyl]-porphyrinato-iron[III]. These observations suggest peroxynitrite has myodepressant properties, possibly arising through protein nitration. This may be of pathophysiological relevance

in situations of cytokine activation, such as heart failure.

Peroxynitrite may also be metabolised to other reactive oxidative intermediates, such as the hydroxyl radical ($^{\cdot}OH$), which may also affect vascular tone. This radical has, for example, been demonstrated to induce activation of soluble guanylate cyclase in VSMC (Mittal and Murad 1977).

1.5.3 ROS-mediated regulation of vascular cell growth and hypertrophy

In vitro studies have demonstrated that both $^{\cdot}O_2^-$ and hydrogen peroxide affect VSMC growth. Rao and Berk (1992) showed that in VSMC exposed to ROS generated by the xanthine/xanthine oxidase reaction there was an increase in DNA synthesis, which could be attenuated by the addition of either catalase, which removes hydrogen peroxide, or SOD, which scavenges $^{\cdot}O_2^-$. These effects were mediated through an increase in protein kinase C-dependent proto-oncogene expression. Similar studies have also demonstrated that this effect is concentration dependent, such that hydroxyl radicals may also induce apoptosis (Li *et al* 1997a). These *in vitro* data suggest that these growth-regulating activities may operate *in vivo*.

1.5.4 ROS activity in host defence mechanisms

ROS are generated by and participate in a variety of biological reactions *in vivo*. Phagocytes generate $^{\cdot}O_2^-$, as part of the 'respiratory burst' reaction, originally described by Balridge and Gerrard (1933). The bactericidal activity of quiescent neutrophils and macrophages is activated on exposure to pathogens. This results in the rapid consumption of NAD(P)H reducing equivalents, by non-mitochondrial NAD(P)H oxidase, and a burst of $^{\cdot}O_2^-$ production. Abnormalities in the structure and composition of the NAD(P)H oxidase protein complex result in deficiencies of cell killing, a feature of chronic granulomatous disease (Segal and Jones 1978). This raises the possibility that, in teleological terms, constitutive ROS production by vascular cells may have a cell-protective effect.

ROS activity is also implicated in a variety of other pathophysiological processes, such as neurodegenerative diseases, and aging (Warner 1994).

1.6 Pathophysiological effects of increased vascular ROS activity

Excess ROS activity, also known as 'oxidative stress', is associated with a variety of pathophysiological processes. Vascular smooth muscle cell proliferation and hypertrophy, processes known to be important in atherosclerosis, may be promoted by increased $\cdot O_2^-$ activity (Miller *et al* 1998). Other processes which are redox-sensitive and are believed to be important for the development of atherosclerotic plaques include activation of matrix metalloproteinases (Rajagopalan *et al* 1996a), mitogen-activated kinases (Baas and Berk 1995; Clerk *et al* 1998), and vascular cell adhesion molecules (VCAM) (Marui *et al* 1993). Excess ROS activity may also promote increased rates of vascular cell apoptosis, which may have deleterious effects in vascular remodelling (McMurray *et al* 1990).

1.6.1 ROS as intracellular signalling messengers

ROS are involved in modulating a variety of intracellular signalling pathways for vascular cell growth regulation (Irani 2000). For example, Frank *et al.* (Frank *et al* 2000a) recently demonstrated in VSMC that the activity of PYK2, an intracellular tyrosine kinase, is redox sensitive, suggesting that ROS regulate tyrosine kinase-induced trophic effects.

ROS may also serve as second-messengers for AT_1 receptor activation. Activation of these pathways is an integral link between AT_1 receptor activation and nuclear transcriptional changes leading to cell growth (Marrero *et al* 1997). However, the mechanism whereby activation of the G-protein coupled AT_1 receptor might lead to activation of tyrosine kinases, such as JAK2, is not clear. Although JAK2 may bind with the AT_1 receptor, this physical association does not directly lead to JAK2 activation (Ali *et al* 1998). Alternatively, Simon *et al* (1998) demonstrated that JAK2 activation is dependent on the activity of ROS. These observations raise the possibility that ROS may act as intermediates between distinct signalling pathways. The janus kinase (JAK) and signal transducers and activators of transcription (STAT) signalling pathway activates early growth response genes, and may be a mechanism whereby Ang II influences vascular and cardiac growth, remodelling and repair (Berk and Corson 1997; Hefti *et al* 1997).

For this reason, Schieffer *et al* (2000) recently investigated the possibility that ROS

may act as signalling messengers for AT_1 receptor activation of JAK and STAT factors in rat aortic VSMC. Treatment of these cells with 10 μmol/L of Ang II stimulated an increase in the concentrations of both $\cdot O_2^-$ and the cytokine, interleukin 6 (IL-6). Both of these effects were abolished by co-treatment with either 10 μmol/L of the AT_1 receptor antagonist, losartan, or DPI (maximal inhibition attained at a concentration of 100 μmol/L), or by inhibition of p47phox by electroporation of p47phox antibodies into these cells. Similarly, treatment of these cells with Ang II led to JAK2, STAT1α/β and STAT3 tyrosine phosphorylation, which could also be inhibited by treatment with losartan, DPI or electroporation of p47phox antibodies. In other studies, these investigators demonstrated that treatment of rat VSMC with either 10 μmol/L of AG940, a selective antagonist of JAK2, or STAT1α/β antisera, prevented Ang II-induced synthesis of IL-6. These studies demonstrated that in rat VSMC, Ang II-induced, NADP(H) oxidase dependent $\cdot O_2^-$ production may be important for activation of the JAK/STAT cascade. This may, in turn, lead to an increase in production of the cytokine IL-6.

In other studies in rat VSMC, Viedt *et al* (2000) reported that AT_1 receptor-induced ROS production stimulated JNK and p38 MAP kinase, but not ERK1/2, leading to an increase in AP-1 binding DNA. Inhibition of p22phox activity by treatment with either a specific antibody or antisense DNA abolished AT_1 receptor-induced JNK and p38 MAP kinase activation, and reduced AP-1 DNA binding. In this study, treatment with Ang II – induced ERK1/2 activation by a tyrosine kinase, PKC- and MEK-dependent pathway. Taken together, these findings demonstrate that ROS are critical signalling factors for apparently uncoupled signalling cascades, such as the G-protein coupled AT_1 receptor and soluble tyrosine kinases, such as JAK2 or MAPK. Interestingly, the involvement of ROS in non-receptor kinase activation (Rao 1996), raised the possibility that ROS may mediate AT_1 receptor-induced epidermal growth factor receptor (EGFR) activation (Eguchi and Inagami 2000a).

This possibility has recently been explored by Ushio-Fukai *et al.* (Ushio-Fukai *et al* 2001). In these studies, pretreatment of VSMC with anti-oxidants prevented Ang II-induced tyrosine phosphorylation of the EGFR, but not EGF-induced phosphorylation of its own receptor. Alternatively, direct treatment of these cells with hydrogen peroxide, and the superoxide generating compound LY83583, in the absence of any other ligand, was associated with a concentration-dependent increase in EGFR phosphorylation. These observations suggest that ROS may induce EGFR

phosphorylation through activation of an upstream intermediary, rather than activation of EGFR-kinase. In this case, redox-sensitive candidates include Ca^{2+} (Suzuki and Ford 1999), PYK2 (Frank *et al* 2000b) and c-Src (Eguchi and Inagami 2000b; Griendling *et al* 2000). Further studies by Ushio-Fukai *et al.* (2001) in VSMC demonstrated that EGFR transactivation could be prevented by inhibition of either tyrosine kinases, or c-Src kinases, or by Ca^{2+} chelation, but not by Jak2 kinase or PI3K inhibtion. In addition, transfection of these cells with an adenovirus containing DNA for a kinase-inactive form of c-Src lead to inhibition of the activity of c-Src compared to inactive (Ad.LacZ) control transfected cells. These data suggest that c-Src is an upstream effector for Ang II-induced EGFR transactivation by tyrosine phosphorylation.

The activity of nuclear factor kappa B (NFκB) is also regulated by ROS (Barnes and Karin 1997), suggesting the possibility that AT_1 receptor-induced ROS production (Rajagopalan *et al* 1996b) may lead to activation of NFκB. This thesis has been recently investigated by Pueyo *et al* (2000), who demonstrated that AT_1 receptor-induced activation of NFκB, which was associated with enhanced VCAM-1 expression, is a redox sensitive pathway. In these studies, NFκB activation was associated with the breakdown of cytoplasmic IκB proteins, this being inhibited by treatment with either the non-specific anti-oxidant, pyrrolidinedithiocarbamate, or rotenone, which is an inhibitor of mitochondrial respiration. By contrast, treatment with SOD failed to attenuate Ang II-induced IκB degradation. The mechanism of ROS-induced IκB degradation is not known. Taken together these findings suggest that AT_1 receptor activation stimulates mitochondrial ROS production, which in turn may trigger IκB degradation, NFκB activation and endothelial cell recruitment of monocytes. This is one process whereby AT_1 receptor activation could contribute to vascular inflammation and atherosclerosis.

1.7 ROS in vascular disease states: mechanisms of production and functional importance

ROS have been implicated in the pathogenesis of a variety of disease states, and may be particularly important in the pathophysiology of cardiovascular disease. Increased vascular O_2^- production can lead to reduced bioavailable nitric oxide and impaired endothelium-dependent relaxation (Grunfeld *et al* 1995; Nakazono *et al* 1991; Rubanyi and Vanhoutte 1986b) which can be evident in disease states such as hypertension (Nakazono *et al* 1991), hypercholesterolaemia (Creager *et al* 1990), atherosclerosis (Cox *et al* 1989), diabetes (Calver *et al* 1992a), and heart failure (Kubo *et al* 1991). In

this section, the role of ROS in a variety of vascular disease states will be discussed.

1.7.1 Renin angiotensin aldosterone system activation and vascular superoxide production

Some of the vasoactive effects of hormones, such as Ang II, are similar to those of ROS. For example, treatment of vascular smooth muscle cells with Ang II will result in hypertrophy and hyperplasia of these cells (Berk *et al* 1989; Haller *et al* 1996). Furthermore, chronic treatment of experimental animals with Ang II results in not only hypertension, but also vascular hypertrophy and endothelial dysfunction in these animals (Griendling *et al* 1997). These overlapping physiological effects raise the possibility of a functional interaction between Ang II and ROS production.

This hypothesis was initially explored by Wilson (1990), who observed that chronic treatment of rats with Ang II resulted in hypertension and vascular hypertrophy, which was associated with increased vascular ROS production. The possibility that Ang II might directly stimulate ROS production was explored by Griendling *et al.* (1994). In these studies, treatment of cultured rat VSMC resulted in increased $\cdot O_2^-$ production, which could, in turn, be prevented by co-treatment with an inhibitor of NAD(P)H oxidase. Further studies identified this enzyme to be bound to the plasma membrane of these cells (Griendling *et al* 1994).

In rats chronically infused with pressor and non-pressor doses of Ang II, increased vascular NAD(P)H oxidase $\cdot O_2^-$ production contributed to both the impaired endothelium-dependent vasorelaxation and hypertension evident in these animals (Rajagopalan *et al* 1996b). Importantly, these effects were abolished by co-treatment with an Ang type 1 receptor antagonist (ARA), implicating this receptor in Ang II-stimulated superoxide production. In a similar study, the hypertensive effects of Ang II were attenuated by co-infusion with liposomal SOD (Laursen *et al* 1997).

Further studies by Zafari *et al* (2000) from Griendling's group demonstrated that the Ang II-stimulated activation of NAD(P)H oxidase occurred through release of arachidonic acid metabolites, which triggered protein kinase C activation. This in turn led to phosphorylation of the phox subunits and activation of NAD(P)H oxidase. Further work by Ushio-Fukai *et al* (1996) in the same group demonstrated that transfection of anti-sense p22phox cDNA into rat cultured VSMC abrogated the Ang II-stimulated increases in $\cdot O_2^-$ concentrations, hypertrophy of these cells or subsequent

H_2O_2 production. These observations implicated p22phox subunit as a key element in NAD(P)H oxidase-dependent $\cdot O_2^-$ production and H_2O_2 as a mediator of the trophic effects of Ang II. Furthermore, work in cultured rat VSMC treated with the SOD inhibitor, DETCA, demonstrated that $\cdot O_2^-$ conversion to H_2O_2 was also important for Ang II-stimulated VSMC hypertrophy (Zafari *et al* 1998). Similar observations were made in studies of VSMC which stably over-expressed catalase, an enzyme which catalyses the breakdown of H_2O_2 to water (Zafari *et al* 1998). These studies clearly demonstrated a novel, functionally important relationship between vasoactive hormones and ROS generation, and raise the possibility that renin-aldosterone-angiotensin system (RAAS) activation evident in human vascular disease states may result in enhanced ROS activities.

Interestingly, Ang II-stimulated ROS generation may not be endothelium dependent. In studies performed by Rajagopalan *et al* (1996b), denudation of the endothelium from the aortic rings of Ang II-infused rats failed to prevent the rise in $\cdot O_2^-$ production stimulated by treatment with this hormone. That is to say, Ang II-stimulated $\cdot O_2^-$ production may occur within the smooth muscle and/or adventitial layers. This thesis is supported by comparable findings from studies by Miller *et al.* (1998) in a rabbit model of atherosclerosis.

Recently, Lang *et al.* (2000) demonstrated an association between increased circulating plasma Ang II concentrations and enhanced NAD(H) oxidase activity in a guinea pig model of left ventricular hypertrophy. These findings suggest that in disease states where chronic activation of the renin angiotensin aldosterone system (RAAS) is evident, increased plasma and tissue concentrations of Ang II may contribute to the increased ROS activity that has been observed in chronic heart failure and coronary heart disease (McMurray *et al* 1990).

Pagano *et al.* (1997a; 1998) explored the potential importance of adventitial $\cdot O_2^-$ production in the modulation of vascular tone (Pagano *et al* 1997b; Wang *et al* 1999; Wang *et al* 1998). This group identified adventitial fibroblast NAD(P)H oxidase to be an important source of $\cdot O_2^-$ in both rabbit and rat aortae. Studies of particulate fractions of rabbit aortae demonstrated that immunodepletion of p67phox markedly reduced NAD(P)H oxidase activity, this being restored by the addition of recombinant p67phox (Pagano *et al* 1997a). These studies demonstrated that p67phox has an obligate role for electron transport in this enzyme. Furthermore, incubation of Ang II-treated aortae with actinomycin D, an inhibitor of transcription, and cycloheximide, an inhibitor of protein synthesis, both attenuated Ang II-stimulated $\cdot O_2^-$ production (Wang *et al* 1999). These data suggest that Ang II augments NAD(P)H oxidase-mediated $\cdot O_2^-$ production by

enhancing the abundance of messenger ribonucleic acid (mRNA), through an increased rate of transcription. This in turn may lead to increased amounts of the protein and enhanced capacity of the enzyme for $\cdot O_2^-$ production (Pagano *et al* 1998).

These investigators also demonstrated using rat aortae under isometric tension that adventitial $\cdot O_2^-$ production, by inactivating $\cdot NO$ and therefore modulating vascular tone, is functionally important source of ROS generation (Wang *et al* 1998). Furthermore, the angiotensin type 1 receptor antagonist (ARA), losartan, inhibited Ang II-stimulated superoxide production in rat thoracic aortae, however, losartan had no such effect in rabbits (Pagano *et al* 1997a; Wang *et al* 1999). That Ang II-stimulated $\cdot O_2^-$ production in rabbits could be inhibited by the non-specific (non-AT_1 non AT_2 receptor) receptor antagonist, [sar^1,Thr^8]-Ang II suggests that important species differences may exist in this pathway. These observations raise the question as to whether or not Ang II might stimulate $\cdot O_2^-$ production in human blood vessels. This question remains to be answered and is therefore the subject of the present investigation.

1.7.2 ROS activation in hypertension

1.7.2.1 Studies in experimental animals

In a model of genetic hypertension, the stroke prone spontaneously hypertensive rat (SHRSP), investigations in our laboratory, demonstrated that markedly decreased $\cdot NO$ bioavailability is due to an excess production of $\cdot O_2^-$ within the endothelium, compared with normotensive control animals (Grunfeld *et al* 1995; McIntyre *et al* 1997; Kerr *et al* 1999).

Zalba *et al* (2000) recently demonstrated in the SHR, that increased $\cdot O_2^-$ production in homogenates of aorta is associated with an increased abundance of p22phox mRNA, as determined by reverse transcription polymerase chain reaction (RTPCR) techniques. Furthermore, the observed activation of NAD(P)H oxidase and impaired endothelium-dependent responses in the aortae of these animals were normalised by treatment with the ARA, irbesartan. As discussed above, Rajagopalan *et al* (1996b) demonstrated the functional importance of increased vascular $\cdot O_2^-$ production in an Ang II-induced model of hypertension.

These observations were recently supported in studies by Brovkovych *et al.* (1999) in which a porphyrinic microsensor was used to quantify $\cdot NO$ production in endothelium obtained from spontaneously hypertensive (SHR) and Wistar-Kyoto (WKY) control

rats. This electrochemical method was originally developed by Malinski and Taha (1992) for the direct electrochemical detection of ·NO either *in vitro* or *in vivo*. The microsensor is 2-3 μm in diameter and 5-7 μm in length and selectively detects ·NO on the basis of current generated by the oxidation of ·NO. The sensor can be positioned up to 10 μm from a single cell and the specificity of the signal can be confirmed by inhibition of NOS on treatment with L-*N*-monomethyl-L-arginine (L-NMMA). Using these techniques, Brovkovych *et al* (1999) found that after treatment with calcium ionophore to stimulate NOS, ·NO concentrations were higher for WKY than SHR (910 ± 40 and 580 ± 35 nmol/L, respectively; n=6, $p<0.005$). In the presence of 100 U/mL of polyethylene glycol-SOD (PEG-SOD), ·NO concentrations in the endothelium of WKY rats increased by 8% however, an increase of 83% was observed for ·NO concentrations in the endothelium of SHR rats. This observation supports the concept that vascular, or endothelial, cell $\cdot O_2^-$ production is greater in hypertensive rats, compared with normotensive controls, leading to a reduction in bioavailable ·NO in the SHR/SHRSP.

1.7.2.2 Studies in humans

In humans, *in vivo* strain gauge plethysmography studies demonstrated that the ·NO - dependent forearm vasodilator response to intrabrachial arterial infusion of acetylcholine (ACh) was impaired in 18 hypertensive patients compared to controls (Panza *et al* 1990). In this particular study, the maximal forearm blood flow response to infusion of ACh was 9.1±5 ml/min in hypertensive patients compared to 20.0±8 ml/min in the controls ($P<0.0002$). This difference was not due to decreased sensitivity of guanylate cyclase to ·NO, because the vasodilator response to the exogenous ·NO donor, sodium nitroprusside, was preserved. These observations suggest that in hypertensive subjects there is an abnormality in endothelium-dependent vasorelaxation. These findings were supported by observations in other studies by Calver *et al* (1992b), who demonstrated that the forearm vasoconstrictor response to L-NMMA infusion was reduced in untreated hypertensive patients compared to controls, suggesting a reduction in basal ·NO bioavailability in hypertension. These observations raise the possibility that reduced ·NO bioavailability in the blood vessels of hypertensive patients may be due to increased removal of ·NO, as a result of enhanced ROS generation in essential hypertension.

The reasons for enhanced vascular ROS activity in human hypertension may be due to genetic abnormalities causing altered expression of the enzymes responsible for ROS production or removal (see section 1.7). This possibility is supported by recent

observations which suggested a heritable basis for the impaired endothelium-dependent vasodilation which may be present in the offspring of hypertensive parents (Zizek *et al* 2001). These authors studied flow-mediated brachial artery dilatation in both hypertensive and normotensive individuals (normotensive offspring of hypertensives and normotensive controls), and found it to be impaired in both the patients and in their normotensive offspring, compared to controls. The reasons for this are not entirely clear. Although the blood pressure of the hypertensive offspring was within the normal range, it was significantly higher than that of controls. These observations suggest a close relationship between blood pressure and endothelial dysfunction, and raise the question as to whether there may be a genetic basis, or heritable risk, for endothelial dysfunction in hypertension.

The possibility that the presence of hypertension *per se*, might directly result in abnormal endothelial function was investigated in studies by Paniagua *et al* (2000). They isolated human subcutaneous resistance arteries (SRA) from gluteal fat biopsies, and then cannulated and perfused these arteries at 37°C in a pressure-flow organ bath. SRA diameter was measured directly from amplified digital images. Endothelium – dependent and –independent vasodilator responses were assessed by treatment of SRA with ACh (10^{-9} to 10^{-4} mol/L) and sodium nitroprusside (10^{-9} to 10^{-4} mol/L) after exposure of SRA to incremental vascular pressures of 50, 80, and 120 mmHg for 1 hour. The dilator response of SRA to ACh was attenuated by exposure to higher intravascular pressures (mean vasodilation, 62%, 49%, and 26% at 50, 80, and 120 mmHg, respectively), whereas the responses to SNP were not affected. These observations suggest that exposure to increased intravascular pressures, such as occurs in hypertension, may directly lead to endothelial dysfunction.

One mechanism underlying hypertension-induced endothelial dysfunction may relate to pulsatile stretch of vascular cells. Studies by Howard *et al* (1997) in porcine aortic endothelial cells (PAEC) and VSMC demonstrated that cyclic, or pulsatile, stretch of cells grown on elastic membranes resulted in an increased production of lipid peroxides, which could be further enhanced by treatment with NADH/NADPH. These observations suggest that the activity of NAD(P)H oxidase is upregulated by mechanical stimuli, such as pulsatile stretch. This may be one further pathway underlying enhanced vascular ROS production in hypertension.

1.7.3 Diabetes

Diabetes is one other vascular disease in which oxidative stress is recognised (Maxwell *et al* 1997; Opara *et al* 1999). Hattori *et al* (1991) observed that ACh –dependent vasorelaxation was impaired in aortic segments obtained from streptozotocin-induced diabetic rats, compared to normal controls. This impairment was improved by pre-treatment of these blood vessels with SOD (60 U/ml), but not catalase (600 U/ml) or allopurinol (10 mmol/L). By contrast, vasorelaxation responses to the endothelium – independent vasodilator, sodium nitroprusside, were similar in the two groups. These observations suggest that impaired endothelium-dependent vasorelaxation in these diabetic animals may be due to increased vascular $\cdot O_2^-$ concentrations, as a result of either increased ROS generation or reduced removal, leading to in turn, a reduction in bioavailable $\cdot NO$. The lack of effect of either catalase or allopurinol suggests that neither H_2O_2 or xanthine oxidase contribute to the impaired endothelium-dependent responses in these animals.

Impaired endothelium-dependent vasorelaxation may also be present in diabetic patients (Calver *et al* 1992a). In fact, hyperglycaemia directly induces ROS production in vascular cells and anti-oxidant treatment of these cells prevents the protein glycation that occurs on their exposure to high glucose concentrations (Giardino *et al* 1996; Nishikawa *et al* 2000). Nishikawa *et al* (2000) recently demonstrated in bovine aortic endothelial cells that the deleterious effects of hyperglycaemia, namely protein kinase C activation, protein glycation and increased glucose flux, were attenuated by normalising mitochondrial ROS concentrations through inhibition of mitochondrial electron transport. Experimental studies in human cultured aortic endothelial cells have also demonstrated that exposure to high glucose concentrations stimulates increased vascular $\cdot O_2^-$ production by eNOS (Cosentino *et al* 1997). Furthermore, in experimental rats, hyperinsulinaemia has been demonstrated to directly increase vascular $\cdot O_2^-$ production (Kashiwagi *et al* 1999).

1.7.4 Hypercholesterolaemia

Increased vascular $\cdot O_2^-$ production also contributes to endothelial dysfunction in hypercholesterolaemic rabbits (Mugge *et al* 1994; Ohara *et al* 1993). This appears to be due, in part, to increased removal of $\cdot NO$ by $\cdot O_2^-$ (Mugge *et al* 1994). Furthermore, treatment of these rabbits with PEG-SOD for 1 week, therefore reducing vascular $\cdot O_2^-$ concentrations, was associated with an improvement in endothelium-dependent

vasodilator responses in these animals (Mugge *et al* 1991b). Hypercholesterolaemia in humans is associated with abnormal vascular function, even in the absence of end-organ damage, such as, for example, atherosclerosis. Creager *et al* (1990) used strain-gauge plethysmography techniques to study forearm blood flow in 13 young, hypercholesterolaemic patients (age range 24 – 45) and 11 healthy controls (age range 22 – 43). Basal forearm blood flow was comparable between the two groups. They also found, however, that the vasodilator responses to infusion of either methacholine, an endothelium-dependent vasodilator, or sodium nitroprusside, an endothelium-independent vasodilator, were impaired, compared to controls. These observations suggest that abnormalities in both endothelial and vascular smooth muscle cell function may be present in hypercholesterolaemia, predating the development of atherosclerosis. One possible reason for this may be due to increased vascular ROS production in hypercholesterolaemia (Mugge *et al* 1994; Ohara *et al* 1993).

Stokes *et al* (2001) recently characterised the importance of NAD(P)H oxidase-dependent ROS production as a pro-inflammatory pathway in an animal model of hypercholesterolaemia. In these studies, mice transgenic for either p47phox (heterozygous p47phox +/- or homozygous p47phox -/-) or CuZn-SOD (SOD TgN) were placed on either a normal diet (ND) or high cholesterol diet (HCD) for 2 weeks. The microvascular damage associated with hypercholesterolaemia involves leucocyte-endothelial cell adhesion (Scalia *et al* 1998), which are processes known to be redox sensitive (Marui *et al* 1993). Stokes *et al.* (2001) studied the effects of hypercholesterolaemia in these transgenic mice using intravital microscopy of cremasteric muscle postcapillary venules. Venular leucocyte adherence and emigration were quantified by videoimaging. They demonstrated that wild-type, SOD non-TgN and SOD TgN HCD mice had 2-3-fold higher serum cholesterol concentrations than ND mice. This was associated with more than doubling of the number of adherent leucocytes in the postcapillary venules of both WT and SOD non-TgN HCD groups, compared with the ND mice. By contrast, TgN mice that overexpressed CuZn-SOD exhibited a significantly reduced number of adherent and emigrated leukocytes when placed on the HCD diet, compared to the wild type and non-TgN mice. In order to investigate the role of NAD(P)H oxidase in hypercholesterolaemia-induced leucocyte adherence and emigration, mice heterozygous and homozygous for p47phox were placed on HCD or ND. The HCD-induced increases in leucocyte adherence and emigration in WT and heterozygous TgN p47phox mice did not occur in p47phox -/- mice. Interestingly, mean cholesterol concentrations were higher in the p47phox TgN mice than in WT. The observations that enhanced $O_2^{\cdot-}$ scavenging, as occurred in the

SOD TgN mice, and inhibition of NAD(P)H oxidase-dependent $\cdot O_2^-$ generation, as occurred in the p47phox homozygous TgN mice, prevented the microvascular inflammatory changes associated with hypercholesterolaemia, suggests that NAD(P)H oxidase-dependent $\cdot O_2^-$ generation is involved in mediating hypercholesterolaemic vasculopathy and atherosclerosis.

1.7.5 Atherosclerosis

Individuals with hypertension and high plasma concentrations of cholesterol and glucose are at risk of developing atherosclerosis. In pathological terms, atherosclerosis is a focal abnormality which occurs at a site of high shear stress in a large conduit or elastic artery. An atherosclerotic plaque is characterised by an accumulation of macrophages and T lymphocytes and extracellular matrix tissue, hyperplasia of intimal smooth muscle cells and neovascularisation (Alexander 1995). The pathogenesis of atherosclerosis involves accumulation and modification of lipids within the blood vessel wall (Steinberg *et al* 1989), induction of pro-inflammatory genes such as vascular cell adhesion molecule (VCAM) (Marui *et al* 1993), increased cellular proliferation (Gong *et al* 1996), alteration in VSMC phenotype (Rao and Berk 1992) and impaired endothelial function (Mugge *et al* 1991b; Ohara *et al* 1995; White *et al* 1994). All of these processes may be promoted by an increase in ROS activity (Alexander 1995). For example, low-density lipoprotein (LDL) is not readily sequestered by macrophages. After exposure to endothelial cells, however, LDL becomes oxidised (oLDL), and in this form, is recognised by the macrophage scavenger LDL receptor. Macrophages can, therefore, readily sequester oLDL, which results in their transformation into lipid-laden 'foam cells', which are a pathological feature in atherosclerosis (Alexander 1995). Lipid peroxidation by-products, such as lisophosphatidylcholine, are amphipahthic compounds that directly impair G-protein dependent signal transduction, leading to impaired endothelium-dependent vasorelaxation (Flavahan 1992).

1.7.5.1 Studies of atherosclerosis and ROS activity in animal models

Studies in animal models of atherosclerosis demonstrated that activation of the RAAS may be one important mechanism leading to increased ROS activity, atherosclerosis and vascular damage (Warnholtz *et al* 1999). These investigators used an animal model of atherosclerosis, in which healthy rabbits were fed either a high cholesterol diet or normal diet in the presence or absence of concomitant treatment with either an ARA or a calcium channel blocker. The latter treatment served as a positive anti-hypertensive

control. Vascular $\cdot O_2^-$ production, as measured by lucigenin-enhanced chemiluminescence, was greater in hypercholesterolaemic rabbits compared to controls. This difference was abolished by either de-endothelialisation of the aortic segments from hypercholesterolaemic rabbits, or treatment of these segments with an inhibitor of NAD(P)H oxidase, DPI. In isometric tension studies, ACh-induced relaxation in vascular rings pre-constricted to PE was attenuated in hypercholesterolaemic animals, compared to controls. This difference was not evident in high cholesterol animals treated with the ARA. Endothelium-dependent vasodilation was not modified by calcium channel blockade in cholesterol-fed animals. Receptor binding studies demonstrated that aortic membrane AT_1 receptor density was greater in high cholesterol animals compared to controls. In addition, histological analysis of atherosclerotic plaques in arteries from cholesterol fed animals demonstrated a reduction in lipid content and macrophage infiltration in animals treated with an ARA, compared to those that were not. These findings suggest that hypercholesterolaemia is associated with increased vascular $\cdot O_2^-$ production, endothelium-dependent dysfunction and atherosclerosis. The mechanism underlying this appears to be cholesterol-induced AT_1 receptor-dependent, NAD(P)H oxidase activation, principally occurring within the vascular endothelium.

One other mechanism which may also contribute to enhanced vascular $\cdot O_2^-$ production in atherosclerotic arteries is the presence of shear stresses, which are known to be potent stimuli for vascular $\cdot O_2^-$ production (DeKeulenaer *et al* 1998b; Howard *et al* 1997). *In vitro* studies in cultured HAEC and VSMC plated in a closed loop flow system demonstrated that alteration in physiological levels of laminar shear stress (0.6 to 15 dyne/cm^2) increased rates of gene transcription and protein synthesis of CuZn SOD in HAEC, compared to control cells (Inoue *et al* 1996). This was not the case, however, in VSMC, which suggests that in some circumstances, certain vascular cells may have inadequate anti-oxidant scavenging properties.

The effect of SOD deficiency on lipid peroxidation has been investigated using a rat model of dietary copper restriction (Lynch *et al* 1997). Male weanling Sprague-Dawley rats were fed a diet deficient in copper and zinc and received either copper replacement in their drinking water, or copper-deficient drinking water. They were then sacrificed after 5 weeks. Isometric tension studies in aortic segments obtained from these animals demonstrated comparable contractile responses to potassium and PE, but deficient relaxation responses to ACh. This was associated with a 68% reduction in vascular CuZnSOD activity and a 58% increase in vascular $\cdot O_2^-$ concentrations in the copper

deficient animals compared to controls. In addition, the plasma concentrations of esterified F_2-isoprostanes, which are stable products of arachidonic acid autoxidation (Morrow *et al* 1990), were also measured. Plasma F_2-isoprostane concentrations in control animals and copper-deficient animals were 263 ± 40 pg/mL and 665 ± 156 pg/mL ($p<0.05$ vs control), respectively. This study may have benefited from additional investigations to assess whether or not treatment with exogenous SOD might have prevented these abnormalities in copper-deficient rats. Nevertheless, it does provide information on how a functional deficiency in SOD, leading to an excess activity of ROS, may lead to lipid peroxidation, which is a critical process in atherosclerosis.

Studies by Fukai *et al* (1998) have suggested, paradoxically, that vascular SOD protein content and activity may be increased in atherosclerosis. In this study, vascular tissues obtained from either apo E-deficient mice, a model of atherosclerosis, or controls, were homogenised. EC-SOD protein quantification studies were undertaken by western analysis and SOD activity was determined by monitoring the inhibition of the rate of XOR-mediated reduction of cytochrome *c*. These investigators found that although total aortic SOD activity was similar between apo E-deficient mice and controls, specific assays of EC-SOD activity revealed greater activity of this enzyme in the aortae of apo E-deficient mice. Western analysis demonstrated a three-fold increase in the amount of this isoenzyme in the aortae of apo E-deficient mice, compared to controls. Immunodetection studies demonstrated EC-SOD to be most abundant in intimal macrophages in the aortae of apo E-deficient mice, which was in contrast to control aortae, in which macrophage EC-SOD staining was absent. The observation that vascular EC-SOD may be increased in atherosclerosis suggests a compensatory, adaptive process. In this case, the principal oxidants generated from these macrophages would be H_2O_2 and $^{\cdot}OH$. These products are more stable than $^{\cdot}O_2^-$, and as H_2O_2 is uncharged, may penetrate more readily into cells. Importantly, however, total vascular SOD activity was similar between the apo E-deficient mouse and controls, suggesting that a deficiency in ROS scavenging is not a primary phenomenon, at least in this model of atherosclerosis.

1.7.5.2 Studies of atherosclerosis and ROS activity in human blood vessels

Atherosclerosis results in impaired endothelium-dependent vasodilator responses in human arteries. *In vivo* studies of coronary artery vasomotor responses by Ludmer *et al* (1986) demonstrated that in segments narrowed by the presence of atherosclerosis,

intra-coronary infusion of ACh resulted in vasconstriction, whereas normal segments dilated in response to ACh. In other studies by this group, coronary artery flow-mediated dilatation in response to intra-coronary infusion of adenosine, a direct smooth muscle vasodilator, was less in segments affected by atherosclerosis, compared to non-affected segments (Cox *et al* 1989). By contrast, the dilator response to infusion of nitroglycerin was comparable between the diseased and normal segments. This suggests that rather than a generalised impairment of dilator function, a selective abnormality in endothelial function was present in atherosclerotic arteries. Further studies by this group demonstrated that selective endothelial dysfunction may also be evident at bifurcation points, compared to straight sections, in atherosclerosis-free coronary arteries (McLenachan *et al* 1990). This observation raised the question of whether endothelial dysfunction may pre-date the development of atherosclerosis, which is prone to occur at the branch points of these arteries.

Huraux *et al* (1999) first quantified $\cdot O_2^-$ concentrations in human IMA and investigated the relationships between IMA $\cdot O_2^-$ concentrations, endothelial function and risk factors for atherosclerosis. Superoxide concentrations were quantified by lucigenin-enhanced chemiluminescence and endothelial function was assessed by isometric tension studies in IMA preconstricted with the thromboxane A_2 analogue, U46619, followed by cumulative concentration response curves (CRCs) with ACh (10^{-8} to 10^{-5} mol/L). In parallel studies, endothelial-independent relaxation was assessed using CRCs with nitroglycerin (10^{-10} to 10^{-6} mol/L). Immunohistochemical analyses of IMA from 26 patients demonstrated that that the endothelium of these blood vessels was uniformly intact and free of atherosclerosis. Huraux *et al* (1999) found that vascular $\cdot O_2^-$ concentrations ranged from 669 to 12,309 counts per minute per mg tissue (mean 4589 ± 554 Counts/min/mg) and endothelium-dependent relaxation to ACh were highly variable, with values ranging from 0% to 89%. Step-wise multivariate analysis was reported to reveal that $\cdot O_2^-$ concentrations were related to hypercholesterolaemia, although the numeric data were not actually reported in the paper. Overall, however, the variability observed in vascular $\cdot O_2^-$ concentrations and vasorelaxation responses could not be explained qualitatively or quantitatively by either risk factors or drug therapy (Huraux *et al* 1999).

Activation of the RAAS may also be important for the development of atherosclerosis in human arteries. Immunohistochemical studies in normal and atherosclerotic human coronary arteries have demonstrated increased immunoreactivity of angiotensin converting enzyme (ACE) in diseased arteries (Diet *et al* 1996). In normal arteries, ACE

was only evident in luminal and adventitial vasa vasorum endothelium, whereas in arteries affected by atherosclerosis, ACE immunoreactivity was also evident in macrophages (foam cells) and T-lymphocytes. In arteries in which the atherosclerosis was more diffuse, ACE immunoreactivity was also more abundant and immunoreactive Ang II was also evident. This raises the question as to whether ACE inhibition might be associated with anti-atherosclerotic effects. This question was recently investigated in a randomised, placebo-controlled clinical trial of the angiotensin convertin enzyme inhibitor (ACE-I), quinapril (Mancini *et al* 1996). In TREND (Trial on Reversing ENdothelial Dysfunction) 105 patients with atherosclerotic coronary artery disease were randomised to 40mg of quinapril per day (n=51), or placebo (n=54), for 6 months. Coronary endothelial function was assessed by measurement of the change in coronary artery diameter consequent to infusion of ACh using quantitative coronary angiography, both at baseline and after 6 months. The constrictive responses to ACh were comparable in both groups at baseline. After 6 months, only the quinapril group showed any improvement in response to incremental concentrations of acetylcholine (4.5+/-3.0% [mean+/-SEM] versus -0.1+/-2.8% at 10^{-6} mol/L and 12.1+/-3.0% versus -0.8+/-2.9% at 10^{-4} mol/L, quinapril versus placebo, respectively; overall P=0.002). These observations suggest that inhibition of the RAAS can improve endothelium-dependent vasomotor responses in the coronary arteries of patients with coronary atherosclerosis. The mechanisms for this effect may include enhanced bioavailable NO through, for example, anti-oxidant effects, and potentiation of bradykinin (Warnholtz *et al* 1999).

SOD activity may also be abnormal in the coronary arteries of patients with coronary atherosclerotic disease. Landmesser *et al* (2000) studied endothelium-derived EC-SOD isoenzyme activity, stimulated by bolus injection of heparin, both in *ex vivo* atherosclerotic coronary arteries obtained from 10 deceased patients and 10 control subjects, and *in vivo* in cubital venous blood from 35 other patients with CHD and 15 age-matched controls. Flow-mediated, or endothelium-dependent, radial artery dilator responses were also measured in these patients by high-resolution ultrasound. *In vivo* studies were performed after intravenous, systemic infusion of either vehicle, L-NMMA (7 μmol/min), vitamin C (25 mg/min), or both.

In the *ex vivo* studies of vascular EC-SOD activity, infusion of a single bolus of heparin (5000 U or 1000 U) resulted in enhanced EC-SOD activity in the coronary arteries in non-atherosclerotic rings, compared to diseased rings (control subjects: 126 ± 14; CHD: 63 ± 11 U/mg of protein; $p<0.01$). Cubital venous blood EC-SOD activity was reduced

in patients with atherosclerotic CAD compared to healthy controls (control subjects: 14.5 ± 1.1; CHD: 3.8 ± 1.1 U/mL/min; $p<0.01$). Furthermore, the activity of EC-SOD in cubital venous blood correlated positively with flow-dependent vasodilation ($r=0.5$; $p<0.01$) and negatively ($r=0.6$; $p<0.01$) with the effect of vitamin C on flow-dependent vasodilation in radial arteries in these patients. In other words, enhanced endothelium-dependent flow-responses were associated with greater EC-SOD activity. Furthermore, the ROS-scavenging effect of vitamin C was greater in patients with less EC-SOD activity. Limitations of this study include the fact that a bolus injection of heparin will only stimulate the release of a fraction of total vascular EC-SOD. Nevertheless, these observations suggest that vascular EC-SOD activity may be reduced in patients with CHD and this may be related to enhanced ROS activity and abnormal endothelium-dependent blood flow responses in these patients.

1.7.6 ROS and coronary artery vein graft stenosis

Coronary artery bypass graft surgery is performed on symptomatic and prognostic grounds in patients with flow-limiting coronary atherosclerosis (Fisher 1983). At present, optimal long-term graft patency is attained with left internal mammary artery bypass grafting. However, only one such artery may be used, and usually three grafts are required, so the saphenous vein in the leg is the alternative vessel for grafting other stenosed coronary arteries. Unfortunately, long-term outcomes are related to vessel type as graft patency is approximately 90% at 10 years in IMA grafts compared to less than 50% for saphenous veins (Fisher 1983). The reasons for premature vein graft failure are thought to be due to remodelling within the vein wall with the development of neointimal hyperplasia and focal stenoses. Reasons for this include the limited potential of veins for neovascularisation, thus rendering the graft prone to ischaemia, and the arterialised blood pressure within these venous grafts. Vein graft stenosis in CABG patients therefore presents a major clinical problem.

Channon's group developed their interest in the pathobiology of vein conduits, by utilising a rabbit model of venous bypass grafts in normocholesterolaemic normotensive rabbits (West *et al* 2001). Their aim was to study the mechanisms of vascular O_2^- production in these vessels in relation to the development of vein graft stenosis. These experimental venous grafts are characterised by smooth muscle cell proliferation.

Male New Zealand rabbits underwent surgical interposition bypass grafting of the jugular vein onto the ipsilateral carotid artery. In other words, this jugular vein was inserted as a conduit between proximal and distal ends of the carotid artery. In control

studies, other animals underwent surgical isolation and mobilisation of the jugular vein, including ligation and diathermy of side branches, however these veins were replaced, and were not used as venous conduits for arterial blood. The graft and control veins were harvested 28 days after surgery. Vascular $\cdot O_2^-$ concentrations were measured by lucigenin-enhanced chemiluminescence, using lucigenin concentrations of either 5 or 250 μm. *In situ* generation of $\cdot O_2^-$ production was qualitatively evaluated by use of the oxidative fluorescent dye, dihydroethidium, on 30 μm tissue sections. Quantification of NADP(H) oxidase subunits in vascular homogenates was also undertaken by western immunoblotting techniques with monoclonal antibodies to p22phox and p67phox. In this case, portions of vascular homogenates, equalised for protein content, were boiled in loading buffer containing 100 mM dithiothreitol, separated by SDS-PAGE, and transferred to nitrocellulose membranes. Immunohistochemical studies were also performed using these antibodies.

Forty blood vessels from 28 animals were studied. Superoxide concentrations were unchanged in control jugular veins which had undergone surgical manipulation, but not bypass grafting. Alternatively, the native vein grafts were found to have substantially increased vascular $\cdot O_2^-$ concentrations (19.9 ± 3.3 vs. 8.4 ± 2.3 relative light units per second per milligram of tissue [RLU/s/μg]; $p<0.01$; n=11), compared to control jugular veins. This difference in $\cdot O_2^-$ concentrations between graft and control veins was attenuated by treatment with 350 U/mL of SOD for 30 minutes. These observations support the assertion that this difference was a function of increased vascular $\cdot O_2^-$ production. Interestingly, removal of the endothelium had no effect on vascular superoxide concentrations, suggesting that either the media or adventitia, (or both) of the vein grafts were the site of enhanced ROS production.

Further investigations in these tissues demonstrated that inhibition of NAD(P)H oxidase in these vein grafts with 100 μmol/L of DPI was associated with a substantial reduction in $\cdot O_2^-$ concentrations. Studies of substrate-enhanced venous $\cdot O_2^-$ production were also undertaken. In this case, treatment of vascular homogenates with both NADH and NADPH were associated with increases in vein $\cdot O_2^-$ production, which could be inhibited by co-treatment with DPI. In additional studies to identify the subcellular location of NAD(P)H oxidase, almost all of the NAD(P)H oxidase activity was localised to the particulate fraction of the cell (1.27 ± 0.73 [basal cytosolic] vs. 89.0 ± 25.4 [membrane] RLU/s/μg and 3.85 ± 0.58 [cytosolic + NADH] vs. 466 ± 108 [membrane + NADH] RLU/s/μg, $p<0.05$ in each case). Taken together, these data demonstrate that NAD(P)H oxidase is an important source of ROS generation in venous

bypass grafts. This enzyme is predominately located within cell membranes, rather than the cytosol.

Concentrations of p22 and p67phox proteins were three fold greater in vein grafts compared to control jugular veins. Furthermore, correlations were demonstrated between protein p22 and p67phox protein concentrations and vein graft superoxide concentrations (n=9 vessels; for p22phox, r^2=0.50, P<0.05; for p67phox, r^2=0.78, P<0.005), suggesting that the increase in $\cdot O_2^-$ concentrations in vein grafts may be related to the increase in the NAD(P)H oxidase enzyme protein subunits.

Subsequently, immunohistochemistry studies were undertaken to investigate the number, location and type of cells expressing p22phox subunit in jugular veins and vein grafts. Alpha actin is a marker for VSMC, and smoothelin is a marker of smooth muscle cell differentiation. Vein grafts showed marked intimal hyperplasia, with a more modest increase in medial thickness. Staining for smoothelin demonstrated an increase in VSMC differentiation. Cells staining positive for p22phox were present in greatly increased numbers in vein grafts. Dihydroethidine staining was reported to demonstrate greatest superoxide production in the intimal layer. Immunostaining for macrophages and neutrophils with RAM 11 and CD18 revealed similar staining patterns between veins or vein grafts. It was concluded that the predominate cell type responsible for enhanced vascular $\cdot O_2^-$ production in vein grafts is intimal or de-differentiated VSMC. These observations should be interpreted with some caution as hydroethidine fluorescence is a qualitative technique which does not give reproducible measurements of $\cdot O_2^-$ concentrations.

These studies demonstrate that vein grafts are subject to intimal hyperplasia, which may contribute to loss of graft patency. These pathological changes are associated with increased vascular ROS production, predominately due to NAD(P)H oxidase activation in these blood vessels. The reasons for this, although not explored in this study, may be due to an increase in transmural pressure in vein grafts carrying arterialised blood, and the lack of new vessel formation in these blood vessels. This thesis is supported by *in vitro* studies which demonstrated that enhanced cyclic strain can promote enhanced-vascular ROS generation (Howard *et al* 1997). Taken together, these observations raise the question as to whether or not strategies to reduce NAD(P)H oxidase-dependent $\cdot O_2^-$ generation in vein grafts may have therapeutic potential. The clinical importance of vein graft stenosis mandates further human investigations.

1.8 Oxidative stress in chronic cardiac failure

1.8.1 Studies in experimental animals

Enhanced myocardial ROS activity is one of several abnormalities which are implicated in the pathogenesis of left ventricular dysfunction and heart failure (Dhalla *et al* 1996 ; Li *et al* 1995). One such study was undertaken in experimental mutant mice which lacked the gene for Mn-SOD (Li *et al* 1995). Mn-SOD is important for scavenging ROS generated as by-products of oxidative phosphorylation. Homozygous mice fatigued rapidly, and died within 10 days of birth. Pathological abnormalities in these animals included a dilated cardiomyopathy, muscle wasting, hepatic steatosis and metabolic acidosis. Cytochemical analyses demonstrated a reduction in mitochondrial enzymes involved in oxidative phosphorylation, such as succinate dehydrogenase and aconitase. These enzymes are highly sensitive to oxidative damage (Zhang *et al* 1990). Taken together, these observations demonstrate that enhanced mitochondrial ROS production may result in direct damage to mitochondrial enzymes involved in cell respiration. This in turn may lead to metabolic acidosis, abnormal fatty acid metabolism, cardiomyopathy and cardiac failure.

Ide *et al* (1999a) demonstrated that myocardial $\cdot O_2^-$ production was enhanced in a murine model of heart failure, and that one possible source of myocardial ROS production was the mitochondrial electron transport enzyme system. Developing their studies in this model, this group subsequently investigated the role and consequences of myocardial-ROS generation on mitochondria structure and function (Ide *et al* 2001). Mice underwent either left anterior descending coronary artery ligation or sham-operation. The presence of impaired or normal left ventricular function was confirmed 4 weeks later using echocardiography. Myocardial oxidant status was characterised by ESR spectroscopy and quantification of lipid peroxide concentrations. Mitochondrial enzyme activities and DNA were also quantified. Features consistent with oxidant stress were identified in animals with left ventricular dysfunction, compared to controls. Copy number, or DNA template availability, of mitochondrial DNA and mitochondrial RNA expression was also found to be reduced compared to controls. Furthermore, mitochondrial enzyme activities (complexes I, III and IV) encoded by mitochondrial DNA were downregulated, compared to those other mitochondrial enzymes (complex II/Mn-SOD and citrate synthase) encoded by nuclear DNA. Mitochondrial size, shape and number were also abnormal in mice with ventricular dysfunction, compared to

controls. These data demonstrate that in this murine heart failure model, there is evidence of myocardial oxidative stress and mitochondrial lipid peroxidation, in association with abnormalities in mitochondrial morphology and function. Although this study could not establish a causal relationship between ROS activity and mitochondrial damage, other studies have demonstrated that mitochondria lack proteins such as histones (Clayton 2001), which are protective against ROS. Mitochondria are therefore prone to the accumulation of DNA oxidation products under conditions of oxidative stress (Giulivi *et al* 1995). In addition, mitochondria in other cell types, such as cultured vascular endothelial and smooth muscle cells, have also been shown to be sensitive to ROS-mediated damage (Ballinger *et al* 2000; Hill and Singal 1996; Hill and Singal 1997).

Other studies by Dhalla *et al* (1996) in a guinea pig model of left ventricular dysfunction induced by aortic banding, demonstrated that even the early features of compensatory left ventricular hypertrophy were accompanied by a reduction in myocardial content of anti-oxidant enzymes, such as SOD and catalase. These animals were also randomised to receive either vitamin E or placebo. Treatment of these animals with vitamin E improved both myocardial and blood anti-oxidant enzyme concentrations and histological features of cardiomyocyte damage. Although anti-oxidant supplementation did not prevent left ventricular dysfunction in these animals, these data do suggest that oxidative stress may be an early process in the transition from cardiac hypertrophy to cardiac failure.

More recently, Bauersachs *et al* (1999) investigated the pathophysiology of vascular dysfunction in heart failure using a rat model of left ventricular dysfunction secondary to myocardial infarction. They found that there was increased NADH-dependent vascular $^{\cdot}O_2^-$ production and impaired endothelium-dependent vasodilation, which could be improved by pre-treatment with exogenous SOD. Taken together, these findings suggest that endothelial dysfunction in ischaemic heart failure may be in part attributable to enhanced NADH-oxidase dependent ROS production which, in turn, may result in a reduction in bioavailable $^{\cdot}NO$.

1.8.2 Studies in humans which demonstrate evidence of oxidative stress in CHF

In vivo studies of endothelium –dependent and –independent coronary vasodilation in patients with dilated cardiomyopathy, have demonstrated a selective impairment of

ACh-induced increase in coronary blood flow, compared to that obtained after infusion of adenosine (Treasure *et al* 1990). This original observations suggested that endothelial dysfunction may be evident in CHF patients, possibly due to enhanced ROS activity.

Studies by McMurray *et al* (1993) confirmed that ROS activity was increased in the blood of patients with heart failure secondary to coronary artery disease, compared to controls. Plasma malondialdehyde, a marker of lipid peroxidation, is elevated in chronic heart failure (DiazVelez *et al* 1996), and is related to exercise-intolerance (Nishiyama *et al* 1998). In other studies, increased plasma concentrations of malondialdehyde, and decreased concentrations of glutathione, vitamin C and E, were correlated with both NYHA functional class (Charney *et al* 1997; Keith *et al* 1998) and plasma concentrations of the cytokine, tumour necrosis factor alpha (Keith *et al* 1998).

CHF is a state characterised by a number of processes that may promote ROS generation *in vivo*. These pro-oxidant pathways include cytokine activation (Berry and Clark 2000; Cross and Jones 1991; DeKeulenaer *et al* 1998a), mitochondrial dysfunction (Ide *et al* 1999b), recurrent hypoxia-reperfusion (Ferrari *et al* 1998), possibly genetic abnormalities (Guzik *et al* 2000a) and activation of the RAAS (Berry and Clark 2000). There are a number of potential cellular sources implicated in enhanced ROS generation in CHF. For example, it has recently been demonstrated that CHF patients may have increased leucocyte $\cdot O_2^-$ production (Ellis *et al* 2000), which is, in turn, related to severity of disease, as measured by NYHA functional class (Ellis *et al* 1998). Other sources of enhanced ROS generation in human CHF are both the myocardium (Dieterich *et al* 2000) and peripheral blood vessels (DiazVelez *et al* 1996).

1.9 Genetic variation, NAD(P)H oxidase and cardiovascular pathophysiology

A number of polymorphisms have now been identified in the genes which code for the individual proteins which collectively constitute the NAD(P)H oxidase enzyme (Babior 1999; Kenney *et al* 1993; Kuribayashi *et al* 1996; Leusen *et al* 2000). Many of the genetic studies of NAD(P)H oxidase have been in relation to the role of this enzyme in chronic granulomatous disease (Babior 1999).

There are now several reports, which suggest a relationship between variation in the NAD(P)H oxidase gene and cardiovascular risk. Inoue *et al* (1998) performed a case-control study, in which an association was found between the C242T polymorphism of

the p22phox gene and a reduced susceptibility to coronary artery disease (Inoue *et al* 1998). These findings should be considered with some caution because of both the relatively small number of patients included in this study and the low frequency of this polymorphism in the Japanese population. Other studies have also failed to find any association between this polymorphism and the presence of atherosclerotic vascular disease (Gardemann *et al* 1999; Renner *et al* 2000; Saha *et al* 1999).

By contrast, genotyping of the patients from the Lipoprotein and Coronary Artery Study (LCAS) found an association between the C242T allele and the progression of coronary atherosclerosis (Cahilly *et al* 2000). In this prospective study, the sample size totalled 368 subjects, the extent of coronary atherosclerosis was documented using quantitative coronary angiography and the period of follow up was 2.5 years. The presence of this polymorphism has also been associated with an increased risk of stroke in a Japanese population (Ito *et al* 2000).

More recently, other studies have provided evidence a functional effect of the C242T p22phox gene polymorphism in the blood vessels of patients with coronary heart disease. In studies in IMA and SV obtained from patients at the time of coronary artery bypass surgery, Guzik *et al* (2000a) demonstrated that $\cdot O_2^-$ production was reduced in the blood vessels of patients with the CT/TT genotype compared to those with the CC genotype. Most recently, Schachinger *et al* (2001) investigated the vasodilator function of epicardial coronary arteries in patients with coronary heart disease in relation to genotype for the C242T polymorphism of the p22phox gene. They found that patients with the C242T allele had enhanced coronary endothelium-dependent vasodilation compared to those with the CC. genotype. These data suggest that the C242T mutation may lead to a functional inactivation of this protein, resulting in reduced vascular $\cdot O_2^-$ production.

1.10 Rationale for studies of ROS-generation and activity in human blood vessels

At the inception of this thesis, the body of data pertaining to the sources and mechanisms of vascular ROS generation existed primarily as a result of studies undertaken in experimental animal models (Griendling *et al* 1994; Li *et al* 1995; Li *et al* 1995; McIntyre *et al* 1997; Mohazzab and Wolin 1994; Mohazzabh *et al* 1994; Mugge *et al* 1991c; Mugge *et al* 1994; Ohara *et al* 1993; Pagano *et al* 1995; Rajagopalan *et al* 1996b; Wilson 1990; Li *et al* 1995; Li *et al* 1995). Human studies had previously demonstrated both the functional importance of endothelium-dependent vasodilation in

the regulation of normal blood flow *in vivo* (Vallance and Collier 1994), and how this may be abnormal in a variety of cardiovascular disease states (Calver *et al* 1992b; Calver *et al* 1992a; Cox *et al* 1989; Creager *et al* 1990; Panza *et al* 1990).

As discussed above, the sources of $\cdot O_2^-$ generation in the vasculature of a number of animal models of vascular disease, have been documented. The sources and mechanisms of ROS production in human blood vessels, and whether this may be altered in vascular disease, remain incompletely understood.

1.11 Strategies for reducing vascular oxidative stress

1.11.1 Oestrogen

Oestrogen has both rapid and longer-term effects on the blood vessel wall. Current data suggest that oestrogen treatment may enhance bioavailable, endothelial-derived $\cdot NO$ (Mendelsohn and Karas 1999). Huang *et al* (2000) reported that oestrogen, via a receptor – mediated pathway, upregulated eNOS gene expression and restored vascular responses in male SHR. Garcia-Duran *et al* (1999) examined neuronal (nNOS) expression in neutrophils from male and female human subjects. In premenopausal women nNOS expression and plasma oestrogen levels were higher in the ovulatory than the follicular stage of their menstrual cycle, whereas, in postmenopausal women nNOS expression was lower. Transdermal oestradiol therapy in these women increased neutrophil nNOS expression towards those observed in the follicular stage of the cycle in premenopausal women. Neutrophils from male subjects incubated with 17β oestradiol showed enhanced nNOS protein expression which was inhibited by tamoxifen, indicative of a receptor mediated mechanism. It has recently been suggested that oestrogen may also enhance eNOS activity by modulating $\cdot O_2^-$ concentrations (Barbacanne *et al* 1999). Simoncini *et al* (2000) demonstrated that the oestrogen receptor isoform ERα binds to the P85α regulatory subunit of phospatidylinositol -3-hydroxyl-kinase (PI3K). Oestrogen enhanced the ERα–associated PI3K activity, leading to activation of eNOS, independent of gene transcription.

1.11.2 Vitamins

The possibility that vitamin C therapy might enhance bioavailable $\cdot NO$ and therefore improve abnormal endothelium-dependent vasodilator responses in vascular disease has

investigated in a number of experimental animal and human studies. In a pig coronary balloon injury model, Nunes *et al* (1997) investigated whether or not dietary supplementation of these animals with vitamin C, vitamin E, both, or neither, could enhance attenuate vascular $\cdot O_2^-$ concentrations, as measured by lucigenin-enhanced chemiluminescence. Vitamin C & E plasma concentrations were increased in those groups receiving dietary supplements with these vitamins. After 7 days of supplements, animals sustained balloon injury to the left anterior descending (LAD) coronary artery balloon injury and were sacrificed 14 days later. Superoxide concentrations in LAD coronary segments were 2.5 times greater than in segments from either an uninjured part of the LAD, or from the right coronary artery (RCA) of the same animal. Furthermore, vascular $\cdot O_2^-$ concentrations were reduced in both LAD and RCA segments in all vitamin-treated animals, compared with controls. In further studies, this group demonstrated that therapy with vitamins C & E in combination, but not alone, reduced plasma concentrations of lipid peroxides and attenuated intimal hypertrophy (intimal area : vessel area), compared to controls.

Taddei *et al* (1998) investigated whether treatment with vitamin C might improve attenuated in brachial artery endothelial-dependent dilator responses, as measured by forearm strain-gauge plethysmography, in a group of hypertensive patients. This study included 47 patients with essential hypertension (systolic blood pressure 155 ± 7 mmHg; diastolic blood pressure 102 ± 4 mmHg) and 35 age-matched control subjects (systolic blood pressure 121 ± 4 mmHg; diastolic blood pressure 81 ± 3 mmHg). $\cdot NO$, or endothelium-dependent vasodilator responses were assessed by infusion of incremental concentrations of ACh. These studies demonstrated a blunted response to ACh infusion in hypertensive patients. Forearm blood flow (FBF) increased less, from 3.6 ± 0.5 to a maximum of 16.5 ± 2.7 mL/100ml forearm tissue per minute compared to normotensive subjects (3.6 ± 0.6 to a maximum of 22.8 ± 3.3 mL/100mL forearm tissue per minute). Endothelium-independent vasodilator function was assessed by infusion of sodium nitroprusside, with comparable responses obtained in both groups. In additional studies, co-infusion of vitamin C with ACh increased the maximum vasodilator response in hypertensive subjects (FBF increased from 3.5 ± 0.6 (mean ± SEM) to a maximum of 20.8 ± 2.6 mL/100ml forearm tissue per minute; $p<0.05$), whereas vitamin C was without effect in normal subjects.

In further studies (Taddei *et al* 1998), the response to inhibition of NOS, by infusion of L-NMMA, was assessed both in the presence and absence of vitamin C. In healthy

subjects, L-NMMA infusion caused a reduction in basal FBF (from 3.6 ± 0.4 to 2.2 ± 0.2 mL/100ml forearm tissue per minute; $p<0.05$), and also blunted the vasodilating response to ACh (saline, from 3.6 ± 0.5 to 22.7 ± 3.7 mL/100ml forearm tissue per minute; L-NMMA, from 2.2 ± 0.2 to 9.9 ± 1.8 mL/100ml forearm tissue per minute; $p<0.01$ vs ACh alone). Co-infusion of vitamin C had no effect on either of these responses. By contrast, in hypertensive subjects, L-NMMA infusion caused a smaller decrease in FBF (from 3.5 ± 0.5 to 2.6 ± 0.2 mL/100ml forearm tissue per minute; $p<0.001$), compared with normotensive subjects (39% vs 25%, respectively; $p<0.01$). Furthermore, L-NMMA infusion did not change the response to ACh (saline, from 3.6 ± 0.5 to 16.7 ± 2.3 mL/100ml forearm tissue per minute; L-NMMA, from 2.6 ± 0.2 to 9.9 ± 1.9 mL/100ml forearm tissue per minute; $p<0.01$ vs ACh during saline). When the effect of L-NMMA was retested in the presence of vitamin C, the NOS inhibitor attenuated the vasodilating response to ACh (from 2.6 ± 0.2 to 11.4 ± 1.8 mL/100ml forearm tissue per minute; $p<0.01$ vs ACh during vitamin C co-infusion). Taken together, these data suggest that in hypertensive subjects, a reduction in bioavailable vascular NO is present due to increased activity of ROS. This could be improved by treatment with vitamin C. That vitamin C had no effect on basal FBF in either hypertensive or control subjects suggests that ROS are not tonically produced. In other studies of brachial artery FBF responses, vitamin C also improved impaired endothelium-dependent vasodilation in patients with diabetes (Timimi *et al* 1998), hypercholesterolaemia (Ting *et al* 1997) and coronary heart disease (Levine *et al* 1996).

The question as to whether anti-oxidant interventions are beneficial in the primary or secondary prevention of human vascular disease has been investigated in a number of large, randomised, placebo-controlled clinical trials, namely the Cambridge Heart Anti-Oxidant Study (CHAOS) Trial (Stephens *et al* 1996), and the Heart Outcomes PrevEntion (HOPE) study (Yusuf *et al* 2000). However, the potential of vitamin therapies has not translated into actual clinical benefits. By contrast, a recent randomised, placebo-controlled trial of vitamins C & E in 283 patients at risk of pre-eclampsia (PET), reported a reduced incidence of PET in patients treated with vitamin C & E, compared to those given placebo (8% v 17%; $p=0.002$) (Chappell *et al* 1999)

1.11.3 Superoxide dismutase

Initial studies undertaken to investigate the effect of SOD infusion on the impaired endothelium-dependent ACh vasodilator response in hypertensive patients failed to

demonstrate any improvement in this response (Garcia *et al* 1995). Possible explanations for this include a lack of bioavailable ·NO due to decreased production rather than increased breakdown of by $\cdot O_2^-$ scavenging, a thesis recently supported by the findings of Forte *et al* (1997). Alternatively, exogenous SOD may have been inactive due to either its relatively short half-life or its poor intracellular penetrance. This is due to SOD being electrostaticically repelled from cell membranes as a result of its negative charge (Omar *et al* 1992).

For this reason, other studies were undertaken with SOD combined with polyethylene glycol (PEG-SOD). Initial investigations with this compound demonstrated that PEG-SOD can increase cultured porcine endothelial cell SOD activity therefore preventing oxidative damage by activation of XOR (Beckman *et al* 1988). Mugge *et al* (1991b) investigated whether or not treatment with PEG-SOD might improve impaired endothelium-dependent vasorexlaxation in atherosclerotic aortae obtained from cholesterol fed New Zealand White rabbits. The presence or absence of atherosclerosis was confirmed by histological analyses using Verhoeff and van Gieson stains. Isometric tension studies were undertaken and endothelium-dependent responses in aortae pre-constricted to phenylephrine. In addition, total vascular SOD activity was also assessed using spectrophotometric techniques. PEG-SOD treatment increased total vascular SOD activity in both cholesterol fed and normal rabbits. Endothelium-dependent relaxations in response to ACh and the calcium ionophore A23187 were markedly impaired in atherosclerotic arteries (43 ± 7% and 54 ± 6%, respectively), compared with normal controls (80 ± 2% and 89 ± 2%, respectively). Treatment of cholesterol-fed rabbits with PEG-SOD improved the maximal relaxation in response to ACh and A23187 (65 ± 2 % and 73 ± 3%; $p<0.05$), respectively, whereas PEG-SOD had no vasorelaxant effect in normal rabbits.

Lipid permeable SOD analogues, however, have been shown to have acute vasodilator properties in rabbits, rats and most recently mice (Nakamura *et al* 1998). In one other study, treatment of SHR with tempol (4- hydroxy –2, 2, 6, 6- tetra methyl piperidinoxyl) resulted in reductions in blood pressure, glomerular filtration rate and renal excretion of 8-iso- prostaglandin F2α - a marker of oxidative stress (Schnackenberg and Wilcox 1999).

A number of studies have been undertaken to investigate the possible cardioprotective effects of EC-SOD therapy in ischaemia/reperfusion injury. For example, Marklund *et*

al (Wahlund 1992) demonstrated that EC-SOD reduced creatine kinase release in rats subjected to 10 minutes of coronary occlusion and 24 hours of reperfusion. Hatori *et al* (Hatori 1992) demonstrated that infusion of purified, recombinant EC-SOD into the great cardiac vein of pigs was associated with a reduction in infarct size.

1.11.4 Gene transfer

Several groups have now used vascular gene transfer in an effort to reduce ROS activities in the cardiovascular system, however, only some of these studies have demonstrated beneficial effects. One early study by Fang *et al* (Fang 1998) investigated the possibility that transfer of CuZn-SOD and Mn-SOD cDNA into bovine aortic endothelial cells (BAEC) could protect these cells from lipid peroxidation. Incubation of BAEC with 200 μg/mL of LDL for 18 hours resulted in a four-fold increase in the concentration of lipid peroxides, such as malondialdehyde, compared to controls (22.5 ± 1.1 vs 6.3 ± 0.2 nmol malondialdehyde/mg LDL protein, respectively; $p<0.05$). In further studies, treatment of these cells with media containing a concentration of 100 μg/mL of Cu/Zn-SOD reduced concentrations of oxidised LDL by 79%. In order to determine whether enhanced activity of intracellular SOD could prevent lipid peroxidation, these cells were transfected with either adenoviral vectors containing cDNA for CuZn-SOD and Mn-SOD, or no foreign DNA. In this case, incubation of CuZn-SOD and Mn-SOD-transfected cells with LDL resulted in a reduction in malondialdehyde concentrations by 77% and 32% respectively. These studies suggest that lipid peroxidation in cultured vascular cells is a $^{\cdot}O_2^-$ -mediated effect, which can be prevented by manipulations to increase exogenous or endogenous SOD activity through gene transfer.

These observations raised the question of whether SOD gene transfer might improve abnormal endothelium-dependent responses in intact blood vessels obtained from animal models of vascular disease. However, *ex vivo* gene transfer of either CuZn-SOD (Adenovirus cytomegalovirus (AdCMV) Cu Zn-SOD) or extracellular SOD (Ad CMV EC-SOD) to aorta from Watanabe heritable hyperlipidaemic rabbits failed to improve the impaired relaxation to ACh or calcium ionophore, despite successful transfer of the virus (Miller *et al* 1998). Similarly when segments of thoracic aorta obtained from rabbits made hypertensive by Ang II infusion were incubated with adenoviral vectors containing CuZn- or EC-SOD cDNA, no improvement in endothelium-dependent vasorelaxation was observed, compared to control arteries incubated with adnovirus

containing cDNA beta-galactosidase (Nakane *et al* 2000). Furthermore, in studies performed in our laboratory, *in vivo* adenoviral gene transfer of CuZn-SOD to carotid arteries of SHRSP also showed no beneficial effect (Alexander *et al* 2000).

By contrast, gene therapy with EC-SOD has protective effects in rabbit models of reperfusion injury (Li *et al* 1998b) and myocardial infarction (MI) (Li *et al* 1998c). In this latter study, healthy rabbits were randomised into four groups. Animals in the first group (I) underwent instrumentation and coronary occlusion only. Ischaemic preconditioning has a cardioprotective effect (Bolli *et al* 1991), and therefore a second group (II) was included to serve as positive control. The animals in this group were exposed to a period of ischaemic preconditioning prior to coronary occlusion, which involved a sequence of six 4-minute coronary occlusions interspersed with 4 minutes of reperfusion performed 24 hours before the 30 minute coronary inclusion. Animals in the third group (III) served as controls, receiving replication-deficient adenovirus with LacZ reporter gene, prior to coronary inclusion. Intravenous heparin was infused 2 hours before coronary occlusion in order to release hepatic EC-SOD into the systemic circulation, with intravenous protamine being subsequently infused immediately prior to the occlusion to reverse the effects of heparin. The fourth group (IV) of animals received human EC-SOD cDNA incorporated into adenovirus and the herparin/protamine infusion prior to coronary occlusion. In this study, the liver was the principal target for gene transfer in order to both exploit the efficiency of adenoviral transfection of hepatocytes and to avoid the possibility of an inflammatory response within the heart. Myocardial infarction was defined on the basis of new ST-segment elevation and QRS changes on the electrocardiogram, and systolic wall thinning as detected by cardiac ultrasound. Northern blot analyses demonstrated that hepatocyte EC-SOD gene expression was substantially increased by this treatment. Myocardial infarct size was comparable between control groups I and III (57 ± 6% and 58 ± 5%, respectively), whereas infarct size was reduced in the EC-SOD gene therapy-treated animals by 25 ± 4% compared to the control virus-treated animals ($p<0.01$). This reduction was comparable to that which occurred in preconditioned animals, compared to instrumented controls [29 ± 3%, $p<0.01$]. This study was the first to demonstrate a beneficial effect of EC-SOD gene therapy in a model of either reperfusion or MI. The discrepancy between this and other studies which have investigated the same question may be due to the use of EC-SOD, rather than CuZn or MnSOD, as the former is excreted by cells and binds more readily to intra and extracellular membranes.

An alternative approach involves vascular gene transfer of NOS. Nakane *et al* (Nakane 2000) showed that transfer of eNOS gene into aortic segments from rabbits that had been chronically infused with Ang II improved endothelium-dependent vasorelaxation. Similarly, studies in our laboratory have demonstrated that *in vivo* transfer of the eNOS gene improved vasomotor responses in the SHRSP (Alexander *et al* 2000). It seems that strategies aimed at modification of vascular function could also be targeted to the adventitia, as demonstrated by Tsutsui *et al* (Tsutsui 1998). In the future, use of vectors devoided of any immune or inflammatory responses, might facilitate these mechanistic studies.

1.11.5 Pharmacolgical agents with antioxidant effects

Some therapies which improve prognosis in hypertension and coronary heart disease, such as beta-blockers and angiotensin converting enzyme (ACE) (Lechat *et al* 1999; Yusuf *et al* 2000) may exert beneficial effects, in part, through anti-oxidant mechanisms (Chopra *et al* 1992; Lysko *et al* 2000). For example, these treatments are associated with improvements in endothelium-dependent vasodilation (Anderson *et al* 2000; Hornig *et al* 1997). In the following section, the current and potential therapeutic anti-oxidant interventions will be reviewed.

1.11.5.1 Inhibition of the RAAS

ACE-I have anti-oxidant properties *in vitro* (Benzie and Tomlinson 1998; Chopra *et al* 1992; McMurray *et al* 1989; McMurray *et al* 1990; McMurray *et al* 1990). The mechanism for this effect is related to a sulphydryl (-SH) group present in some, but not all, of these drugs (Figure 1.7).

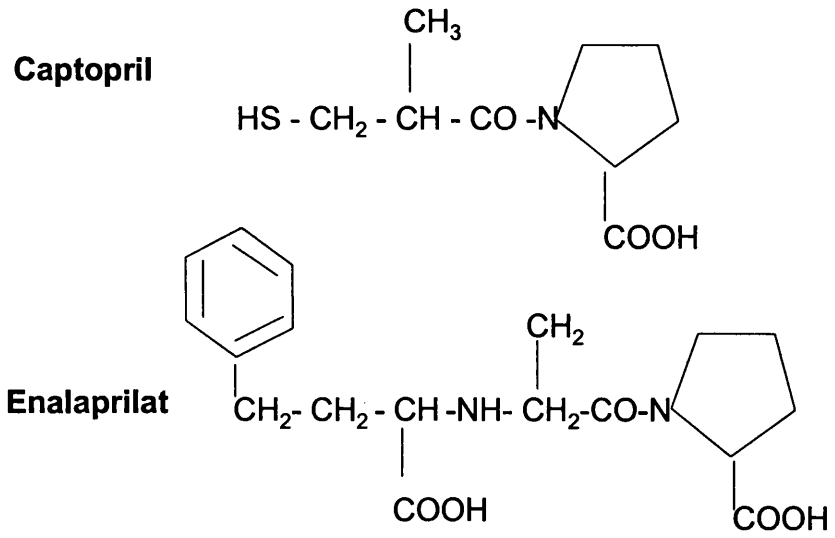


Figure 1.7 Chemical structure of the angiotensin converting enzyme inhibitors, captopril and enalaprilat. Captopril has a sulphydryl (-SH) group whereas enalaprilat does not.

For example, in photo-oxidation studies undertaken by Chopra *et al* (1992) captopril, an SH-containing ACE-I, did not exhibit any significant scavenging of $^{\cdot}O_2^-$, but effectively removed H_2O_2, singlet oxygen and HOCl. In this case, the SH group was oxidised on interaction with certain ROS. Blockade of the –SH group in captopril attenuated its ability to scavenge ROS. By contrast, both SH-containing and non-SH containing ACE-I (e.g. enalaprilat) inhibited ascorbate-induced lipid peroxidation in rat liver microsomes. This may be due to the ability of these compounds to chelate metal ions.

In addition, controlled clinical trials have recently shown that ACE-I therapy may improve endothelium-dependent vasodilation in patients with CHD (Anderson *et al* 2000; Mancini *et al* 1996). Although these data support an anti-oxidant effect of ACE-Is, there are several other mechanisms, such as potentiation of bradykinin, which may explain this effect. In addition, ACE-Is reduce the production of Ang II. Results from experimental animal studies have demonstrated that Ang II is pro-oxidant (Rajagopalan *et al* 1996b). Consequently, this may be one additional mechanism whereby ACE-Is, and ARAs, may exert anti-oxidant effects. However, as yet, there are no data on the potential anti-oxidant effects, if any, of these drugs, in humans.

1.11.5.2 Beta-blockers

Carvedilol is a non-specific, beta adrenergic receptor antagonist with alpha-blocking properties, whereas metoprolol is a $beta_1$-specific receptor antagonist. The anti-oxidant properties of carvedilol, and its metabolites, are attributed to the ROS scavenging properties of the carbazole moiety in this drug (Christopher *et al* 1998; Intengan and Schiffrin 2000). Carvedilol, by nature of its chemical structure rather than any receptor-mediated property, can scavenge free radicals and inhibit lipid peroxidation. *In vitro,* carvedilol is a more potent anti-oxidant than other beta-blockers which do not contain a cabazole moiety, such as metoprolol (Lysko *et al* 2000).

Certain studies in animal models of hypertension have demonstrated that carvedilol, but not metoprolol, results in reduced ROS activity and improved endothelium-dependent vasorelaxation (Intengan and Schiffrin 2000). These *in vitro* differences in experimental animal models have not been consistently reproduced *in vivo*. For example, one recent randomised, controlled trial of the effects of carvedilol, compared to metoprolol, on left ventricular ejection fraction (LVEF), exercise capacity and plasma concentrations of thiobarbituric acid-reactive substances (TBARS) (indirect markers of plasma anti-oxidant capacity) in patients with CHF (Kukin *et al* 1999). In this study, LVEF and exercise capacity improved, and plasma TBARS concentrations decreased, to a similar extent in both groups. These observations confirm that beta-blockers have anti-oxidant properties *in vivo*, however, this may not be specific to one particular drug. The mechanisms for this anti-oxidant effect may include direct ROS scavenging, inhibition of the sympathetic nervous system, improvement in cardiac function, and/or anti-cytokine effects (Berry and Clark 2000).

Nebivolol is one other beta-blocker which, in addition to beta-adrenergic receptor blockade, is reported to enhanced endothelium-dependent vasorelaxation through stimulation of eNOS activity (Bowman *et al* 1994; Broeders *et al* 2000; Dawes *et al* 1999; Parenti *et al* 2000). Beta-blockers are sympathetic nervous system antagonists and consequently, inhibit the production of renin, leading in turn to a reduction in plasma Ang II concentrations. Consequently, this may, also lead to a reduction in Ang II-stimulated $\cdot O_2^-$ production, which could be one putative, additional anti-oxidant effect of this class of drug.

1.11.5.3 Aspirin

Aspirin has, in addition to its known anti-platelet effects, anti-oxidant properties (Podhaisky *et al* 1997). *In vitro* studies with aspirin have shown that treatment of cultured bovine pulmonary artery endothelial cells with this drug leads to protection of these cells from the toxic effects of H_2O_2. This mechanism is thought to occur through removal of iron by an aspirin-induced increase in ferritin synthesis (Oberle *et al* 1998). Ferritin sequesters free cytosolic iron, preventing its participation in ROS-generating reactions.

1.11.5.4 HMG-CoA reductase inhibitors

Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) enzyme inhibitors, or statins, have effects beyond inhibition of hepatic cholesterol synthesis (Maron *et al* 2000). Statins enhance vascular production of $^{\cdot}NO$, through up-regulation of eNOS gene expression (Amin-Hanjani *et al* 2001; Hernandez-Perera *et al* 1998). The mechanisms involved in this effect may be mediated through both direct effects of the drug, independent of any reduction in plasma cholesterol concentration (Amin-Hanjani *et al* 2001), and indirect effects, mediated for example, through a reduction in plasma concentrations of oxidised LDL. This latter effect may be explained by results from i*n vitro* studies, which have demonstrated that oxidised LDL, but not LDL, exerts an inhibitory effect on eNOS mRNA expression (Hernandez-Perera *et al* 1998). In this case, enhanced vascular production of $^{\cdot}NO$, may lead to increased removal of ROS.

Statins may also have a direct inhibitory effect on ROS generation in vascular cells. Recently, *in vitro* studies in cultured human VSMC demonstrated that simvastatin, pravastatin, fluvastatin, and its metabolites, attenuated lysophosphatidylcholine-stimulated ROS production through inhibition of a phospholipase D/protein kinase Cα-mediated pathway (Yasunari *et al* 2001). Furthermore, this inhibition of ROS generation was associated with a reduction in VSMC migration. Finally, some statins, such as fluvastatin, may also have direct ROS-scavenging properties (Suzumura *et al* 1999).

1.12 Aims

The purpose of this investigation was to characterise the cellular and enzymatic sources of vascular ROS generation in humans. Specifically, I sought to determine the contribution of NAD(P)H oxidase, XOR and NOS to the basal production of $^{\cdot}O_2^-$ in

IMA and SV obtained at the time of coronary artery revascularisation surgery. Secondly, I sought to determine which clinical and demographic parameters might be associated with basal ROS-generation in these blood vessels. The third aim was to investigate whether or not Ang II might stimulate $\cdot O_2^-$ generation in human blood vessels, and if so by what mechanism. Finally, I sought to characterise the functional effects of ROS generation which may be stimulated by Ang II.

2 Methods

2.1 Summary

This chapter provides a detailed description of how and from whom blood vessels were obtained, and secondly the laboratory and clinical techniques used in the studies described in this thesis. Laboratory studies were undertaken within the British Heart Foundation Blood Pressure Group, Department of Medicine and Therapeutics, and the Autonomic Physiology Unit, University of Glasgow.

2.2 Patients and healthy volunteers

The University of Glasgow is located adjacent to the Western Infirmary which is a tertiary healthcare centre. Healthy human blood vessels are extremely difficult to obtain. For this reason, we sought an alternative source of human vascular tissue. Coronary artery bypass surgery is a common procedure performed in the Western Infirmary, with approximately 1000 operations being performed per annum. Usually, distal segments of left internal mammary artery (IMA) and saphenous vein (SV) are surplus to requirement and are consequently discarded. In certain cases, the radial artery (RA) from the non-dominant arm were used. This situation therefore presents an opportunity to obtain human blood vessels for the purposes of scientific research. Although these conduit blood vessels are obtained from patients with atherosclerotic vascular disease, these blood vessels are, in general, not affected by this process. All studies were fully approved by the West Ethics Committee of the Western Infirmary, on behalf of the North Glasgow Hospitals University NHS Trust. Healthy volunteers were identified from those patients who were undergoing elective inguinal herniae repair. These patients were invited to consent to an abdominal skin biopsy at the time of their operation. All studies were fully approved by the West Ethics Committee of the North Glasgow Hospitals University NHS Trust.

Clinical details were recorded from case note examination. A history of current cigarette smoking, hypertension (defined as either current anti-hypertensive treatment or a blood pressure > 140/90 mmHg), diabetes mellitus (insulin treated or non-insulin treated) and hypercholesterolaemia (plasma cholesterol > 5.4 mmol/L) were considered as risk factors for CHD. Information on current medication was also documented at this point.

2.3 Handling of human tissue samples

2.3.1 Conduit arteries and veins

IMA, RA or SV were collected on a twice-daily basis from the Cardiac Surgical Theatre Suites within the Western Infirmary. Distal segments of these blood vessels were immediately taken to the laboratory in Krebs buffer on ice. At this point, and in these conditions, the blood vessels were then carefully dissected free of loose connective tissue, divided into 4-5mm segments and weighed. The vessels were then incubated in Krebs buffer at pH 7.4 ± 0.22 and maintained in atmospheric conditions (pO_2 19 ± 4 kPa; pCO_2 3 ± 4 kPa) at 37 °C. In the case where it was planned to isolate RNA, then the blood vessels were collected in RNAlater (AMS Biotechnology [Europe] Ltd) on ice and stored at 4°C for 24 – 48 hours prior to isolation of RNA.

In some studies, the IMA were denuded of endothelium by gentle rubbing within the lumen with forceps. In the case where IMA were collected with a view to being used in molecular studies, biopsies of arteries within connective tissue were placed in a sterile universal container containing human culture media maintained at 37°C in a thermoflask. The connective tissue was removed by careful dissection. Endothelial cells were obtained from these arteries by careful dissection and removal of the endothelial cell monolayer under high-powered microscopic view. The studies in which IMA and SV were used are summarised in Figure 2.1.

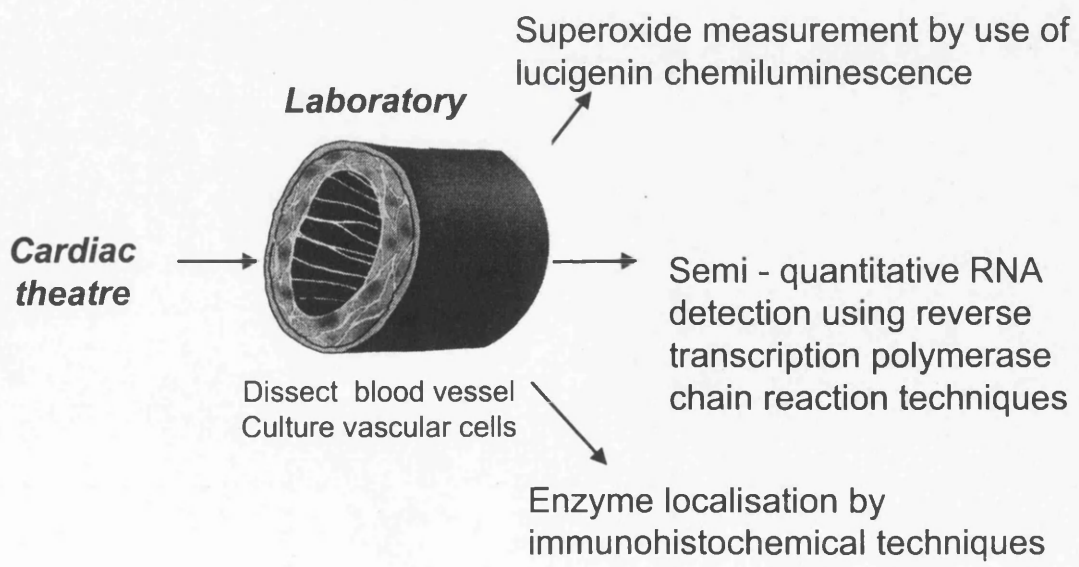


Figure 2.1 Schematic diagram of handling and experimental use of conduit arteries and veins obtained at the time of coronary revascularisation surgery

2.3.2 Skin biopsies

Small resistance arteries (SRA) from healthy subjects were obtained from skin biopsies. These biopsies were taken by from the abdominal wall of healthy male volunteers by the operating surgeon at the time of inguinal hernia repair. The biopsies were taken at the point of skin incision and prior to the use of electrocautery. Once made available, the biopsies were collected from the operating theatre and taken to the laboratory (less than 5 minutes by foot) in normal saline on ice.

At this point, the skin biopsy was transferred from cold 0.9% NaCl solution to ice cold, Krebs buffer solution (composition in mmol/L: NaCl 118.4, KCl 4.7, $MgSO_4$ H_2O 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24.9, $CaCl_2$ 2.5, glucose 11.1, and EDTA 0.023, which gives a pH of 7.4 when aerated with a 5% CO_2/95% O_2 mixture). Fresh Krebs buffer was prepared each day.

SRA can be identified by their helical shape and opaque wall, in contrast to veins which are rather more elongated, flaccid and transparent. On arrival in the laboratory, SRA were isolated from the skin biopsy by careful dissection using surgical grade instruments with the aid of a high power microscope. This was done with the biopsy placed in a Petri dish filled with ice cold Krebs buffer, which was regularly changed during the dissection process. A thorough inspection of the skin biopsy, and dissection of any SRA that were identified, could take anything up to two or three hours to complete. Thereafter, the SRA were placed in a universal container and stored in a fridge at 4°C overnight. Storage of resistance arteries in this way has been previously demonstrated to have no effect on the vasoactive properties of these blood vessels (McIntyre *et al* 1998).

2.4 Cell culture

Human aortic endothelial cells (HAEC; Clonetics), human coronary artery smooth muscle cells (HCASMC; Biowhittaker) were grown to subconfluence (80-90%) at 37°C under 5% CO_2, 95% air. The HAEC and HCASMC were obtained from primary cultures in appropriate culture media containing bovine serum in 25 ml culture flasks and used between passages four and six in serum free conditions.

2.4.1 Isolation of total cellular RNA from blood vessels or cultured cells

RNA was extracted from fresh, intact blood vessels and cultured cells. This was done according to a standard protocol, which involved four basic steps. The first involved homogenisation of blood vessels using RNAzol™ B (2 ml/100 mg tissue). The second step involved RNA extraction using 1 volume of homogenate: 0.1 volume of choloroform, which was then followed by RNA precipitation with 1 volume of isopropanol. The fourth step involved washing the RNA with 75% ethanol.

All glass and plasticware had been sterilised by treating with Diethylamine Pyrocabonate (DEPC) as 0.1% v/v solution to minimise degradation of RNA due to endogenous or contaminating RNAses, then autoclaved for approximately 15 minutes to remove traces of DEPC. All materials were handled using disposable rubber gloves.

The biopsies containing internal mammary arteries (and adherent tissue) were obtained immediately at the conclusion of revascularisation during CABG surgery. The tissue was placed on ice-cold saline, transferred to the laboratory and carefully cleaned of surrounding connective tissue under high-powered microscopic view. The blood vessel was then inserted into 1ml of RNAzol™ B (Biogenesis, UK) and placed on dry-ice.

RNAzol™ B is an RNA extraction agent that contains guanidinium thiocyanate and phenol. RNA molecules form complexes with the guanidinium thiocyanate and water molecules, preventing hydrophilic interactions with any DNA and protein present. The frozen blood vessel was then placed into a disposable cell culture dish (Nunclon™) on dry ice and manually macerated using a scalpel blade, and the frozen homogenate was then scraped into a microfuge tube (Townsend *et al* 1999). This mass of tissue was then further homogenised with a Kinematica polytron® homogeniser (Philip Harris Scientific, Aberdeen, UK). The polytron head was washed in 3% hydrogen peroxide then DEPC-treated distilled water (dH_2O) between samples. Choloroform was then added to the homogenate in a ratio of 1:10.

In order to extract RNA from the homogenate, the samples were vortexed for 10 seconds and then left to stand on ice for 15 minutes. After this, the samples were centrifuged for 15 minutes at 12,000 g (4°C). This resulted in separation of the sample into an upper, colourless aqueous phase containing RNA, an intermediate DNA phase

and a lower, blue organic phase, containing phenol-chloroform and protein. The upper aqueous phase was removed by careful pipetting and added to an equal volume of isopropanol (Fisher Scientific, UK). The sample was then vortexed and left to stand on ice for a minimum of 15 minutes, which was followed by centrifugation for 15 minutes at 12,000-g (4°C). The RNA precipitate forms a yellow-white pellet at the bottom of the tube. The RNA was then washed in 75% ethanol – 0.8 ml of ethanol per 50 – 100 μg RNA (Fisher Scientific, UK), vortexed, and subsequently centrifuged for 5 minutes at 7500 g (4°C). The supernatant was then removed by careful pipetting, and the RNA pellet was air dried (1 minute at 37°C) and mixed with 15-20 μL of nuclease-free H_2O (Promega, UK).

Similar steps were followed in the case of extraction of RNA from cultured cells. RNAzol™ B was added to cells in culture flasks in a ratio of 0.2 ml of RNAzol™ B: 1 x 10^6 cells. It is estimated that once grown to confluence, there will be 1 x 10^7 cells in a 250-300 ml flask (growth area = 75 cm^2) and 2 x 10^7 cells in a 650-750 ml (growth area = 162-175 cm^2). It is recognised that these values are approximations as animal cells can vary in length from 10-100 μm. After the addition of RNAzol™ B, the flasks were agitated and the cell suspension scraped off and aspirated into a 1.5ml Eppendorf tube. Total cellular RNA was then obtained by the phenol: choloroform extraction technique described above.

Each of the RNA samples was quantified by use of the RiboGreen™ RNA quantification assay (Molecular Probes, Europe). This is a fluorescent assay based on the Ribogreen™ reagent used in conjunction with a fluorescence microplate reader (Wallac 1420 Multilabel Counter). This assay has high sensitivity, compared to other techniques such as ethidium bromide or absorbance-based spectrophotometry assays. The RiboGreen™ assay affords detection of RNA concentrations as low as 1ng/ml, with a linear range extending to 1μg/ml. This linearity is maintained in the presence of several compounds known to contaminate nucleic acid preparations, including nucleotides, salts and urea. The RiboGreen™ reagent is a dye solution dissolved in anhydrous dimethylsulfoxide (DMSO). A ribosomal RNA standard curve was prepared using a working solution of 2 μg/ml solution of RNA in 20X TE (200 mM Tris-HCL, 20 mM EDTA, pH 7.5 in DEPC-treated dH_2O). The ribosomal RNA standard was 16S and 23S rRNA from *E. coli* at a concentrated stock solution (100 μg/ml). Most single-stranded RNA molecules yield approximately equivalent signals. The assay was performed at room temperature in disposable dark-adapted, plastic microplates.

Qualitative assessment of RNA obtained from cell and tissue homogenates was also undertaken by electrophoresis on a 1.5% agarose gel (Life Technologies, UK).

2.4.2 Removal of contaminating DNA

Genomic DNA contamination of the RNA would interfere with both the RNA quantification and rt-PCR quantification reaction. For example, the PCR reaction will amplify templates of genomic DNA as well as reverse-transcribed complementary DNA (cDNA). In order to remove contaminating genomic DNA, each RNA sample underwent treatment with RQ1 deoxyribonuclease (DNase). This endonuclease enzyme digests both double- and single-stranded DNA, but has no RNase activity, and therefore leaves the RNA intact. In the presence of Mg^{2+}, DNase I cleaves each strand of DNA independently, and the sites of cleavage are random. Alternatively, in the presence of Mn^{2+}, DNase I cleaves both strands of DNA at approximately the same site to yield fragments that are blunt ended, or have protruding termini only one or two nucleotides in length. The experiments in the present investigation were performed with RQ1 RNase-Free DNase (Promega, UK) in the presenceof Mg^{2+} .The DNase digestion reaction was as follows:-

RNA in dH_2O or TE buffer	1-8 μL
RQ1 RNase-Free DNase 10X reaction buffer	1μL
RQ1 RNAse-FreeDNase	1μL/μg RNA
Nuclease-free dH_2O to a final volume of	10 μL

The reaction mixture was incubated at 37°C for 45 minutes at which point 1μL of RQ1 DNase Stop Solution was added to terminate the reaction. The reaction mixture was then incubated at 65°C for 10 minutes to inactivate the DNase.

2.5 Reverse Transcription Polymerase Chain Reaction - semi-quantitative measurement of RNA

2.5.1 Principles of Reverse Transcription Polymerase Chain Reaction

Reverse transcription of messenger RNA, coupled with polymerase chain reaction of cDNA, is a technique which affords information on gene expression. The reverse transcription polymerase chain reaction (RT-PCR) technique may be used to determine whether or not a particular gene is expressed in a cell type or tissue. Furthermore, RT-PCR may also yield quantitative information (Ferre *et al* 1994), and is sensitive to very low levels of mRNA abundance (e.g. femtomolar concentrations) (Chelly and Kahn 1994). RT-PCR, therefore, may usefully detect very low levels of gene expression.

RT-PCR is based on the activity of a retroviral reverse transcriptase enzyme e.g Avian Myoblastosis Virus (AMV) (Goodman and MacDonald 1979). This enzyme utilises oligo deoxythymidine [oligo $(dT)_{15}$] as a primer which hybridises to the poly (A) tail of total or poly (A)+ isolated mRNA to produce cDNA transcripts. The concentration of the reverse transcription reaction reagents should be adjusted to the type of reaction being performed. AMV Reverse Transcriptase can only hybridise to mRNA in its elongated secondary form, therefore the mRNA in its native coiled tertiary structure must be heated to temperatures of 45 - 50°C, which causes a conformational change to the secondary form. RT-PCR is sensitive to the concentration of magnesium sulfate. A magnesiun sulfate concentration in the range 1.0 - 2.5 mM is suitable for most applications, although this may require to be optimised for any particular experiment. The activity of the reverse transcriptase enzyme is optimal at 42°C. The hybridisation reaction should take place for 15 - 45 minutes. Subsequently, the sample should be heated to 99°C for 5 minutes to inactivate the AMV Reverse Transcriptase and prevent it binding to the cDNA (Chelly and Kahn 1994; Ferre *et al* 1994).

PCR is an efficient and rapid *in vitro* method for the amplification of specific DNA sequences (Mullis *et al* 1986). The principle of PCR is based on the properties of thermostable Taq polymerase, which may amplify DNA by adding nucleotides (dNTPs) according to specific forward and reverse primer sequences which flank the target DNA sequence. In RT-PCR, second strand synthesis takes place using first strand cDNA, generated from mRNA, as a template. These sequences should be designed to amplify

the specific region of genomic DNA of interest. The primer sequences are complementary to that of the template cDNA.

PCR involves a repetitive series of cycles which, because the strand synthesised in one cycle serves as the template for the next, can result in a million-fold increase in the DNA amount from a series of 20 cycles. PCR involves initially a template denaturation step, typically by heating to 94°C for 2 minutes, prior to the initiation of the PCR cycle (Mullis *et al* 1986). This is then followed by second cDNA strand synthesis and amplification. This process consists of a cycle of denaturation at 94°C, a template/primer annealing step (range - 42° - 60°C) and an extension step (68°C). The annealing temperature (T_m) of a primer is determined by its nucleotide content. For primers with a high T_m it may be advantageous to increase the suggested annealing temperatures. Higher temperatures minimise non-specific primer-template, thereby increasing the amount of specific product produced. The PCR cycle is repeated, up to a maximum of 40-50 cycles, which results in amplification of the two-stranded cDNA product. The increase in cDNA product is initially linear, reaching a plateau. A minimum of 1 minute is usually required for every 1kb of amplimer. A final period of extension at 68°C improves the quality of the final product by extending the truncated product to full length (Mullis *et al* 1986).

Accurate determination of absolute abundance of a specific transcript by RT-PCR necessitates the use of competitive RT-PCR (Chelly and Kahn 1994). This technique makes use of exogenous RNA transcript added during the RT reaction. This technique requires equal efficiency of the PCR conditions for both the competitor and target RNA. It is a more rigorous technique and is costly in terms of both time and consumables. Semi-quantitative PCR may be undertaken whereby the RT-PCR cDNA of the target mRNA product resolved on an agarose gel may be expressed as a ratio of an RT-PCR product of an internal control, such as β-Actin or glyceraldehyde – 3 – phosphate dehydrogenase.

2.5.2 Reverse transcription experimental protocols

The RT reaction was performed using total cellular RNA according to a standard protocol (Promega 2001a). A 20 µl reaction mixture was prepared, consisting of 4 µl of $MgCl_2$ (25 mmol/L), 2 µl of Reverse Transcription 10X Buffer, 2 µl of dNTP mixture (10 mmol/L), 0.5 µl of Recombinant RNasin® Ribonuclease Inhibitor, 15U of AMV

Reverse Transcriptase, 0.5μg of Oligo $(dT)_{15}$ primer, ≤ 1μg of total RNA. The reaction was made up to a final volume of 20 μl by the addition of nuclease-free dH_2O. First strand cDNA synthesis was typically performed using 100ng of total RNA. The reaction was set up with parallel negative controls, where the AMV RT was replaced with an equal volume of nuclease-free dH_2O. Samples were incubated at 42°C for 30 minutes, then heated at 99°C for 5 minutes and finally kept at 4°C.

The final concentration of the reaction components was therefore: 5mM $MgCl_2$, 1X Reverse Transcription Buffer (10 mM Tris-HCL [pH 9.0 at 25°C], 50 mM KCl, 0.1% Triton® X-100, 1 mM dNTP, 1 μ/μl Recombinant Rnasin® Ribonuclease Inhibitor, 15μ/μg AMV Reverse Transcriptase (High Concentration), 0.5μg $Oligo(dT)_{15}$ Primer per microgram of RNA. Pipetting steps were minimised by making up a master mix of these reagents using sterilised RNase-free and DNAse-free pipette tips (Gilson, U.K.).

2.5.3 PCR experimental protocols

PCR was performed, using cDNA products generated by the RT reaction, according to standard protocols (Promega 2001b). The first strand cDNA obtained from the RT reaction was diluted to 100μL with nuclease-free dH_2O. A 50μL PCR amplification reaction mix was prepared. This was made up with 10μL of first strand cDNA, 2μL of $MgCl_2$ (25 mmol/L), 4 μL of Reverse Transcription 10X Buffer, 50 pmol upstream primer, 50 pmol of downstream primer, 2.5 units of *Taq* DNA polymerase and made up to a final volume of 50 μL by the addition of nuclease-free dH_2O. Individual PCR preparations were aliquoted into DEPC-treated, autoclaved PCR tubes and covered with molecular biology-grade mineral oil (Sigma, U.K.) prior to thermal cycling. This took place in a Hybaid Omnigene thermal cycler according to programmes specific for each primer sequence.

The final concentration of the reaction components was therefore: <10ng/μL first-strand cDNA reaction, 200μM cDNA reaction dNTPs (carry-over from the first-strand RT reaction), 2mM $MgCl_2$ (with contribution from the first-strand cDNA reaction), 1X Reverse Transcription Buffer (10mM Tris-HCL [pH 9.0 at 25°C], 50mM KCl, 0.1% Triton® X-100).

Specific primer sequences were selected for vascular cell p22 phox, p67 phox, p47phox and gp 91phox. Human neutrophil and vascular cell phox DNA sequences have been

cloned and have a high degree (>90%) of homology (Bayraktutan *et al* 2000). The forward and reverse phox primer sequences were therefore selected according to published DNA sequences for human neutrophil phox proteins (Jones *et al* 1995), and synthesised by Oswel DNA service, University of Southampton.

PCR primer sequences were designed for amplification of p22 phox, p67 phox, p47phox and gp 91phox, based on the published human sequences. The primer sequences used in the present investigation were as follows:

p22 phox	forward -	5′-GTTTGTGTGCCTGCTGCAGT-3′
	reverse-	5′-TGGGCGGCTGCTTGATGGT-3′
p47 phox	forward -	5′-ACCCAGCCAGCACTATGTGT-3′
	reverse-	5′-AGTAGCCTGTGACGTCGTCT-3′
p67 phox	forward -	5′-TACTTCCAACGAGGGATGCTC-3′
	reverse -	5′-AGCTTTCCTCCTGGGGCT-3′
gp91 phox	forward -	5′-GGTGCGGTTTTGGCGATCTCA
	reverse -	5′-GGCATGTGGTCCCGGCACAG-3′

Control studies with the house-keeping genes glyceraldehyde – 3 – phosphate dehydrogenase (GAPDH) and β-Actin were undertaken by simultaneously using a PCR reaction with specific forward and reverse primer sequences for either one or the other control. In this case, the primers sequences for GAPDH and β-Actin were:

GAPDH	forward -	5′-ACCACAGTCCCATGCCATC-3′
	reverse -	5′-TCCACCACCCTGTTGCTG-3′
β-Actin	forward -	5′-TCATGAAFTGTGACGTTGACATCCGT-3′
	reverse -	5′-CCTAGAAGCATTTGCGGTGCACGATG-3′

The annealing temperature (T_m) was calculated according to the following formula:

$$T_m = 81.5 + [16.6 \times (\log_{10}[Na^+]) + 0.41 \times (\%G+C) - 675/n$$

Where $[Na^+]$ is the molar salt concentration; $[K^+] = [Na^+]$ and n = number of bases in the oligonucleotide.

PCR conditions were denaturation at 94°C for 5 minutes, annealing at 55 - 58°C for 1 minute, and extension at 72°C for 2 minutes. This cycle was repeated 30 times, and was

then followed by an extension at 72°C for 10 minutes. Initial RT-PCR studies were undertaken using cycles (n=10 - 30) in order to establish the conditions for a linear range. In all cases, PCR products were electrophoresed on a 1.5% agarose gel (Life Technologies, UK). Semi-quantative analysis was undertaken using phosphoimaging analysis (Biorad), with expression of the amplicon products as a ratio of either GAPDH or actin products.

2.6 Oxidative Fluorescent Microtopography

This technique allows the *in situ* localisation of $\cdot O_2^-$ in intact blood vessels (Carter *et al* 1994; Miller *et al* 1998). Hydroethidine (HEt) is a dye which in the presence of $\cdot O_2^-$ is oxidised to ethidium bromide (Et). Fluorescence can then be assessed microscopically after exciting at 585nm wavelength. HEt is made up in dimethylsulphoxide (DMSO) as a $2x10^{-4}$ mol/L solution and diluted to $2x10^{-6}$ mol/L in PBS before use. IMA and SV segments (5μm) were placed on coverslips and the dye topically applied. The sections were then incubated at 37°C for 30 min before visualising the fluorescence under a microscope. The localisation of the oxidised Het, which reflects $\cdot O_2^-$ production, was then observed.

2.7 Immunohistochemistry

2.7.1 Background

Immunohistochemistry (IHC) is an *in vitro* technique which affords the detection of proteins within isolated tissues, by treatment with specific anti-bodies (Harlow and Lane 1988). Monoclonal antibodies are generated from unique hybridoma cell lines, which are immortal somatic cells, typically generated by the fusion of an antibody secreting cell and a myeloma cell (Kohler and Milstein 1975). Monoclonal antibodies are characterised by specific binding properties, homogeneity and their ability to be synthesised in unlimited amounts. IHC is, therefore, a powerful technique for the identification of a specific epitope within different tissues and cell types. Monoclonal antobodies may be expensive, difficult to use and have a low antigen affinity. As such certain monoclonal antibodies may not be sensitive for epitope detection in certain tissue preparations. In such instances, polyclonal antibodies may be a more suitable alternative (Harlow and Lane 1988). Antibody-antigen binding should take place in a solid phase matrix, unless the primary antibody is biotinylated, in which case it may

bind to the antigen in solution. In this case, the primary antibody can be detected by labelled reagents, such as avidin or streptavidin, which are biotin-binding proteins. Immunodetection studies may be direct, in which a primary antibody is labelled, or indirect, in which case the primary antibody must be bound by a secondary, labelled, anti-immunoglobulin. The direct technique may be less sensitive, but easier to use, whereas the indirect technique utilises primary antibodies which have not been modified (and therefore have optimal activities) and secondary, labelled antibodies with a wide range of applications. In terms of chromogenic labels, enzymes such as horse-radish peroxidase (HRP), have a high degree of sensitivity, a reasonably long shelf life and direct visualisation is possible, although may not yield sufficient resolution for cytochemical analyses. In this case, HRP-labelled antibodies can be revealed by treatment with substrates such as diaminobenzidine (DAB), which readily reacts with HRP, yielding a brown reaction product. Biotin labels have high sensitivity, a long shelf-life and have universal application in different tissues and cells (Kohler and Milstein 1975). Avidin and streptavidin have tetravalent binding sites which means the additional binding sites can be used to increase the strength of the detection complex.

2.7.2 Immunodetection methods

Immunodetection studies were performed in frozen and paraffin-embedded 5 μm sections of intact IMA. P22phox and gp91phox were identified using specific mouse monoclonal antibodies (kindly provided by Dr MT Quinn, Montana State University, MT) whereas, p47phox and p67phox proteins were identified by use of rabbit anti-sera (Upstate Biotechnology, N.Y.). Slides were placed in acetone for 15 minutes then washed in phosphate buffered saline (PBS; Sigma, UK). Sections were demarcated on the glass slide by a ring made with a wax pen. Endogenous peroxidase activity in the sections was quenched by incubation with 3% hydrogen peroxide:methanol (1:1) for 30 minutes. Non-specific antibody binding was blocked by incubation of the tissue sections with antigen unmasking solution. This blocking buffer was made with 10% serum of the species in which the secondary antibody was raised. Secondary antibodies (biotinylated anti-mouse and biotinylated anti-rabbit) were applied for 30 minutes. The sections were then treated with streptavidin-horseradish peroxidase (Sigma), diluted to 1:400 in PBS and treated with 3,3′-diaminobenzidine (Sigma, UK) in 0.01% hydrogen peroxide in PBS (pH 7.6) for 1 hour in the dark. Finally, slides were treated with haematoxylin and eosin and fixed with dibutylpthalate xylene (DPX) (BDH Laboratory Supplies, UK). Experiments were performed between 3 and 5 times with each vessel providing its own,

untreated control.

In addition, immunohistochemical studies were undertaken in order to determine the presence or absence of macrophages and granulocytes within sections of IMA. Immunocytochemical detection of CD68, (1:100) Mac 387 (1:500) and Kpl (1:100) was performed on formalin fixed paraffin embedded sections of IMA along with light microscopic evaluation of haematoxylin and eosin stained sections (Townsend *et al* 1999).

2.8 Isometric tension studies in small resistance arteries

Resistance arteries are those blood vessels which contribute the greatest resistance to blood flow, and are therefore most involved in regulating blood flow and capillary pressure (Mulvany and Aalkjaer 1990). SRA wire myography is an *in vitro* technique which allows resistance arteries with a diameter of 100 – 500μm to be studied under precise and standardised conditions. Use of this technique yields information on the contractile or relaxant properties, and morphology, of SRA under isometric tension (Mulvany and Aalkjaer 1990; Mulvany and Halpern 1977). In the present investigation, functional studies were undertaken with SRA because of the physiological importance of these blood vessels. Wire myography was employed for these studies because this is an established technique in our laboratory (Hillier *et al* 1999; Padmanabhan *et al* 1999), in which several arteries may be studied at any one time, using one or more protocols.

Human SRA were isolated from within the subcutaneous fat obtained at the time of inguinal hernia repair. A single biopsy may yield several blood vessels (average 2 – 4). Isolated SRA were divided into segments approximately 2mm long. When possible, four resistance arteries, were carefully mounted on two 40 μm-diameter stainless steel wires and mounted in the bath of a 4-channel myograph (Halpern & Mulvany, Aarhus), in which the wires are attached to a force transducer and micrometer, respectively. The bath contained Krebs buffer which had been gassed and pre-heated at 37°C, and these conditions were maintained for the duration of the experiment. In addition the Krebs buffer was regularly changed throughout the experiment.

2.8.1 Set-up and normalisation procedures

After a rest period of 30 minutes, a normalisation procedure was followed for each artery to determine the normalised internal diameter (ID), L0, at which contraction is

thought to be optimal, and the vessel was set to that diameter (Mulvany and Halpern 1977). In the present study, arteries with a normalised ID of 200 – 400μm were used. After a rest period of 30 minute each artery was stretched at 1 minute intervals to determine the passive exponential, wall tension-internal circumference (L) relationship. From the Laplace equation, where P=T/r (P is the effective pressure, T is the wall tension and r is the internal radius), the equivalent circumference (L_{100}) for a transmural pressure of 100mmHg, was calculated for each artery by an iterative computer method. Each artery was then set to the normalised internal diameter, $L_1=0.9 \times L_{100}/\pi$, at which contraction is thought to be optimal (Mulvany and Aalkjaer 1990; Mulvany and Halpern 1977).

Following normalisation, the vessels were left for a further hour. They were then exposed to a high (123 mM) concentration of potassium (KPSS, solution identical to PSS except that sodium was replaced by potassium on an equimolar basis) for a series of 5 minute periods until repeatable maximal contractions were achieved, and then once to 10 μmol/L of norepinephrine (NE). After a plateau contraction had been attained with NE, 3 μmol/L of acetylcholine (ACh) was added to stimulate endothelium-dependent vasodilatation. Arteries that were unable to contract to either KPSS or NE or showed no relaxation to ACh (and were therefore considered to have no functionally intact endothelium) were discarded. The arteries were then incubated for a further 30 minutes in Krebs solution prior to the commencement of the concentration-response curves (CRC) incorporated in the study protocol.

2.9 Measurement of free radical concentrations in human blood vessels

Of the variety of free radicals which are generated within vascular tissue, the $^{\cdot}O_2^-$ and $^{\cdot}OH$ anion radicals are reported to be present in physiologically important concentrations (Wolin 2000). Superoxide, in particular, is reported to be an important determinant of bioavailable $^{\cdot}NO$.

This chapter describes studies undertaken to characterise the nature of lucigenin-enhanced chemiluminescence. Lucigenin-enhanced chemiluminescence has recently been criticised due to the potential for redox cycling by lucigenin. In this case, *in vitro* studies have suggested that in certain conditions, lucigenin may act as a reducing agent, by serving as a potential source of electrons for the reduction of molecular oxygen to

O_2^- (Liochev and Fridovich 1997; Liochev and Fridovich 1998). At the inception of this study, the author was not aware of any data on the direct measurement of O_2^- concentrations in human blood vessels using lucigenin-enhanced chemiluminescence. For these reasons, a series of systematic validation studies were undertaken in order to characterise lucigenin enhanced chemiluminescence as a measure of O_2^- production in intact human blood vessels.

2.9.1 Chemiluminescence

Lucigenin-enhanced chemiluminescence is a widely-used technique for the measurement of O_2^- concentrations *in vitro*. Previous studies used lucigenin-enhanced chemiluminescence to measure O_2^- concentrations *in vitro* using either cultured cells or in vascular tissues obtained from experimental animals.

The amount of lucigenin-enhanced chemiluminescence in vascular tissue is a function of both the rate of O_2^- production by endogenous enzymes, and the rate of O_2^- removal, by for example, dismuation by SOD. Brandes *et al* (1997) attempted to characterise O_2^- production in intact pig coronary artery segments, using lucigenin-enhanced chemiluminescence. In these studies, artery segments were approximately 3 mm long, were placed in 600 μL of aerated Krebs buffer at pH 7.4 and the light reaction between O_2^- and lucigenin (250 μM) was detected in a scintillation counter during a 5 minute period. The counts for this period were corrected by background subtraction. Counts per minute were plotted against a xanthine- xanthine oxidase calibration curve and expressed as production of O_2^- (pmol) per minute per milligram of dry-blotted tissue.

The experimental conditions in this system were characterised and validated (Brandes *et al* 1997). A cell-free xanthine-XOR assay for O_2^- generation was investigated and found to generate O_2^- with linear increments in association with increasing concentrations of xanthine, provided the concentration of XOR was low (< 1mU/ml). This signal could be completely inhibited by co-treatment with the XOR inhibitor, oxypurinol, or the specific O_2^- scavenger (4-5 dihydroxy-1, 3-benzene disulphonic acid salt [Tiron], 10 mmol/L). In addition the protocols also included measurement of the effect of pH, and stimulation or inhibition of vascular O_2^- production. Acid-base status of the phosphate buffer was adjusted by the addition of either NaOH or HCl to various pH values in a range from 7.0 to 8.0. An increase in pH was associated with an increase in chemiluminescence signal with a change of 200% across this range. In studies in intact pig coronary arteries, these investigators also observed that treatment with

substrates for enzymes believed to be involved in $\cdot O_2^-$ production, namely NAD(P)H oxidase and XOR, were associated with increases in the chemiluminescence signal, these being inhibited by co-treatment with Tiron. There are few data on the direct measurement of ROS concentrations in human blood vessels. Given the findings of Brandes *et al* (1997), the experimental conditions in relation to the measurement of $\cdot O_2^-$ concentrations in human blood vessels using lucigenin-enhanced chemiluminescence were also characterised and validated.

2.9.2 Characterisation of lucigenin-enhanced chemiluminescence

An initial subset of twenty conduit blood vessels underwent analysis by an experienced vascular pathologist, who confirmed the presence of an intact endothelium and the absence of atherosclerotic disease.

In order to investigate the effects of temperature, pH, lucigenin concentration and incubation time on $\cdot O_2^-$ concentrations in vascular tissues, a number of exploratory studies were also performed. Given the limited availability of human vascular tissue, fresh aortic segments obtained from Sprague Dawley rat were studied. As a first step, the effects of environmental factors, such as pH and temperature, on $\cdot O_2^-$ concentrations, as measured by lucigenin-enhanced chemiluminescence, were determined in both a cell/tissue-free system, and in other studies using rat aortic segments. Lucigenin (Sigma, UK) was added to Krebs buffer, which had been maintained at either room temperature (18°C) or body temperature (37°C), in liquid scintillation vials (Packard, U.K.) in order to make a final lucigenin concentration of either 5 μM or 250 μM. The vials were then maintained at either room temperature or were placed in an incubator at 37°C prior to being placed in a scintillation counter.

2.9.3 Effects of pH, temperature and lucigenin concentration on superoxide concentrations in a cell-free system

I first sought to determine the effect of pH, temperature and lucigenin concentration on the chemiluminescence signal measured in the absence of cells or tissues. This 'background signal' is represented by ROS generation within the aqueous buffer. The data in Table 1. represent results of chemiluminescence studies undertaken using Krebs buffer alone. Liquid scintillation vials were filled with 2 ml of Krebs buffer and placed in an incubator and maintained at 37°C for either 1 hour or 4 hours prior to the addition

of lucigenin and quantification of counts in a liquid scintillation counter (Hewlet Packard Tricarb 2100TR).

In these studies (Table 2.1), buffer pH was comparable between vials maintained at either 18°C or 37°C. By contrast, there was a tendency for the pH of the buffer to rise with increasing concentrations of lucigenin. At 37°C, counts tended to fall, be it in the absence or presence of either 5 μmol/L or 250 μmol/L lucigenin, with duration of incubation. Furthermore, when studies were performed after incubation of the vials for 1 hour, counts in vials allowed to stand on the bench till the buffer temperature fell 18°C tended to be lower than those measured in vials maintained at 37°C. These data suggest that chemiluminescence counts may be affected by duration of incubation and buffer temperature.

2.9.4 Effect of pH, temperature and lucigenin concentration on superoxide concentrations in vascular tissues

In order to determine whether or not pH, temperature and lucigenin concentration affected chemiluminescence counts from vascular tissues, experiments were undertaken using rat aortae. In this case, rat tissues were used given the limited availability of human blood vessels. Fresh aortae, which had been harvested from sacrificed, Sprague Dawley rats, were divided into segments approximately 5 mm length, weighed and placed into scintillation vials containing 2 ml of Krebs buffer. The vials were then incubated at 37°C for either 1 hour or 4 hours which was then followed by the addition of lucigenin and quantification of counts in the scintillation counter. Data are presented as either the results of single measurements or in summary form (mean +/- standard error of the mean).

The results of these studies are presented in Table 2.2. In every case, the pH was higher in vials maintained at 18°C compared to those at 37°C, which was in contrast to earlier studies undertaken with buffer alone. As before, buffer pH tended to rise with duration of incubation. Although absolute counts increased with duration of incubation, no differences were observed when these values were corrected for tissue weight.

Table 2.1 Effect of lucigenin concentration, pH of the incubation buffer, and temperature on chemiluminescence counts in the absence of vascular tissue.

Time (hours)	Lucigenin concentrations (μmol/L)	pH at 37°C	pH at 18°C	CL count at 37°C	CL count at 18°C
0	0	7.55	7.65	83312	
	5	7.68	7.68	110018	
	250	7.72	7.72	108612	
1	0	7.56	7.57	81304	78325
	5	7.45	7.45	90056	84172
	250	7.76	7.76	80240	76596
4	0	7.55	7.55	52248	
	5	7.82	7.82	51348	
	250	7.55	7.55	51892	56700

CL - chemiluminescence

2.9.5 Selective augmentation and depletion of superoxide concentrations in human blood vessels

It has been previously reported that lucigenin-enhanced chemiluminescence is both a sensitive and specific measure of $\cdot O_2^-$ production from neutrophils (Gyllenhammar 1987). I sought to determine, therefore, whether this might also be the case in human blood vessels.

Simple pharmacological studies were undertaken in order to determine whether

selective augmentation and depletion of vascular $^{\cdot}O_2^-$ concentrations in human blood vessels could be predictably measured using lucigenin-enhanced chemiluminescence techniques. Lucigenin itself has been reported to generate $^{\cdot}O_2^-$ at higher concentrations (Liochev and Fridovich 1997; Liochev and Fridovich 1998). Therefore, a range of lucigenin concentrations was also used to assay $^{\cdot}O_2^-$ in a series of samples from single vessels using xanthine/xanthine oxidase (Sigma, UK) calibration curves, with the appropriate concentration of lucigenin in each sample.

Fresh IMA and SV were divided into 5 mm segments and weighed. Initially, studies were undertaken to determine whether or not lucigenin-enhanced chemiluminescence might increase when $^{\cdot}O_2^-$ concentrations are enhanced. Paired segments from the same blood vessel were placed in scintillation vials in either the absence (2 ml of Krebs buffer) or presence 100 μmol/L of the SOD inhibitor, DETCA (Sigma, UK). Superoxide concentrations were then quantified by lucigenin-enhanced chemiluminescence. In both IMA and SV, counts were increased by this treatment (Figure 2.2). Treatment with 100μmol/L of DETCA increased $^{\cdot}O_2^-$ steady state concentrations in IMA (control 853 ± 208, DETCA 100 μmol/L 1492 ± 347; n=8, p=0.021, 95% CI 186, 1149 and SV (control 551 ± 145, DETCA 100 μmol/L 945 ± 245; n=14, p=0.002, 95% CI 67, 694).

These data demonstrate that pharmacological inhibition of SOD in IMA and SV results in an increase in lucigenin-enhanced chemiluminescence, in keeping with the rise in vascular $^{\cdot}O_2^-$ concentrations that will have occurred with this treatment. Furthermore, these data also demonstrate that $^{\cdot}O_2^-$ production is greater in conduit arteries, than in veins. One reason for this may be because these arteries have a proportionately greater content of VSMC, than do veins.

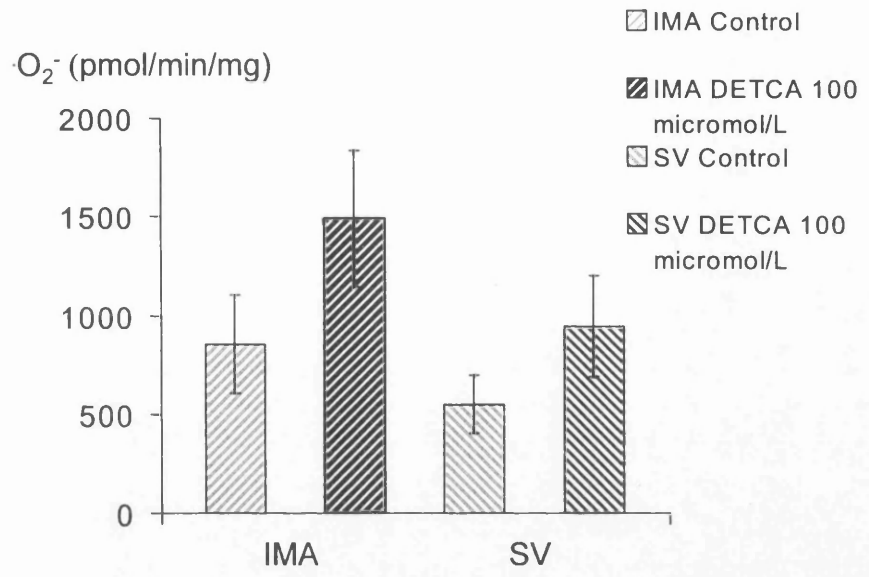
Table 2.2 Effect of buffer pH, temperature and incubation time on lucigenin-enhanced chemiluminescence in fresh rat aortic segments.

Time (hours)	Concentration of lucigenin	Weight	pH at 37°C	pH at 18°C	CL count	CL count – background*	CL count / milligram of tissue	CL count/ minute/ milligram of tissue
1	5	0.00787	7.39	7.56	64452	5920	7533	753
	250	0.01100	7.40	7.69	73844	68682	5390	539
4	5	0.0104	7.32	7.71	80844	75682	7277	727
	250	0.0828	7.31	7.84	82992	77830	9399	940

CL – chemiluminescence; * Average background CL count = 51620

There is considerable inter– and intra–individual variation in $\cdot O_2^-$ production by both arteries, and veins. This is reflected by the considerable variability in $\cdot O_2^-$ concentrations that was observed in these vascular tissues. This intra- and inter-subject variation in vascular $\cdot O_2^-$ concentrations may arise for a number of reasons. These tissues are *ex vivo*. In addition, there are several 'handling' steps, including surgical manipulation, variation in time to harvest, transfer to the laboratory, and subsequent handling, including, for example, debridement of surrounding connective tissue and variation in time to measurement. For these reasons, every effort was made to standardise the handling steps for these blood vessels in the laboratory.

In other studies, undertaken to determine whether or not lucigenin-enhanced chemiluminescence might detect a reduction in vascular $\cdot O_2^-$ concentrations, paired blood vessels were incubated in the absence (2 ml of Krebs buffer) or presence of the free radical scavenger (4-5 dihydroxy-1, 3-benzene disulphonic acid salt [Tiron], 10 mmol/L; Sigma, UK) and incubated at 37°C for 1 hour. In this case, the counts were reduced by this treatment (Figure 2.3). Tiron 10 mmol/L, reduced basal $\cdot O_2^-$ concentrations in both IMA (control 1937 ± 412, Tiron 10mmol/L 901 ± 94; n=9, p=0.018, 95% CI –1919, -238) and SV (350 ± 85, Tiron 10mmol/L 149 ± 39; n=13, p=0.002, 95% CI –311, -95). In this case, treatment of these blood vessels with a scavenger of $\cdot O_2^-$, was associated with a reduction in $\cdot O_2^-$ concentrations in both IMA and SV.



DETCA - diethylenethiocarbamate, an inhibitor of superoxide dismutase;
IMA - internal mammary artery; SV - saphenous vein;

Figure 2.2 The effect of inhibition of SOD in IMA (n=8) and SV (n=14) by treatment with 100 μmol/L of DETCA. Each column represents mean O_2^- generation in pmol/min/mg tissue. Error bars represent standard error of the mean (SEM).

2.9.6 Effect of different concentrations of lucigenin on superoxide concentrations in human blood vessels

In other studies, O_2^- concentrations were compared in samples obtained from a single blood vessel. Superoxide production in IMA was similar with either a lucigenin concentration of 5 μmol/L (1715 ± 343 pmol/min/mg) or 250 μmol/L (1410 ± 93 pmol/min/mg; n=6, p=0.59). In SV, sufficient tissue was available to assess the effect of lucigenin on O_2^- production at a range of lucigenin concentrations (5, 15, 50, and 250 μmol/L). In this case, in each experiment all SV segments were obtained from a single length of blood vessel such that variation in O_2^- concentrations between samples was minimised given. Superoxide production* was 311 ± 67 pmol/min/mg with 5μmol/L lucigenin, 208 ± 27 pmol/min/mg with 15 μmol/L lucigenin, 300 ± 33 pmol/min/mg with 50 μmol/L lucigenin and 241 ± 50 pmol/min/mg with 250 μmol/L (n=10 for each

lucigenin concentration; NS between all groups).

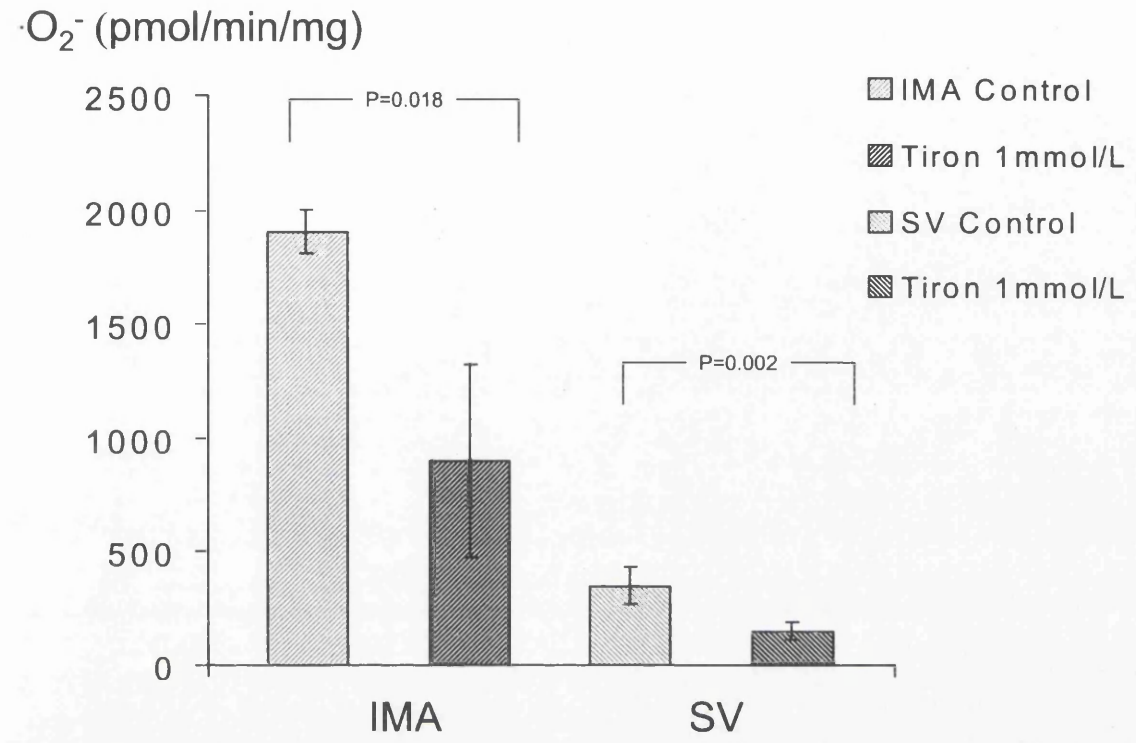


Figure 2.3 The effect of superoxide scavenging by treatment of IMA (n=9) and SV (n=13) with 1 mmol/L of Tiron. Each column represents mean $\cdot O_2^-$ generation in pmol/min/mg tissue. Error bars represent standard error of the mean (SEM).

2.9.7 Summary of validation studies of lucigenin-enhanced chemiluminescence in human blood vessels.

The data obtained from the studies of lucigenin-enhanced chemiluminescence in a cell and tissue free system, suggested that the lucigenin-enhanced chemiluminescence counts fell with duration of incubation. Higher concentrations of lucigenin tended to be associated with higher values of pH, although this was not consistently the case with 250 μM of lucigenin after 4 hours incubation. Interestingly, in these studies, the counts were not greater with higher concentrations of lucigenin. This latter observation contrasts with other reports which observed that counts increased with increasing concentration of lucigenin, which could be attributed to redox cycling by lucigenin itself (Liochev and

Fridovich 1997; Liochev and Fridovich 1998).

In the present study, the data obtained from using different concentrations of lucigenin in human IMA and SV demonstrate that $\cdot O_2^-$ production in these blood vessels was not affected by the concentration of lucigenin under these conditions. Lucigenin-enhanced chemiluminescence has been previously used in our laboratory in order to measure vascular $\cdot O_2^-$ concentrations in other experimental animal studies (Grunfeld *et al* 1995; Kerr *et al* 1999). The data obtained from measurements using this technique have generated reproducible results which have been utilised in a variety of pharmacological studies of vascular ROS in the SHRSP and WKY rats.

In other studies, pharmacological manipulation of vascular $\cdot O_2^-$ concentrations resulted in predictable increases and reductions in chemiluminescence counts. These conclusions are based on the fact that treatment with DETCA causes specific inhibiton of SOD, and therefore a selective increase in $\cdot O_2^-$ concentrations, whereas treatment with Tiron results in selective scavenging of $\cdot O_2^-$. DETCA is a copper-chelating compound which augments vascular $\cdot O_2^-$ concentrations through inhibiton of both intra – and extracellular Cu/Zn SOD. *In vitro* studies in rabbit aortae by Mackenzie and Martin (1998) confirmed that low concentrations (300 μmol/L) of DETCA selectively inhibit extracellular Cu/Zn SOD, as impaired ACh-induced, NO-dependent vasorelaxation could be restored by treatment with SOD (250 U/ml). At higher concentrations of DETCA (e.g. 3 mmol/L), the predominant effect of this treatment was inhibition of intracellular Cu/Zn SOD, as impaired vasorelaxation could only be reversed by treatment with a membrane permeant SOD mimetic (MacKenzie and Martin 1998). Only a very minor component of the pro-oxidant effect of DETCA is due to non-selective effects, such as depletion of glutathione and generation of lipid peroxides (Kelner *et al* 1989). On the other hand, Tiron is a selective, membrane permeant scavenger of $\cdot O_2^-$ (Ledenev *et al* 1986). Lucigenin-enhanced chemiluminescence, has therefore, resulted in observations in keeping with the expected pharmacological effects of these treatments. Taken together, these data indicate that lucigenin-enhanced chemiluminescence is both a sensitive and specific tool for the detection and measurement of $\cdot O_2^-$ concentrations in human IMA and SV.

3 Sources of superoxide production in human blood vessels

3.1 Summary

The enzymatic sources, and distribution, of ROS generation, in human blood vessels are not known. In this chapter, experiments undertaken to characterise the enzymes involved in $\cdot O_2^-$ production, and their location in human conduit blood vessels, will be described. In addition, studies were undertaken to characterise endogenous ROS scavenging systems in these blood vessels.

3.2 Methods

IMA, RA and SV samples were prepared for measurement of $\cdot O_2^-$ production by lucigenin-enhanced chemiluminescence according to methods described in section 2.1, 2.2.1 and 2.5.3. Firstly, $\cdot O_2^-$ concentrations were measured in a consecutive series of IMA, RA and SV. Secondly, a series of basic pharmacological studies were undertaken using IMA and SV, in which inhibitors of NAD(P)H oxidase, XOR and eNOS were used to determine whether or not these enzymes contributed to basal $\cdot O_2^-$ production. Thirdly, oxidative microtopography studies were undertaken in IMA and SV in order to characterise the location and distribution of $\cdot O_2^-$ production within the wall of these blood vessels. Finally, an additional aim was to quantify the amount of SOD protein present in arteries and veins using western blotting techniques in order to assess whether SOD expression might account for any differences in $\cdot O_2^-$ measured.

3.2.1 Pharmacological studies

In order to investigate the enzymatic sources of O_2^- generation in human blood vessels, IMA and SV were incubated for 1 hour at 37°C in the absence (control) or presence of an inhibitor of NAD(P)H oxidase, diphenyleneiodonium ([DPI; Sigma, UK], 10 μmol/L, 100 μmol/L and 200 μmol/L), an inhibitor of eNOS (N^{ω}-Nitro-L-arginine methyl ester,

[L-NAME; Sigma, UK] 100 μmol/L), or an inhibitor of XOR (allopurinol [ALP; Sigma, UK] 1mmol/L). DPI and ALP were dissolved in dimethylsuphoxide (DMSO), whereas all other drugs were dissolved in Krebs buffer. For studies with DPI and ALP, the appropriate concentration of DMSO was added to control samples. In addition, some vessels were denuded of endothelium by careful rubbing of the vessel lumen with fine forceps. The absence of the endothelium and integrity of the vessel wall was confirmed by histological analysis in a subset of 20 vessels.

3.2.2 Protein quantification studies

Five millimeter segments of vessels were homogenised in 200 μl of a boiling vanadate buffer (1% SDS, 1 mmol Na_3VO_4, 10 mM Tris, pH 7.4). Following centrifugation at 14,000 g for 60 seconds the supernatant was withdrawn and the protein concentration measured. Ten micrograms of protein and pre-stained molecular weight standards were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis using a 12% gel (Life Technologies, UK). The proteins were transferred overnight onto a polyvinylidene difluride (PVDF) membrane and sequentially probed with sheep monoclonal anti-CuZn SOD, or rabbit polyclonal anti -MnSOD or anti-actin antibodies. Protein bands were visualised using enhanced chemiluminescence (GS-525 Biorad-Laboratories Ltd). Anti-human MnSOD was a gift from Professor Taniguchi (University of Osaka, Japan) and anti- human CuZn SOD was purchased from Calbiochem (UK).

3.2.3 Oxidative microtopography

IMA and SV segments (5μm) were placed on coverslips and the hydroethidine dye topically applied. The sections are then incubated at 37°C for 30 mins before visualising the fluorescence under a microscope. The localisation of the oxidised Het, which reflects $\cdot O_2^-$ production, was then observed.

3.2.4 Statistical analyses

Data are described as mean +/- S.E.M or as % change from controls to facilitate comparison between groups. Statistical analyses of vascular $\cdot O_2^-$ concentrations were undertaken using the Wilcoxon Signed-Rank Test. The relationships between risk factors and basal vascular $\cdot O_2^-$ concentrations were determined by use of the Pearson's correlation coefficient (r). Results of statistical analyses are presented with both a probability (P) value and 95% confidence intervals (95% CI). A P value of < 0.05 was considered statistically significant.

3.3 Results

3.3.1 Patient characteristics

The study population consisted of two hundred and forty four patients with CAD who consecutively underwent CABG in our hospital over a 14 month period. Data on age, sex, risk factors for atherosclerotic vascular disease, and drug therapy are given in Table 3.1. Patient age ranged from 33 to 80 years. Seventy five percent of patients had one or more risk factor for CHD and 92% of patients were on one or more type of anti-anginal therapy.

Table 3.1 Patient characteristics, including risk factors and therapy.

Males, n (%)	179 (73)
Females, n (%)	66 (27)
Mean age, y	62±8
Risk factors, n (%)	
Smoking	43 (18)
Hypertension	82 (34)
Diabetes mellitus	26 (11)
Hypercholesterolemia	180 (74)
Plasma cholesterol, mmol/L (mean±SD)	5.4 ± 1.4
Medication, n (%)	
Aspirin	203 (83)
β-Blockers	136 (56)
Calcium channel blockers	145 (60)
HMGCoA Reductase Inhibitors	127 (52)
Nitroglycerin	148 (61)
Renin angiotensin system inhibitors	54 (22)

3.3.2 Measurement of superoxide anion concentrations in IMA, RA and SV

Mean $^{\cdot}O_2^-$ production was greater in arteries than in veins: IMA 1922±235 pmol/min/mg (n=55), SV 662±179 pmol/min/mg (n=58); $p<0.0001$ (Figure 3.1).

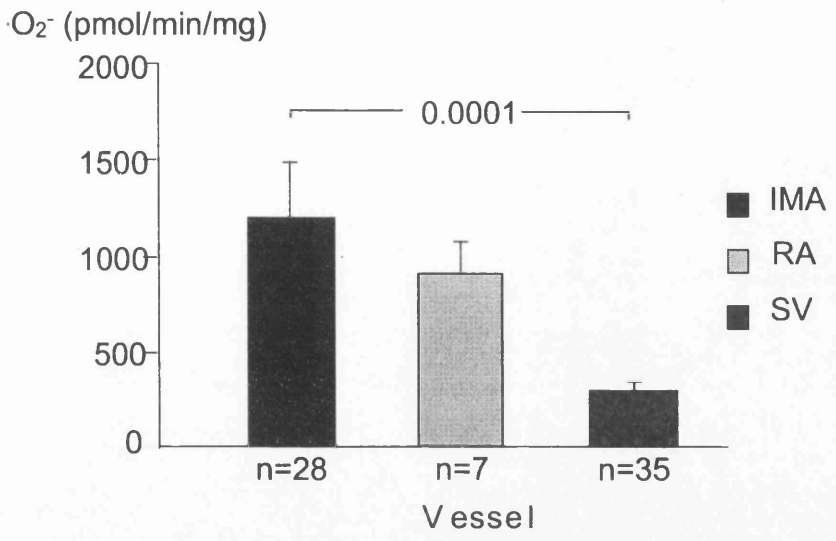


Figure 3.1 Mean $\cdot O_2^-$ production in internal mammary arteries (IMA), radial arteries (RA) and saphenous veins (SV). Error bars represent standard error of the mean (SEM).

3.3.3 Characterisation of the sources of superoxide anion production in IMA and SV

The NAD(P)H oxidase inhibitor, DPI (concentration range 10-200 μmol/L), attenuated $\cdot O_2^-$ generation in both arteries (Figure 3.2; Table 3.2) and veins (Table 3.3). The effects of endothelial denudation by rubbing, and inhibition of eNOS by incubation with L-NAME, on $\cdot O_2^-$ concentrations in IMA and SV were more variable (Table 3.2 & 3.3). NOS inhibition (Figure 3.4) was associated with a small reduction in basal $\cdot O_2^-$ concentrations in both IMA and SV, which approached statistical significance. Incubation of IMA with 100 μmol/L of L-NAME and endothelial denudation reduced $\cdot O_2^-$ concentrations in 7/10 and 6/10 patients respectively. In SV, these treatments

corresponded with a reduction in $\cdot O_2^-$ concentrations in 12/16 and 10/14 patients. Sufficient tissue was available in veins to study the effects of L-NAME treatment and endothelium removal by rubbing in vessel segments from the same patients. In these studies, both manipulations had similar effects on $\cdot O_2^-$ concentrations in individual patients ($r = 0.85$; $n=12$ $p<0.001$). Furthermore, the difference in $\cdot O_2^-$ concentrations between IMA and SV was maintained after both endothelial denudation and eNOS inhibition.

Table 3.2 Effect of inhibition of NAD(P)H oxidase, xanthine oxidase and nitric oxide synthase and of endothelial denudation on $\cdot O_2^-$ production in IMA.

		$\cdot O_2$- generation (pmol/min/mg)			
Treatment	n	Control	Treated	% Change	p value and 95% CI
DPI 10μM	8	965±150	616±212	-36±17	0.14; -808, 239
DPI 100μM	8	2723±696	1568±430	-39±13	0.03; –2515, -137
ALP 1mmol/L	9	2120±629	1024±290	-42±10	0.013; –2020, -330
L-NAME 100μM	10	1990±508	1420±304	-29 ±16	0.1; –1421, 67
ED	10	643±134	647±145	-3±10	0.55; –183, 153

Data are shown as mean ± SEM. Saphenous vein (SV). Diphenyleneiodonium (DPI), allopurinol (ALP) and N^{ω}-Nitro-L-arginine methyl ester, (L-NAME) are inhibitors of NAD(P)H oxidase, xanthine oxidase and endothelial nitric oxide synthase enzymes, respectively. ED – endothelial denudation

Table 3.3 Effect of inhibition of NAD(P)H oxidase, xanthine oxidase and nitric oxide synthase and of endothelial denudation on $\cdot O_2^-$ production in SV.

		$\cdot O_2$- generation (pmol/min/mg)			
Treatment	n	Control	Treated	% Change	p value and 95% CI
DPI 10μM	10	184±25	116±28	-34±16	0.037; -124, -7
DPI 100μM	14	759±140	469±94	-37±6	0.001; –519, -103
DPI 200μM	8	452±126	228±73	-47±7	0.014; -394, -59
ALP 1mM	13	759±173	426±107	-32±8	0.003; –673, -57
L-NAME 100μM	16	284±44	214±36	-20 ±13	0.06; –148, 5
ED	14	324±48	250±40	-15 ±9	0.04; –144, -8
ED + DPI 100μM	9	787±259	477±136	-29±9	0.018; -671, -3

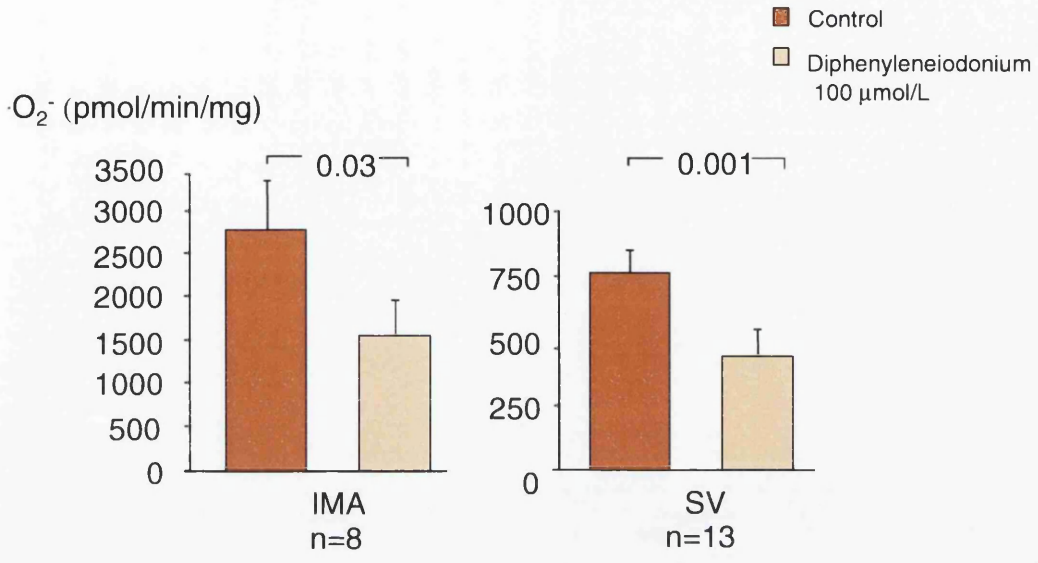


Figure 3.2 Effect of NAD(P)H oxidase inhibition on mean O_2^- concentrations in IMA and SV.

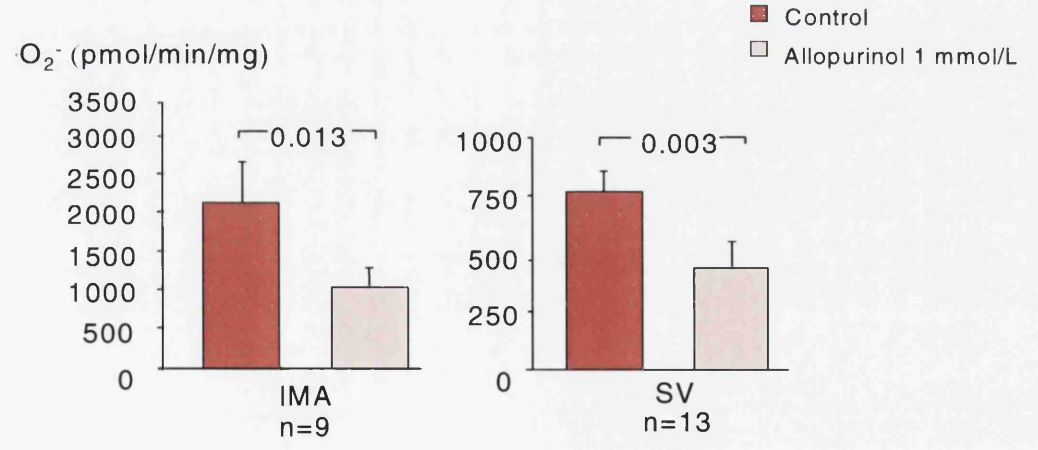


Figure 3.3 Effect of XOR inhibition on O_2^- concentrations in IMA and SV. Treatment allopurinol (ALP, 1 mmol/L), attenuated O_2^- production in both IMA and SV.

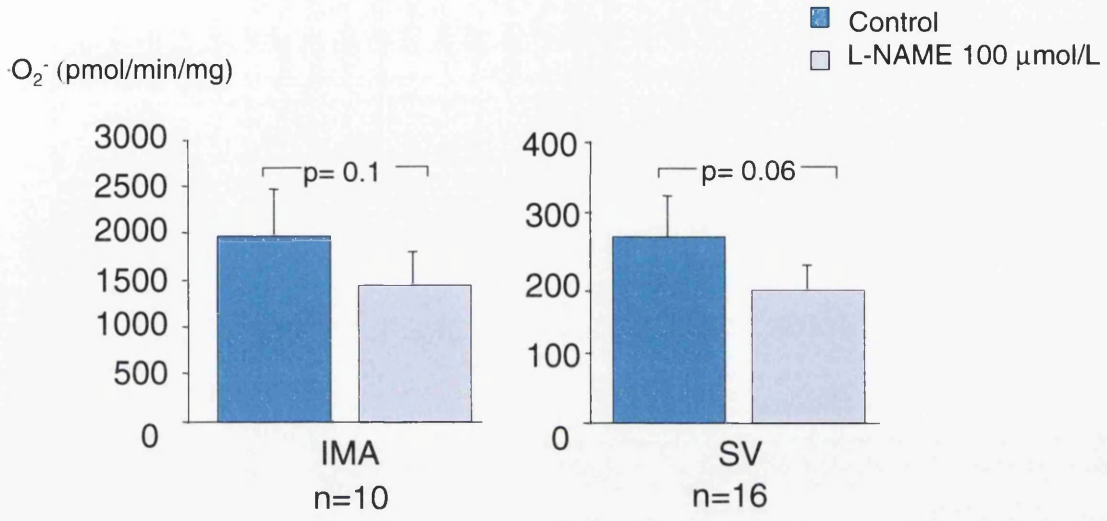


Figure 3.4 Effect of NOS inhibition on superoxide concentrations in IMA and SV.

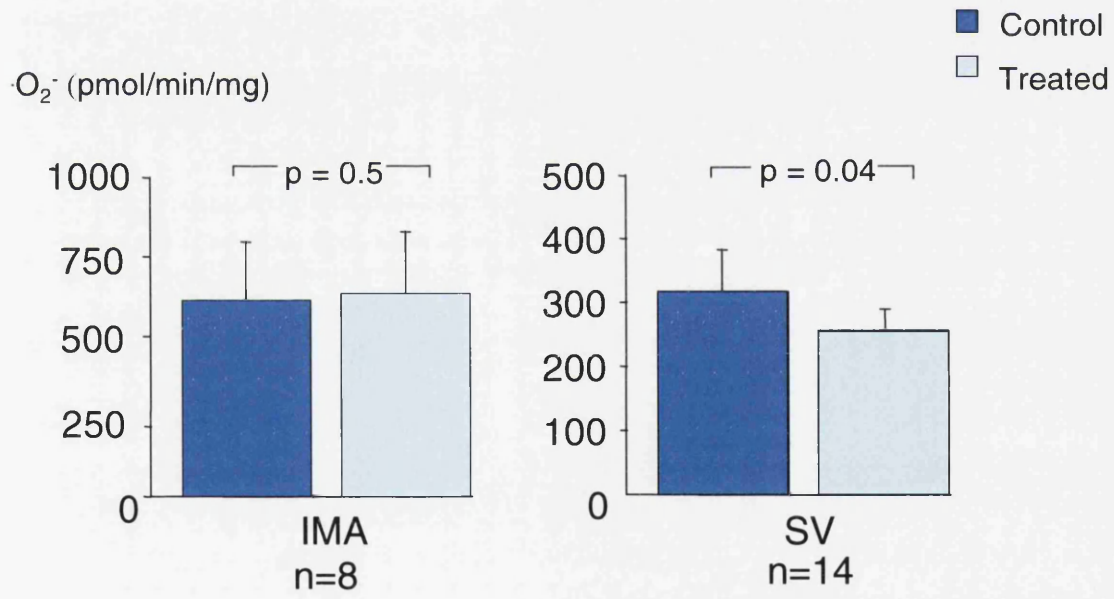


Figure 3.5 Effect of endothelial denudation on $\cdot O_2^-$ concentrations in IMA and SV. Endothelial denudation had no effect on $\cdot O_2^-$ concentrations in either IMA or SV

3.3.4 Oxidative microtopography

Superoxide production was demonstrated throughout the wall of both IMA and SV. These images suggest that ROS generation occurs throughout the vascular wall, including the adventitia and endothelial layers (Figure 3.6).

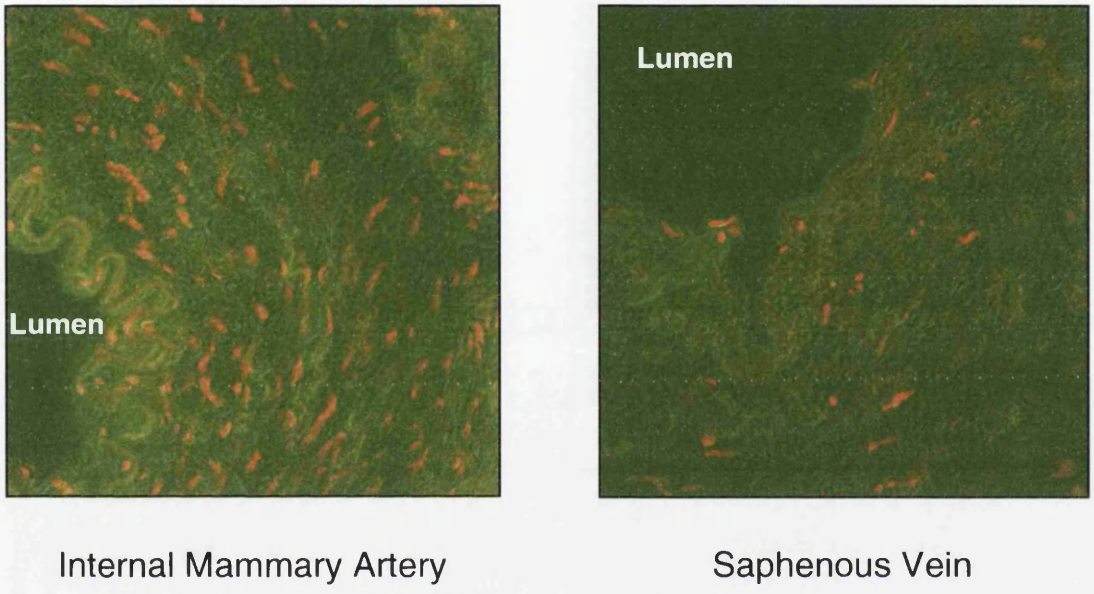


Figure 3.6 Identification of $O_2^{\cdot-}$ production in a section IMA and SV with hydroethidine fluorescence.

3.3.5 Quantification of SOD proteins

Immunoblotting showed that single bands were detected for both MnSOD and CuZn SOD (Figure 3.7). The intensity of CuZn SOD bands relative to an actin control was 4.04 ± 0.31 in arteries vs. 3.59 ± 0.23 in veins (p=0.055). Mn SOD expression was found to have relative intensities of 3.14 ± 0.25 vs. 3.87 ± 0.42 (p=0.056) in arteries and veins respectively.

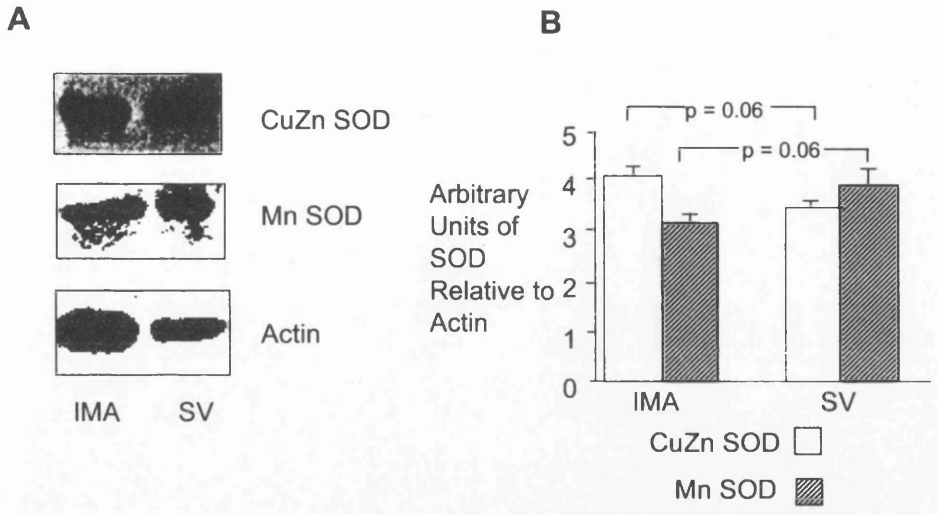


Figure 3.7 Quantification of intracellular SOD proteins in human IMA and SV. (A) Scanned images of representative western blots of CuZn SOD, MnSOD and actin proteins. (B) Densitometric quantification of CuZn SOD and MnSOD western blots expressed as a ratio of actin.

3.4 Discussion

We have shown that basal $^{\cdot}O_2^-$ concentrations are greater in human IMA than SV and have demonstrated that both NAD(P)H oxidase and XOR enzymes contribute to basal $^{\cdot}O_2^-$ production in these vessels. Furthermore, we report that SOD proteins are quantitatively similar in human IMA and SV.

We sought to characterise the mechanisms of $^{\cdot}O_2^-$ production in human IMA and SV. Endothelial NOS activity was inhibited by removal of the endothelium by rubbing and by incubating vessels with L-NAME, however, these treatments failed to reduce $^{\cdot}O_2^-$ steady state concentrations in all patients. The lack of effect of these treatments on $^{\cdot}O_2^-$ production in the blood vessels of some patients suggests that VSMC and adventitial fibroblasts were alternative sources of $^{\cdot}O_2^-$ generation. Conduit arteries have a

proportionately greater content of VSMC than conduit veins, which may explain the fact that $\cdot O_2^-$ generation is greater in arteries, compared to veins. Inhibition of NAD(P)H oxidase and XOR, by incubation of arteries and veins with DPI and ALP respectively, resulted in similar reductions in basal $\cdot O_2^-$ steady state concentrations in both of these tissues. This suggests that both NAD(P)H and XOR are sources of $\cdot O_2^-$ generation in human arteries and veins.

The balance between $\cdot O_2^-$ generation and degradation determines $\cdot O_2^-$ steady state concentrations. In this study, the levels of MnSOD and CuZn SOD proteins were qualitatively different in arteries and veins, but overall, total SOD protein was quantitatively similar. Taken together with our results this would suggest that the elevated concentrations of $\cdot O_2^-$ observed in arteries compared with veins were not a consequence of a reduced capacity for enzymatic removal, but occurred through increased $\cdot O_2^-$ production.

Observations from *in vitro* studies suggest that the activity of XOR may be increased in endothelial cells subject to ischemia-reperfusion injury (Harrison 2000; Phan *et al* 1989). Findings from *in vivo* human studies suggest that patients with risk factors for CAD also have increased vascular XOR-mediated $\cdot O_2^-$ production, which may contribute to impaired endothelium dependent vasodilation in these patients (Cardillo *et al* 1997). When considered with our findings, this suggests that XOR may be an important source of vascular $\cdot O_2^-$ production in patients who have CAD.

4 Effect of angiotensin II on superoxide anion concentrations in human blood vessels

4.1 Summary

Given that the enzymatic sources of $^{\cdot}O_2^-$ production in human blood vessels had been described, I subsequently sought to determine whether or not vascular $^{\cdot}O_2^-$ concentrations might be augmented by vasoactive hormones, such as angiotensin II (Ang II). This chapter provides a detailed description of the experiments performed using lucigenin-enhanced chemiluminescence to characterise the effect of Ang II on $^{\cdot}O_2^-$ concentrations in human IMA and SV.

4.2 Methods

4.2.1 Lucigenin-enhanced chemiluminescence

IMA and SV samples were prepared for measurement of $^{\cdot}O_2^-$ production by lucigenin-enhanced chemiluminescence according to methods described in Chapter 2 (sections 2.5.1 and 2.5.6). Blood vessels were incubated in the absence (control) or presence of 1 pmol/L, 1 nmol/L and 1 μmol/L of Ang II for 1 and 4 hours. Functional integrity of the vessels was not compromised by this incubation. IMA were also incubated with 1 μmol/L of Ang II for 15 minutes. In order to assess the effects of a positive control, IMA were incubated in the absence or presence of 1μmol/L of norepinephrine (NE), for 4 hours.

Additional studies were undertaken in order to determine whether or not any effect of Ang II on $^{\cdot}O_2^-$ production could be receptor specific. IMA were co-incubated with 1 μmol/L of Ang II and either an AT_1 specific and competitive receptor antagonist (losartan 1 μmol/L) or a non-specific receptor antagonist (sar^1 $thre^8$ Ang II 1 μmol/L) (Criscione *et al* 1990). NE and [sar^1, $thre^8$]-Ang II and were purchased from Sigma

(Poole, UK), whereas, losartan was obtained as a gift from Merck, Sharpe and Dome (UK). In subsequent studies, the role of the AT_2 receptor in relation to vascular ROS generation was also explored. In this case, IMA were treated with 1 μmol/L of Ang II, in the presence or absence of PD 123319, an AT_2 selective antagonist (Garcha *et al* 1999).

Finally, in studies designed to investigate intracellular pathways, the effect of inhibition of NAD(P)H oxidase was investigated by co-treatment of IMA with Ang II and DPI. In other studies, the effect of inhibition of protein synthesis was also investigated by treatment of IMA with cyclohexamide (Sigma, UK), a protein synthesis inhibitor. Due to the limitation in availability of IMA biopsies, and their relatively small length, IMA were divided into two and treated with either an ARA, an inhibitior of NADP(H) oxidase, or an inhibitor of protein synthesis, in the presence or absence of Ang II.

4.2.2 Statistical analyses

All data are presented as mean +/- S.E.M or as % change from controls to facilitate comparison between groups. Statistical analyses of vascular $\cdot O_2^-$ concentrations were undertaken using the Wilcoxon Signed-Rank Test.

4.3 Results

4.3.1 Effect of Ang II on superoxide anion concentrations in human arteries and veins

In IMA, Ang II stimulated an increase in $\cdot O_2^-$ production in both a concentration (Table 4.1, Figure 4.1) and time (Table 4.1, Figure 4.2) dependent manner. Ang II had no effect on $\cdot O_2^-$ production after 15 minutes incubation (control 1173±239 pmol/min/mg tissue, 1 μmol/L of Ang II 918±170 pmol/min/mg; n=11 p=0.12; 95% CI –680, 155), but increased $\cdot O_2^-$ production after 1 and 4 hours, respectively (Figure 4.2). Ang II stimulated an increase $\cdot O_2^-$ production in IMA but not SV (Figure 4.3, Table 4.1). NE (1 μmol/L), which was used as a vasoconstrictor control, had no significant effect on $\cdot O_2^-$ generation

in IMA (control 1581±899 pmol/min/mg, NE 738±241; n=11, p=0.45, 95% CI –3590, 164) or SV (control 360±218 pmol/min/mg, NE 249±118 pmol/min/mg; n=7 p=0.18 95% CI –391, 45).

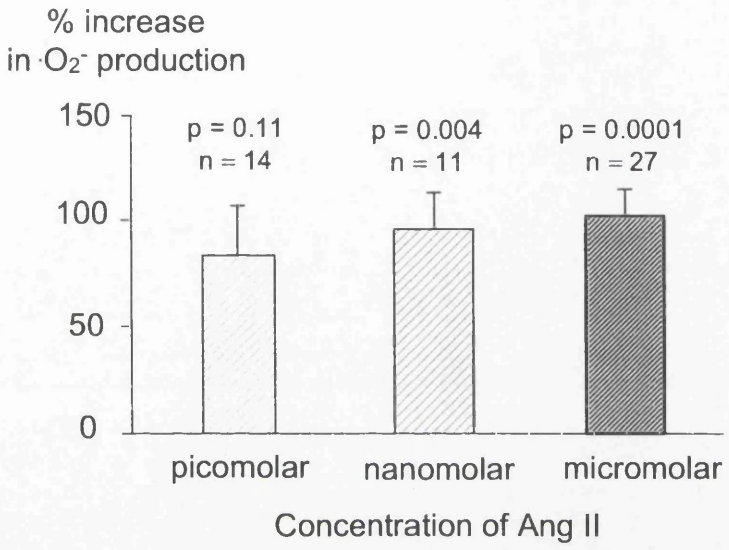


Figure 4.1 Effect of treatment of IMA with different concentrations of Ang II for 4 hours on vascular O_2^- production, compared to artery rings from the blood vessel treated with vehicle. Data are expressed as a percent change from control. The effect of Ang II on O_2^- production in IMA occurred at pharmacological concentrations (1 μmol/L, 1 nmol/L), whereas a trend approaching statistical significance was observed with physiological concentrations (1 pmol/L) of Ang II.

Table 4.1 Effect of ang II concentration (1 pmol/L, 1 nmol/L and 1 μmol/L) on $\cdot O_2^-$ production in IMA after incubation periods of 1 and 4 hours.

	$\cdot O_2^-$ generation (pmol/min/mg)*					
	1hour			4 hours		
n	1pmol/L (10)	1nmol/L (9)	1μmol/L (11)	1pmol/L (15)	1nmol/L (11)	1μmol/L (27)
Control	1516±253	1604±322	1331±232	1511±324	1360±293	978±117
Angiotensin II	1380±138	1752±323	2572±708	2090±358	2285±502	1690±213
p value	0.8	0.52	0.012	0.057	0.004	0.0001
95% Confidence Interval	-690, 396	-430, 540	170, 2672	–25, 1098	315, 1545	336, 925
% Change	12±22	15±16	76±22	83±30	93±27	99±24

* Data are shown as mean±SEM

Internal mammary artery (IMA)

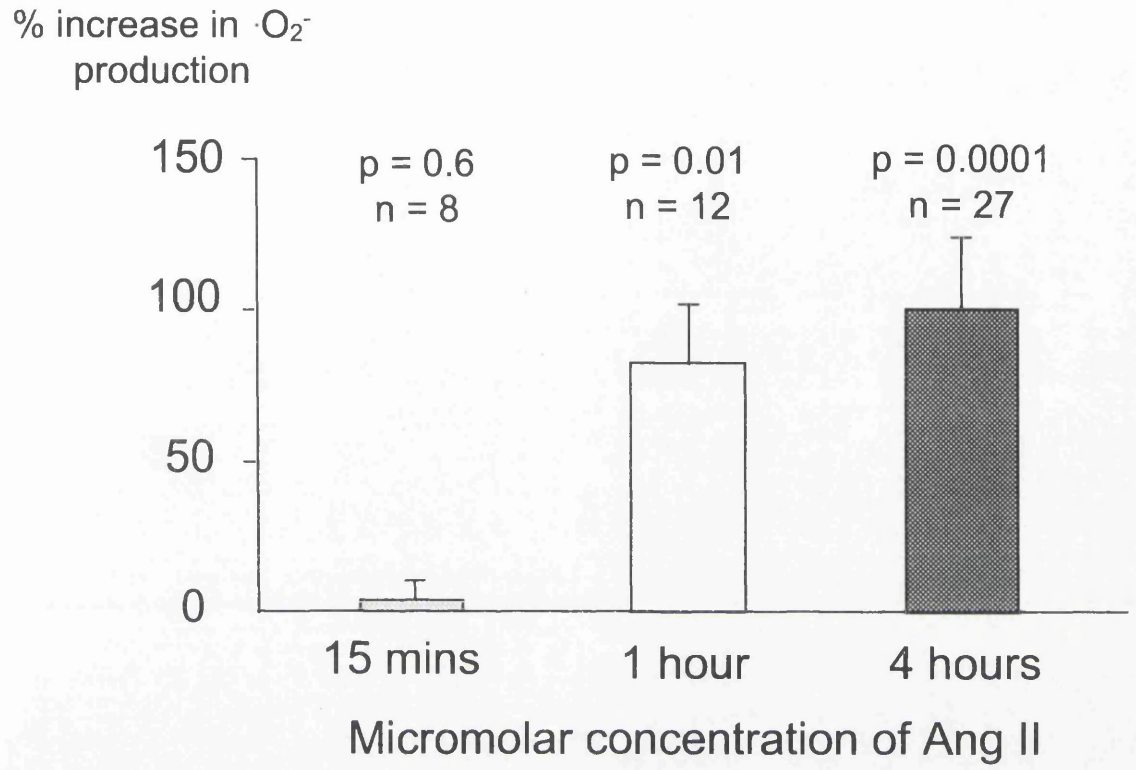


Figure 4.2 Effect of duration of exposure with 1 μmol/L of Ang II on $\cdot O_2^-$ production in IMA. Data are expressed as percent change from untreated controls ± SEM.

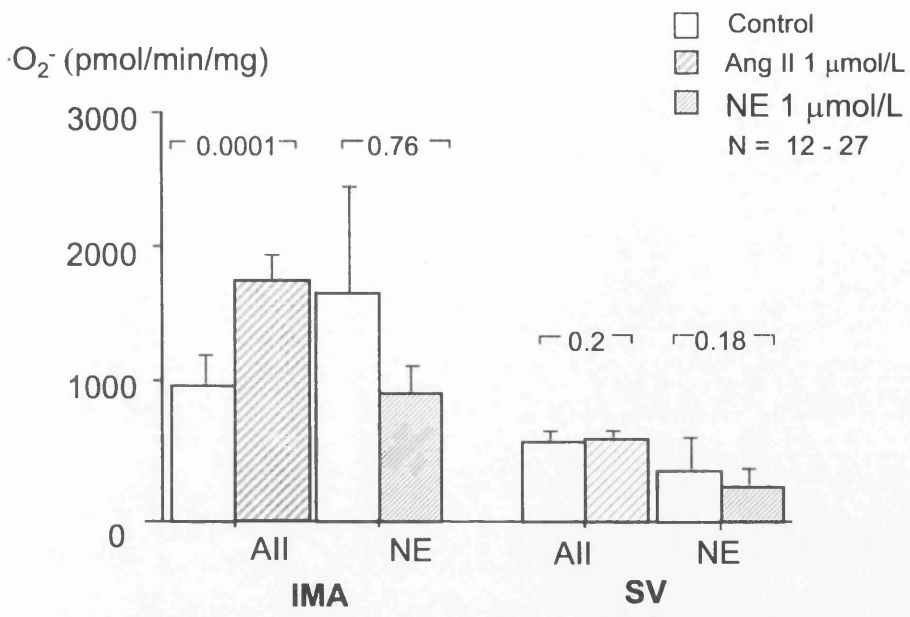


Figure 4.3 Effect of 4 hrs treatment with 1 μmol/L of either Ang II or norepinephrine (NE) on O_2^- concentrations in internal mammary arteries and saphenous veins. Although Ang II stimulated an increase in O_2^- production in IMA, it had no effect on O_2^- production in veins.

4.3.2 Mechanisms of Ang II – stimulated superoxide anion production in human arteries and veins

Losartan, a competitive AT_1 specific receptor antagonist, at a concentration of 1 μmol/L, had no effect on basal O_2^- production but blocked the Ang II – mediated increase of O_2^- production (Figure 4.4). [Sar^1, $thre^8$]-Ang II 1 μmol/L, a non-specific Ang II receptor antagonist, also blocked Ang II – mediated increase of O_2^- production (1 μmol/L of [sar^1, $thre^8$]-Ang II 1252±276 pmol/min/mg, 1 μmol/L of [sar^1, $thre^8$]-Ang II + 1 μmol/L of Ang II 1281±204; n=15, p=0.63, 95% CI –200, 305). Incubation of IMA for 4 hours with DPI 100 μmol/L, but not DPI 10 μmol/L, blocked Ang II – mediated increase of O_2^- production (Figure 4.5). Treatment with an AT_2 receptor antagonist had no effect on Ang II-stimulated O_2^- production in IMA (PD 552±127 counts/min/mg; PD + Ang II

665±138 counts/min/mg; n=16, P = 0.1).

Finally, in order to determine whether or not Ang II – stimulated NAD(P)H oxidase activation might involve an increase in the synthesis of this multimeric protein, IMA were treated with the protein synthesis inhibitor, cyclohexamide. Treatment of IMA with cyclohexamide had no effect on basal $\cdot O_2^-$. IMA were therefore treated with 1 μmol/L of Ang II, in the presence or absence of 100 μmol/L of cyclohexamide. Inhibition of protein synthesis by treatment with 100 μmol/L of cyclohexamide completely abrogated Ang II – stimulated $\cdot O_2^-$ production (cyclohexamide 460 ± 100 pmol/min/mg and cyclohexamide plus Ang II 430 ± 130 pmol/min/mg of tissue; n=8; Figure 4.6).

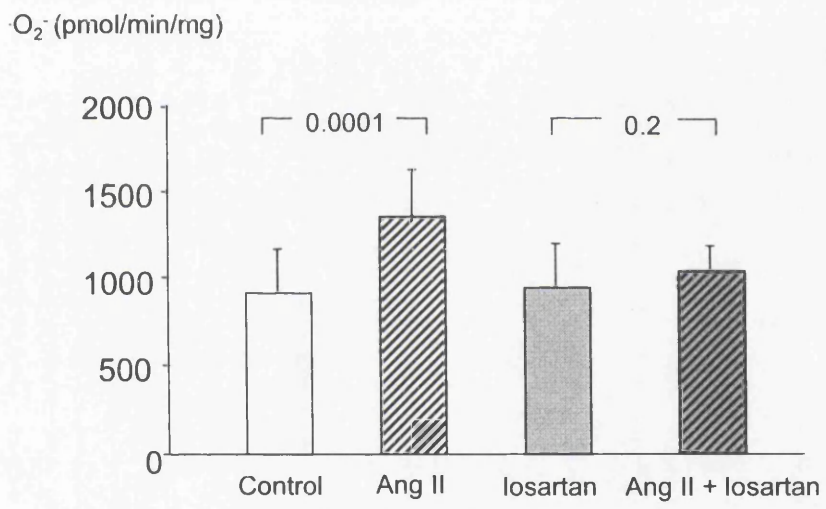


Figure 4.4 Effect of the AT_1 specific receptor antagonist, losartan, on Ang II - stimulated $\cdot O_2^-$ production. Paired segments of IMA were treated for 4 hours in the presence or absence of 1 μmol/L of Ang II. In parallel studies, IMA were treated with 1 μmol/L of the angiotensin type 1 receptor antagonist, losartan, in the presence or absence of Ang II.

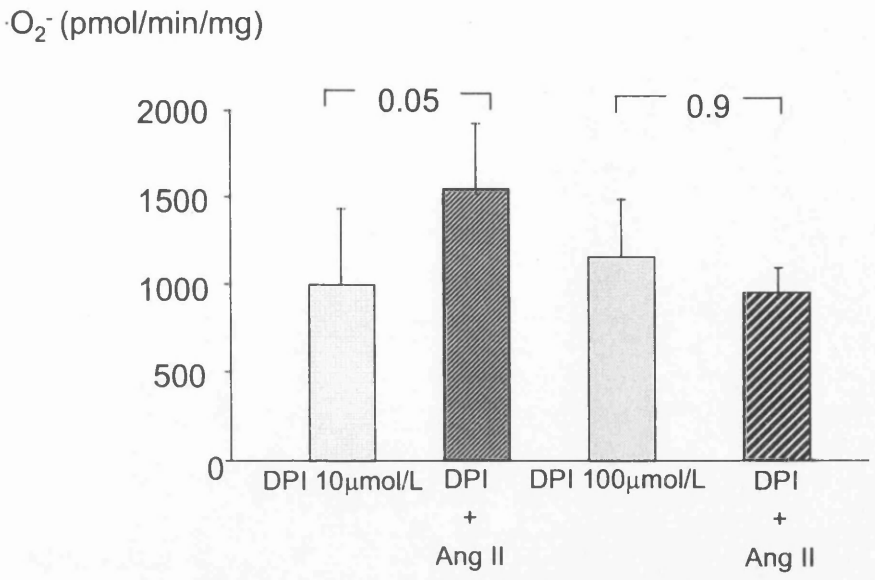


Figure 4.5 Effect of NAD(P)H oxidase inhibition on Ang II-stimulated $\cdot O_2^-$ production in IMA. Paired arteries were treated with either 10 μmol/L or 100 μmol/L of diphenyleneiodonium, in the presence or absence of 1 μmol/L of Ang II. Data are expressed as percent change from untreated controls ± SEM.

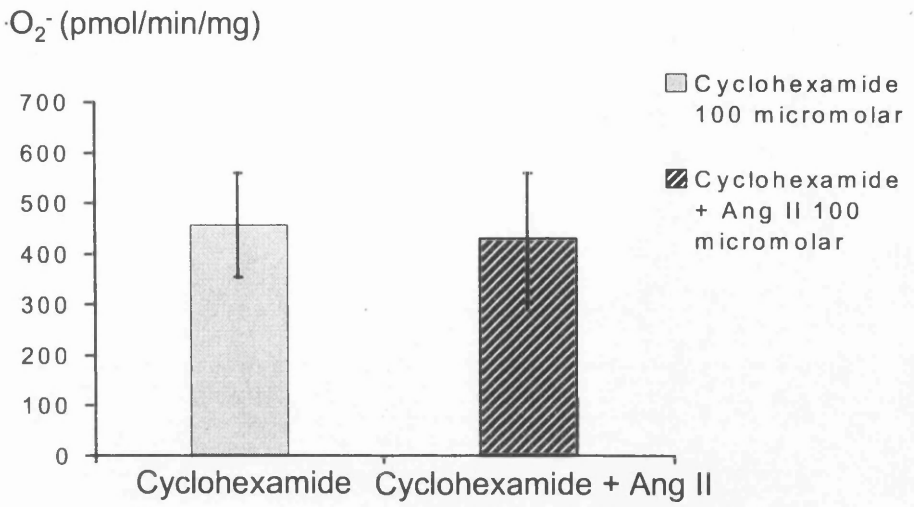


Figure 4.6 Effect of protein synthesis inhibition on Ang II-stimulated O_2^- production in IMA. Paired segments (n=8) of IMA were treated with a 100 micromolar concentration of cycloheximide, in the presence or absence of 100 micromolar concentration of Ang II, for 1 hour.

4.4 Discussion

This is the first demonstration that Ang II can increase O_2^- production in human arteries. This effect is AT_1 receptor-dependent as it was completely blocked by an AT_1 receptor antagonist. We have also demonstrated that this Ang II – mediated increase of O_2^- is mediated by NAD(P)H oxidase as it was inhibited by DPI. This is a clinically important observation as physiological concentrations (pmol) of Ang II tendend to increase O_2^- production in human arteries, and this effect was blocked by losartan.

Ang II increased O_2^- production in arteries, but not in veins. These effects are unlikely to be due to a reduction in AT_1 receptor expression in veins as the constrictor effect of Ang II in human saphenous veins, which is known to be AT_1 dependent (Li *et al* 1997b), may

be greater in SV than IMA (Borland *et al* 1996). Ang II exerts different effects in distinct vascular beds. For example, in experimental animal models of hypertension, Ang II has renal vasodilator effects mediated through AT_2 receptor activation (Siragy *et al* 1999; Siragy and Carey 1998). One explanation for our observations may be that the intracellular mechanisms by which Ang II activates NAD(P)H oxidase may differ in human arteries and veins. The mechanism of Ang II – mediated increase of $\cdot O_2^-$ production may also differ between species. AT_1 dependent Ang II – mediated increase of $\cdot O_2^-$ production has been reported in the aortae (Rajagopalan *et al* 1996b), and mesangium (Jaimes *et al* 1998) of Sprague Dawley rats and aorta of hypercholesterolemic rabbits (Pagano *et al* 1997a). However, Ang II – induced increase in $\cdot O_2^-$ production in normocholesterolemic rabbit aortic adventitial fibroblasts is mediated by a non AT_1-non AT_2 receptor mechanism (Pagano *et al* 1998). The species differences in the mechanism of Ang II – mediated increase in $\cdot O_2^-$ production make human investigations important.

Experimental studies have demonstrated that Ang II – mediated increase in $\cdot O_2^-$ production is of functional importance. Ang II – stimulated increase in $\cdot O_2^-$ production contributes to its trophic effect of on rat VSMC (Griendling *et al* 1994) and mesangial cells (Jaimes *et al* 1998) and also contributes to the pressor effect of this hormone in a hypertensive rat model (Rajagopalan *et al* 1996b). The observations in the present study may also be substantiated by a report that the pressor effect of intra-brachial artery infusion of Ang II in humans is attenuated by the co-infusion of vitamin C (Dijkhorst-Oei *et al* 1999). This study however failed to identify a specific Ang II receptor or to evaluate a positive control (i.e. another vasoconstrictor such as norepinephrine).

Treatment of IMA with the protein synthesis inhibitor, cyclohexamide, prevented Ang II stimulated increase in $\cdot O_2^-$ concentration in these arteries. This observation supports the conclusion that Ang II mediates NAD(P)H oxidase activation through an increase in synthesis of one, or more, of the subunits of this enzyme. In other studies, inhibition of the AT_2 receptor had no effect on vascular $\cdot O_2^-$ concentrations, suggesting that this receptor does not contribute to $\cdot O_2^-$ generation in IMA. Alternatively, the trend to an

increase in $\cdot O_2^-$ concentrations in IMA co-treated with PD123319 and Ang II suggests that the AT_2 receptor may have a tonic inhibitory effect on AT_1 receptor-dependent $\cdot O_2^-$ generation in human arteries.

In conclusion, we have characterised cellular and enzyme sources of $\cdot O_2^-$ production in human arteries and veins. We have demonstrated that Ang II increases $\cdot O_2^-$ production at pharmacological concentrations in human arteries. Furthermore, physiological concentrations of Ang II tended to increase $\cdot O_2^-$ production in IMA. This effect, which is NAD(P)H oxidase mediated, is completely inhibited by the AT_1 receptor antagonist losartan. These observations suggest a putative therapeutic role for AT_1 receptor antagonists in reducing oxidative stress in cardiovascular disease.

5 Immunodetection studies for NAD(P)H oxidase and xanthine oxidoreductase proteins in human blood vessels

5.1 Summary

This chapter provides a description of the immunohistochemical studies undertaken to identify the presence and distribution of the subunits of NAD(P)H oxidase in human IMA. Using monoclonal antibodies and antisera, p22phox, gp91 phox (or a homologue), p67phox and p47 phox are identified within the endothelium, vascular smooth muscle cell layer and adventitia. Xanthine oxidoreductase was identified in the endothelium and adventitial layers of IMA. These observations suggest that enzymes capable of generating ROS are distributed throughout the wall of human conduit arteries.

5.2 Methods

5.2.1 Blood vessel preparation and cell culture

Left IMA was obtained at the time of coronary artery revascularisation surgery and taken to the laboratory in Krebs buffer on ice. Segments of IMA were carefully dissected free of loose connective tissue and cut into 4 – 5 mm lengths, and incubated for 4 hours at 37°C in Krebs buffer, in the presence or absence of 1 μmol/L of Ang II. After this, the blood vessels were either immediately embedded in frozen in Tissue Tek® (O.C.T. compound, Miles Scientific, IL), or embedded in paraffin, whilst taking care to ensure that the blood vessel was not deformed in any way. Sections, approximately 5 μm thick, were then cut using a microtome.

Human coronary artery smooth muscle cells (HCASMC; Biowhittaker) were grown in 25 ml culture flasks and used between passages 4 – 6 in serum free conditions.

5.2.2 Immunohistochemistry

Immunohistochemical studies were performed in both frozen and paraffin-embedded 5μM sections of intact IMA, according to the methods described in chapter 2 (Section 2.7.1). P22phox and gp91phox were identified using specific mouse monoclonal antibodies (kindly provided by Dr MT Quinn, Montana State University, MT) at a dilution of 1:100. P47phox and p67phox proteins were identified by use of rabbit anti-sera (Upstate Biotechnology, N.Y.), at a dilution of 1:500. In each experiment, negative controls treated with non-immune antibody or antisera were included to assess for non-specific staining. Biotinylated secondary anti-mouse antibody, in the case of p22phox and gp91phox, and anti-rabbit antibody, in the case of p47phox and p67phox, were used. Ten percent horse or goat block was used according to the species that the antibodies were raised in. XOR was identified using a specific mouse monoclonal antibody (kindly provided by Professor R. Harrison, University of Bath) at a dilution of 1:100. In each experiment, negative controls treated with non-immune mouse IgG antibody were included to assess for non-specific staining. Secondary antibodies were biotinylated anti-mouse (1:50) with 10% horse block. Experiments were performed between 3 and 5 times with each vessel providing its own, untreated control. In addition, immunocytochemical studies were undertaken in order to determine the presence or absence of macrophages and granulocytes within sections of IMA (Section 2.7.1).

5.2.3 Western Blotting

Western blotting was performed by extracting samples in lysis buffers containing protease inhibitors. Homogenates were centrigufed at 14,000 g at 4°C. The supernatant fraction (20 μg of protein) was loaded onto 12% of SDS polyacrylamide gel and transformed to PVDF membrane (Boerhinger Mannheim). Membranes wer blocked in 5% non-fat milk and incubated with mase monoclonal antibody diluted 1:1000 for 1 hour at room temperature. They were then washed and incubated with an anti-mase horseradish peroxidase – conjugated antibody, diluted 1:5000 for 1 hour at room temperature and washed extensively. The membranes were then exposed to ECL

(Enhanced chemiluminescence, [Amersham,UK]), exposed to film and developped.

5.3 Results

5.3.1 Patient characteristics

Table 5.1 Clinical characteristics. The clinical information presented in this table represent data describing patients who provided blood vessels that were used in Chapters 5 and 6.

	Number (%)
Mean age, yrs	62±12
Males, n (%)	8 (57)
Females, n (%)	6 (43)
Risk factors, n (%)	
Diabetes mellitus	2 (14)
Hypertension	6 (43)
Hypercholesterolaemia	7 (50)
Smoking	4 (29)
Medication, n (%)	
Aspirin	13 (93)
β-Blocker	11 (79)
Calcium channel blocker	6 (57)
HMGCoA Reductase Inhibitor	7 (57)
Nitroglycerin	11 (86)
Angiotensin converting enzyme inhibitor	5 (36)

β-Blockers – beta-blocker; HMGCoA Reductase Inhibitor - 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitor.

5.3.2 Immunohistochemical identification of NAD(P)H oxidase subunits in IMA

Figure 5.1 shows typical immunohistochemical features of a frozen section of IMA. In this case, the IMA was treated with non-immune mouse antibody (control), or a monoclonal antibody specific for gp91 phox (treated).

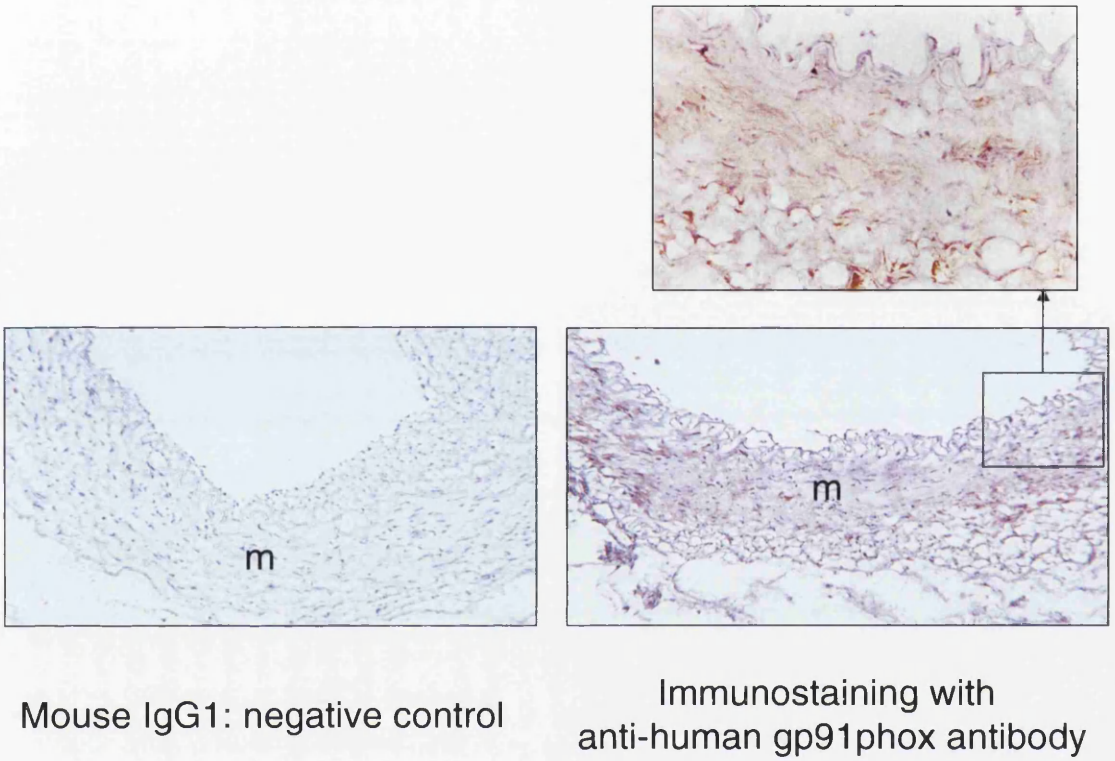


Figure 5.1 Immunohistochemistry of IMA treated with specific monoclonal antibodies to gp91phox. The sections are shown at a magnification of x20 and **m** represents the media. The inset image shows staining for gp91phox, at a magnification of x40. The image on the left is a negative control where the primary antibody has been replaced with non-immune mouse IgG1. Brown staining represents the chromogenic reaction product arising from the interaction between HRP-labelled secondary antibodies (bound to to the primary anti-gp91phox anti-bodies) and diaminobenzidine (DAB). This brown staining therefore represents immunodetection of gp91phox. Results shown are typical of those seen in 4 experiments.

It is apparent that there is dense staining throughout the vessel wall, including the media, in contrast to the control section from the same vessel. In the case of gp91phox, staining appeared most abundant within the vascular smooth muscle layer,

but was also evident within the adventitia and endothelium. Wax sections of IMA failed to demonstrate any staining with this antibody. One explanation for this is that immunohistochemical studies undertaken on wax sections are much less sensitive for the detection of candidate proteins than is the case with frozen sections, this being due to the effect of parraffin on the tissue sections.

Additional immunohistochemistry studies using wax-embedded sections of IMA, treated with anti-sera for either p47phox (Figure 5.2) or p67phox (Figure 5.3) proteins revealed staining throughout the blood vessel wall, which was particularly marked within the adventitial and endothelial layers. Control studies, in which studies were performed with the appropriate primary non-immune antibody or anti-sera were performed in every case (not shown).

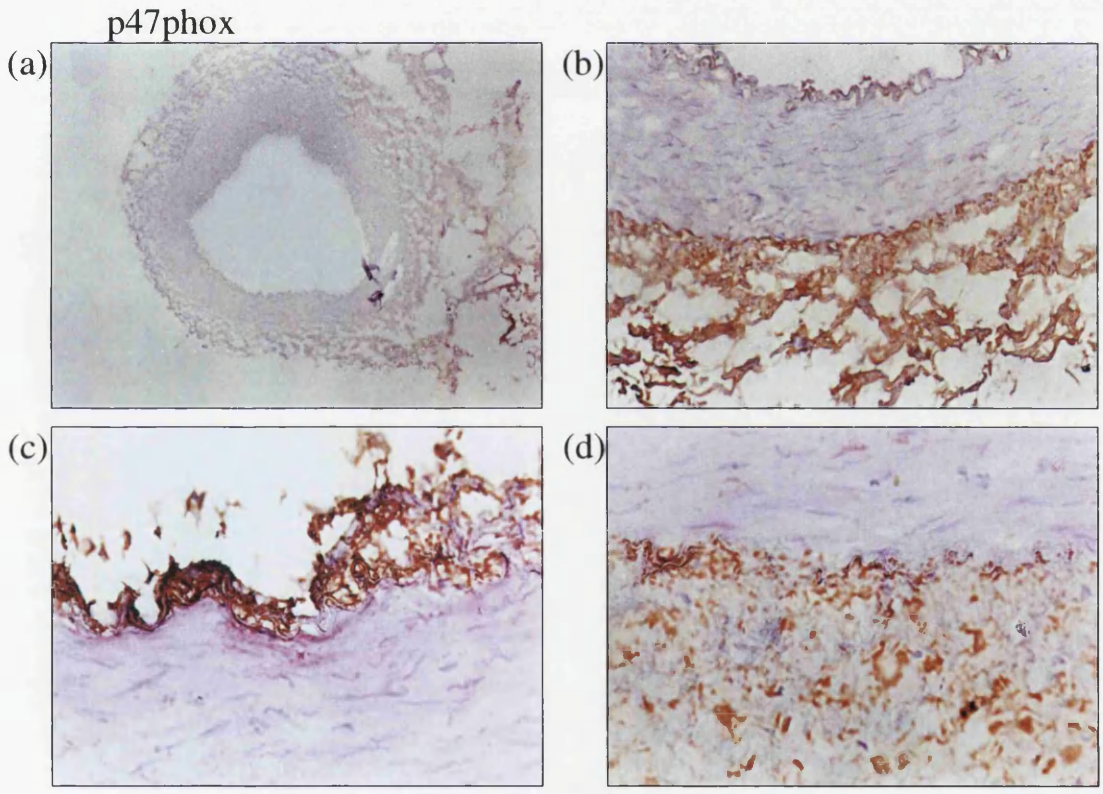


Figure 5.2 Coronal, paraffin-embedded sections of IMA treated with anti-sera for p47phox protein. (a) x4 magnification (b) x10 magnification (c & d) x40 magnification. In this case, the sections were also stained with haematoxylin and eosin.

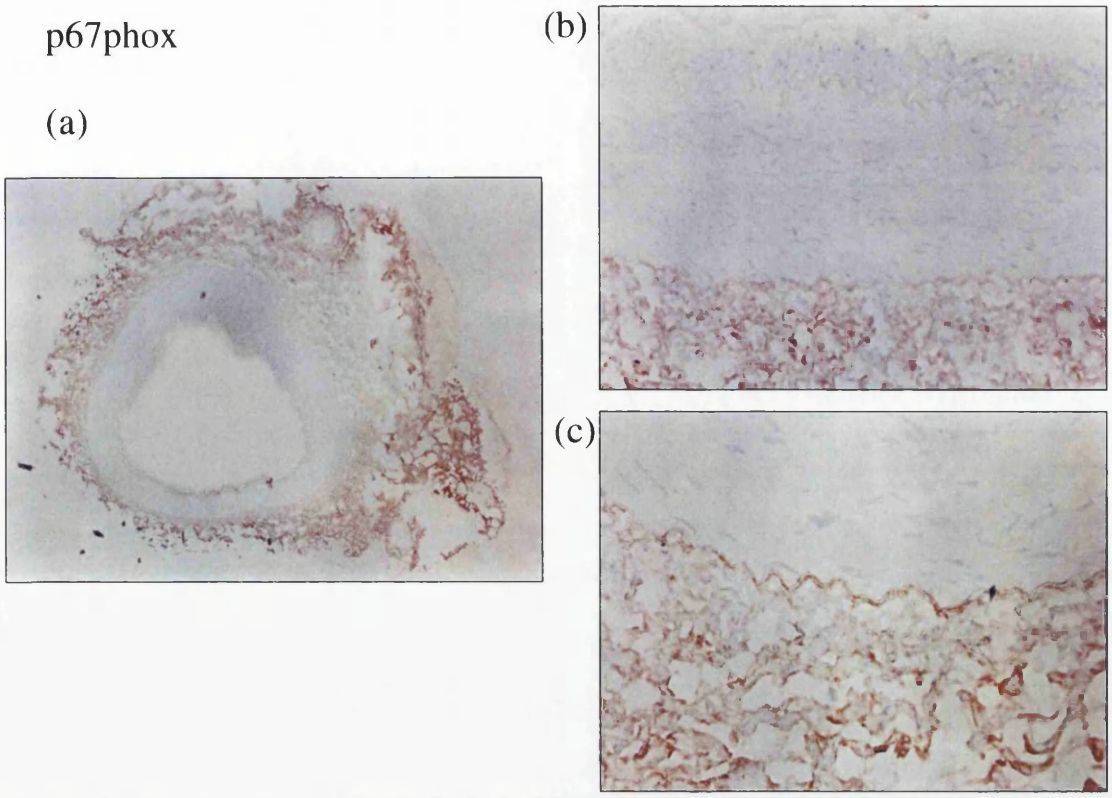


Figure 5.3 Coronal, paraffin-embedded sections of IMA treated with anti-sera for p67phox protein. (a) x4 magnification (b) x10 magnification (c) and x40 magnification.

Immunodetection studies for p22phox protein demonstrated staining within the smooth muscle layer and endothelium (Figure 5.4 [a & b]).

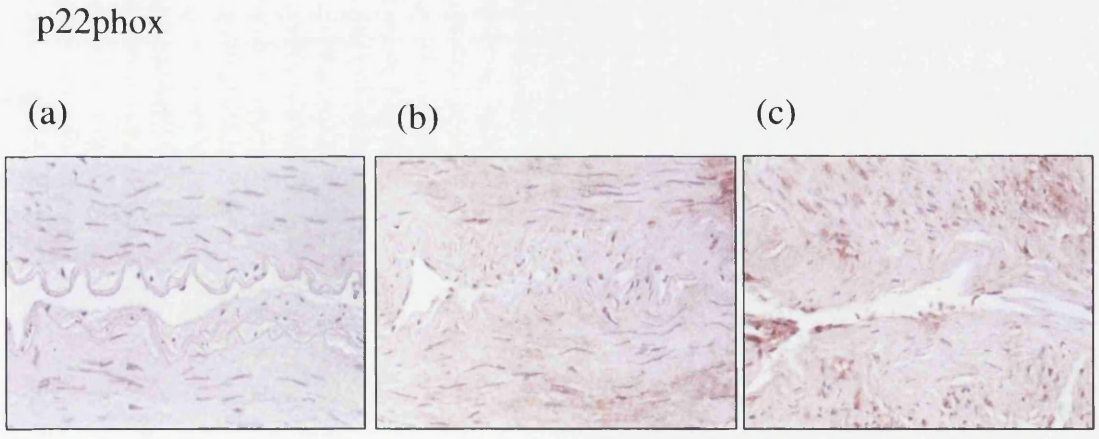


Figure 5.4 Coronal paraffin-embedded sections of IMA treated with anti-p22phox monoclonal antibody. (a) - x4 magnification (b) x10 magnification (c) and (d) x40 magnification.

Segments of IMA were also treated with 1 µmol/L of Ang II for 4 hours. These

experiments were performed on paired samples from between 3 – 5 vessels with representative data shown. Control experiments with either non-immune mouse IgG or rabbit sera showed no staining. Qualitative assement of the effect of this treatment on the abundance of p22phox protein (Figure 5.4) in IMA sections was performed by scoring the density of immunostaining by an independent observer who was unaware of the nature of each slide. Representative images for p22phox (Figure 5.4) immunostaing are shown below. In this case, the intensity of this staining appeared to be greater after treatment with Ang II.

In separate studies, no light microscopic or immunocytochemical positivity for macrophages (anti-CD68, anti-mac387 or anti-Kp1), or granulocytes were identified in any of the vessels investigated (data not shown).

5.3.3 Immunodetection of gp91phox (or a homologue) by western blotting techniques

Figure 5.7 shows typical results of western blotting of cultured HCASMC. A major band was detected at approximately 90 kD, which was absent when the primary antibody was omitted.

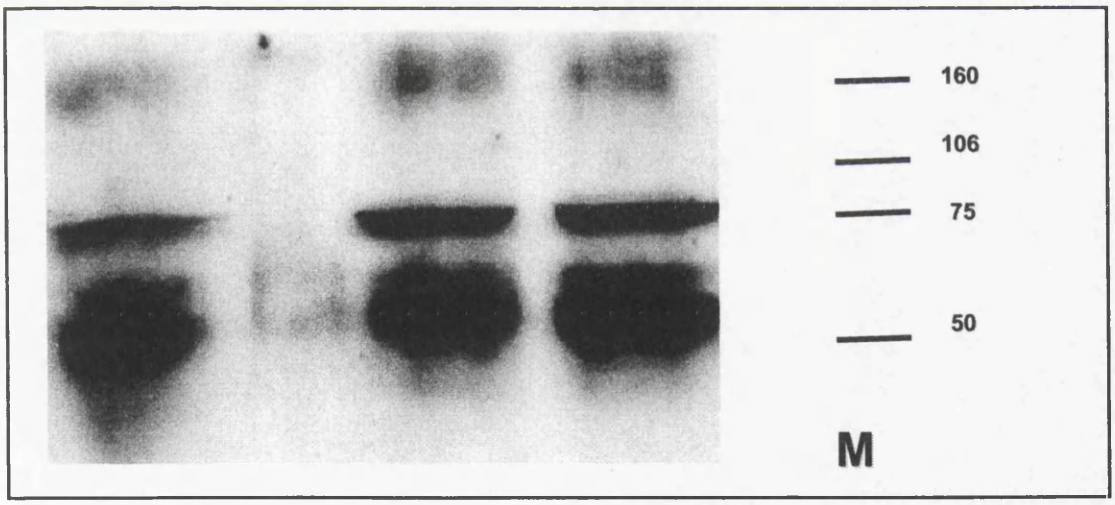
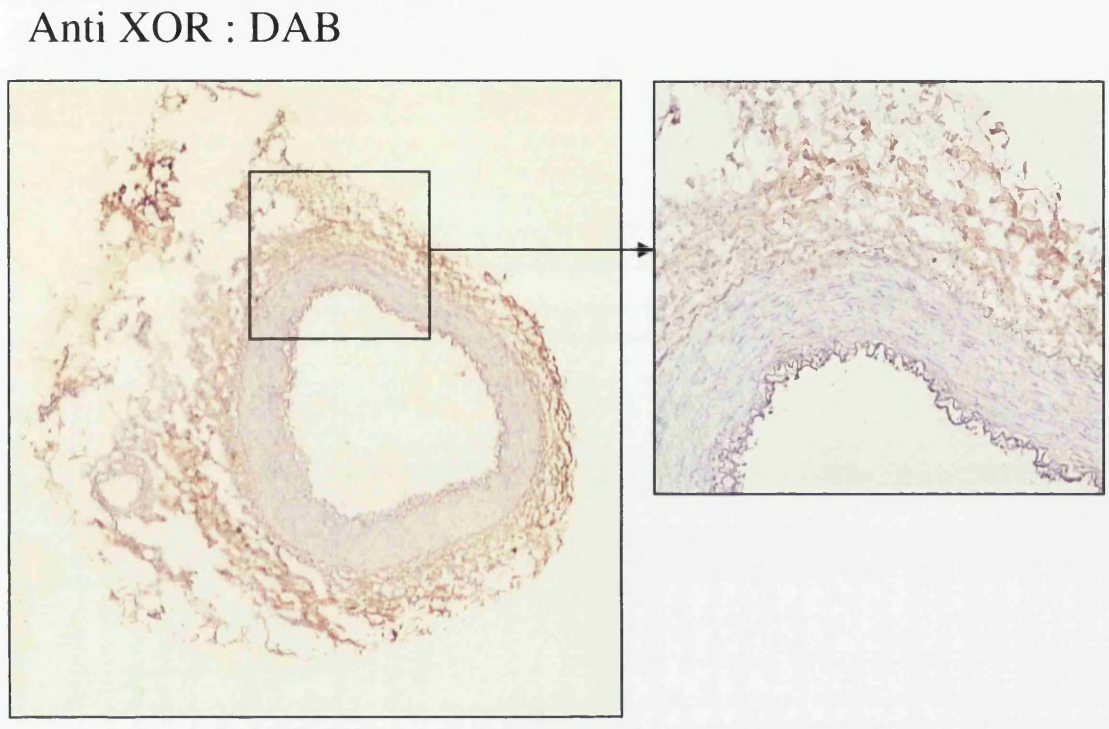


Figure 5.5 Western blotting of gp91phox in human coronary VSMC using a monoclonal antibody against gp91phox. M represents molecular weight markers.

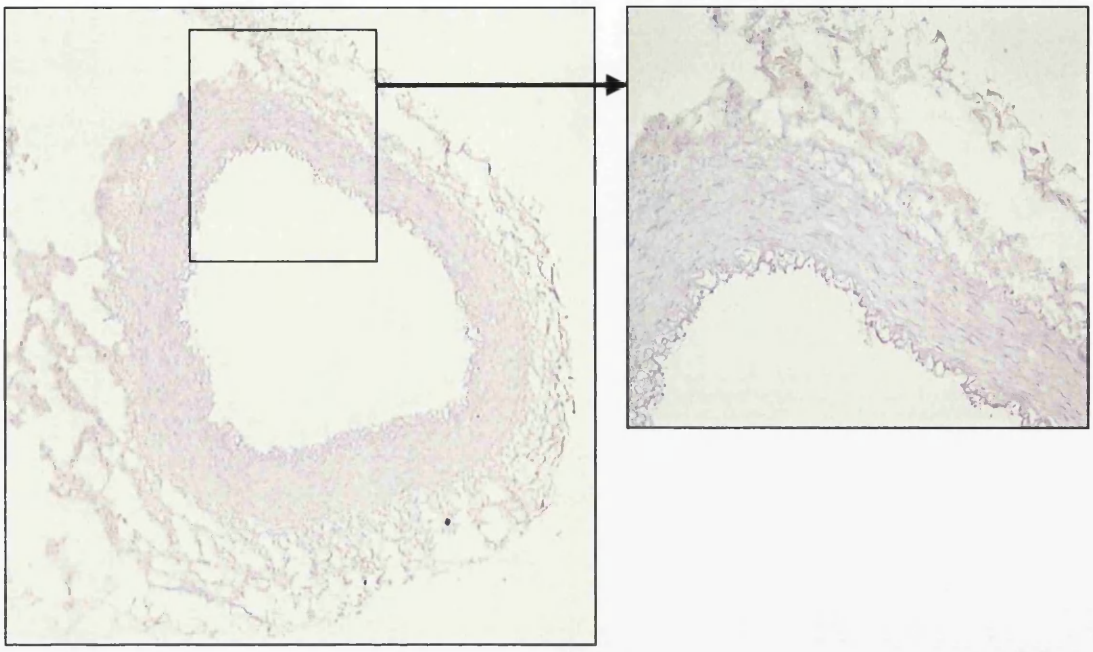
5.3.4 Immunodetection of xanthine oxidase in IMA

Figure 5.4 shows a representative, wax-embedded section of an IMA. In this case the IMA was treated with non-immune mouse IgG antibody (control), or a monoclonal antibody specific for XOR (treated). There is staining evident within the endothelium and adventitia, but by contrast, the smooth muscle layer is spared. These features contrast those of the control section from the same vessel, in which staining is absent.



(a)

Mouse IgG1 : Negative Control



(b)

Figure 5.6 (a) Immunohistochemistry of IMA treated with specific monoclonal antibodies to xanthine oxidase. The sections are shown at a magnification of x20 and **m** represents the media. The inset image demonstrates staining for xanthine oxidase, at a magnification of x40. (b) Staining was absent in negative control section, where the primary antibody has been replaced with non-immune mouse IgG1. Results shown are typical of those seen in 4 experiments.

5.4 Discussion

In these studies, p47phox, p22phox and p67phox proteins were identified in the endothelium, media and adventitial layers of IMA. Furthermore, gp91phox, or a protein homologue, was detected using a monoclonal antibody directed against gp91phox. This protein was evident throughout the artery wall including the medial layer.

The data in the present study suggest that gp91phox is abundant within human VSMC. This conclusion is dependent on the specificity of this antibody for gp91phox, rather than one of its homologues. In order to verify this result, western blotting was performed, using the same monoclonal antibody, in cultured HCASMC. In these

studies, protein bands of the anticipated molecular weight for gp91phox protein were identified. This observation supports the assertion that gp91phox is present in human VSMC. Although it might be argued that the expression of this protein in IMA could be ascribed to the disease status of these patients, the cultured VSMC were obtained from individuals with no history of cardiovascular disease. This is in agreement with observations by Suh *et al.* (Suh *et al* 1999) who reported that the nox1 protein (formerly mox-1), a homologue of gp91phox, was present in rat VSMC.

More recently, Lassegue *et al.* (Lassegue *et al* 2001) characterised the expression of three nox family members, nox1, nox4 and gp91phox in rat VSMC. Using quantitative PCR, they found that the number of RNA molecules of nox1 and nox4 were approximately 3000 times greater than gp91phox, which was just above the limit of detection in this assay. This result contrasts the earlier findings by this group (Suh *et al* 1999). In these studies, northern blotting techniques failed to identify any gp91phox mRNA, however, this technique is much less sensitive than PCR. The nox family of proteins are functionally important, not only for $\cdot O_2^-$ production, but also for the regulation of vascular growth and hypertrophy, and belong to a growing number of Nox family members (Lambeth 2000). Taken together, these data suggest that nox1 and nox4, rather than gp91phox, are functionally active homolgues in NAD(P)H oxidase in this cell type. Studies of the relative expression of the nox family members in human vascular cells have not yet been reported.

Initial studies with wax-embedded sections of IMA failed to demonstrate any staining using the anti-gp 91phox monoclonal antibody, in contrast to subsequent studies undertaken in frozen sections. This may be due an effect of processing. Wax, or paraffin-embedded, sections typically reveal greater definition of tissue architecture than is the case with frozen tissue sections. By contrast, this latter type of processing affords greater sensitivity for immunohistochemical identification of target molecules and cells (Harlow and Lane 1988).

A striking difference was observed between the distribution of gp91phox and p22phox proteins, which were evident throughout the artery wall, and the distribution of p47phox and p67phox, which appeared to be concentrated within the endothelium and adventitial layers. There are several possible explanations for this observation. Firstly, it may be that p47phox and p67phox proteins are much less abundant within

vascular smooth muscle cells, than is the case with gp91phox and p22phox. Secondly, these latter proteins together constitute the cytochrome b558 membrane complex, whereas, by contrast, p47phox and p67phox are both cytosolic proteins. Thus, the difference in cellular location of these proteins may give rise to the observed difference in staining arising with this technique. Finally, one additional explanation for these appearances may also be that, in the case of gp91phox and p22phox, a monoclonal antibody was used for immunodetection of these proteins, whereas in the case of p47phox and p67phox proteins, anti-sera were used. It may be, for example, that these anti-sera are less sensitive to binding phox proteins within the media, than is the case with monoclonal antibodies.

In other immunohistochemical studies, XOR protein was identified within sections of IMA, with staining most pronounced within the endothelium and adventitia. Interestingly, the distribution of this protein is comparable with that of p47phox and p67phox. In this case, immunodetection studies for XOR were undertaken with a monoclonal antibody for this protein. Taken together, these observations suggest that the observed distribution of p47phox and p67phox proteins in human IMA is valid, rather than being a function of the methods (and antisera) employed.

That XOR protein could be readily demonstrated in IMA by immunodetection techniques supports the earlier findings of a functional enzyme, capable of generating ROS in these arteries. This raises the question as to the source of XOR in these arteries. One possibility is that XOR, synthesised within the liver, is released into the systemic circulation whereupon this protein may bind with the endothelium, and may also penetrate into the adventia by passing from adventitial vasa vasorum. One alternative possiblity is that vascular cells, and endothelial and adventitial cells in particular, can synthesise this protein. This latter possibility could be addressed in further studies using RT-PCR techniques to detect XOR mRNA in these cells.

6 Effect of Ang II on the abundance of messenger RNA for NAD(P)H oxidase phox subunits in human vascular cells and blood vessels

6.1 Summary

In this chapter, studies of the effects of Ang II on the abundance of cDNA transcripts of the subunits of NAD(P)H oxidase are reported. Here we demonstrate that treatment with Ang II is associated with an increase in the abundance of cDNA transcripts of p22phox, which was attenuated by co-treatment with either actinomycin D, an inhibitor of gene transcription, or losartan, an AT_1 receptor antagonist. These data support the possibility that Ang II stimulates an increase in the synthesis of the subunits of NAD(P)H oxidase in human vascular cells by an AT_1 receptor –dependent increase in gene transcription.

6.2 Methods

6.2.1 Blood vessel preparation and cell culture

IMA were obtained from patients at the time of coronary artery revascularisation surgery. In this case, however, the arteries were collected from the cardiac theatre in sterile universal containers containing serum-free cell culture medium (Clonetics), maintained at 37°C inside a thermos flask. The flasks were taken directly to the laboratory where the arteries were dissected free of surrounding connective tissue and cut into 4 – 5 mm lengths whilst bathed in pre-warmed culture media. The arteries were then immediately placed in an incubator.

Human aortic endothelial cells (HAEC; Clonetics) and coronary artery smooth muscle cells (HCASMC; Biowhittaker) were grown in 25 ml culture flasks and used between passages 4 – 6 in serum free conditions.

6.2.2 Pharmacological studies

IMA were divided into pairs of segments 4 – 5 mm lengths and were incubated for 4

hours at 37 °C in serum-free culture media in the presence or absence of 1 μmol/L of Ang II. In other studies, cultured HCASMC and HAEC were incubated in 25 ml flasks in a similar fashion, but in this case, these cells were also pre-treated and co-incubated with 1 μmol/L of the AT_1 receptor antagonist, losartan, or actinomycin D, an inhibitor of transcription. The studies with cultured cells were performed in triplicate on each occasion.

6.2.3 Reverse transcription polymerase chain reaction

In order to generate first strand cDNA, total RNA underwent reverse transcription which resulted according to methods described in Chapter 2 (section 2.4). This product was used as a template for the polymerase chain reaction using primers for p22phox and GAPDH. Qualitative assessment of RNA obtained from cell and tissue homogenates was undertaken by electrophoresis of the cDNA products on a 1.5% agarose gel (Life Technologies, UK). Semi-quantitative analysis was undertaken using phospho-imaging analysis (Biorad), by expression of the ratio of p22phox amplicons to GAPDH or actin cDNA products.

6.2.4 Statistical analyses

All data are presented as mean +/- S.E.M or as % change from controls to facilitate comparison between groups. Statistical analyses of vascular O_2^- concentrations were undertaken using a paired Student's t-test. A probability value of $P<0.05$ was taken as significant.

6.3 Results

RT-PCR studies undertaken in HAEC (Figure 6.1), in HCASMC (Figure 6.2), and IMA (Figure 6.3) demonstrated the abundance of p22phox cDNA transcripts to be increased by treatment with 1 μmol/L of Ang II for 4 hours.

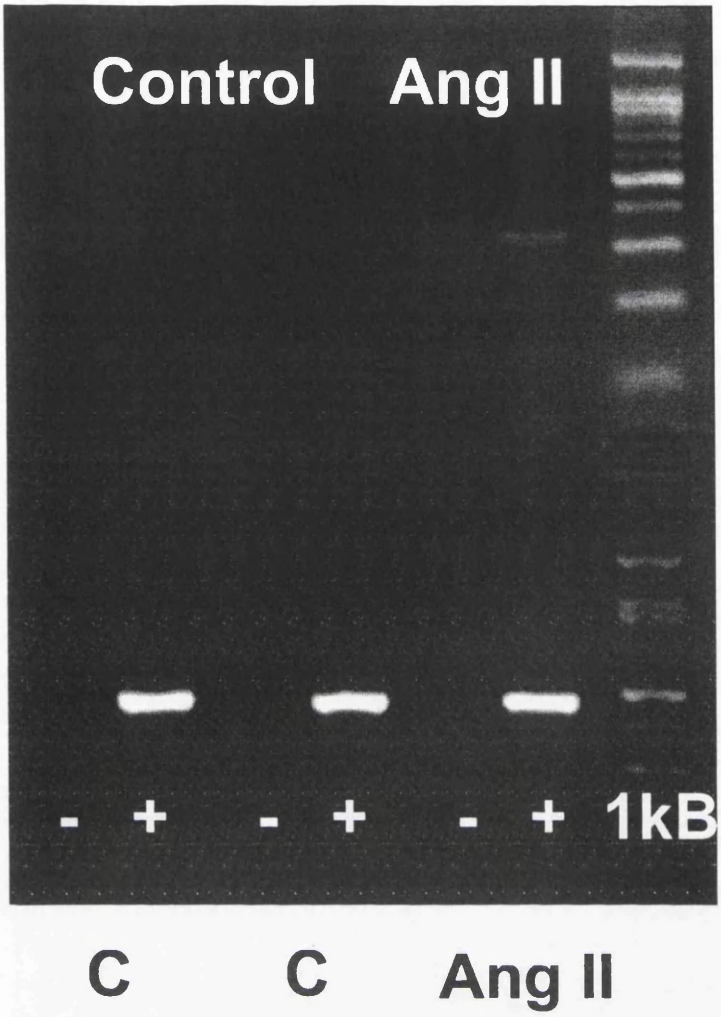


Figure 6.1 Effects of treating human aortic endothelial cells for 4 hours with either 1 μmol/L of Ang II, or vehicle, on the abundance of p22phox mRNA. (C, control; ′+` represents cDNA which was formed through treatment of mRNA with reverse transcriptase, whereas ′-` represents controls, which were treated with vehicle, instead of reverse transcriptase; 1kB represents the molecular weight DNA marker).

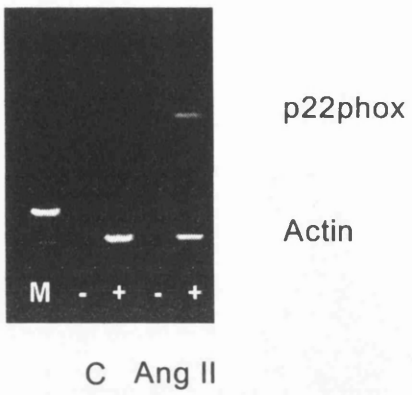


Figure 6.2 Effects of treating HCSMC for 4 hours with either a 1 μmol/L of Ang II, or vehicle, on the abundance of p22phox cDNA transcripts in these cells (C, control; '+' represents cDNA formed through treatment of mRNA with reverse transcriptase, whereas '-' represents controls, treated with vehicle, instead of reverse transcriptase; M represents the molecular weight DNA marker).

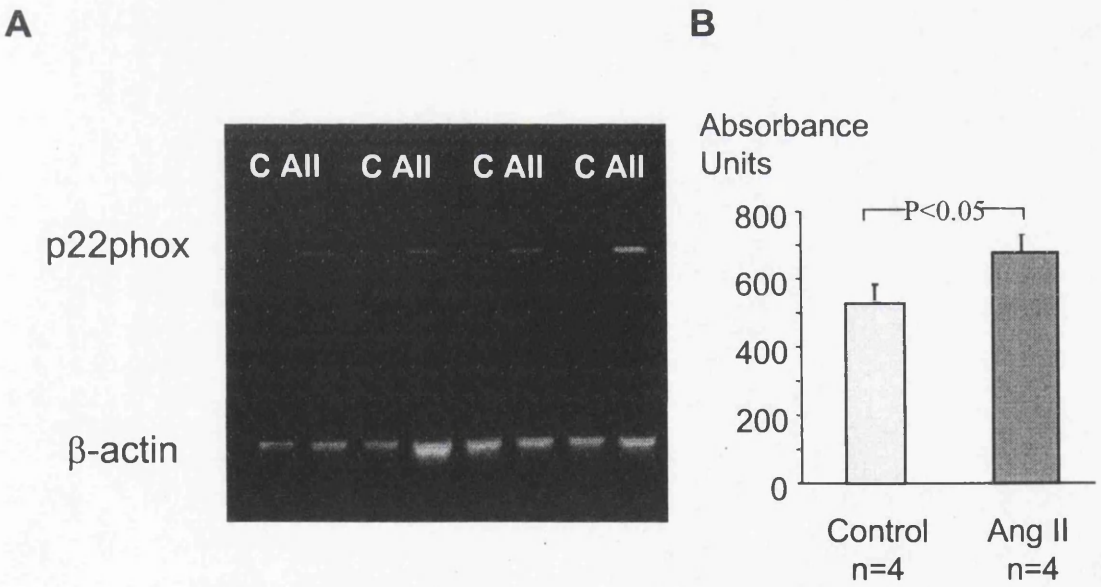
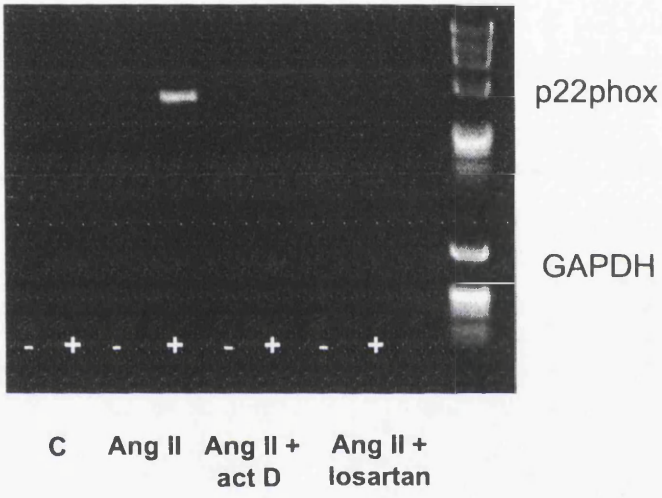


Figure 6.3 Effects of treatment with either 1 μmol/L of Ang II, or vehicle, for 4 hours, on the abundance of p22phox mRNA in the IMA from 4 different patients. (A) A scanned image of RT-PCR cDNA transcript products for p22phox and β-actin after agarose gel (1.5%) electrophoresis. (B) This data was quantified using the phosphorimager, as described in the text (C, control).

The different baseline levels of p22phox are striking, despite the relatively even amplification of the house-keeping gene. In the case of IMA, each treated segment was compared with an untreated segment derived from the same vessel. In unstimulated IMA, p22phox expression relative to actin, in arbitary densitometric units, was 527 ± 31 whereas in Ang II – stimulated arteries this was 680 ± 33 (p=0.048, 95% confidence interval 2.7 to 302, n=5). In similar studies in HAEC, the increase in abundance of p22phox transcripts by treatment with 1 μmol/L of either losartan or actinomycin D, completely prevented the previously observed Ang II – induced increase in abundance of p22phox transcripts (Figure 6.4).



C - control; Ang II - angiotensin II; act D - actinomycin D; GAPDH glyceraldehyde-3 phosphate dehydrogenase

Figure 6.4 Effects of treatment of HAEC with either 1 μmol/L of Ang II, or vehicle, for 4 hours, in the presence or absence of either 1 μmol/L of actinomycin or losartan, on the abundance of p22phox mRNA in human aortic endothelial cells. A scanned image of RT-PCR cDNA transcript products for p22phox and GAPDH after agarose gel (1.5%) electrophoresis (C, control; Act D, actinomycin D).

6.4 Discussion

We investigated the molecular mechanisms that may be involved in Ang II - stimulated activation of NAD(P)H oxidase in human arteries and cells. In previous

studies, we demonstrated that Ang II stimulates increased $\cdot O_2^-$ production by an AT_1 receptor and NAD(P)H oxidase – dependent mechanism.

In the present *in vitro* study, the effect of treatment with Ang II on the abundance of p22phox cDNA transcripts in human vascular cells and tissues was investigated. These transcripts are representative of the amount of p22phox mRNA present in these cells. Messenger RNA is the transcribed molecular product formed as a result of gene expression. The abundance of mRNA is determined by the rate of gene transcription, and the rate of mRNA degradation (or half-life). Messenger RNA provides the template for translation and synthesis of the protein product, and consequently, increased abundance of mRNA will lead to an enhanced amount of the protein product. In the present case, NAD(P)H oxidase is a multimeric protein, which when activated, is composed of p22phox, gp91phox, p47phox, p67phox and, p40phox proteins. An increase in the abundance of these subunits, and in particular those involved in electron transport (such as p22phox), is associated with enhanced abundance of the 'activated' NAD(P)H oxidase multimer. This, in turn, will augment electron transport and $\cdot O_2^-$ production.

Treatment of both HAEC and HCASMC with 1 μmol/L of Ang II for 4 hours, was associated with an increase in the abundance of p22phox cDNA transcripts in these cells. The mechanism for this Ang II effect may be due to either an increase in the rate of gene transcription, leading to enhanced production of p22phox mRNA, or prolongation of the half life of this molecule. In further studies, using IMA obtained from 4 different patients, treatment of vascular tissue with 1 μmol/L of Ang II, or vehicle, for 4 hours, was also associated with a significant increase in the abundance of p22phox cDNA transcripts. This suggests that the effect of Ang II on p22phox mRNA is operative in intact human arteries.

Further molecular studies sought to determine the mechanisms that may be involved in the effect of Ang II on p22phox mRNA. HAEC were again treated with 1 μmol/L of Ang II, or vehicle, for 4 hours in the presence or absence of either the AT_1 receptor antagonist, losartan, or an inhibitor of transcription, actinomycin D. In this case, co-treatment with losartan, prevented the observed increase in abundance of p22phox cDNA transcripts associated with Ang II treatment. This observation is in keeping with the earlier observations, by this laboratory and elsewhere (Griendling *et al* 1994;

Rajagopalan *et al* 1996b), that AT_1 receptor blockade prevents Ang II – stimulated $\cdot O_2^-$ production. In addition, inhibition of gene transcription by treatment of these cells with actinomycin also prevented the previously observed increase in abundance of p22phox cDNA transcripts arising from treatment with Ang II.

Recently, studies by Lassegue *et al.* (2001) investigated the effects of treatment of rat VSMC with Ang II on the abundance of nox (i.e. gp91phox and homologues) mRNA abundance. Treatment of these cells with different concentrations of Ang II lead to a dose- and time-dependent increase in the abundance of nox1 (gp91phox), but not nox4, mRNA (EC_{50} 3nmol/L and a maximal response at 1μmol/L). The increase in nox1 mRNA was detectable within the first hour and increased to a maximum at 4 hours. These observations are in keeping with those of the present study.

Taken together, these findings suggest that exposure of human vascular cells to Ang II leads to an increase in the abundance of p22phox cDNA. Consequently, this may lead to enhanced synthesis of p22phox protein in human vascular cells and blood vessels. These observations also support the thesis that Ang II may augment $\cdot O_2^-$ production in human blood vessels through an AT_1 receptor – dependent activation of NAD(P)H oxidase.

7 Functional significance of Ang II – stimulated superoxide anion production in human resistance arteries

7.1 Summary

Ang II-stimulated $\cdot O_2^-$ production contributes to the vasopressor effect of this hormone in rats made hypertensive by infusion of Ang II (Rajagopalan *et al* 1996b). In this study, we tested the hypothesis that enhanced ROS production might contribute to the vasoconstrictor effect of Ang II in human subcutaneous resistance arteries (SRA). The results did not support this hypothesis.

7.2 Methods

Resistance arteries are, in functional terms, the most important type of blood vessel for the regulation of peripheral vascular resistance, and therefore, blood flow (Mulvany and Aalkjaer 1990). For this reason, I sought to explore whether or not Ang II – stimulated $\cdot O_2^-$ production might contribute to the vasopressor effect of this hormone in human SRA.

Subcutaneous resistance arteries (SRA) were isolated from abdominal wall skin biopsies, according to the methods described in Chapter 2 (section 2.2.2). Isometric tension studies were undertaken in these arteries according to the methods described in Chapter 2 (section 2.8) (Mulvany and Aalkjaer 1990; Mulvany and Halpern 1977). The SRA underwent a standard normalisation process and 'wake-up protocol'. As part of this protocol, the SRA were initially exposed to a 123 mM of KPSS for a series of 5 minute periods until repeatable maximal contractions were achieved, and then once to 10 μmol/L of norepinephrine (NE). After a plateau contraction had been attained with NE, 3 μmol/L of acetylcholine (ACh) was added to stimulate endothelium-dependent vasodilatation. Arteries that were unable to contract to either KPSS or NE or showed no relaxation to ACh (and were therefore considered to have no functionally intact endothelium) were discarded. The arteries were then incubated for a further 30 minutes in Krebs solution prior to the commencement of the concentration-response curves (CRC) incorporated in the study protocol. SRA were

treated with 1mmol/L of the ROS scavenger, Tiron, or vehicle, and a cumulative concentration response curve (CRC) for NE was performed, followed by a further treatment with 3 μmol/L of ACh. SRA were then co-treated with 1 μmol/L of Ang II for 1 hour to stimulate O_2^- production, and a second CRC for NE was performed (for summary of protocol see Figure 7.1).

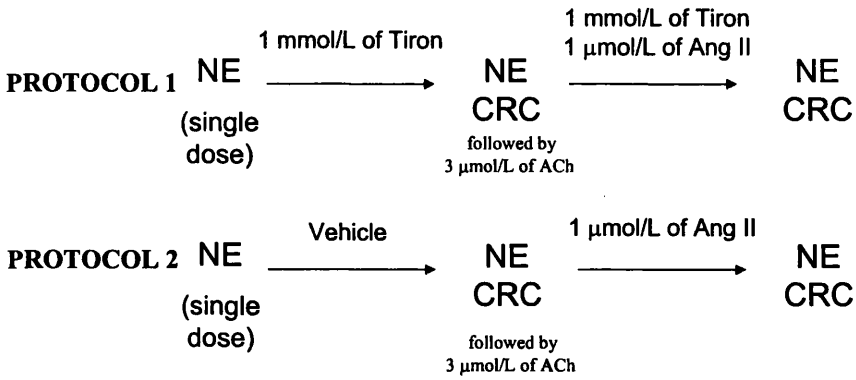


Figure 7.1 Schematic representation of the protocol undertaken in human small resistance arteries.

These studies were undertaken with paired vessels from the same patient in every case. Statistical analyses were undertaken using a paired t test with log-transformed data.

7.3 Results

After initial preconstriction with 10 μmol/L of NE, mean relaxation of all SRA to 3 μmol/L of ACh was 86±3% (n=20). SRA which did not relax fully, and therefore did not have functional endothelium, were not included in this study. Contractile responses obtained after each NE CRC are expressed as a percentage of the maximum response obtained after initial treatment with a single dose of 10 μmol/L of NE. After treatment with vehicle, or Tiron, the maximum contraction (E_{max}) to NE, compared to

the initial response obtained after treatment NE, was 94 +/- 7 % and 98 +/- 1.7%, respectively (Figure 7.2).

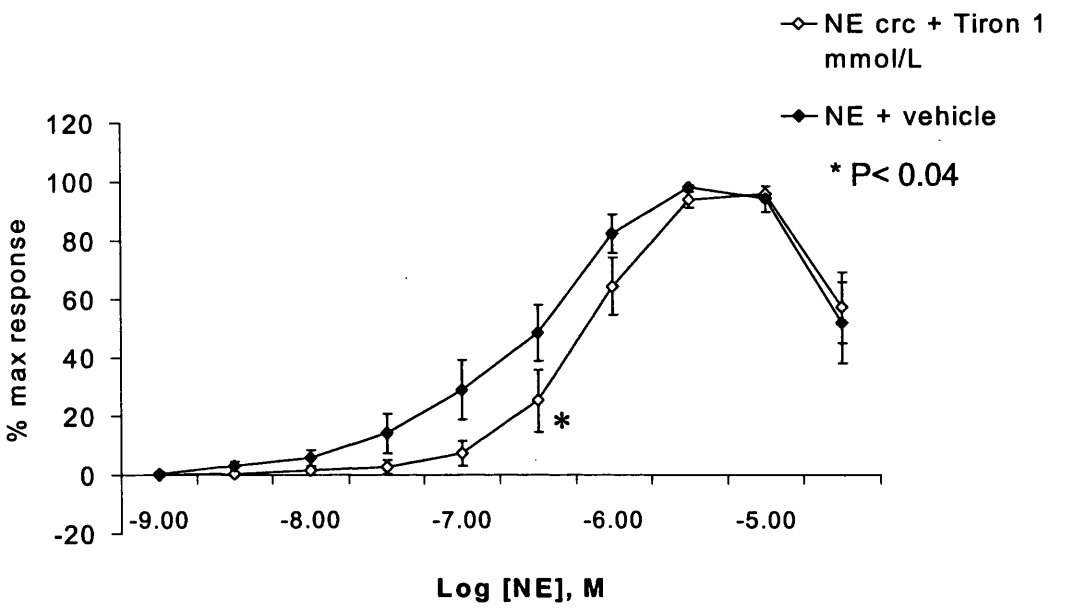


Figure 7.2 This figure shows concentration response curves for NE in SRA incubated in the presence or absence of 1 mmol/l of Tiron.

PD_2 (-log EC_{50}) values for the CRCs for NE undertaken in the presence and absence of 1 mmol/L of Tiron were (6.5 +/- 0.03 vs. 6.1 +/- 0.03, Tiron vs. vehicle, respectively; P=0.036). After incubation with either vehicle or 1 μmol/L of Ang II for 1 hour, the maximum contraction (E_{max}) to NE was 98.4 +/- 6 % and 84.6 +/- 6.6%, respectively (P=0.1) [Figures 7.3].

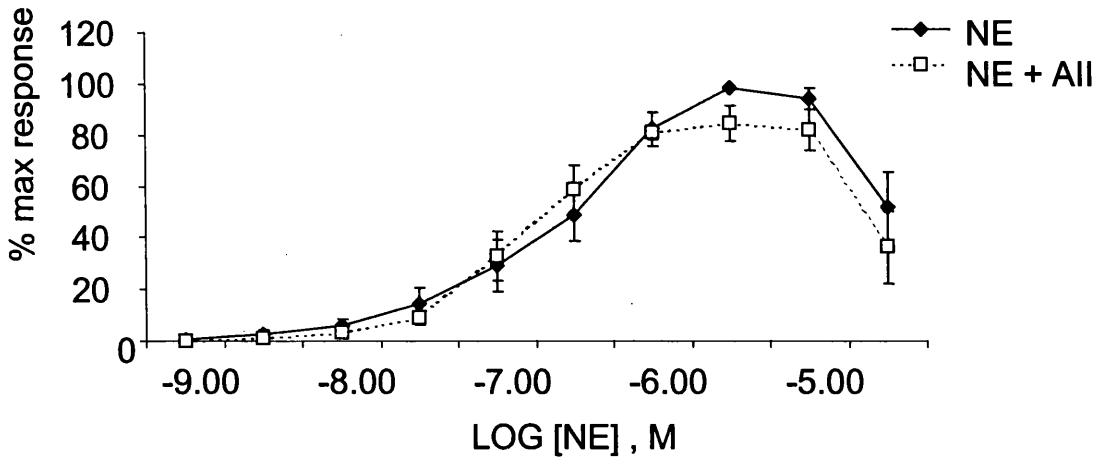


Figure 7.3 Concentration response curves to NE after treatment with vehicle or 1 micromole of Ang II for 1 hour (n=8)

In this case, the pD_2 values for the NE CRCs in the absence and presence of Ang II were 6.5 +/- 0.06 and 6.3 +/- 0.08, respectively (P=0.5). After incubation of SRA with 1mmol/L of Tiron, in the absence or presence of 1 μmol/L of Ang II for 1 hour, the E_{max} of CRC for NE was 94.9 +/- 2.3% and & 97.5 +/- 7.4%, respectively (Figure 7.4).

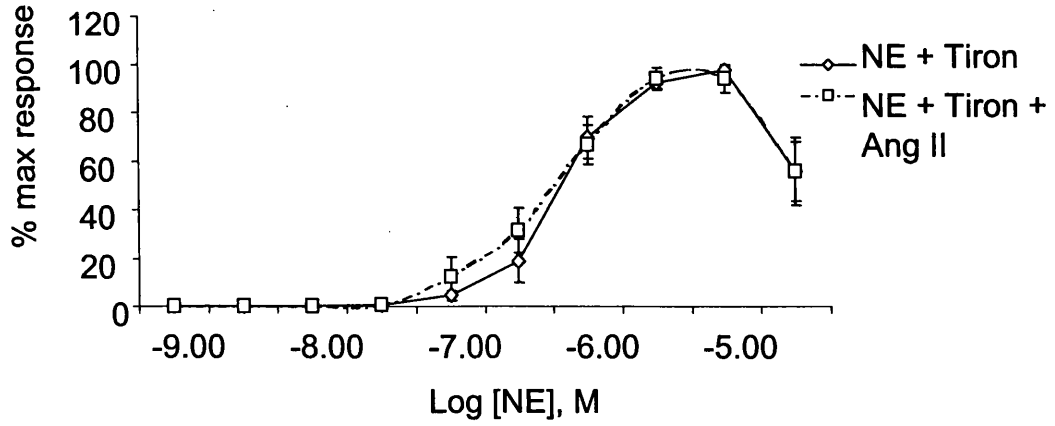


Figure 7.4 Concentration response curves to NE after treatment with vehicle or 1 μmol/L of Ang II for 1 hour in the presence of 1mmol/L of Tiron (n=10).

7.4 Discussion

In this study, treatment of SRA with the 1mmol/L of the ROS scavenger, Tiron, resulted in a shift to the right of the CRC for NE. The maximum contractile responses for these two groups were comparable. Alternatively, treatment with 1 μmol/L of Ang II for 1 hour failed to augment either the sensitivity of SRA, or their maximum contractile response, to NE, compared with responses to vehicle treated SRA. In fact, if anything, there appeared to be a trend in favour of an attenuated NE CRC response by SRA after treatment with Ang II, compared to those treated with vehicle. Finally, incubation of Tiron-treated SRA with 1 μmol/L of Ang II for 1 hour had no effect on the CRC for NE.

These observations do not support the hypothesis that Ang II – stimulated $\cdot O_2^-$ production might contribute to the constrictor effect of this hormone in human SRA. This hypothesis was based on the supposition that NO is an important vasodilator in these arteries, and that bioavailable NO would be modulated by ROS activity. The complex protocol in this study (Figure 7.1) was designed to test this hypothesis using SRA obtained from a single skin biopsy. The prótocol included an initial CRC for NE to be undertaken in the presence or absence of Tiron, followed by an incubation period of 1 hour in which SRA were exposed to a pharmacological concentration of Ang II in order to stimulate vascular NAD(P)H oxidase activation and ROS production.

Only SRA which relaxed fully to ACh, therefore indicating the presence of functional endothelium, were included in this study. Treatment of SRA with the ROS scavenger, Tiron, resulted in a shift to the right of the NE CRC, compared to that of vessels treated with vehicle. This observation suggests that a reduction in ROS activity, and therefore less NO scavenging, led to an increase in bioavailable NO. In this case, higher concentrations of NE were required to achieve a given increment in tension, compared to SRA treated with vehicle. These findings suggest that ROS activity may contribute to resting vascular tone in human SRA, possibly through removal of bioavailable NO.

The lack of potentiation of NE – induced vasoconstriction by treatment with Ang II in

these studies is in contrast to observations in some, but not all (Creager *et al* 1984; Nicholls *et al* 1981), studies in both experimental animals and humans. For example, *in vivo* studies by Qiu *et al.* (1994) demonstrated that superfusion of exteriorised rat mesenteric arteries with PE was associated with a reduction in both vessel diameter and blood flow within these arteries. This effect was attenuated by co-treatment (by topical superfusion) of these arteries with an ACE-I, or ARA, or both.

Evidence for adrenergic facilitation by Ang II has been provided from studies in both normotensive and hypertensive subject, whereas evidence against this effect has also been provided. Studies by (Seidlin *et al* 1991) provided some information which helped to clarify the possible effect of Ang II on adrenergic tone. In these studies healthy human subjects were exposed to lower-body negative pressure (15 mmHg) which resulted in a rise in blood pressure, without any change in heart rate. In this case, forearm vasoconstriction, measured by strain-gauge plethysmography, occurred due to an increase in sympathetic activity. Intra-brachial artery infusion of norepinephrine (NE; 37.5 – 150 pmol/min) was also undertaken, which induced comparable reductions in forearm blood-flow. In subsequent studies, infusion of a non-pressor concentration of Ang II (320 fmol/min) was associated with an enhanced reduction in forearm blood flow to lower-body negative pressure (i.e. sympathetic nervous system activation), whereas Ang II had no effect on NE-induced vasoconstriction. These observations suggest that in human blood vessels, Ang II – induced adrenergic facilitation is a pre-synaptic phenomenon. This effect may be mediated through augmentation of NE release, or perhaps through an inhibition of NE re-uptake. These data, taken together with observations from the present study, suggest species differences may exist in the regulation of adrenergic control of vascular tone.

Given this lack of potentiation, subsequent manipulation of ROS activity in these arteries was unlikely to have yielded any data which might have been informative as regards the question of whether Ang II – stimulated $\cdot O_2^-$ production might contribute to vasomotor tone in human blood vessels. Given the microscopic size of these arteries, it was not possible to quantify ROS concentrations either within the wall of these SRA, or in the bath. The lack of information on whether or not ROS activity was actually enhanced in these SRA by treatment with Ang II makes interpretation of these findings difficult. Nevertheless, there are several possible explanations for our

findings. Firstly, the oxidant-generating properties of human SRA are poorly characterised. It is possible, for example, that NAD(P)H oxidase may not be quantitatively or functionally important in these blood vessels. The effect of ROS scavenging in these SRA by treatment with Tiron does suggest that basal ROS generation has a tonic constrictor effect in these SRA, possibly through removal of NO.

The importance of NO in the regulation of vascular tone in human SRA is a subject of current debate (Buus *et al* 1998). In these studies, human SRA were threaded with stainless steel wires and mounted in a wire myography. NO concentrations in the lumen of the artery were measured by use of a electrochemical microelectrode which had a high selectivity for NO and a sensitivity of approximately 1 nmol/L of NO. The diameter of this microelectrode was approximately 30 -50 μm. The SRA were pre-constricted with 5 μmol/L of NE, and then treated with incremental, cumulative concentrations of ACh. Stimulation of these arteries with ACh resulted in a mean relaxation of 64 ± 7% (n=4), however, no detectable increases in NO concentration were observed. By contrast, treatment of these SRA with the NO donor, S-nitroso-*N* – acetylpenicillamine (SNAP*)* resulted in a comparable relaxation, and an increase in NO concentration by 32 ± 8 nM. These effects were attenuated by co-treatment with the NO scavenger, oxyhaemoglobin. Further studies of intracellular electrical potentials in these arteries demonstrated that ACh-induced vasorelaxation resulted in hyperpolarisation of the VSMC in these arteries, which was not inhibited by co-treatment with the NO synthesis inhibitor, L-NOARG. These observations confirm the presence of an L-arginine/NO pathway in human SRA, but demonstrate that ACh – induced relaxation in these arteries is predominately mediated by hyperpolarisation, which is NO-independent, suggesting the involvement of an endothelium-dependent hyperpolarising factor (EDHF).

These findings are supported by observations in our own laboratory. Kelly *et al.* (Kelly *et al* 2000) undertook studies in human SRA obtained by gluteal fat biopsy from healthy subjects and patients with polycystic ovarian syndrome. These investigators studied the mechanisms of insulin-mediated vasodilation in NE-preconstricted SRA. Concentration response curves for NE were undertaken in the presence and absence of the NO synthesis inhibitor, L-NMMA. They found that the contractile responses to NE in SRA treated with L-NMMA were no different to those

of SRA treated with vehicle. This suggests that NO-mediated vasodilation is not important in human SRA. This conclusion has also been supported by observations in Hillier's laboratory in which EDHF has been demonstrated to be the dominant vasodilator in human SRA (Coats *et al* 2001). That NO-mediated vasodilatation may not be important in human SRA suggests that Ang II-stimulated $^{\cdot}O_2^-$ production does not modulate at least short-term changes in vasomotor tone, which is in keeping with the findings of the present study.

Future studies of the functional effect of Ang II-stimulated $^{\cdot}O_2^-$ production in human blood vessels should focus, therefore, on studies using alternative blood vessel types and methods of investigation. One alternative blood vessel to study might have been a conduit artery, such as the IMA. Endothelium-dependent vasorelaxant responses in these arteries are recognised to be poorly reproducible. Other explanations for our findings may be related to the experimental procedures involved in the present study. It may be, for example, that this *in vitro* technique is insufficiently sensitive to elicit any functional component of Ang-II stimulated $^{\cdot}O_2^-$ generation. In addition, the period of incubation with Ang II may have been too short to result in any important activation of NAD(P)H oxidase in these SRA. Alternatively, the use of a pharmacological concentration of Ang II may have had adverse effects on the contractile properties of these arteries. The concentration of Ang II used in this study was selected on the basis of our earlier findings in IMA, in which it was demonstrated that for a treatment period of 1 hour, only micromolar, but neither nanomolar or picomolar, concentrations of Ang II were associated with a statistically significant increase in vascular $^{\cdot}O_2^-$ concentrations.

In vitro contractile responses of human arteries to Ang II are associated with tachyphylaxis (Thomson and Johnson 1987). This is an attenuation of the contractile response with increasing concentrations and duration of exposure to Ang II. The mechanisms giving rise to tachyphylaxis are poorly understood, but may involve desensitisation of the AT_1 receptor as a result of agonist-induced phosphorylation, and receptor internalisation (Hunyady 2001). The pharmacological concentration of Ang II used in this study may therefore have resulted in desensitisation of these blood vessels to additional agonist-induced contraction. The fact that the cumulative concentration response curves were undertaken with NE, rather than Ang II, mitigates against this being an important factor in the present investigation. The effect of Ang

II-stimulated $^{\cdot}O_2^-$ production in human blood vessels could also be investigated using different functional techniques. One such technique that is thought to represent a more physiological method of vascular assessment is perfusion myography (Falloon *et al* 1995). This technique differs from wire myography in a number of ways. In perfusion myography, the resistance artery is cannulated and pressurised. In this case, vascular responses to pharmacological agents are enhanced, compared to those observed from arteries under isometric tension (Falloon *et al* 1995). One disadvantage of pressure myography studies is that only one resistance artery may be studied at any one time. Clearly, *in vivo* techniques such as studies of blood flow in either the forearm, or dorsal hand veins, using forearm strain-gauge venous occlusion plethysmography.

In conclusion, therefore, this study fails to support the hypothesis that Ang II-stimulated $^{\cdot}O_2^-$ production contributes to the constrictor effect of this hormone in human SRA. The reasons for this are not clear. Further studies are warranted, ideally using *in vivo* techniques.

8 Relationship between IMA superoxide anion concentrations and clinical risk factors and treatment type

8.1 Summary

Angiotensin converting enzyme (ACE) inhibitors have recently been demonstrated to reduce major atherosclerotic events in patients at risk of coronary heart disease (CHD). In this prospective study of consecutive patients undergoing coronary artery bypass grafting (CABG) we found vascular free radical concentrations were lower in those patients who were treated with a renin-angiotensin-aldosterone system (RAAS) inhibitor compared to those who were not. This may be one additional mechanism which contributes to the beneficial effects of these drugs in patients with CHD.

8.2 Methods

The aim of the present study was to determine, which, if any, risk factors and drug therapies, were associated with altered free radical concentrations in the arteries of CHD patients undergoing CABG. Seventy-nine consecutive patients who were undergoing CABG were prospectively included in this study. Patient clinical characteristics and risk factors for cardiovascular disease (defined in Chapter 2, section 2.1) were determined by review of case records.

Superoxide concentrations in distal segments of IMA obtained at the time of CABG were measured by lucigenin-enhanced chemiluminescence in a liquid scintillation counter, as described in section 2.5.6. Statistical analyses of vascular $\cdot O_2^-$ concentrations after log-transformation were undertaken using the non-parametric Mann-Whitney Test. Step-wise multiple regression analysis was also performed. A probability value of $P< 0.05$ was considered statistically significant.

8.3 Results

Data on age, sex, risk factors and drug therapy are given in Table 1.

Table 8.1 Patient characteristics, including risk factors and therapies.*

	RAAS Inhibitor	No RAAS Inhibitor
N	19	60
Mean age, y	61±9	62±8
Males, n (%)	13 (68)	45 (75)
Females, n (%)	6 (33)	15 (25)
Risk factors, n (%)		
Smoking	3 (16)	10 (17)
Hypertension	9 (47)	21 (35)
Diabetes mellitus	2 (10)	4 (7)
Hypercholesterolaemia	14 (73)	41 (68)
Plasma chol, mmol/L (mean±SEM)	5.9±1.2	5.2±0.9
Medication, n (%)		
Aspirin	14 (74)	48 (80)
β-Blockers	13 (68)	33 (55)
Calcium channel blockers	11 (58)	34 (57)
HMG-CoA† reductase inhibitors	7 (37)	31 (52)
Nitroglycerin	12 (63)	34 (57)

Data on different classes of drug therapies in relation to the presence or absence of an ACE-I or ARA are presented in Table 2.

* Renin-angiotensin system inhibitor (includes 16 patients taking an ACEI and 3 patients taking an ARA); † Hydroxymethyl-glutaryl-CoA (HMGCoA) reductase

Table 8.2 This table shows the proportions (n, %) of patients from the ACEI/ARA group and the group not taking these therapies, in relation to all other classes of anti-ischaemic therapies.*

	Aspirin		β-Blockers		Calcium channel blockers		HMGCoA† reductase inhibitors		Nitroglycerin	
	RAAS inhibitor	No RAAS inhibitor	RAAS inhibitor	No RAAS inhibitor	RAAS inhibitor	No RAAS inhibitor	RAAS inhibitor	No RAAS inhibitor	RAAS inhibitor	No RAAS inhibitor
Aspirin	14 (74)	48 (80)	11 (57)	33 (55)	11 (57)	33 (55)	7 (37)	30 (50)	10 (53)	28 (47)
β-Blockers	11 (57)	33 (55)	13 (68)	33 (55)	8 (42)	24 (40)	5 (26)	20 (33)	8 (42)	19 (32)
Calcium channel blockers	11 (57)	33 (55)	8 (42)	24 (40)	11 (58)	34 (57)	6 (32)	20 (33)	8 (42)	21(35)
HMGCoA† reductase inhibitors	7 (37)	30 (50)	5 (26)	20 (33)	6 (32)	20 (33)	7 (37)	31 (52)	4 (21)	20 (33)
Nitroglycerin	10 (53)	28 (47)	8 (42)	19 (32)	8 (42)	21 (35)	4 (21)	20 (33)	12 (63)	34 (57)

* Renin-angiotensin aldosterone system (RAAS) inhibitor (includes 16 patients taking an ACEI and 3 patients taking an ARA); † Hydroxymethyl-glutaryl-CoA (HMGCoA) reductase.

Basal $^{\cdot}O_2^-$ production in human IMA was weakly associated with patient age (r = 0.19, p<0.1). No other relationships existed between basal $^{\cdot}O_2^-$ production in either IMA or SV, and any other patient risk factor. The profiles of risk factors and different classes of drug therapies were similar between patients who were taking an ACE-Ior ARA, compared to those who were not taking these therapies. An ACEI or ARA was prescribed in 16 and 3 patients, respectively. The median rate of production of $^{\cdot}O_2^-$ in IMA was 1137 (interquartile range, IQR 1290) pmol/min/mg. Superoxide concentrations were lower in those patients taking either an ACE-I or an ARA (857, IQR 670 pmol/min/mg; n= 19) compared to those who were not (1600, IQR 1511 pmol/min/mg; n= 60; p=0.002; 95% confidence interval for median difference 487, 1228 pmol/min/mg [Figure 8.1]).

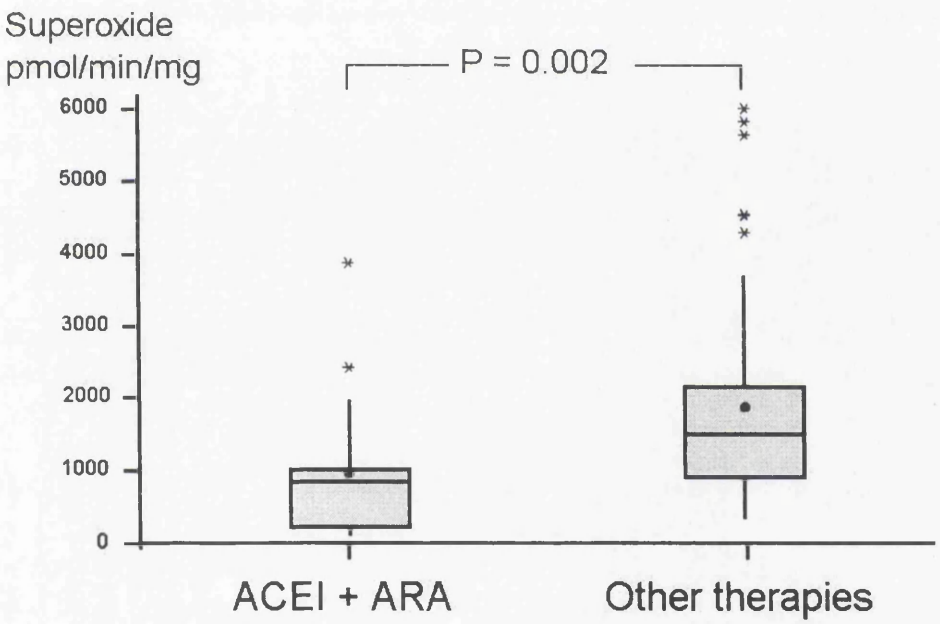


Figure 8.1 Box-plot graphical representation of superoxide concentrations (pmol/min/mg) in IMA from patients undergoing coronary artery bypass surgery who were taking either an ACE-I or an ARA, compared to those patients not taking these therapies. Means are indicated by solid circles, rectangles represent the lower and upper limits of the interquartile range and median values are demarcated inside the

rectangles. The vertical lines (or "whiskers") represent the spread of the data. The upper line represents the upper, or 3rd quartile, plus 1.5 (interquartile range) and the lower line represents the lower, or 1st quartile, minus 1.5 (interquartile range). The asterisks (*) represent outlying values which lie between 1.5 and 3 times away from the middle 50% of the data.

No other associations between age, sex, risk factors, or individual drug therapies, and $\cdot O_2^-$ concentrations were identified.

8.4 Discussion

In this analysis, we demonstrated that $\cdot O_2^-$ concentrations were lower in patients treated with either an ACE-I or an ARA, compared to those who were not. The clinical characteristics of both of these groups were similar such that the observed differences in vascular $\cdot O_2^-$ concentrations were unlikely to be explained by any other patient characteristic or therapy. It is of interest that despite the fact that a large proportion of these patients were taking drugs with putative anti-oxidant properties, such as aspirin, beta-blockers and HMGCoA reductase inhibitors, vascular free radical concentrations were detected at physiologically important concentrations. The absence of any important anti-oxidant effect of these other therapies suggests that the sample size may not be sufficiently large to detect what may be a lesser anti-oxidant effect of these drugs.

In the current study, basal $\cdot O_2^-$ concentrations in IMA were weakly related to patient age. The variation in basal vascular $\cdot O_2^-$ concentrations observed in this and other human studies (Guzik *et al* 2000b; Huraux *et al* 1999), and the lack of correlation of $\cdot O_2^-$ production with some atherosclerotic risk factors, treatment with nitrates and other therapies, may be due to the heterogeneous clinical characteristics of patients with CAD. Such patients have differences in their genetic background, their atherosclerotic risk factors, their disease duration and severity, and their drug therapy.

Our observations raise two questions. The first is how does such treatment exert this effect? Though some ACE-Is may have direct free radical scavenging properties this effect has been difficult to show at therapeutic concentrations in humans (Chopra *et al* 1992). A more plausible explanation is that the anti-oxidant effect of this therapy is

due to inhibition of the effects of Ang II. The second question is what, if any, might be the therapeutic significance of this effect of RAAS inhibitors? A reduction in vascular free radical production associated with RAAS inhibition, as is the case in the current study, may lead to enhanced bioavailable nitric oxide.

Taken together, these observations suggest that RAAS inhibition leads to a reduction in oxidative stress in patients with CHD. Given the damaging effects of increased free radical activity in the vasculature, the anti-oxidative effects of these therapies may be one further mechanism which may contribute to their beneficial effects in patients with CHD.

9 General discussion

The objectives of this thesis were to characterise the sources of $\cdot O_2^-$ production in human blood vessels, and the mechanisms involved in ROS generation. The preliminary findings of this investigation demonstrated that $\cdot O_2^-$ concentrations could be measured in human conduit blood vessels *ex vivo* in a reproducible fashion. The principal findings of this thesis include evidence of higher basal $\cdot O_2^-$ concentrations in human arteries compared to those in veins, and that both NAD(P)H oxidase and XOR enzymes contribute to basal $\cdot O_2^-$ production in these vessels. By contrast, NOS appeared to contribute to vascular $\cdot O_2^-$ production only in some individuals. In addition, it was demonstrated that SOD protein levels are quantitatively similar in human IMA and SV. In subsequent studies, it was shown that Ang II augments vascular $\cdot O_2^-$ production by AT_1 receptor-dependent, NAD(P)H oxidase-mediated pathway. Immunodetection studies demonstrated this enzyme to be present throughout the vascular wall. Additional observations from other molecular investigations support the thesis that this pathway involves an enhanced abundance of mRNA of the subunits of NAD(P)H oxidase, and an increase in protein synthesis. Furthermore, inhibition of the AT_2 receptor did not prevent Ang II-stimulated increase in $\cdot O_2^-$ production in IMA, suggesting that this receptor does not contribute to $\cdot O_2^-$ generation in human arteries. *In vitro* studies failed to demonstrate any contractile effect of Ang II-stimulated $\cdot O_2^-$ production in human subcutaneous resistance arteries. By contrast, multivariate analyses of the relationship between risk factors and treatments for CHD demonstrated that treatment with an ACE-I or ARA was associated with reduced vascular $\cdot O_2^-$ concentrations.

Superoxide production may be greater in IMA, compared to SV, because of the proportionally greater content of vascular smooth muscle in these arteries. One reason for this difference between conduit arteries and veins may be due to the presence of a thicker, muscular tunica media in arteries, where NAD(P)H oxidase was demonstrated to be abundant. Furthermore, that endothelial denudation had no effect on basal $\cdot O_2^-$ concentrations suggests that the endothelium is not an important source of $\cdot O_2^-$ generation overall. Alternatively, the observation that inhibition of eNOS, by treatment of blood vessels with L-NAME, and endothelial denudation, tended to

reduce $\cdot O_2^-$ concentrations in the same patients ($r = 0.85$; $n=12$, $p<0.001$. [see Section 3.3.3), suggests that the endothelium, and eNOS in particular, may be an important source of $\cdot O_2^-$ generation in some, but not all, individuals.

This possibility is supported by studies of endothelial function in both experimental animals and humans. In studies of the SHRSP compared with normotensive control animals, Kerr *et al* (1999) demonstrated that the vascular endothelium was a source of enhanced $\cdot O_2^-$ generation in aortic segments from the SHRSP, in which there was an enhanced abundance of NOS mRNA. Treatment with tetrahydrobiopterin, a co-factor for L-arginine biosynthesis, attenuated the difference in $\cdot O_2^-$ generation. Earlier investigations by our group (Grunfeld *et al* 1995; McIntyre *et al* 1999) demonstrated impaired endothelium (NO)-dependent vasorelaxation in the SHRSP, compared to normotensive control animals. Taken together, these observations suggest that in this model of genetic hypertension, abnormalities in vascular function may be due to enhanced $\cdot O_2^-$ generation within the endothelium. In humans, endothelial dysfunction, or impaired NO-mediated vasodilatation, has been demonstrated a variety of disease states such as hypertension (Nakazono *et al* 1991), hypercholesterolaemia (Creager *et al* 1990), atherosclerosis (Cox *et al* 1989), diabetes (Calver *et al* 1992a), and heart failure (Kubo *et al* 1991). A reduction in bioavailable NO may be due to impaired production, increased removal, or an attenuation in its activity. Our observations suggest that, in this case, endothelium-dependent $\cdot O_2^-$ generation in human conduit blood vessels may be less important for NO breakdown than in other species.

By contrast, $\cdot O_2^-$ generation by VSMC and adventitial cells may be more important in terms of NO degradation in human blood vessels. This is supported by observations from our immunohistochemical studies which demonstrated that NAD(P)H oxidase and XOR appeared are most abundant within the vascular smooth muscle and adventitial layers in IMA and SV. The pro-oxidative activity of VSMC (Griendling *et al* 1994; Mohazzab and Wolin 1994), and the consequent effect on vascular tone (Nowicki *et al* 2000; Rajagopalan *et al* 1996b), is well recognised, however, the role of the adventitia as a source of ROS production has only recently emerged.

Pagano *et al* (1997a; 1998) explored the potential importance of adventitial $\cdot O_2^-$ production in the modulation of vascular tone. In one of these studies (Wang *et al*

1998), rat thoracic aortic rings were mounted in an organ bath such that either the adventitial or endothelial surfaces of the artery were orientated outwards. This was achieved by carefully inverting the artery over a rubber tube. In this study, therefore, there were three groups, including a control group of arteries which had been inverted twice to control for any cellular injury induced by this manipulation. The presence of an intact endothelium was determined by treatment with ACh (10^{-6}mol/L). Arteries were stretched to attain a resting tension of 5g, and after equilibration for 1 hour, they were pre-contracted with PE(10^{-6}mol/L). In order to assess NO-dependent and independent vasorelaxation in conditions of normal or reduced vascular ROS activity, cumulative concentration response curves were performed with either exogenous NO (10^{-10} to 10^{-6}mol/L) or sodium nitroprusside (10^{-10} to 10^{-6}mol/L), in the presence and absence of SOD (150 U/mL). Studies in control vessels demonstrated comparable vasorelaxant responses were no different before or after inversion. NO-dependent vasorelaxation was less in rings with the adventitia facing outward, compared with those in which it faced inward, whereas the responses to sodium nitroprusside were not affected by the orientation of artery. This suggests that NO is inactivated to a greater extent when the adventitia is exposed externally, than when it is inverted internally. Furthermore, in the presence of SOD, this difference was attenuated. Further studies using DPI demonstrated that NAD(P)H oxidase was an important source of adventitial $\cdot O_2^-$ generation. This study suggests that the adventitia regulates bioavailable NO through generation of ROS. Other studies by this group in different animal species demonstrated NAD(P)H oxidase within adventitial fibroblasts to be a source of O_2^- generation (Pagano *et al* 1997b; Pagano *et al* 1998; Wang *et al* 1999).

These observations are consistent with our own findings. We failed to detect cytochemical markers for leucocytes within the adventitia of IMA. This suggests that fibroblasts are the cellular source of ROS generation in the adventitia of human conduit blood vessels. The adventitia has also been the focus of interventional studies, designed to modulate vascular tone. For example, Tsutsui *et al* (1998) demonstrated that *in vivo* infusion of an adenoviral vector encoding recombinant eNOS (AdCMVeNOS) results in high levels of transgenic expression in adventitial fibroblasts of infused arteries. This was associated with preserved vasorelaxation responses to bradykinin in endothelium-denuded arteries and increases in cGMP production.

The enzymatic sources of $\cdot O_2^-$ generation were also investigated in this study. Inhibition of either NAD(P)H oxidase or XOR, was associated with a reduction in $\cdot O_2^-$ concentrations in both arteries and veins. DPI is, however, an inhibitor of flavin-dependent enzymes, which include both NOS, and XOR (Sanders *et al* 1997). It is plausible, therefore that DPI may also have inhibited $\cdot O_2^-$ generation by these enzymes. This possiblity is unlikely, given that, for example, selective inhibition of eNOS by treatment with L-NAME, had no effect on $\cdot O_2^-$ concentrations in either IMA or SV. Our observations are also supported by those of Guzik *et al* (2000b) who demonstrated that treatment of IMA with similar concentrations of DPI resulted in comparable reductions in basal $\cdot O_2^-$ concentrations in both IMA and SV. The lack of specificity of this agent may be one limitation of this study, however, DPI remains the standard pharmacological tool used to investigate vascular $\cdot O_2^-$ generation by NAD(P)H oxidase. Current investigations in our laboratory to test the effects of apocynin, an antagonist which may cause more specific inhibition of NAD(P)H oxidase, in both rat and human blood vessels, confirm the central role of this enzyme for vascular $\cdot O_2^-$ production (Hamilton *et al* 2001).

Studies in animal models of vascular disease have provided evidence of the central role of NAD(P)H oxidase-mediated ROS generation in relation to vascular dysfunction animals (Rajagopalan *et al* 1996b; Zalba *et al* 2000). For example, in studies undertaken in genetically hypertensive rats, Zalba *et al* (2000) demonstrated that the enhanced $\cdot O_2^-$ generation measured in the aortae of these animals, compared with that of age-matched, normotensive controls, was NAD(P)H oxidase-dependent. Furthermore, this was also associated with increases in the abundance of p22phox mRNA, media thickness and cross-sectional area. Studies by Hamilton *et al* (2001) recently demonstrated that NAD(P)H oxidase – dependent $\cdot O_2^-$ generation was greater in old rats, compared to young rats. This in turn was associated with enhanced immunostaining for p22phox protein and reduced bioavailable NO in the old rats, compared to the young rats.

One of the principal findings of the present investigation was that treatment of human arteries with Ang II leads to enhanced $\cdot O_2^-$ production, by an AT_1 receptor-dependent mechanism. This effect was dependent on both the concentration of Ang II and the duration of exposure. Furthermore, we observed that treatment with even

physiological, or picomolar, concentrations of Ang II tended to increase $\cdot O_2^-$ production in IMA, suggesting that this mechanism may occur *in vivo.* These observations are consistent with other investigations of Ang II-stimulated $\cdot O_2^-$ production in cultured rat VSMC (Griendling *et al* 1994), and *in vivo* studies in rat models of hypertension (Rajagopalan *et al* 1996b; Zalba *et al* 2000) and a rabbit model of atherosclerosis (Warnholtz *et al* 1999). In studies of rat models of hypertension, co-treatment with an ARA improved elevated blood pressure and endothelium-dependent vasorelaxation (Rajagopalan *et al* 1996b; Zalba *et al* 2000), whereas in the rabbit atherosclerosis model, co-treatment with an ARA improved endothelial dysfunction, normalised vascular $\cdot O_2^-$ concentrations and NAD(P)H-oxidase activity, decreased macrophage infiltration, and reduced early plaque formation (Warnholtz *et al* 1999).

AT_1 receptor activation triggers a variety of intracellular signaling cascades with differing temporal courses, which result in the vasoactive effects of this hormone (Berry *et al* 2001a). Acute effects, for example vasoconstriction, are due to altered protein kinase C and phospholipase enzyme activity and increases in intracellular Ca^{2+} ion concentrations, whereas slower, more sustained effects, such as promotion of gene transcription, are due to the effects of tyrosine kinase activation and intracellular protein phosphorylation (Berry *et al* 2001a). In the case of of AT_1 receptor-induced NAD(P)H oxidase activation, both in cultured rat VSMC (Griendling *et al* 1994) and in intact human blood vessels (Berry *et al* 2000a), increased $\cdot O_2^-$ concentrations were detectable within one hour, and were sustained for over 4 hours.

The present study also included an investigation of the biochemical and molecular mechanisms that may be involved in Ang II – stimulated $\cdot O_2^-$ production in human blood vessels. Inhibition of NAD(P)H oxidase by treatment with DPI attenuated Ang II-stimulated $\cdot O_2^-$ production in human IMA, implicating this enzyme as the source of $\cdot O_2^-$ generation. This finding was consistent with those of other investigations in experimental animals (Rajagopalan *et al* 1996b; Warnholtz *et al* 1999). Subsequent molecular investigations in this study demonstrated that treatment of either cultured vascular cells or intact arteries with Ang II was associated with an increase in the abundance of mRNA of p22phox. Furthermore, in HAEC, this was prevented by co-treatment with either losartan, or actinomycin, and inhibitor of gene transcirption.

This latter finding suggests that Ang II may augment the rate of transcription of the p22phox gene in these cells.

Studies in Griendling's laboratory have investigated the intracellular siginaling pathways that may be involved in AT_1 receptor-mediated NAD(P)H oxidase activation. Griendling originally described a temporal relationship between the duration of exposure to Ang II and altered VSMC $\cdot O_2^-$ production (Griendling *et al* 1994). The latter increased within the first few minutes of treatment with 100 nmol/L of Ang II. VSMC $\cdot O_2^-$ production also increased, following a similar timecourse, after treatment with fatty acids, such as arachidonic and linoleic acids (Griendling *et al* 1994). Ang II stimulates prostaglandin metabolism, which results in hypertrophy of VSMC (Natarajan *et al* 1994). Given that ROS activation also results in trophic cellular changes (Rao and Berk 1992), it is plausible that the trophic properties of Ang II (Berk *et al* 1989), may be mediated, in part, by an AT_1 receptor – induced activation of prostaglandin metabolism and ROS production.

This thesis was explored by Zafari *et al* (2000). In these studies, cultured rat VSMC were treated with 100 nmol/L of Ang II, which was associated with increases in [^{3}H] AA and $\cdot O_2^-$ concentrations, and hypertrophy of these cells. These effects were attenuated by co-treatment of these cells with either 5,8,11,14-eicosatetraynoic acid, a general inhibitor of AA metabolism, or nordihydroguaiaretic acid, an inhibitor of lipoxygenase metabolism, but not indomethacin, which is a cyclooxygenase inhibitor. Further experiments demonstrated that these effects were phospholipase A2 dependent (Zafari *et al* 2000). In addition, some VSMC were transfected with antisense p22phox, in order to prevent Ang II-induced NAD(P)H oxidase activation (UshioFukai *et al* 1996). In this case, treatment with Ang II had no effect on AA metabolite or $\cdot O_2^-$ concentrations, and prevented hypertrophy of these cells. Taken together, these data indicate that AT_1 receptor-stimulated vascular cell $\cdot O_2^-$ production occurs by a phospholipase A2-mediated increase in lipoxygenase (and possibly CYP450 monoxygenase) metabolites, which in turn, mediate NAD(P)H oxidase activation. These metabolites induce NAD(P)H oxidase activation, by causing exposure of the phox SH3 binding domains, which is required for the assembly of these subunits (Sumimoto *et al* 1994).

More recently, studies by Lassegue *et al* (2001) in the same laboratory investigated the effects of Ang II on the differential expression of nox family members (i.e. gp91phox homologues) in rat VSMC. Treatment of these cells with different concentrations of Ang II lead to a dose- and time-dependent increase in the abundance of nox1, but not nox4, mRNA (EC_{50} 3nmol/L and a maximal response at 1μmol/L). The increase in nox1 mRNA was detectable within the first hour and increased to a maximum at 4 hours. This was sustained for up to 12 hours after exposure to 100 nmol/L of Ang II. Co-treatment with 12,13-phorbol myristate acetate, an inhibitor of protein kinase C, and transfection of antisense nox1, prevented these effects.

These investigations, taken together with observations from the present study, confirm that Ang II increases the abundance of mRNA transcripts for the phox subunits of NAD(P)H oxidase. It is not entirely clear, however, whether this effect is mediated by an increase in gene transcription, or a reduction in mRNA degradation as a result of enhanced stability of these molecules. In the present study, treatment of HAEC with actinomycin D prevented the Ang II-stimulated increase in abundance of p22phox mRNA, which suggests that Ang II may promote an increase in the rate of p22phox gene transcription. This possiblity is supported by observations in other studies of how Ang II promotes enhanced SOD activity (Fukai *et al* 1999). In these investigations, Ang II enhanced SOD protein expression in rat VSMC, through an increase in the rate of EC-SOD mRNA transcription and a prolongation of the half life of these molecules.

The data presented in this thesis are also supported by observations by Zhang *et al* (1999). In these studies, cultured human umbilical artery endothelial cells (HUVECs) were treated with micromolar and nanomolar concentrations of Ang II for up to 3 hours. In this case, O_2^- production peaked at 60 minutes. Furthermore, treatment of these cells with NADH was associated with a greater rise in O_2^- generation, than was observed by treatment with NADPH. This suggests that human vascular NAD(P)H oxidase has greater affinity for NADH, than NADPH. In summary, therefore, there are now compelling data which collectively underline the importance of vascular NAD(P)H oxidase-mediated ROS generation in vascular damage, both in animal models and in humans.

In the present study, it was also demonstrated that AT_2 receptor activation does not contribute to $\cdot O_2^-$ production in human arteries. Investigations in animal models previously demonstrated that stimulation of the AT_2 receptor is associated with increased generation of bradykinin (Siragy *et al* 1998), NO (Siragy *et al* 1998), and cGMP (Siragy and Carey 1996), all of which have vasodilatory properties. In the present study, the trend toward an increase in $\cdot O_2^-$ production in IMA treated with PD123319 suggests that this receptor may have a tonic, inhibitory effect on AT_1 receptor-dependent $\cdot O_2^-$ production. It is possible that, in humans, AT_2 receptor stimulation will lead to vasodilation through a signalling pathway that involves bradykinin and an increased production of NO. This, therefore, could be one mechanism whereby activation of the AT_2 receptor may lead to scavenging of ROS as result of NO generation. At present, however, data on the presence, distribution and functional activity of AT_2 receptors in human blood vessels are lacking. Recently, Ytterberg and Edvinsson (2001) failed to identify any AT_2 receptor mRNA in human subcutaneous resistance arteries obtained from healthy subjects. Furthermore, AT_2 receptor antagonism, by co-treatment of arteries (which had been denuded of endothelium) with PD 123319 (1, 10 nmol/L), had no effect on ANG II concentration-response curves (Ytterberg and Edvinsson 2001). Taken together, these *in vitro* observations suggest that in human resistance arteries, at least in healthy subjects, the AT_2 receptor may have little functional importance in these blood vessels. Other studies of this receptor elsewhere in the human cardiovascular system nevertheless remain important.

One other purpose of this investigation was to investigate the functional significance, if any, of Ang II-stimulated $\cdot O_2^-$ generation in human blood vessels. As discussed earlier (Chapter 7), data obtained from *in vitro* studies in subcutaneous resistance arteries did not support this thesis. The reasons for this are not entirely clear. As discussed in Chapter 7, the lack of any demonstrable effect of Ang II-stimulated to $\cdot O_2^-$ production in these protocols may be due to the vessel type, experimental conditions, or the fact that this pathway does not contribute to vasomotor tone in these arteries. Forearm blood flow studies in healthy subjects demonstrated that the reduction in forearm blood flow with infusion of Ang II was attenuated by co-infusion of vitamin C (Dijkhorst-Oei *et al* 1999). Although these observations suggest that the vasoconstrictor effect of Ang II may be partly due to enhanced ROS production, this

study did not quantify ROS concentrations in the blood of these patients. Further studies are therefore required to confirm this hypothesis.

Ang II has well documented trophic effects (Berk *et al* 1989), which are predominately mediated through tyrosine kinase activation, which in turn, promote enhanced gene transcription and protein synthesis (Berk and Corson 1997; Berry *et al* 2001a). Recently, however, a number of investigations have demonstrated that many of the intracellular trophic signalling pathways which are activated by Ang II, are in fact, regulated by oxidant activity (Berry *et al* 2001a). For example, AT_1 receptor-induced receptor transactivation, is effected by cSrc-mediated tyrosine phosphorylation, a process which is dependent on oxidant activity (Ushio-Fukai *et al* 2001). ROS are pleiotropic intracellular signals, which modulate a variety of pathways involved in vascular cell growth regulation (Irani 2000; Frank *et al* 2000a), and ROS may also be second-messengers for AT_1 receptor activation (Viedt *et al* 2000; Rao 1996; Eguchi and Inagami 2000a) (see Chapter 1, section 1.4.1). The activity of NFκB is also regulated by ROS (Barnes and Karin 1997). Recent studies have demonstrated that AT_1 receptor-induced ROS production can result in activation of NFκB which lead, in turn, to enhanced VCAM-1 expression and monocyte activation (Pueyo *et al* 2000). These observations arising from *in vitro* and experimental animal studies suggest that Ang II-stimulated $\cdot O_2^-$ generation in human blood vessels may well have non-pressor effects which could contribute to vascular inflammation and atherosclerosis.

Other observations in this study confirm that xanthine oxidase is important source of $\cdot O_2^-$ generation in human blood vessels. The chemical structure of xanthine oxidase incorporates both molybdenum and iron/sulphur and FAD redox centres, such that this enzyme may utilise both xanthine and NAD(P)H as reducing substrates for $\cdot O_2^-$ generation (Harrison 2000; Sanders *et al* 1997). Electron donation from reducing substrates occurs at the molybdenum site (see Chapter 1, section 1.1.2.1, Figure 1.4). The observation that XOR may contribute to basal $\cdot O_2^-$ generation in human blood vessels is perhaps surprising, given previous reports that XOR was present only under conditions of ischaemia-reperfusion (Gimpel *et al* 1995; Harrison 2000; Tan *et al* 1995). Under these conditions, circulating XDH is converted to XO, which has a greater potential for $\cdot O_2^-$ generation. It is possible that the blood vessels used in this

study may have been subject to ischaemia during surgical handling and laboratory manipulation, and under such conditions, XO would be activated. Nevertheless, our demonstration of XOR within the wall of IMA supports the concept that this protein is ubiquitously distributed throughout the cardiovascular system, and that under certain conditions, XOR can be an important source of $\cdot O_2^-$ generation. This thesis is supported by evidence of a functional activity of XOR both from studies in animal models (de Jong *et al* 2000; Ishimoto *et al* 1997; Nielsen *et al* 1994) and from *in vitro* (Patetsios et al 2001) and *in vivo* human studies (Cardillo *et al* 1997; Hellsten *et al* 1997).

Most recently, studies undertaken by Patetsios *et al* (2001) demonstrated uric acid and XOR protein to be present in carotid arteries obtained from human cadavers during organ harvesting. Furthermore, studies undertaken in excised carotid endarterectomy specimens demonstrated uric acid and XOR protein to be more abundant in carotid arteries affected by atherosclerosis, than in unaffected arteries obtained from cadavers. Only a limited amount of immunohistochemical data was presented in this paper, however, these authors did demonstrate staining with their polyclonal anti-XOR antibody within the medial layer of these arteries. These investigations should be interpreted with some caution as the specificity of this polyclonal antibody for XOR was not reported. By contrast, a monoclonal antibody to XOR was used in the present study. Nevertheless, taken together with our own observations, these findings do support a role for XOR in the pathogenesis of atherosclerotic vascular disease. This raises the question as to whether or not therapeutic inhibition of XOR, for example with allopurinol, might be might be cardioprotective in humans.

Given that XOR has NAD(P)H reducing properties, it is plausible that Ang II may augment vascular ROS production by activation of this enzyme. This possibility was explored by Zhang *et al* (1999). In these *in vitro* studies, however, the addition of xanthine to human vascular endothelial cells pre-treated with Ang II had no effect on O_2^- generation, suggesting that the activity of XOR may not affected by Ang II.

I also sought to characterise the endogenous ROS scavenging systems in human IMA and SV. In these studies, it was demonstrated that the total amount of EC and MnSOD proteins were quantitatively similar in human IMA and SV. This suggests that the

observed differences in $\cdot O_2^-$ concentrations between arteries and veins are due to enhanced $\cdot O_2^-$ production in arteries, rather than reduced scavenging in veins. One limitation of this study is the lack of information on SOD activity in these blood vessels. By contrast, other studies have demonstrated that the activity and amount of EC-SOD protein are significantly higher in human arteries than veins (Oury *et al* 1996). The differences between this and the present study could be explained by the use of different blood vessels and methods of protein quantification.

The fact that Ang II augments vascular ROS production raises the question as to whether or not this property might be shared by other vasoactive peptides. One candidate is endothelin, which is a pleiotropic hormone with potent trophic and pressor effects. Deuerrschmidt *et al* (2000) recently investigated whether or not endothelin (ET) might also have pro-oxidative effects. In this study, HUVECs were treated with 10 nmol/L of endothelin-1 (ET-1) for a up to 24 hours. ROS production by these cells was quantified using chemiluminescence techniques using both lucigenin (10 μmol/L), and the novel luminophore, coelenterazine. Treatment with ET-1 was associated with enhanced ROS production, which peaked at approximately 3 hours. This effect was attenuated by co-treatment with both SOD and DPI, suggesting that ET-1 stimulated O_2^- production by an NAD(P)H oxidase-dependent mechanism. In addition, RNA expression studies were also performed, using competitive RT-PCR techniques. Treatment of these cells with ET-1 resulted in a dose-dependent increase in the abundance of gp91phox, which was attenuated by co-treatment with the ET_B receptor antagonist BQ-788. These *in vitro* studies provide mechanistic information which suggests that ET may also promote ROS activity in human blood vessels.

The possibility that ET might stimulate $\cdot O_2^-$ production in human blood vessels was also investigated in other studies in our laboratory (Brett *et al* 2001). IMA and SV were incubated at 37°C with 100 nmol/L of ET-1 for 4 hours. The vasopressor effects of ET used in this study were confirmed in isometric tension studies undertaken in these blood vessels. Superoxide production was quantified by both lucigenin and coelenterazine - enhanced chemiluminescence. Using two different methods, $\cdot O_2^-$ concentrations in both arteries and veins treated with ET were found to be similar to vehicle-treated controls from the same blood vessels. The lack of effect of ET may be

due to a number of reasons. Firstly, it may be that although ET has been shown to stimulate ROS production in cultured HUVECs, this effect does not occur in intact human blood vessels. One other reason may be due to the possible ET-stimulated increase in vascular NO production (Hirata and Emori 1993; Ikeda *et al* 1996), leading to scavenging of O_2^-. Vascular NO concentrations were not quantified in this study. Other alternative explanations for the lack of effect of ET on vascular O_2^- concentrations include the concentration of ET used, or the duration of treatment. These observations do raise the possibility that the ability to stimulate vascular FR production may be a property specific to some (e.g. Ang II) but not all, vasoactive hormones.

Other findings in this study included the observation that patients who were treated with either an ACE-I or ARA had lower vascular $\cdot O_2^-$ concentrations compared with other patients who were not treated with these therapies (Berry *et al* 2000b). This finding supports our *in vitro* observations of AT_1 receptor-dependent $\cdot O_2^-$ generation in IMA. These observations are clinically relevant as physiological concentrations (pmol) of Ang II tended to increase $\cdot O_2^-$ production in human arteries and this effect was blocked by losartan. Inhibition of the RAAS has been proven to have beneficial therapeutic effects in a variety of cardiovascular disease states. However, ACE-I are used in the treatment of hypertension (Gohlke and Unger 1994), attenuate the progression of nephropathy in diabetic renal disease (Tripathi 1997), and reduce morbidity and mortality in chronic heart failure (Swedberg 1987; Yusuf 1991). ACE-I are now recognised to have therapeutic effects beyond simple blood pressure reduction. Evidence of this possibility was provided in data from the Studies of Left Ventricular Dysfunction Treatment Trial (SOLVD-T) (Yusuf 1991). In this trial, 2569 patients with mild to moderate CHF were randomised to either enalapril (n=1285) at doses of 2.5 to 20mg per day, or placebo (n=1284). During the follow-up period of 41 months, there were 510 deaths in the placebo group (39.7 %), as compared with 452 in the enalapril group (35.2 %) (relative risk reduction, 16 %; 95% CI, 5 to 26 %; p = 0.0036). Although reductions in mortality were observed in several categories of cardiac deaths, the largest reduction occurred among the deaths attributed to progressive heart failure (251 in the placebo group vs. 209 in the enalapril group; relative risk reduction, 22 percent; 95 % CI, 6 to 35 %). The addition of enalapril to conventional therapy reduced both mortality and hospitalisations for heart failure in patients with CHF. Surprisingly, however, there was a trend to a reduction in death

from myocardial infarction. In the placebo group, 53 deaths occurred, whereas in the enalapril-treated group deaths 40 deaths occurred (relative risk reduction 28% ; 95% CI, -8 to 52%, $p<0.07$). This unexpected observation suggested that ACE-I may have anti-atherogenic properties.

The cardiovascular protective properties of ACE-I were formally addressed in the Heart Outcomes Prevention and Evaluation (HOPE) study. The HOPE study (Yusuf *et al* 2000), was a randomised, controlled study of the effects of the ACE-I, ramipril, with or without vitamin E, compared to placebo, in patients at high risk of future cardiovascular events. In this trial, ramipril alone was associated with a 22% reduction (95% CI, 0.7 to 0.86; $p<0.001$) in all-cause mortality. There were significantly fewer patients who had a myocardial infarction in the ramipril-treated group (460), compared to the placebo group (567; relative risk 0.8; 95% CI 0.71 to 0.91 $p<0.001$). These results support the assertion that ACE-I have an anti-atherosclerotic effect. The mechanisms for this may include atherosclerotic plaque stabilisation, a reduction in vascular cell apoptosis, or an increase in bioavailable NO. All of these effects may also be promoted by a reduction in ROS activity, as may occur through treatment of patients with RAAS inhibitors, thereby attenuating the pro-oxidant vascular effects of Ang II (Berry *et al* 2001b).

The conclusions of the HOPE study have been challenged by others who have argued against the investigators' conclusion that the beneficial effects of ramipril were largely due to the non-pressor effect of this treatment (Gavras 2000; O'Rourke and Nichols 2000). However, a reduction of 3/2 mmHg in the ramipril-treated group, compared to the placebo group, could at most account for 25% of the difference in the primary endpoint between the two groups (Yusuf *et al* 2000). This suggests that the non-pressor, or anti-atherogenic, effects of this ACE-I may account for some of its beneficial effects in this study.

Ang II-stimulated $\cdot O_2^-$ generation may contribute to a variety of trophic and pro-atherogenic processes, which in turn, are involved in the pathogenesis of cardiovascular disease. The observations in the present study support the thesis that Ang II-stimulated $\cdot O_2^-$ generation may occur in human blood vessels. This suggests an additional pathophysiological effect of this hormone. Importantly, the observation that

ARAs may attenuate Ang II-stimulated $\cdot O_2^-$ generation in human blood vessels suggests a putative therapeutic role for ARAs in reducing oxidative stress in patients with cardiovascular disease.

9.1 Future studies

At present, the only study which has investigated the putative vasopressor effects of Ang II - stimulated O_2^- generation in humans *in vivo,* was that undertaken by (Dijkhorst-Oei *et al* 1999). These investigators used forearm strain gauge plethysmography to study the vasoactive effects of Ang II in the forearm circulation of healthy volunteers. They found that the vasoconstrictor effect of Ang II was attenuated by co-infusion of vitamin C. Further studies might attempt to reproduce these findings, both in health and disease. In addition, other studies might also be undertaken in order to investigate the importance, if any, of this pathway in the regulation of normal blood flow in other vascular beds, such as in the heart and skin.

Future studies may also investigate further the exact mechanism of how Ang II induces an increase in the abundance of phox mRNA in vascular cells. In order to address this question, other techniques which may be informative include, nuclear run-on analyses and RNA protection assays. Nuclear run-on analysis is a technique which yields specific information on gene transcription, whereas RNA protection assays yield information on RNA stability. Other studies may also investigate the effects, if any, of Ang II on translational, or post-translational pathways, which may also contribute to Ang II – induced NAD(P)H oxidase activation.

Finally, recent reports suggest that HMGCoA reductase inhibitors, in addition to inhibiting cholesterol synthesis, also have anti-oxidant properties. One mechanism for this effect, in addition to other established anti-oxidant properties (see Section 1.11.5.4) may be through direct inhibition of phox gene expression (Wassmann *et al* 2001). These preliminary findings in animal models merit further investigation in humans.

10 References

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