Nitric Oxide and Superoxide in Genetic Hypertension:

Effects of Age and Gender

by

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List of abbreviations

ACh: Acetylcholine

BAEC: Bovine aortic endothelial cells

BH_4: Tetrahydrobiopterin

$[Ca^{2+}]_i$: Intracellular calcium concentration

CaM: Calmodulin

cAMP: Adenosine-3',5'-cyclic monophosphate

cDNA: Complementary DNA

cGMP: Guanosine-3',5'-cyclic monophosphate

DNA: Deoxyribonucleic acid

EDRF: Endothelium derived relaxing factor

FAD: Flavin adenine dinucleotide

FISH: Fluorescent *in situ* hybridisation

FMN: Flavin mononucleotide

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GTP: Guanosine 5'-triphosphate

Hb: Haemoglobin

HUVEC: Human umbilical vein endothelial cells

LDL: Low density lipoprotein

L-NA: N^G-nitro-L-arginine

L-NAME: N^G-nitro-L-arginine methyl ester

L-NMMA: N^G-monomethyl-L-arginine

LPS: Lipopolysaccharide

mRNA: Messenger RNA

NADH: Nicotinamide adenine dinucleotide, reduced form

NADPH: Nicotinamide adenine dinucleotide phosphate, reduced form

NANC: Non-adrenergic non-cholinergic

NMDA: N-methyl-D-aspartate

NO: Nitric oxide

NOS: Nitric oxide synthase

$O_2^{\cdot}$: Superoxide anion

$ONOO^{-}$: Peroxynitrite

ox-LDL: Oxidised LDL

RNA: Ribonucleic acid

SHRSP: Stroke prone spontaneously hypertensive rats

SOD: Superoxide dismutase

UTR: Untranslated region

VSMC: Vascular smooth muscle cell

WKY: Wistar-Kyoto

Summary

Nitric oxide (NO) is now recognised as an important regulatory molecule in the cardiovascular system. NO is synthesised from the amino acid L-arginine by a family of three isoenzymes, the nitric oxide synthases (NOS). NOS III is the isoform constitutively expressed in the endothelium, but its expression can be regulated by shear stress and oestrogens among other factors. Endothelium derived NO seems to play a role in preventing atherosclerosis, but its role in hypertension is less clear. Some animal and human studies favour an important role, whereas others do not. Gender differences in cardiovascular disease, endothelial function and NO are well recognised, but the underlying mechanism remains unclear. There are theoretical reasons, and indeed experimental evidence, to implicate oestrogens, although again there is some debate about possible mechanism(s). The process of ageing is thought to be related to oxidative stress and hypertension has been described as an accelerated form of ageing, but the role of superoxide anion (O_2^-) and superoxide dismutase (SOD) in hypertension and ageing is only now becoming clearer.

To determine blood pressure and gender differences in NO availability, rings of thoracic aorta from 16 week old males and females, normotensive Wistar-Kyoto rats (WKY) and stroke prone spontaneously hypertensive rats (SHRSP) were studied in classic organ bath experiments. Stimulated NO availability was assessed by carbachol relaxation (10^{-8} - 10^{-5} mol/L) and basal NO availability was assessed by contraction to the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 100μmol/L). The contribution of the O_2^-/SOD system was assessed in the organ bath by examining the effects of inhibiting endogenous SOD with diethyldithiocarbamate (DETCA, 10 mmol/L) and adding exogenous SOD (45U/mL). *In vitro* NOS III activity was assessed in the 4 groups using the L-arginine/L-citrulline assay. Endothelial NOS gene (*Nos3*) expression was measured by reverse transcription-polymerase

chain reaction. Expression of intracellular copper zinc SOD (*Sod1*) and mitochondrial manganese SOD (*Sod2*) mRNA was measured by northern blotting and expressed as a ratio to glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA. The effect of age on endothelial function was assessed by measuring the response to L-NAME (100μmol/L) in aortic rings from the same 4 groups of rats at 4, 6 and 12 months of age. The effect of oestrogen was assessed by administering 17β-oestradiol (20μg per day, intraperitoneally, for 2 weeks) to a group of 1 year old WKY females and comparing basal and stimulated NO availability and *Nos3* gene expression, as above, with a group of controls.

In the organ bath, maximum relaxation to carbachol (E_{max}) was significantly greater in aortic rings from WKY compared to rings from SHRSP (p=0.015). Within the SHRSP strain the carbachol concentration causing 50% relaxation (EC_{50}) was significantly less in females compared to males (p=0.003). There were no other significant gender differences in carbachol E_{max} or EC_{50}. These data suggest that stimulated NO availability may be greater in WKY compared to SHRSP and perhaps SHRSP females compared to SHRSP males. The contraction of aortic rings to L-NAME was significantly greater in WKY compared to SHRSP (p=0.015). Further, this contraction in rings from WKY females was significantly greater than WKY males (p=0.00004). Similarly, the contraction in rings from SHRSP females was significantly greater than SHRSP males (p=0.0006). These data suggest that basal NO availability is greater in WKY compared to SHRSP and in females compared to males in both strains. The SOD inhibitor DETCA attenuated the relaxation to carbachol of SHRSP significantly more than WKY (p=0.0008). There were no strain or gender differences in the effect of adding exogenous SOD to the water bath. These data suggest that in aortas of SHRSP, SOD is more active, or O_2^- accumulates faster, or a combination of both.

NOS III activity was significantly greater in the aortas from SHRSP compared to WKY ($p=0.016$). In both strains NOS III activity tended to be higher in males compared to females, but the differences did not reach statistical significance. *Nos3* mRNA expression was greater in SHRSP compared to WKY, although the difference just failed to reach statistical significance when corrected for triple comparisons ($p=0.02$). Again there was a tendency for *Nos3* expression to be greater in males compared to females in both strains. These data combined suggest that the reduced NO availability in SHRSP is not due to reduced NO production. *Sod2* gene expression was significantly greater in SHRSP males compared to WKY males ($p=0.02$), suggesting that *Sod2* deficiency is not responsible for strain differences in NO availability. *Nos3* and *Sod2* may be upregulated to compensate for increased O_2^- in SHRSP.

In WKY females the response to L-NAME was significantly lower at 12 months compared to 4 months ($p=0.0009$). In the SHRSP females the response to L-NAME was also significantly lower at 12 months compared to 4 months ($p=0.0054$). In males from both strains there was no significant difference in the response to L-NAME between 4 months and 12 months. When 1 year old WKY females were treated with 17β-oestradiol, the response to L-NAME was greater compared to the control group ($p=0.05$). There was no significant difference in the carbachol E_{max} or EC_{50} when oestrogen treated animals were compared to controls. *Nos3* mRNA expression was significantly greater in aortas from WKY females at 12 months of age compared to aortas from WKY females at 4 months of age ($p=0.01$). 17β-oestradiol did not significantly affect *Nos3* expression in 1 year old animals.

It can be concluded from these studies that basal NO availability is reduced in SHRSP, in males and in older females. This does not appear to be due to reduced NO production,

suggesting increased scavenging by O_2^- instead. Differences in O_2^- are not explained by differences in SOD gene expression, and are most likely due to increased O_2^- generation within the vessel wall. Increased O_2^- is likely responsible for the age related decline in endothelial function. Gender differences in NO are likely due to oestrogen, but mechanisms other than increased *Nos3* expression are likely to contribute.

1 Introduction

1.1 Nitric oxide

For many years nitric oxide (NO) was regarded as a noxious pollutant in car exhaust fumes, fossil fuel smoke and cigarette smoke, responsible for acid rain and depletion of the ozone layer. However, interest in the physiological role of this simple diatomic molecule has risen exponentially in the 10 years since the endothelium derived relaxing factor (EDRF), first proposed by Furchgott and Zawadski in 1980[1], was identified by Palmer *et al* as NO[2]. Between 1981 and 1986, 10 papers relating to NO biosynthesis were published. In the next 5 years, there were more than 500 such publications. A computer search for the keyword 'nitric oxide' generated more than 4,500 publications in the world literature during the calendar year 1996. In 1992, interest was such, that NO was voted "molecule of the year" by *Science*.

Several independent lines of investigation led to this explosion. As far back as 1916, it had been realised from dietary balance studies that humans excrete more nitrate than they ingest[3]. This excess had been thought to be produced by intestinal micro-organisms and was thought to be of little relevance to mammalian biology. Several decades later in 1981, Green and colleagues showed that urinary excretion of nitrates was maintained in germ-free rats[4], and could therefore not be solely derived from bacterial metabolism. They also showed that the nitrate was being biosynthesised mainly outside the intestine. A marked increase in nitrates was detected in rats exposed to bacterial endotoxin, but was abolished in mice with a genetically determined absence of macrophages. A ninefold increase in excretion of nitrate was observed in a human subject during an episode of fever and diarrhoea. Thus, interest in this area was renewed. Around the same time, studies on carcinogenesis revealed that

mammalian cells metabolised L-arginine to produce a reactive nitrogen oxide compound[5]. Moreover, the cytotoxic and cytostatic actions of activated macrophages were found to require L-arginine as a substrate and N^G-substituted analogues of L-arginine or NO scavengers blocked cytotoxicity. Indeed, NO was identified as the effector molecule of these cytostatic functions[6].

In the field of neuroscience, interest was also growing. It had been known since the mid-1970's that neurotransmitters, especially excitatory amino acids, produce increases in guanosine-3',5'-cyclic monophosphate (cGMP) concentrations in many regions of the brain. This cGMP accumulation was abolished in the absence of extracellular calcium. Cyclic GMP is synthesised by the enzyme guanylate cyclase (EC 4.6.1.2), which is not regulated by calcium, therefore the mechanism underlying the cGMP rise was not clear. In 1988, Garthwaite *et al* [7] demonstrated that stimulation of N-methyl-D-aspartate (NMDA) receptors led to Ca^{2+}-dependent production of an NO-like material in brain slices which, in turn, stimulated soluble guanylate cyclase in the tissue. Also in the 1970's, work on autonomic non-adrenergic non-cholinergic (NANC) neurotransmission was also fitting into the jig-saw. The neurotransmitter turned out to be NO[8].

In the meantime, another fascinating story had developed in the cardiovascular area. The discovery of EDRF by Furchgott and Zawadski[1] and its subsequent identification as NO[2] fuelled the increasing interest. NO had brought together the disparate fields of immunology, neurobiology and vascular biology.

The physiological roles of NO are at least as protean as those discovered for corticosteroids in the 1940s-1960s and eicosanoids in the 1960s-1980s - all three the products of haem containing oxygenases. In addition to being an important immunological effector molecule[9],

NO has now been confirmed as a new class of neurotransmitter[10] and paracrine signalling molecule[11]. Classical mediator molecules have complex structures, are frequently stored in granules, released specifically, and depend for their action on a complimentary fit to a specific receptor. However, NO is a simple diatomic gas which is not stored and diffuses freely to its site of action, where it binds covalently to produce its effect. Carbon monoxide (CO) is another diatomic gaseous molecule in this class, which may act as a neurotransmitter and a regulator of cardiovascular function.

The property of NO which allows it to diffuse so easily in the environment of the cell is its solubility in water (2 mmol/L at 20°C and 1 atmosphere) and lipid. In solution, NO undergoes rapid oxidation to nitrite and nitrate with an estimated half-life of <4 minutes. In biological systems, NO has an estimated half-life of only 3-5 seconds as it interacts with superoxide anion and haem-containing proteins.

Because of this very short half-life *in vivo*, there is some debate about whether NO can exist long enough to accomplish its biological actions. However, NO has a diffusion coefficient 1.4-fold higher than O_2, and can diffuse over the 100μm or so from the endothelium to the underlying vascular smooth muscle cells within a few seconds. Nonetheless, some investigators feel that the molecule must be stabilised in some way by binding to high or low molecular weight thiol groups. There are theoretical reasons why NO cannot be transported in a more stable form. Classical receptors can easily distinguish between two closely related molecules produced by distant endocrine systems (e.g. adrenaline and noradrenaline) because of subtle differences in their shape. With only two atoms, NO encodes information not by its shape, but by changes in its local concentration. If NO were transported in a more stable form, the target guanylate cyclase would become saturated, thus obliterating any

information being transferred. Therefore, stabilisation of NO is not necessary and may even be detrimental.

Although not thought to be stored, there is evidence that NO can exist in the circulation bound to macromolecules such as albumin and haemoglobin (Hb), which retain some biological actions by releasing active NO via low molecular weight S-nitrosothiols. The interaction of NO with haemoglobin is via two highly conserved sulphydryl groups. The formation of nitroso-Hb is favoured when haemoglobin is in the oxy-Hb state, whereas dissociation of NO is more likely in the deoxy-Hb state. This interaction with haemoglobin may permit NO to become associated with haemoglobin in the pulmonary vasculature and to be released from haemoglobin in the systemic circulation[12].

The vascular regulatory and neurotransmitter functions of NO share a common mechanism. NO diffuses from the endothelium to the vascular smooth muscle layer or across the synapse. After entering the target cell, NO binds to the haem moiety of guanylate cyclase and activates the enzyme[13] by inducing a conformational change that displaces iron out of the plane of the porphyrin ring. Guanylate cyclase then catalyses the production of cGMP from guanosine 5'-triphosphate (GTP). Direct evidence has now been obtained that NO mediates glutamate stimulation of cGMP formation in the cerebellum via NMDA receptors[14].

Cyclic GMP is believed to elicit vascular smooth muscle relaxation through several mechanisms. One important mechanism involves reducing $[Ca^{2+}]_i$, but the precise mechanism for this remains poorly understood. Cyclic GMP-dependent kinase is thought to be involved through inositol 1,4,5-triphosphate (IP_3) production and action; Ca^{2+}-ATPase activation; activation of Ca^{2+}-activated K^+ channels; and even via alteration of the

cytoskeleton. Another proposed mechanism again involves cGMP-dependent protein kinase phosphorylation, and thus inactivation, of the enzyme myosin light chain kinase (MLCK). This, in turn, reduces cross-bridge cycling of the 20kDa myosin light chain (LC_{20}), and hence causes relaxation.

A recent, elegant study by Bolotina *et al* [15] demonstrate a novel direct action of NO on Ca^{2+}-dependent K^+ [K^+(Ca)] channels. They showed that, even when guanylate cyclase was inhibited by methylene blue, considerable NO-dependent relaxation of rabbit aorta persisted. This relaxation was blocked by the specific K^+(Ca) channel inhibitor, charybdotoxin. They consolidated these findings by showing that, in cell-free membrane patches, NO can directly activate K^+(Ca) without requiring cGMP.

Endothelium-derived NO can also diffuse into the lumen of the vessel where it can prevent platelet aggregation[16] and their adhesion to the endothelium[17,18] by a cGMP-dependent mechanism[18]. This is thought to be one mechanism whereby NO prevents atherogenesis and will be discussed in more detail in section 1.1.3.4.

Interestingly, guanylate cyclase is inhibited by physiological concentrations of $[Ca^{2+}]_i$, which would minimise cGMP production in cells stimulated to produce NO by agonist induced increases in $[Ca^{2+}]_i$. This may, therefore, represent a control mechanism by which activated cells release NO to stimulate guanylate cyclase selectively in target cells.

The effects of the relatively large amounts of NO produced by macrophages are more complex and less well understood. These effects will be discussed in section 1.1.1.2.

1.1.1 Nitric oxide synthases (EC 1.14.13.39)

NO is synthesised from the abundant amino acid L-arginine by the nitric oxide synthase (NOS) family of oxidoreductases[19], through a hitherto unrecognised pathway - namely the L-arginine-nitric oxide pathway. In the presence of molecular oxygen and reducing equivalents in the form of nicotinamide adenine dinucleotide phosphate (NADPH), one of the guanidino nitrogen atoms of L-arginine undergoes a five-electron oxidation to yield the free radical NO and L-citrulline[19] via an N^G-OH-L-arginine intermediate (Figure 1). Interestingly, L-citrulline has been shown, in endothelial cells at least, to be recycled back to L-arginine by the incorporation of one nitrogen atom from urea[20], which not only regenerates substrate for further NO production, but also eliminates excess nitrogen created by the cell's metabolism (Figure 1).

There are three known isoforms of NOS (Table 1, Figure 2), which are products of three distinct genes[21-23]. The unique characteristics of each isoform will be discussed in detail below, but as they each catalyse the same reaction, it is no surprise that they share several common features. The C-terminal domain of each isoform shows significant homology with NADPH cytochrome P-450 reductase (Figure 2). For this reason, and also because of its rare spectral characteristics, NOS was thought to belong to the P-450 superfamily of enzymes. However, NOS lacks the decapeptide around the haem binding cysteine present in almost all P-450s characterised, which is not surprising considering that the substrates for the P-450s are hydrophobic molecules, whereas L-arginine is very hydrophilic. It would therefore appear that NOS lies outside the P-450 superfamily and has converged on using the P-450 chromophore for the chemistry that it carries out. All NOS isoforms have binding sites for, and rely for their action on, the cofactors flavin mononucleotide (FMN)[21], flavin adenine dinucleotide (FAD)[21], haem[24], calmodulin (CaM)[25] and tetrahydrobiopterin (BH_4)[26].

As NOS is known to be a haemoprotein and NO is known to interact with such haemoproteins[12], it is thought that NO exerts a negative feedback inhibition of NOS[27].

The importance of NOS is indicated by its evolutionary conservation. NOS enzymes have been identified in many vertebrates and even invertebrates. A Ca^{2+}/CaM-dependent NOS has been identified in Apis mellifera and Drosophila melanogaster. In Drosophila, the gene has been cloned and termed dNOS. The dNOS cDNA encodes a protein of 152kDa with 43% amino acid identity with rat neuronal NOS. Like the mammalian protein, dNOS contains putative binding sites for CaM, FAD, FMN and NADPH. In Drosophila NO has been shown to regulate fluid secretion in the Malpighian tubule and is also involved in controlling the size of body structures during development. NOS also shares some homology with flavohaem proteins found in yeasts and bacteria, suggesting conservation for 1.8 billion years, provided that cross-species gene transfer has not occurred.

The mammalian genes are very highly conserved. In general, the same isoforms from different species are essentially identical. For example, there is 94% sequence identity between bovine and human NOS III[28]. By contrast, the different isoforms from the same species share only 50-55% sequence identity with each other[28-30]

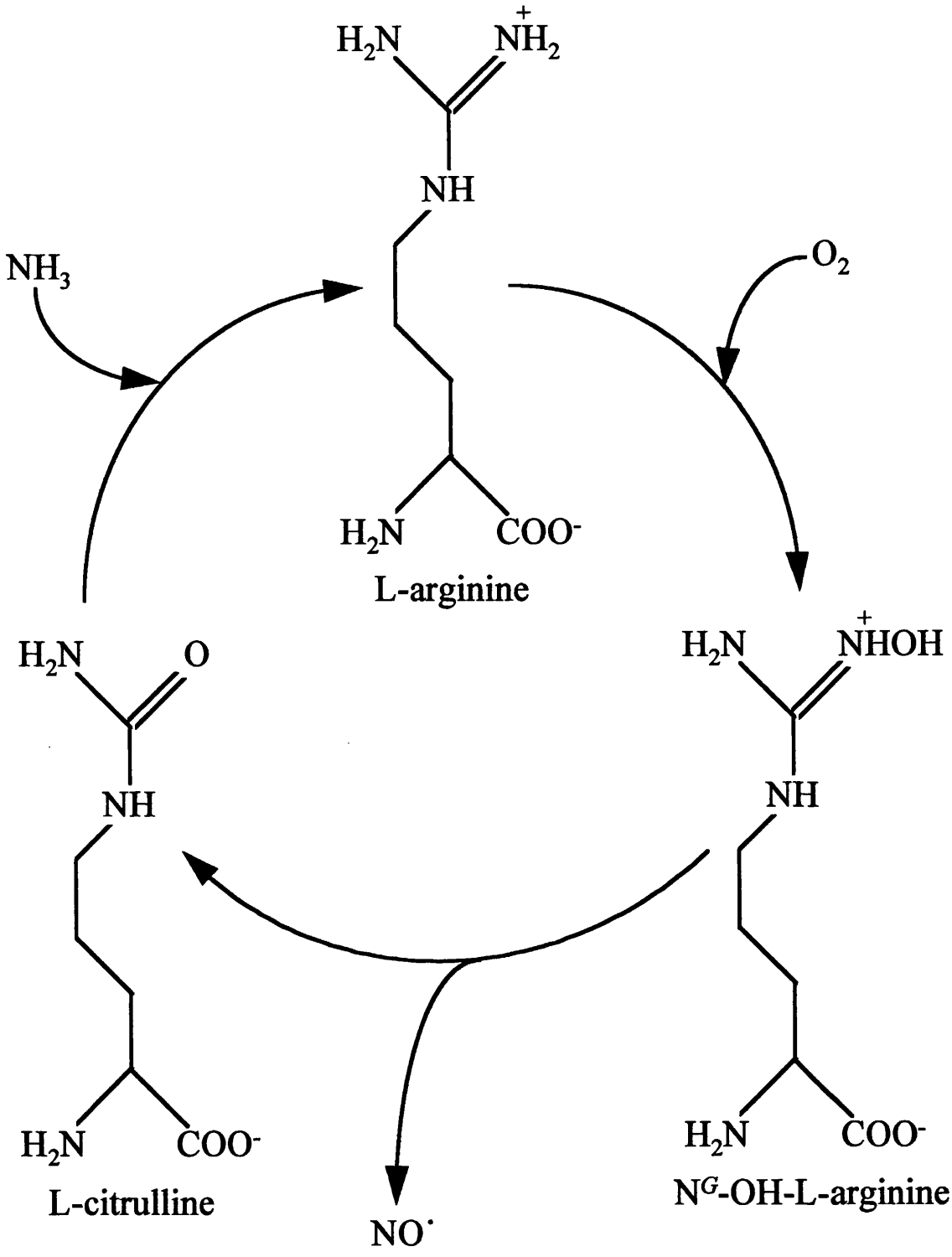

Figure 1 - Biosynthesis of nitric oxide (NO) from L-arginine, via N^G-OH-L-arginine, by incorporating molecular oxygen (O_2). L-citrulline can be recycled back to L-arginine by incorporation of ammonia (NH_3).

Table 1 - Characteristics of the three human nitric oxide synthase isoforms

Gene	Enzyme Nomenclature		Expression	Chromosomal Localisation	Gene Structure	Protein M_r (kDa)	Enzyme Structure
	Old	New					
Nos1	nNOS, bNOS	NOS I	Constitutive	12q24.2 - 24.31	≈160kb, 29 exons	155	Dimer
Nos2	iNOS, macNOS	NOS II	Inducible	17cen - q11.2	≈37kb, 27 exons	125-135	Dimer
Nos3	eNOS, ecNOS	NOS III	Constitutive	7q35 - 36	≈21kb, 26 exons	135	Dimer

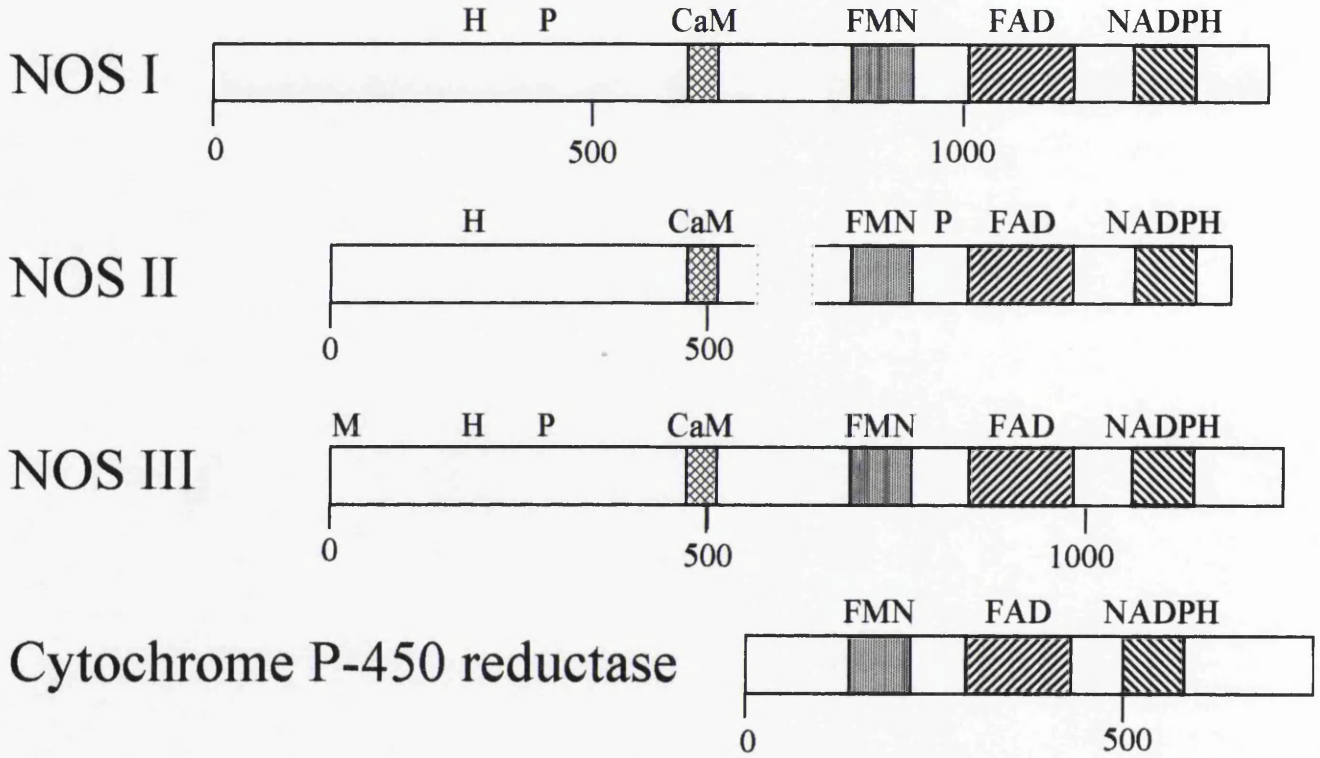

Figure 2 - Schematic representation of the three isoforms of nitric oxide synthase (NOS) and cytochrome P-450 reductase, showing alignment of the consensus binding sites for haem (H), calmodulin (CaM), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and reduced nicotinamide adenine dinucleotide phosphate (NADPH). Potential sites for phosphorylation by cAMP-dependent protein kinase (P) are shown. Note that a potential site for N-terminal myristoylation (M) is only present on the NOS III isoform.

1.1.1.1 Neuronal nitric oxide synthase (NOS I)

1.1.1.1.1 Biochemistry

Neuronal NOS was the first isoform of NOS to be isolated, and consequently the enzyme is termed NOS I, while the gene is termed *Nos1*, under the newly agreed nomenclature[31]. It was purified 6000-fold to homogeneity from rat cerebellum by a 2',5'-ADP column eluted with NADPH[25] and almost simultaneously isolated by another group from porcine cerebellum by ammonium sulphate precipitation and affinity chromatography on 2',5'-ADP-Sepharose[32]. The purified enzyme migrated as a single 150kDa band on sodium dodecyl sulphate/polyacrilamide gel electrophoresis (SDS/PAGE). The native enzyme initially appeared to be a monomer[25], although subsequent evidence suggests that the enzyme is cytosolic and exists as a homodimer under native conditions[33].

1.1.1.1.2 Physiology

NOS I is a highly regulated enzyme. As mentioned above, it was found to have an absolute requirement for CaM[34], which is the first indication that its activity is dependent on $[Ca^{2+}]_i$. The gene for the enzyme (*Nos1*) is constitutively expressed[25] centrally in discrete neuronal populations of the brain and posterior pituitary; and peripherally in the non-adrenergic non-cholinergic (NANC) nerves of the myenteric plexi of the intestine, adrenal medulla and perivascular nerves[34]. Therefore, in these cells the enzyme is always present, but inactive at the resting $[Ca^{2+}]_i$ of ≈80 nmol/L. It becomes active when the $[Ca^{2+}]_i$ increases to above 500 nmol/L, allowing CaM to bind to, and activate, the enzyme.

A further potential mechanism for regulating NOS I is phosphorylation. The enzyme can be phosphorylated stoichiometrically by adenosine 3',5'-cyclic monophosphate (cAMP)-dependent protein kinase, protein kinase C and calcium/calmodulin-dependent protein kinase, with each kinase phosphorylating a different serine residue on NOS I. Activation of protein kinase C in cells transfected with *Nos1* cDNA reduces activity of NOS I by ≈77% in intact cells and by ≈50% in protein homogenates from these cells. As the enzyme is cytosolic, phosphorylation must reduce NOS I activity independently of membrane binding.

In the brain, the stimulus for the increase in $[Ca^{2+}]_i$ is glutamate binding to the N-methyl-D-aspartate (NMDA) receptor[35]. In the hippocampus, this is thought to be associated with long-term potentiation (LTP) and memory. In the spinal cord, it is thought to be involved in nociception.

1.1.1.1.3 Pathology

In addition to these neuortrasmitter functions, NO is thought to be responsible for neurotoxicity in certain circumstances. The first indication that this was the case came from the observation that NOS containing neurons (identified by NADPH diaphorase staining and *in situ* hybridisation) are relatively resistant to the toxic effects of excitatory amino acids such as quinolinic acid and glutamate and ischaemia. These cells also selectively survive the degenerative processes in the corpus striatum of Huntington's Disease, suggesting that the gene defect is modifiable by the local biochemical environment. They are also relatively resistant to the neurodegenerative processes of Alzheimer's disease and aluminium-induced neurofibrillary degeneration, which is thought to be a very similar disease. It was postulated that NOS containing neurons released NO to the surrounding cells in toxic quantities as many of the neurotoxic insults could be limited by NOS inhibitors, although ischaemic insult

seems to be affected less by NOS inhibition. The NOS containing neurons themselves may be protected by their high levels of NADPH, which is required for NOS function and could reduce oxidative neurotoxins.

Further evidence that NO is important in neurotoxicity comes from mice with targeted disruption of the *Nos1* gene[36]. These *Nos1* 'knock out' mice are resistant to neurotoxicity and sustain smaller infarct volumes 24 and 72 hours post-middle cerebral artery occlusion (MCAO). The infarct volume increased if the knock out mice were pre-treated with the NOS inhibitor nitro-L-arginine. This suggests that, whereas NOS I may be deleterious, NOS III may be protective. Indeed, *Nos3* 'knock out' mice sustain larger infarcts 24 hours post-MCAO, even when blood pressure is normalised with hydralazine. This effect was attenuated by nitro-L-arginine, confirming the neuroprotective effect of NOS III.

1.1.1.1.4 Molecular biology

The human *Nos1* gene was originally localised to 12q14-qter on chromosome 12 by using human-rodent hybrid cell line panels[37]. The same group later used fluorescent *in situ* hybridisation (FISH) to more accurately localise human *Nos1* to 12q24.2 - 24.31[38]. The structural organisation of the full length genomic sequence of human *Nos1* has been determined by restriction enzyme mapping, subcloning and DNA sequence analysis of bacteriophage- and yeast artificial chromosome-derived genomic DNA[29]. The open reading frame is encoded by 29 exons spanning at least 160kb, with translation initiation in exon 2 and termination in exon 29. The deduced amino acid sequence of human NOS I revealed 93-97% identity with rat NOS I[21,39], with binding sites for all of the co-factors being present.

1.1.1.2 Inducible nitric oxide synthase (NOS II)

1.1.1.2.1 Background

Mammals, including humans, excrete more nitrate in their urine than they ingest[3]. The original perception that this nitrate imbalance represented synthesis by intestinal flora, proved incorrect when it was determined that germ-free rats synthesised the same amount of nitrate as control animals. Subsequent reports demonstrated that injection of rats with *Escherichia coli* lipopolysaccharide (LPS) markedly increases urinary nitrate excretion[40]. This triggered the search for mammalian cells capable of synthesising nitrate which, in 1985, led to the discovery that macrophages could be induced with LPS to produce significant amounts of both nitrite and nitrate[41]. Further work demonstrated that L-arginine is the substrate for this pathway[9] and that the effector molecule is NO[6,9]. The pathway was also found to be stimulated by the endogenous cytokine interferon-γ (IFN-γ)[42]. The enzyme responsible for NO synthesis in LPS/IFN-γ-stimulated macrophages was reported to have been localised in the cytosolic fraction[42].

1.1.1.2.2 Biochemistry

This cytosolic NOS activity was purified 426-fold from an LPS/IFN-γ-stimulated mouse macrophage cell line by sequential anion-exchange, affinity, and gel filtration chromatography[43]. SDS/PAGE of the purified NOS, later termed NOS II[31], gave three closely spaced silver-staining protein bands between 125 and 135 kDa. L-arginine was confirmed as the substrate and, like NOS I, NADPH, BH_4, FAD and FMN were necessary for activity. Gel filtration chromatography indicated that the induced NO synthase was

catalytically competent as a dimer of ≈250 kDa, but could be dissociated into inactive monomers of ≈130 kDa in the absence of L-arginine, FAD, and BH_4.

1.1.1.2.3 Physiology

Unlike NOS I, addition of Ca^{2+} with or without CaM did not increase the activity of the purified enzyme, and NO synthesis was not altered by CaM inhibitors[43]. The apparent lack of dependence on Ca^{2+} and CaM for activity came to distinguish this so-called 'inducible' NOS from the 'constitutive' NOS I and NOS III. Despite this, when the gene encoding NOS II (*Nos2*) was cloned and sequenced from activated mouse macrophages[22], researchers were surprised to find a consensus CaM recognition site[44].

The answer to this puzzle was revealed by Cho *et al*, who showed that NOS II contains, as a tightly bound subunit, a molecule with the immunologic reactivity, high performance liquid chromatography retention time, tryptic map, partial amino acid sequence, and exact molecular mass of CaM[45]. In contrast to most CaM-dependent enzymes, NOS II binds CaM tightly without a requirement for elevated Ca^{2+}. This explains why NOS II appears to be regulated by transcription and activated simply by being synthesised.

1.1.1.2.4 Molecular biology

Nos2 cDNA has been cloned and sequenced from a variety of species and tissues[22,30,46-49]. When the *Nos2* cDNA was cloned from rat hepatocytes[46], and the sequence compared to that cloned from mouse macrophages[22], the predicted amino acid sequences were 94% identical. This was very suggestive that the NOS II enzyme was produced in different tissues by a single *Nos2* gene, and that the 6% difference observed was a species difference.

The *Nos2* cDNA was subsequently cloned from rat vascular smooth muscle cells[47] and the amino acid sequence was found to be identical to rat hepatocyte NOS II[46], confirming the existence of a single *Nos2* gene.

NOS II activity was demonstrated in human macrophages (RAW 264.7 cells) in 1992[50], but human *Nos2* cDNA was first cloned and sequenced from human chondrocytes[48] and hepatocytes[30], then later in vascular smooth muscle cells[49]. *Nos2* mRNA has now been identified in so many different tissues in different species that one may speculate that any mammalian cell can produce NOS II if the cocktail of pro-inflammatory agents and cytokines is right.

The full length genomic human *Nos2* gene was isolated on overlapping cosmid clones from a human genomic library using both the murine macrophage and the human hepatocyte *Nos2* cDNAs as probes[51]. All isolated cosmids were part of a single genomic locus and no other genomic loci were identified or isolated. Analysis of this locus indicated that the human *Nos2* gene is ≈37 kb in length and consists of 26 exons and 25 introns. Polymerase chain reaction analysis of a human/rodent genomic DNA somatic cell hybrid panel and FISH indicated that the human *Nos2* gene is located on chromosome 17 at position 17cen-q11.2. Similar analysis by another group[52] revealed the *Nos2* open reading frame to be encoded by 27 exons, with translation initiation and termination in exon 2 and exon 27, respectively. The 5'-flanking region contains several consensus sequences for the binding of transcription factors involved in the inducibility of other genes by cytokines. These include an IFN-γ responsive element and binding consensus sequences for nuclear factors NF-IL6 and NF-κB[49]. Interestingly, the human *Nos2* gene contains a shear-stress responsive element, which was also found to exist in human NOS III (see section 1.1.1.4), but not in murine NOS II.

NOS II differs from NOS I (and, as we shall see later, NOS III) in that the enzyme activity is negligible in unstimulated macrophages[41,42]. Enzyme activity appears about 4-6 hours after induction[42] and relies on RNA transcription and protein synthesis. When the enzyme has been induced, it produces large quantities of NO and its activity can be present for many days[42]. The large quantity of NO produced by NOS II is involved in host defence.

1.1.1.2.5 Immunology

The immunological properties of NO may have originated as an ancient first-line defence for metazoan cells against intracellular parasites (e.g. Leishmania major, Toxoplasma gondii, and Mycobacterium leprae), evolving only later for signalling. Knowledge of this antimicrobial principle can be traced back to the Sumerians, who used nitrite to cure meat and inhibit Clostridium botulinum.

In addition to immunity to parasitic infections, NO is involved in destroying tumour cells. NO gas under anaerobic culture conditions has been shown to be cytotoxic to guinea pig hepatoma cells, by causing intracellular iron loss, inhibition of mitochondrial respiration, inhibition of aconitase, inhibition of DNA synthesis and hence cytostasis in the tumour cells[6].

Despite these apparent toxic effects, NO does not directly attack DNA, inhibit the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), inhibit mitochondrial respiration, or inactivate iron/sulphur centres of tumour cells, unless it is allowed to oxidise to higher nitrogen oxides. The higher oxide of nitrogen most commonly implicated is peroxynitrite ($ONOO^-$)[53].

Peroxynitrite is formed by the diffusion limited reaction of O_2^- with NO[54] with the release of 22 kcal/mol Gibbs energy (thermodynamically equivalent to the hydrolysis of two ATPs), making the reaction essentially irreversible. The reaction rate for the formation of peroxynitrite has recently been determined to be $6.7 \pm 0.9 \times 10^9$ $M^{-1}.s^{-1}$, which is over 3-fold faster than the rate at which superoxide dismutase (SOD) removes O_2^- at physiological ionic strength (Figure 3)[55]. Only when NO is produced in micromolar concentrations, such as after NOS II induction, can it compete with SOD for O_2^- to produce toxic amounts of peroxynitrite. Similarly, NOS I can also produce micromolar concentrations of NO, and hence toxic amounts of peroxynitrite, when enough cells in one region produce NO simultaneously, such as during cerebral ischaemia. This would explain the neurotoxic effects of NOS I described in section 1.1.1.1.3. Therefore, peroxynitrite can be described as a binary toxin, which is assembled when cells make large amounts of both NO and O_2^-, as is the case with activated macrophages and neutrophils.

1.1.1.2.6 Pathology

As an adverse consequence of this immune function, NO has been implicated in a number of chronic inflammatory diseases including rheumatoid and osteoarthritis, inflammatory bowel disease and asthma. Indeed, treatment with NOS inhibitors reduces the degree of inflammation in rats with acute inflammation or adjuvant arthritis, whereas L-arginine increases it.

NO has now been established as contributing to septic shock[56]. Sepsis and its associated syndromes represent a potentially devastating systemic inflammatory response estimated to occur in 1% of hospitalised patients, 10-20% of whom die. Mortality approaches 60-90% in those who develop septic shock. Patients and animals with septic shock lose peripheral

vascular tone, and the responsiveness of vessels to constricting agents, both *in vitro* and *in vivo*, is diminished[56,57]. In patients with septic shock, the levels of NO metabolites in plasma are significantly elevated[58], and the infusion of NOS inhibitors in such cases[59] and in animal models of septic shock[60] can lead to a rapid and reproducible rise in systemic vascular resistance[61] where other vasoconstrictors are ineffective. However, this treatment can be associated with increased mortality[60], even to 100%[62].

Glucocorticoids have been used as anti-inflammatory agents since the early 1950's, but the precise mechanism of their anti-inflammatory action was poorly understood. As NO is involved in inflammation, the role of NOS II in glucocorticoid action was a theoretical possibility. This involvement has now been confirmed. Glucocorticoids have been shown to prevent NOS II induction, and hence NO production, in intact organs of the rat, macrophages, neutrophils, vascular smooth muscle cells and endothelial cells. This effect is via specific glucocorticoid receptors, and is not elicited by other steroid hormones such as progesterone. This effect can be shown clinically by normalisation of the increased NO exhalation in asthma by systemic or inhaled glucocorticoids. In contrast to inhibiting endotoxin induced NOS II by L-arginine analogues where mortality increases[60,62], inhibiting NOS II induction in rats with glucocorticoids 1 hour before and 2 hours after endotoxin administration prevented hypotension and reduced mortality from 40% to 0% in one animal study[62].

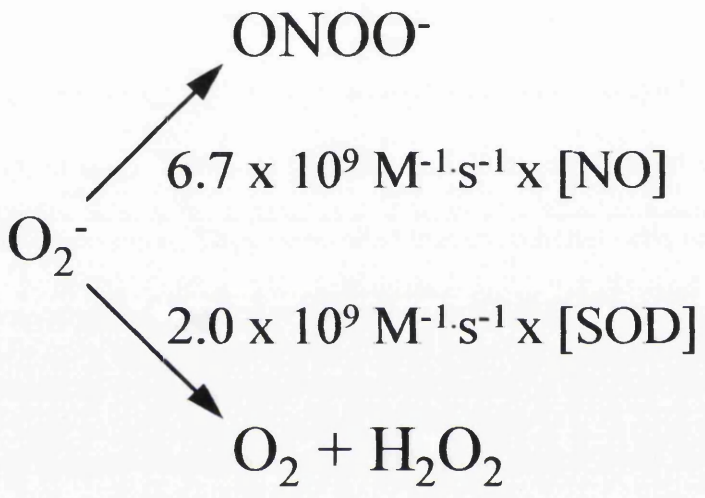

Figure 3 - The fate of superoxide (O_2^-) depends on its reaction rates with target molecules and the concentrations of those molecules. Superoxide dismutase (SOD) is usually present in micromolar concentrations, therefore this is the major route of O_2^- elimination. However, if nitric oxide (NO) concentrations rise into the micromolar range, such as after NOS II induction, then peroxynitrite ($ONOO^-$) will form preferentially due to the high rate constant.

1.1.1.3 Endothelial nitric oxide synthase (NOS III)

1.1.1.3.1 Background

For a long time the endothelium had been regarded as a simple inert barrier between the blood in the lumen and the underlying vascular smooth muscle cells. However, the endothelium is now known to be a metabolically and physiologically dynamic tissue with multiple functions. The first clue that the endothelium and NO may be important in cardiovascular regulation came in 1980, when Furchgott and Zawadski[1] demonstrated that an intact endothelium was essential for the vasodilator action of acetylcholine (ACh) in isolated arterial strips or rings. Removal of the endothelium prevented the relaxant effects of ACh and even led to contraction. They concluded that endothelial cells release a substance, which they called endothelium-derived relaxing factor (EDRF). They also demonstrated that its action was destroyed by oxyhaemoglobin and enhanced by superoxide dismutase. Unlike the other known endogenous vasodilator, prostacyclin, its activity was not inhibited by indomethacin.

Throughout the early 1980's the nature of this novel substance remained elusive. NO was a candidate[16], but there was much debate about whether such a simple molecule could be responsible for what would turn out to be such an important function[63]. Other candidates included S-nitrosocysteine, dinitrosyl-iron-cysteine complexes, nitrosyl and hydroxylamine[63]. Several studies suggested that EDRF and NO were identical[63], but others studies found differences, albeit subtle, between the two[64]. The definitive study by Palmer and colleagues in 1987 showed that NO exhibited all of the properties of EDRF[2]. In their study NO was determined by chemiluminescence and the biological activity of EDRF was determined by bioassay. EDRF and NO produced identical relaxation in bioassay tissues.

Both substances were equally unstable. Bradykinin caused dose-dependent release of NO from the cells in amounts sufficient to account for the biological activity of EDRF. The relaxations induced by EDRF and NO were inhibited by haemoglobin and enhanced by superoxide dismutase to a similar degree. All of theses observations were taken as sufficient evidence that EDRF was in fact NO.

1.1.1.3.2 Biochemistry

Despite the fact that endothelial cells were the first cell type described to carry out NO synthesis[2], the endothelial isoform (NOS III) was the third and last to be isolated. The main reason for this delay was that, whereas the other isoforms are present in the cytosolic fraction of the cell, NOS activity was located in the particulate fraction of endothelial cells. It was eventually purified 3419-fold to homogeneity from the crude particulate fraction of cultured and native bovine aortic endothelial cells by a 2',5'-ADP-Sepharose column followed by Superose 6 gel electrophoresis[65]. This resulted in a single protein band after denaturing PAGE that corresponded to ≈135kDa. Later studies suggested that NOS III exists as a homodimer[66].

This was the first example of a membrane bound NOS. The nature of the membrane binding was initially unclear, but when the bovine *Nos3* gene was cloned and sequenced[23], a myristoylation consensus sequence was identified at the N-terminus, which was not present on NOS I or NOS II. It was subsequently confirmed that endothelial cells metabolically incorporate myristate into NOS III[67]. As no recognised transmembrane domains were identified[23], this fatty acylation of the protein was thought to 'anchor' the enzyme into the membrane, either directly or via another membrane-bound protein. Further studies, using oligonucleotide-directed mutagenesis of the myristoylation consensus sequence of *Nos3*

cDNA, convincingly showed that myristoylation is critical for the membrane association of this isoform[68].

It is natural to assume that the membrane, with which NOS III associates, is the plasma membrane. However, recent electron-micrographic studies on endothelial cells from guinea-pig ileum submucosal arterioles, capillaries and venules showed NOS III immunoreactivity predominately in association with the Golgi complex and membranes of some vesicles. Only small amounts of immunoreactivity were present in association with the plasma membrane and rough endoplasmic reticulum[69].

NOS III has a consensus sequence for phosphorylation by cyclic AMP dependent protein kinase[23]. As alluded to with NOS I in section 1.1.1.1.2, Michel *et al* have demonstrated that NOS III in bovine aortic endothelial cells is serine phosphorylated when the cells are stimulated by a NO dependent agonist such as bradykinin[70]. They also showed that such phosphorylation changes the subcellular location from particulate to cytosolic. If, as suggested above, membrane association is important for NOS III activity , then phosphorylation and dissociation from the Golgi complex would result in reduced activity. This may represent a novel control mechanism to inactivate the enzyme once it is activated by an agonist. In addition to this potential post-translational control of NOS III function, there are many well described transcriptional control mechanisms, which will be discussed in section 1.1.1.5.

1.1.1.3.3 Molecular biology

The full length *Nos3* cDNA was first cloned from a bovine aortic endothelial cell (BAEC) cDNA library[23]. Sequencing revealed a 3615 bp transcript with consensus sequences for

CaM, FMN, FAD and NADPH binding, myristoylation, and phosphorylation by the cAMP-dependent protein kinase (PKA)[23]. Human *Nos3* cDNA was subsequently cloned from a human umbilical vein endothelial cell (HUVEC) cDNA library[28]. Two years later, the full length genomic sequence of human *Nos3* was determined[71]. Later that year *Nos3* was localised to the 7q35 - 7q36 region of human chromosome 7 by Southern blot hybridisation of human-rodent somatic cell hybrid lines and FISH[72], and independently by mapping a highly polymorphic dinucleotide repeat $(CA)_n$ within intron 13[73].

The human *Nos3* gene contains 26 exons spanning ≈22 kb of genomic DNA, encodes a messenger RNA of 4052 nucleotides, and is present as a single copy in the haploid human genome. The 5' promoter region is "TATA-less", which has been described for some housekeeping genes with multiple transcription start sites. The *Nos3* mRNA does not correspond to these criteria, but the presence of multiple other potential transcription factor binding sites within the promoter region could account for the lack of a TATA-like element. These other potential transcription factor binding sites include, among others, a shear-stress responsive element (SSRE)[71,73], which has been identified in the promoter region of other shear-stress inducible endothelial genes, and at least two oestrogen receptor binding elements[71], which have also been identified in the promoter region of bovine *Nos3*[74]. The significance of these particular response elements, with regard to *Nos3* expression, will be discussed in section 1.1.1.5.1.

1.1.1.3.4 Pathology

Alterations in the endothelial L-arginine NO pathway have been implicated in hypertension and other cardiovascular diseases. These will be discussed in greater detail in sections 1.1.2-3, but NOS III appears to be involved in pathology over and above cardiovascular control.

The role of NOS I in neurodegenerative diseases has been discussed in section 1.1.1.1.3. However, recent work has also implicated NOS III in the neuronal pathology of end stage Alzheimer disease and Down's syndrome[75].

1.1.1.4 The arginine paradox

There is an increasing body of evidence suggesting that L-arginine supplementation, either by feeding or intravenous infusion, is beneficial in animal models[76,77] and humans[78]. These effects of L-arginine appear to involve the NOS pathway as they are associated with increased tissue cGMP[76,77], and can be inhibited by the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA)[78]. It is tempting to conclude from these studies that a deficiency of L-arginine exists, such that this amino acid is rate limiting in terms of NOS activity. However, in cultured endothelial cells, intracellular concentrations of L-arginine are in the range 0.1-0.8mmol/L[20]. This is 35-275 times greater than the substrate concentration at which the NOS reaction velocity is half maximal (Km), which is 2.9μmol/L *in vitro*[65]. Therefore, it seems unlikely that that L-arginine could ever be rate limiting *in vivo*. The term "arginine paradox" has been coined for this discrepancy.

A clue to the solution may come from the fact that the L-arginine effect is usually only apparent when added *in vivo*, and not detected in *in vitro* studies[19]. A number of possible explanations exist as described below.

1.1.1.4.1 Asymmetric dimethyl arginine

An endogenous inhibitor of the enzyme may be present *in vivo*, resulting in an increased requirement for L-arginine. One such endogenous NOS inhibitor may be the L-arginine

metabolite asymmetric dimethyl arginine (ADMA). This has been shown to accumulate in hypercholesterolaemia[79] and chronic renal failure, perhaps contributing to the hypertension associated with this latter condition[80]. Indeed, part of the beneficial effect of oral L-arginine supplementation in hypercholesterolaemic rabbits is thought to be produced by correcting the plasma L-arginine/ADMA ratio[79].

1.1.1.4.2 L-Glutamine

As discussed in section 1.1.1, it has recently been discovered that endothelial cells can recycle L-citrulline back to L-arginine, which would prevent L-arginine becoming rate limiting for NOS even during sustained NO production[20]. The same researchers demonstrated that this recycling could be inhibited by the amino acid L-glutamine at concentrations below those encountered in plasma[20]. Therefore, the reduced L-arginine production in the presence of *in vivo* concentrations of L-glutamine may make L-arginine rate limiting.

1.1.1.4.3 Insulin

Another possible explanation for the *in vivo*, *in vitro* discrepancy exists. Giugliano *et al* recently demonstrated that when L-arginine is administered *in vivo* intravenously, insulin secretion is stimulated from the pancreatic β cells[81], which is a well recognised phenomenon. Insulin is known to have a vasodilator effect, which is NO dependent[82]. However, Giugliano *et al* have shown that the other beneficial effects of L-arginine, including reduced platelet aggregation, are insulin mediated, as they are abolished by octreotide, a somatostatin analogue which inhibits insulin secretion[81].

1.1.1.4.4 NOS independent NO generation

A further twist in the story has recently been added by Nagase *et al*, who have shown that L-arginine (and D-arginine) can interact with hydrogen peroxide (H_2O_2) to produce NO non-enzymatically[83]. H_2O_2 accumulates under conditions of increased oxidative stress such as hypercholesterolaemia, which may explain some of the beneficial effects of L-arginine on endothelial function under these conditions. The concentrations of L-arginine and H_2O_2 required to drive this reaction are in excess of 10 mmol/L, but the authors argue that such concentrations may exist within the atheromatous plaque.

1.1.1.4.5 L-arginine and NOS independent hypotension

There are some studies which suggest that L-arginine can lower blood pressure completely independently from the NOS pathway. The amino acid is also a substrate for arginine decarboxylase, which converts L-arginine to agmatine, an endogenous non-catecholamine ligand for central α_2 adrenoceptors[84]. Therefore, supplemental L-arginine may act as an α-agonist via agmatine.

In addition, Higashi *et al* showed that giving intravenous L-arginine at a rate of 500 mg/kg over 30 minutes reduced blood pressure in healthy young males by significantly reducing serum angiotensin converting enzyme activity and angiotensin II levels, with surprisingly no effect on plasma renin activity[85].

Therefore, there are many possible solutions to the arginine paradox, which are not all mutually exclusive. L-arginine may act via several non-NOS dependent mechanisms. Even as far as the NOS pathway is concerned, there are many possible competitive inhibitors, the

local *in vivo* concentrations of which we do not know, which can interfere with L-arginine transport and/or NOS activity.

1.1.1.5. Regulation of *Nos3* transcription

NOS III has traditionally been classified as a constitutive enzyme, not requiring protein synthesis for its action[19]. However, this has more recently been called into question[86]. As discussed in section 1.1.1.3.3 above, several features of *Nos3* are compatible with a constitutively expressed, poorly regulated gene, including absence of a TATA box and numerous Sp-1 sites. The promoter also contains a number of putative binding domains, which suggest that it may be regulated by a variety of transcription factor mediated signals. NOS III activity and *Nos3* mRNA levels have been found to be influenced by a number of factors indicating that the gene is constitutively expressed, but is subject to a modest degree of regulation by important physiological influences.

1.1.1.5.1 Shear stress

In 1921 Thoma first suggested that blood flow may regulate vessel tone[87]. Twelve years later Schretzenmayr demonstrated this experimentally[88]. It is now accepted that increased blood flow reduces arterial tone, whereas reduced flow results in vessel contraction. Therefore, when an artery is constricted pharmacologically or neurochemically, the increased blood flow and consequent shear stress cause a compensatory vasorelaxation to restore vessel tone. Although the arterial circulation does not consist of rigid tubes of circular cross-section, and blood is a non-Newtonian fluid, Hagen-Poiseuille's Law still holds that, under a laminar steady state flow, shear stress is inversely proportional to the third power of the vessel radius, but directly proportional to changes in flow and viscosity.

Therefore, relatively small changes in vessel diameter at constant flow can markedly affect shear stress within the vessel. The endothelium is now recognised as the principal vascular signal transducer that responds to changes in flow[89]. The major effector molecule of this pathway is NO[89]. The mechanism of mechanotransduction by the endothelium remains obscure. Alterations of the endothelial cytoskeleton have been implicated, and these possibilities have recently been reviewed[89].

There are at least two distinct mechanisms whereby shear stress increases NO production. A sustained increase in flow results in increased transcription of *Nos3* mRNA in endothelial cells and this will be described in detail later. In addition, there are a number of rapid and reversible signalling mechanisms which are responsible for the minute-to-minute adjustments of arterial diameter. Not all of these mechanisms are fully understood, but in cultured endothelial cells, there appears to be an initial Ca^{2+} dependent peak in NO release, which is independent of the magnitude of shear stress. This is followed by a more sustained increase, which is Ca^{2+} independent, but dependent on the magnitude of the shear stress stimulus. However, *in vivo*, it appears that the Ca^{2+} independent phase predominates.

A chronic increase in shear stress induces transcription of a number of genes, most notably *Nos3*. Several studies have been published recently examining the effects of chronic shear stress on *Nos3* expression[90,91]. Again the precise mechanism for this effect is also poorly understood, but Davies' recent, comprehensive review discusses what is known[89]. It seems that shear induced changes in cytoskeletal organisation activate second messenger systems, such as $[Ca^{2+}]_i$, which are coupled to "third messenger" transcription factors, such as immediate early response genes (IERG) including *c-myc*, *c-fos* and *c-jun*. These transcription factors in turn upregulate other transcription factors, such as nuclear factor kappa B (NFκB). *Fos/Jun* heterodimers interact directly with a nuclear factor activator

protein 1 (AP-1) *cis*-acting element, while NFκB may bind to a shear stress response element, both of which have been identified in the promoter region of *Nos3*[71-73] and other shear stress inducible endothelial genes.

There is an abundance of epidemiological evidence that regular aerobic exercise improves cardiovascular morbidity and mortality. Exercise has a number of potentially beneficial effects, which have recently been reviewed[92], including lowering percentage body fat, improving lipoprotein profile, improving carbohydrate tolerance and insulin sensitivity, reducing neurohormonal release (catecholamines, renin, aldosterone, vasopressin) and reducing blood pressure. Over and above these effects, exercise increases heart rate, blood flow and hence shear stress, which we now know increases endothelial NO production. This has been shown acutely in healthy subjects, detected by increased forearm vasodilatation and increased exhaled NO, which is likely due to increased activity of existing NOS III. It has also been shown chronically in animal studies in which 10 days of treadmill running increased *Nos3* mRNA expression in canine aortic endothelial cells and NOS III activity in coronary vessels[90].

Interestingly, shear stress may have other beneficial effects in preventing cardiovascular disease, including increased cytosolic copper zinc superoxide dismutase (Cu/Zn SOD) expression[93], which would reduce O_2^- levels (see section 1.2.2). Shear stress also appears to reduce leukocyte adhesion to the endothelium, which is an early event in atherogenesis.

1.1.1.5.2 Oestrogen

The oestrogen response element (ERE) was first described in 1987 by Klock *et al*[94]. They had previously identified the 15 bp glucocorticoid response element, which was also

recognised by the progesterone receptor complex. They subsequently defined another 15 bp palindromic sequence in the promoter region of the frog and chicken vitellogenin gene, which conferred oestrogen inducibility on a heterologous promoter[94]. Oestrogen interacts with a 67kDa intranuclear receptor, causing a conformational change, allowing it to bind with high affinity to EREs upstream from target genes, thus modulating their transcription. The precise binding of the ER to an ERE is realised through two Zn^{2+} binding motifs folded to form a single structural unit. ERE half-palindromic motifs bind the oestrogen receptor weakly, but several copies of the motif separated by more than 100bp can act synergistically to enhance transcription in response to oestrogen[95]

Since the gene for the human oestrogen receptor (ER) was cloned and sequenced over 10 years ago[96], searches for other ERs proved unsuccessful, suggesting only one existed. However, recently a second oestrogen receptor (ERβ) has been cloned from rat prostate and ovary[97] and human testis[98]. The unlikely sources of these cDNAs is perhaps the reason that ERβ has remained so elusive. The discovery of a second ER raises many important questions and these have been recently reviewed[99]. It would appear that the original receptor (ERα) and ERβ have a very similar high affinity for natural oestradiol, although some synthetic ligands show receptor selectivity[100]. The tissue distribution of the two receptors also appears to be different[100].

ERα mRNA and protein have been detected in HUVEC and BAEC[101], therefore this receptor was thought to be responsible for the effect of oestrogen on endothelial function. However, ERβ mRNA has now also been detected in mouse aorta by reverse transcription polymerase chain reaction (RT-PCR), although not specifically localised to the endothelium[102]. Of greater interest was the fact that the same authors showed in ERα

'knock out' mice (homozygous -/-, therefore expressing no ERα), that 17β-oestradiol still inhibited the extent of vascular injury after endothelial denudation of the carotid artery, as much as it did in wild-type mice[102]. They concluded that ERα could not be the sole receptor responsible for the vascular effects of oestrogen, or perhaps ERβ can substitute for ERα. However, the same oestrogen receptor 'knock out' (ERKO) mice have been shown to have reduced basal NO release[103], suggesting that ERα may be responsible for some oestrogen effects and not others. In humans, some of the vascular effects of oestrogen also appear to rely on an intact ERα. Sudhir *et al* have just recently reported that a 31 year old man with oestrogen resistance caused by a disruptive mutation in the ERα gene, has impaired endothelium-dependent vasodilatation of the brachial artery, with intact endothelium-independent vasodilatation[104].

The literature is now abundant in animal and human studies which examine the effects of gender and/or oestrogen on endothelial function and/or cardiovascular disease. These will be discussed in greater detail in section 1.3. *Nos3* has several copies of the ERE half palindrome in its promoter region[71,74], but only a few studies have directly examined the effects of oestrogen on *Nos3* expression (Table 2). Some of these studies show that the effect can be inhibited by tamoxifen, suggesting that it is mediated through the oestrogen receptor[105-107].

Table 2 - Studies examining the effects of pregnancy or 17β-oestradiol on *Nos3* expression.

Model	Intervention	Outcome	Ref.
HUVEC and BAEC	17β-oestradiol (10^{-12} - 10^{-8} mol/L for 8 hours)	Increased *Nos3* mRNA detected by northern blotting	105
Human aortic endothelial cells	17β-oestradiol (200 pg/mL for 8 hours)	Increased NOS III protein detected by western blotting	106
Guinea pigs	Pregnancy (last 13 days of gestation)	Increased *Nos3* mRNA in skeletal muscle detected by northern blotting	107
Guinea pigs	17β-oestradiol (500μg/kg/day i.p. for 5 days)	Increased *Nos3* mRNA in skeletal muscle detected by northern blotting	107
Rats	Pregnancy (last 2 days of gestation)	Two-fold increase in *Nos3* mRNA in aorta detected by reverse transcription polymerase chain reaction	108
Rats	17β-oestradiol (1.0mg/kg/day s.c. for 2 weeks)	70% increase in *Nos3* mRNA in aorta detected by reverse transcription polymerase chain reaction	108

HUVEC: human umbilical vein endothelial cells; BAEC: bovine aortic endothelial cells; i.p.: intra-peritoneally; s.c.: sub-cutaneously

1.1.1.5.3 Other factors

Atherosclerosis impairs endothelium-dependent vasodilatation. Oxidised low-density lipoprotein (ox-LDL), which is a key factor in atherogenesis (see section 1.1.3.3), was thought to be responsible for the impaired endothelium-dependent vasodilatation. However, *in vitro* experiments, using a ribonuclease (RNase) protection assay and western blotting in BAEC, have shown that ox-LDL, but not native LDL, increases *Nos3* transcription and NOS III protein levels[109]. The authors conclude that ox-LDL is not responsible for the impaired endothelium-dependent vasodilatation, although they do not consider the fact that NOS III function may be reduced.

1.1.2 Nitric oxide in hypertension

Human essential hypertension and several animal models of hypertension are associated with increased peripheral vascular resistance. The crucial structures of the circulation that determine peripheral vascular resistance are arteries of diameter <200μm; the so-called 'resistance arteries'. As NO is accepted as an endogenous vasodilator[2], there are theoretical reasons why reduced NO production or bioavailability would lead to vasoconstriction and hence increased peripheral vascular resistance. NO has been found to regulate the tone of normal vessels[110], including resistance vessels[111]. In addition, NO causes renal vasodilatation with consequent diuresis and natriuresis[112]. These actions would tend to lower blood pressure, therefore a reduction in this mechanism is another way in which NO deficiency may theoretically contribute to hypertension. However, there are many conflicting reports about the role of NO deficiency in experimental models of hypertension and human essential hypertension.

1.1.2.1 Nitric oxide in experimental hypertension

Since the identification of NO as an endogenous vasodilator[2], there have been many studies examining the role of NO in experimental hypertension. These have been comprehensively reviewed recently[113]. Some studies indicate reduced NO in hypertensive animals compared to normotensive controls, while others show increased NO or no difference. On balance, it would appear that the NO system may be overactive in the spontaneously hypertensive rat (SHR) model of genetic hypertension, but depressed in the related stroke prone spontaneously hypertensive rat (SHRSP) model and in experimental mineralocorticoid and renal hypertension However, some studies remain contradictory.

The reason for the above discrepancy remains unclear, but may reflect the different methods employed to assess the NO system in different studies. It may be that NO production is normal or even increased in experimental hypertension, but the bioavailability of NO may be reduced by a scavenger such as O_2^-. Grunfeld *et al* used organ bath experiments to demonstrate that excess O_2^- in aortas of SHRSP could exactly account for the reduced bioavailability of NO detected at the cell surface by their porphyrinic microsensor[114]. The following year, Tschudi *et al* confirmed normal NO production, but increased decomposition by O_2^- in the mesenteric resistance vessels of SHRSP[115]. Mian and Martin have shown that basal NO is more sensitive to destruction by O_2^- than stimulated NO[116]. They speculate that under basal conditions, endogenous SOD keeps O_2^- at such low concentrations that only the low levels of basal NO can be scavenged, but when NO is stimulated by ACh, there is insufficient O_2^- to scavenge it all. However, if endogenous SOD is inhibited, then the levels of O_2^- rise to such high levels that even ACh stimulated NO can be scavenged. The precise role of scavenging by O_2^- in other situations of apparent differences in NO, such as gender differences, remains to be determined.

Despite the large number of endogenous vasoconstrictors and vasodilators which can potentially affect blood pressure, the importance of the L-arginine/nitric oxide system is apparent in models where the system is manipulated. Exogenous L-arginine, but not D-arginine, normalises blood pressure in salt-sensitive Dahl/Rapp rats made hypertensive by high salt chow[117]. Conversely, NOS can be inhibited by a variety of L-arginine analogues[118] and this model of hypertension will be discussed in section 1.1.2.3. However, such pharmacological intervention affects all isoforms of NOS, therefore their individual contribution to blood pressure control cannot be distinguished.

Very specific interruption of the endothelial L-arginine/nitric oxide has recently become possible with the advent of mice with targeted disruption of the *Nos3* gene[119,120]. Homozygous -/- mutants have no NOS III protein in their endothelium, which has been confirmed by immunohistochemical staining[120]. These so-called *Nos3* 'knock out' mice have increased blood pressure compared to +/+ homozygotes and +/- heterozygotes[119,120] suggesting that NOS III is indeed important in blood pressure regulation, although they also have increased plasma renin concentration, despite a modest decrease in kidney renin mRNA[120].

An unexpected curiosity was revealed when *Nos3* 'knock out' mice were exposed to the NOS inhibitor L-nitroarginine (L-NA). One might have expected no change in blood pressure as they have no NOS III to block, or an increase in blood pressure if NO from other NOS isoforms is important in blood pressure regulation. Surprisingly, their blood pressure fell when treated with L-NA, while that of the +/+ homozygotes rose as anticipated[119]. The hypotensive effect of L-NA was prevented by L-arginine, but not D-arginine. These observations would suggest that one of the other NOS isoenzymes is producing a vasoconstrictor substance which can be blocked by L-NA. Alternatively, L-NA

may be exerting a completely different and unknown action. A candidate vasoconstrictor is O_2^-, which Pou *et al* have shown to be produced by rat NOS I, albeit when purified *in* [121]. They also showed that O_2^- production is inhibited by L-NAME, but not L-NMMA. It is conceivable that when NOS III is absent, as in *Nos3* 'knock out' mice, NOS I generated O_2^- predominates and contributes to the hypertension. Like L-NAME, L-NA may block NOS I generation of O_2^- and thus lower blood pressure.

Despite the implication from 'knock out' mouse studies that the *Nos3* gene is important in blood pressure regulation, using mapping studies Deng and Rapp failed to link the *Nos3* locus on rat chromosome 4 to hypertension in the Dahl salt-sensitive rat[122]. Instead, they found genetic linkage between hypertension and the *Nos2* locus on rat chromosome 10 in the same model[123], although the *Ace* locus, encoding angiotensin I converting enzyme, also mapped to this region. This implicates *Nos2* in genetic hypertension, but *Nos2* 'knock out' mice have also been generated, and while they have reduced immunity to parasitic infections in keeping with the known role of NOS II, they have normal blood pressure[124].

Another interesting model of genetic hypertension is the hypertension-prone Sabra rat (SBH) versus the hypertension resistant Sabra rat (SBN). SBH are more sensitive to deoxycorticosterone acetate-salt (DOCA-salt) than SBN. In addition, although still within the normal range, SBH have a significantly higher basal mean arterial blood pressure than SBN. Rees *et al* used the pressor response to L-NMMA in this model to convincingly show reduced basal NO in SBH[125]. Using the L-arginine/L-citrulline assay to measure NOS III activity, they demonstrated that the reduced basal NO was due to reduced production, although they did not exclude increased break-down in addition.

1.1.2.2 Nitric oxide in human essential hypertension

Similar controversy exists regarding the role of NO in human essential hypertension. There have been many studies employing forearm venous occlusion plethysmography to examine stimulated NO release in response to intrabrachial ACh. When essential hypertensive patients are compared to matched normotensive controls, the majority of the studies show a reduction in stimulated NO release[126-128], although another study found no difference[129]. Basal NO production, as measured by the reduced forearm blood flow in response to L-NMMA, has also been shown to be reduced in patients with hypertension compared to normotensive controls[130].

The use of forearm venous occlusion plethysmography to measure endothelium dependent vasodilatation may be prone to errors caused by effects of different basal blood flow and conduit vessel length[131]. These effects are seldom considered in these studies and may partly explain some of the above discrepancies. Using flow-mediated vasodilatation of the brachial artery as an alternative method of quantifying stimulated NO production, Laurent *et al* found no difference between essential hypertensives and normotensive controls[132].

In a further attempt to circumvent the limitations of forearm venous occlusion plethysmography, Forte *et al* employed yet another method of estimating NO production[133]. They fed a group of essential hypertensive patients, and a group of matched normotensive controls, a fixed low-nitrate diet for two days and thereafter found 24 hour urinary nitrate excretion to be reduced in the hypertensive group. They also administered the stable isotope L-[$^{15}N]_2$-guanidino arginine intravenously and found ^{15}N excretion also to be reduced in the hypertensive group. This is certainly more direct evidence for reduced NO synthesis in hypertensive patients, but gives no indication that NOS III is involved. NOS II could be

induced in these patients due to subclinical infection or inflammation, although attempts were made to exclude such patients from the study.

A polymorphism in the *Nos3* gene has been associated with smoking related coronary heart disease[134], and this will be discussed in greater detail in section 1.1.3.5. However, Bonnardeaux *et al* failed to find linkage between a highly polymorphic marker in the *Nos3* gene and hypertension in 145 hypertensive pedigrees (269 sib pairs, 346 subjects)[135]. They concluded that their data do not suggest involvement of common molecular variants of the endothelial nitric oxide synthase gene in essential hypertension.

Over and above the methodological problems, another reason for the discrepancies in the above studies may be that patients with essential hypertension represent a heterogeneous group in whom a small, as yet undefined, subgroup may have NO deficiency, while others have normal or even increased NO release as a compensatory mechanism. To distinguish those with NO deficiency from the rest of the hypertensive population in a practical way would require a simple screening test. Such a simple test may have been discovered by Jilma *et al* who measured NO in parts per billion (ppb) in a single breath and found it to be higher in females (34ppb) compared to males (20ppb)[136]. Such gender differences in NO production will be discussed later, but the point is that such a test, while subject to the vagaries of dietary nitrate and non-NOS III production, may be easily employed to screen large numbers of hypertensive patients. Confirmation of these data from other groups would first be necessary.

1.1.2.3 Hypertension induced by inhibition of nitric oxide synthase

There have been many animal studies examining the effects of acute or chronic blockade of NOS by a variety of L-arginine analogues, but they are too numerous to describe in any detail here. Without exception, they demonstrate sustained elevation of blood pressure, suggesting a role for NO, although not necessarily endothelium-derived NO, in blood pressure regulation. This relatively novel model of experimental hypertension has helped shed light on some of the hitherto unrecognised roles of NO on cardiovascular function. For instance, renal blood and glomerular filtration rate are reduced in Sprague-Dawley rats given L-NA for 3 weeks[137]. This would explain why NOS inhibition produces an antidiuresis and antinatriuresis[112], suggesting that part of the hypotensive action of NO is via diuresis and natriuresis.

A peculiarity of this model of hypertension is the relative absence of vascular structural changes. Arnal *et al* gave oral L-NAME to Wistar rats for 8 weeks and found no cardiac hypertrophy in the majority of animals[138]. They did find cardiac hypertrophy in some animals, but these were the animals with the highest blood pressure and stimulated renin angiotensin system (RAS) detected by increased plasma renin activity. Devlin *et al* from our group found cardiac hypertrophy and aortic VSMC polyploidy in WKY rats after only 3 weeks of L-NAME treatment, and again these changes were associated with a stimulated RAS[139]. Chronic NOS inhibition has been shown to produce glomerular damage[140], and the subsequent stimulation of the RAS may be responsible for the vascular structural changes. This is confirmed by Takemoto *et al*, who produced coronary vascular remodelling and myocardial hypertrophy in WKY after 4 and 8 weeks of NOS inhibition with L-NAME[141]. They showed that the cardiovascular structural changes could be prevented by co-

administration of the angiotensin converting enzyme inhibitor temocapril, but not with hydralazine, despite producing a similar reduction in blood pressure.

The different susceptibility of various strains of rats to NOS inhibitor induced vascular changes may reflect altered sensitivity to renal damage and subsequent stimulation of the RAS, rather than altered sensitivity of heart and blood vessels *per se*. This altered sensitivity to renal damage may in turn reflect divergent genetic backgrounds.

As with other forms of hypertension, compensatory mechanisms come into play in an attempt to normalise blood pressure. In a previous study, we demonstrated increased activity of NOS and SOD in the aortas of Sprague-Dawley rats made hypertensive by 2 weeks of oral L-NAME treatment[142]. This may implicate an imbalance in NO and O_2^- contributing to this form of hypertension. A relative excess of other vasoconstrictors has been suggested, although Bank *et al* failed to correct L-NA hypertension in Sprague-Dawley rats with intravenous infusion of receptor antagonists for angiotensin II, thromboxane, adrenaline and endothelin-1[137].

Although local intrabrachial infusions of L-arginine analogues have been given to humans in many studies to investigate endothelial function, a systemic infusion of a pharmacological dose of a NOS inhibitor was given to humans for the first time by Haynes *et al*[143]. A five minute infusion of L-NAME (3mg/kg) in healthy volunteers produced a 10% increase in mean arterial pressure, a 19% decrease in heart rate and 25% decrease in cardiac index, resulting in a 46% increase in calculated total peripheral resistance. Creatinine clearance was unchanged, but in contrast to the animal studies, they found a natriuresis with NOS inhibition, which they attributed to a pressure natriuresis.

1.1.3 Nitric oxide in atherosclerosis

1.1.3.1 History

Manipulation of the L-arginine/nitric oxide pathway has been employed since the days of early Chinese civilisation. *Panax ginseng* is a medicinal plant which has been used in traditional Chinese medicine since antiquity. More recently, it has been discovered that ginsenosides, saponins extracted from the plant, can protect against myocardial and cerebral ischaemia, and that these effects are attributable to increased NO production[144].

Manipulation of the L-arginine/nitric oxide pathway entered more conventional medicine in the management of atherosclerotic disease still more than a century before the L-arginine/ nitric oxide pathway was conceived, or the mechanisms underlying atherosclerosis elucidated.

The disease angina pectoris was described by William Heberton in the Medical Transactions of the College of Physicians as far back as 1768[145]. It remained resistant to treatment with "brandy, ether, chloroform, ammonia, and other stimulants" for nearly a century until, in 1867, Sir Lauder Brunton successfully employed amyl nitrite[146]. In later life, Sir Lauder Brunton himself developed angina pectoris, for which he repeatedly used amyl nitrite to terminate attacks.

Meanwhile in America, nitroglycerine was being used in the treatment of "coronary involvements, angina pectoris, cardiac edema, headache, epilepsy and cerebral involvements, among other disease conditions". In 1897, William Murrell published his first independent paper in *Lancet*[147] comparing and contrasting the effects of nitroglycerine with

the known effects of amyl nitrite. He described in great detail the duration and effect of both compounds on symptoms, pulse rate and volume. In addition, he clearly presented three cases of classical angina pectoris, which were successfully treated with sublingual nitroglycerine.

1.1.3.2 Current concepts

Over the course of the next century, much knowledge has been gained about the pharmacological properties of organic nitrate esters. However, until fairly recently, it was not understood how they worked. Sodium nitrite was known to be a vasodilator, albeit a weak one[148], and it had been known since the last century that inorganic nitrite appears after incubating glyceryl trinitrate (GTN) with blood[149]. Therefore, it was thought that the action of nitroglycerine and other nitrates was via reduction to nitrite.

In the early 1980's, evidence was accumulating that sodium nitroprusside (SNP) and other nitrovasodilators activate guanylate cyclase[150], perhaps involving thiols, but the precise mechanism remained unclear. NO was also known to activate guanylate cyclase[13], therefore biotransformation to NO became a possible mechanism for nitrovasodilator-induced relaxation. It was not until after NO had been identified as an endogenous vasodilator[2], that this mechanism was accepted[151].

The involvement of thiols in the biotransformation of nitrovasodilators was thought to be behind the well described phenomenon of nitrate tolerance. Prolonged use of a nitrovasodilators leads to depletion of reduced thiol groups, which prevents further biotransformation[152]. Vasodilating S-nitrosothiols, which in effect provide their own thiol groups, can circumvent GTN induced tolerance[153]. This is further evidence for the

importance of thiol groups. However, the mechanisms of nitrate tolerance are likely to be more complex, and involve multiple sites on the nitrovasodilator-guanylate cyclase cascade[154].

There is some evidence for the involvement of O_2^- in tolerance. Increased levels of O_2^- have been found in aortic rings of rabbits made tolerant to GTN[155]. Indeed, the aortas of these animals were found to have increased activity of the membrane-bound, O_2^- generating enzyme NADH oxidase[156]. This increased activity may be related to increased circulating angiotensin II, which is found in chronic GTN treatment[157]. Hydralazine, which reduces NADH oxidase activity, has been shown to reduce tolerance to nitrovasodilators in a model of heart failure[156].

1.1.3.3 Mechanisms of atherosclerosis

Many of the complex steps involved in atherogenesis were comprehensively reviewed almost 10 years ago[158] (Figure 4). Endothelial injury is thought to be a very early stage. This need not be denudation, and in fact may consist of altered endothelial function, which is what we would nowadays call 'endothelial dysfunction'. Hyperlipidaemia, hypertension, diabetes mellitus, obesity, smoking, immune injury, and other risk factors may contribute to this endothelial dysfunction in different ways, and sometimes in combination. In the severely hypercholesterolaemic patient, such as one with familial hypercholesterolaemia, hyperlipidaemia is alone sufficient to cause atherosclerosis in the absence of other risk factors. Some factors may cause both endothelial dysfunction and hypertension, thus increasing their risk contribution.

Another early phenomenon appears to be trapping of low density lipoproteins (LDL) within the intima. The resulting increase in residence time may facilitate oxidation of the lipoproteins to oxidised LDL (ox-LDL) by macrophages, which, in turn, may damage the endothelium and act as a chemotactic signal for other monocytes to enter the intima.

The dysfunctional endothelium allows circulating monocytes to adhere and penetrate, at endothelial cell junctions, to the innermost layer of the artery wall, the intima, where they effectively become macrophages. The macrophages take up native LDL and ox-LDL by conventional and ox-LDL receptors respectively. Ox-LDL can also be taken up at an enhanced rate by the 'scavenger pathway' via acetylated LDL (ac-LDL) receptors. The ox-LDL is hydrolysed to form large quantities of cholesterol and cholesterol esters. These lipid laden macrophages are called 'foam cells'. Activated T-lymphocytes are also recruited, and the resultant 'fatty streak' is the earliest recognisable atherosclerotic lesion. Alarmingly, such fatty streaks are found in the coronary arteries of 50% of children aged 10 to 14 at post mortem[158]. Fatty streaks often form at sites of pre-existing vascular smooth muscle cell (VSMC) proliferation, as the latter is also associated with endothelial dysfunction.

VSMC exist in at least two different phenotypic forms[159]. In a contractile phenotype they respond to vasodilator agents such as NO and prostacyclin and vasoconstrictor agents such as endothelin and angiotensin II. However, they can also display a synthetic phenotype, when they are capable of producing a number of growth factors and cytokines and can synthesise extracellular matrix. Within the fatty streak VSMC, together with macrophages and lymphocytes, release such growth factors and cytokines resulting in further monocyte and lymphocyte migration and VSMC proliferation to form an 'intermediate lesion'. VSMC also produce a connective tissue matrix of fibro-elastic proteins, collagen and proteoglycans, which form a 'fibrous cap' on what has become a more advanced, complex,

occlusive lesion called a 'fibrous plaque'. The fibrous plaque contains a central core of necrotic debris and lipid in the form of free and esterified cholesterol from foam cell lysis and continued uptake from the circulation.

The formation of a fibrous lesion can be regarded as a form of wound healing. However, unlike in other tissues, within the arterial wall connective tissue is produced principally by VSMC and the insults (hypertension, hypercholesterolaemia, diabetes mellitus, obesity, smoking etc.) are likely to be chronic. Therefore, the progression from fatty streak to fibrous plaque is unlikely to be interrupted.

As the fibrous plaque grows, the overlying endothelium becomes increasingly damaged, which further propagates the whole process. At the same time the plaque projects further into the lumen and impedes blood flow. In the coronary circulation this would be manifest as stable angina pectoris. As the plaque grows, the large number of macrophages at the margins of the fibrous cap produce areas of weakness. This makes the plaque 'unstable', allowing cracks and fissures to appear, revealing the underlying thrombogenic surface. Platelets adhere forming a thrombus, which may occlude the artery, or the plaque may completely rupture, resulting in haemorrhage into the plaque, thrombosis and occlusion of the artery. Again, in the coronary circulation, this end stage would be manifest as a myocardial infarction[160].

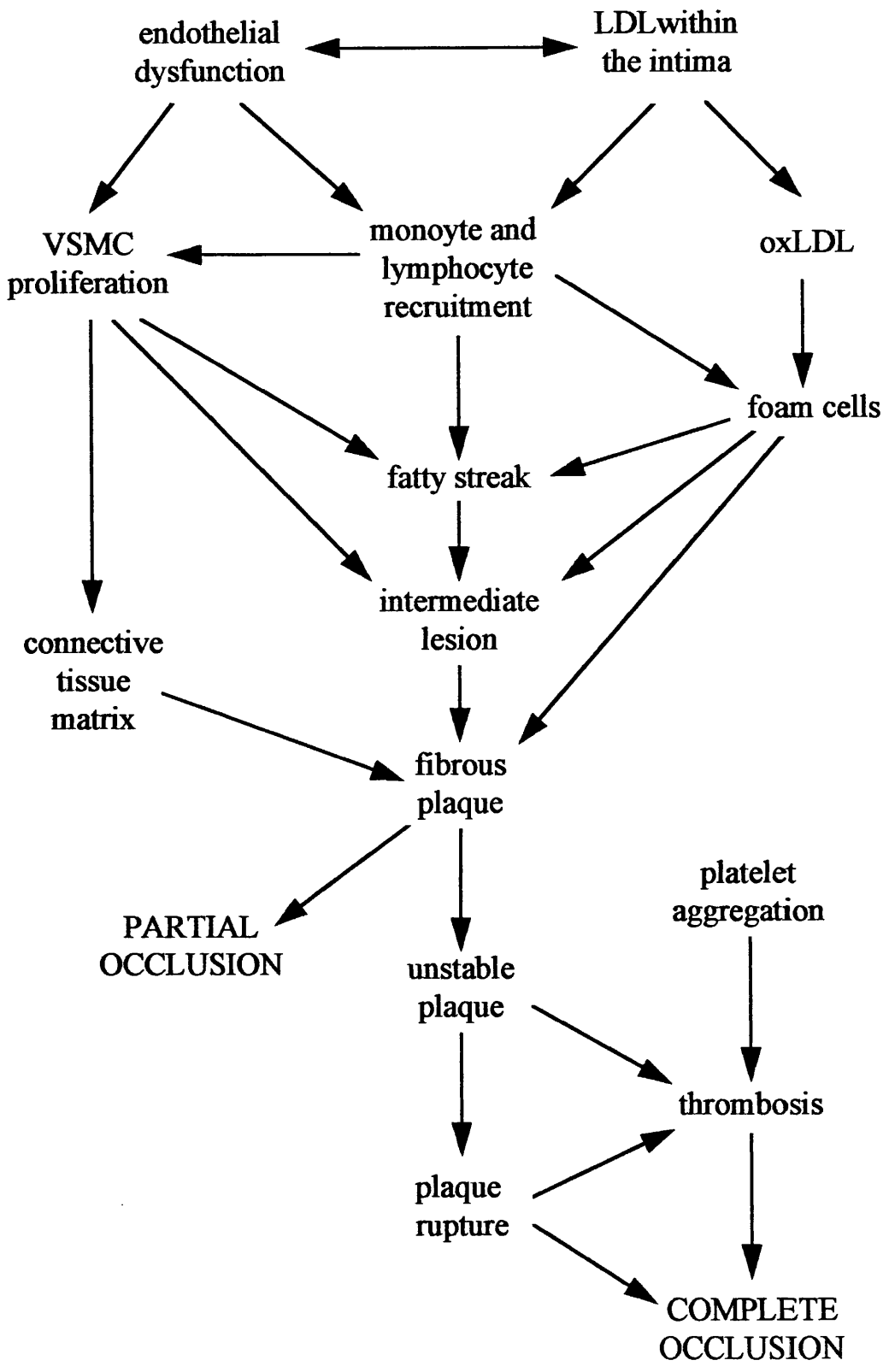

Figure 4 - Schematic representation of the steps towards atherosclerosis.

LDL: low density lipoprotein. ox-LDL: oxidised low density lipoprotein. VSMC: vascular smooth muscle cell.

1.1.3.4 Role of nitric oxide

There are many points in the pathway towards atherosclerosis where NO may have a beneficial effect, which is probably why NO donors have been employed so successfully for over a century. Firstly, the endothelial dysfunction, described as a very early stage in the development of atherosclerosis, refers to impaired release of NO, among other mediators. By virtue of its reaction with superoxide anion, NO is an anti-oxidant that can prevent oxidation of LDL[161], which is another of the early steps in fatty streak formation. Ox-LDL is produced by the enzyme lipoxygenase. NO has been shown to reduce lipoxygenase activity *in vitro*[162], which would further reduce the amount of ox-LDL available for uptake by macrophages.

NO has also been shown to inhibit monocyte adhesion to cultured porcine aortic endothelial cells[163], and together with prostacyclin NO inhibits monocyte migration[163]. These are important steps in the generation of the initial fatty streak. Other than acute vasodilator effects, NO donors can inhibit the proliferation of VSMC in culture[164] and NO itself can inhibit lymphocyte proliferation[165]; both of which are involved in the progression from fatty streak to fibrous plaque. NO can also prevent the aggregation of platelets *in vitro*[16] and their adhesion to endothelial cells in culture[17], which are involved in thrombogenesis later in the pathway.

There are, therefore, many theoretical reasons, backed up with *in vitro* evidence, why NO may act as an antiarthrogenic molecule. Most of the *in vivo* evidence of a beneficial effect of NO comes from extrapolation of the beneficial effects of L-arginine supplementation[76-78]. However, as discussion of the arginine paradox revealed in section 1.1.1.4, there are many possible roles for L-arginine other than acting as a precursor of NO synthesis. More direct

in vivo evidence for the beneficial role of NO in atherogenesis came from two recent studies where NOS inhibition with L-NAME worsened endothelium-dependent relaxation and increased lesional surface area in the thoracic aortas of hypercholesterolaemic rabbits[166,167]. In the study where L-NAME was given orally[166], NOS inhibition was associated with increased serum cholesterol levels, which is interesting in itself, but leads to a confounding effect. In the other study, L-NAME was administered subcutaneously by minipump[167], which did not significantly alter the lipid profile, but still produced the same adverse effects.

Probably the most compelling evidence for a beneficial effect of endothelium derived NO in preventing vascular lesion formation comes from a study by Dzau's group[168]. They used balloon injury to denude rat carotid arteries, then restored NOS III activity by transfecting the arteries with a recombinant bovine *Nos3* gene, using the highly efficient Sendai virus/liposome *in vivo* gene transfer technique. This intervention restored NO production and vascular reactivity to normal, and reduced neointima formation at 14 days by 70% compared to denuded, but non-transfected arteries. This is, of course, a model of neointimal hyperplasia of re-stenosis and not primary atherogenesis, however there are a number of similarities between the two.

Despite these apparent beneficial effects of NO in the early stages of atherogenesis, there is some evidence that NO may be deleterious in the established plaque. All of the cell types within the fibrous plaque (VSMC, macrophages and lymphocytes) can be, and probably are, induced to produce NO via NOS II[169]. NO produced in such great amounts can interact with superoxide anions, also produced by activated macrophages[170], to produce peroxynitrite, which can further oxidise LDL[171] and damage the endothelium[172]. In addition, VSMC within the plaque increase its stability. Therefore, by inhibiting VSMC proliferation, NO may cause plaque instability and subsequent rupture.

1.1.3.5 Smoking, coronary heart disease and NO

Another interesting study which indirectly implicates NO in atherosclerosis looked at a genetic polymorphism within the human *Nos3* gene[134]. The authors identified a rare allele present in only 17% of a control population with no history or symptoms of coronary artery disease. Only 0.7% of the control population were homozygous for the rare allele. However, when they genotyped a group of 549 patients consecutively referred for coronary angiography, they found 3.3% were homozygous for the rare allele i.e. 4.7 times as high as the control population. Not all of the patients referred had significant stenosis (defined as ≥50% occlusion) demonstrable at angiography. In the subgroup of 401 referred who had significant stenosis in 1, 2 or 3 coronary arteries, the frequency of homozygotes for the rare allele rose to 4.2% i.e. 6 times as high as the control population. This represents a significant association between homozygosity for the rare allele and coronary artery disease.

As discussed above, cigarette smoking is a known risk factor for coronary artery disease and also endothelial dysfunction. Therefore, the authors went further and divided the coronary population into smokers (past and present) and non-smokers (never). Among the smokers they found a 35% excess risk for significant stenosis in 1,2 or 3 coronary arteries associated with homozygosity for the rare allele compared to non-smoking homozygotes. While there is as yet no evidence that this polymorphism affects endothelial NOS function, it suggests a gene-environment interaction whereby there is a genetically determined predisposition to increased smoking-associated coronary risk in subjects homozygous for the rare allele. This may partly explain the anecdotes of some heavy cigarette smokers who never succumb to cardiovascular disease, while others succumb at an early age.

1.2 Superoxide anion

Despite being essential for most forms of life, the high content of O_2 in the atmosphere means that oxidation reactions are commonplace in our environment. Although our body uses O_2 and oxidation reactions to good effect for generating energy and killing invaders, unwanted side reactions are unavoidable. Therefore, to support aerobic metabolism, mechanisms had to evolve for the biological control of O_2. Such a mechanism involves its complete reduction to water (Figure 5), which produces the free radical $O_2^{\cdot-}$ by the one electron reduction of molecular O_2 as the first intermediate in this pathway. It has been estimated that a typical human cell metabolises about 10^{12} molecules of O_2 per day and generates some 3×10^9 molecules of H_2O_2 per hour.

Unlike NO, $O_2^{\cdot-}$ is not membrane permeable and is therefore restricted to reacting in the compartment in which it is generated. Although associated with so-called 'oxidative stress', $O_2^{\cdot-}$ is an unusual species in that it can act as a reducing agent, donating its extra electron e.g. to form $ONOO^-$ with NO, or as an oxidising agent, in which case it is reduced to H_2O_2. As described in section 1.1.1.2.5, under normal circumstances the relatively high abundance of SOD enzyme ensures that the latter reaction occurs preferentially. However, when NO is produced in large quantities, a significant amount of $O_2^{\cdot-}$ reacts with NO to produce $ONOO^-$.

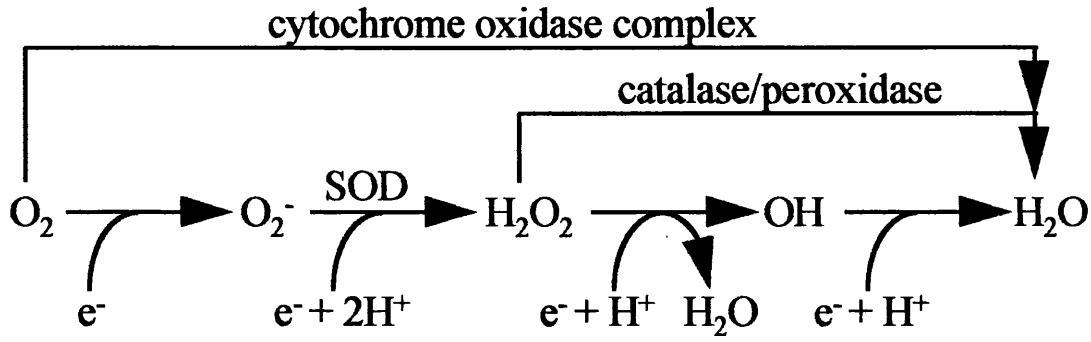

Figure 5 - Steps in the 4 electron (e^-) reduction of molecular oxygen (O_2) to water (H_2O), via superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) intermediates. The majority of O_2 is reduced by the cytochrome oxidase complex, which prevents release of the reactive intermediates. However, the evolution of a variety of superoxide dismutase (SOD) enzymes, catalase and peroxidase, to remove the reactive intermediates, suggests that a significant proportion of O_2 is reduced by this route.

1.2.1 Superoxide anion production

The main sources of O_2^- *in vivo* have been characterised. In the vasculature, it can be generated by xanthine oxidase (XO) (EC 1.1.3.22). Increased vascular O_2^- generation by XO has been implicated in the endothelial dysfunction of hypercholesterolaemia (section 1.2.3.1).

The main intracellular source of O_2^- is the respiratory chain of enzymes in the mitochondria [173], including the reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase and ubiquinone Q-cytochrome b complex. Mitochondrial O_2^- generation is greatest when the respiratory chain carriers are highly reduced, which is regulated by the availability of NAD-linked substrates, succinate, ADP, and O_2 [174]. Mitochondrial O_2^- spontaneously dismutates to H_2O_2 or the reaction is efficiently catalysed by mitochondrial manganese SOD[175]. This enzyme will be further discussed in section 1.2.2.2. Other minor sources of O_2^- include aldehyde oxidase, dihydro-orotic dehydrogenases, flavin dehydrogenases, peroxidases and autoxidation of a large group of compounds including catecholamines, flavins and ferredoxin[176].

Another important source of O_2^- generation within the vasculature is the enzyme NADPH cytochrome P-450 oxidoreductase (EC 1.6.2.4), more commonly termed NAD(P)H oxidase. This membrane bound enzyme has been identified in cultured rat VSMC[177], bovine coronary artery endothelium[178] and more recently in rat aortas[179]. Whereas excess O_2^- generation by XO has been implicated in hypercholesterolaemia, NAD(P)H oxidase has been incriminated in excess O_2^- generation in some forms of hypertension (section 1.2.3.2).

Paradoxically, NOS can also be a source of O_2^-. Purified rat NOS I has been shown to produce O_2^- in a reaction which is inhibitable by L-NAME, but not L-NMMA[121]. Heinzel *et* showed that purified porcine NOS I can produce H_2O_2 under conditions of low L-arginine concentrations[180], and Xia *et al* confirmed this finding in intact human kidney cells stably transfected with the rat *Nos1* gene[181]. NOS III has also been suggested as the source of native LDL (n-LDL) induced O_2^- by HUVEC described below, as it can be inhibited by L-NAME[182].

O_2^- generation can be influenced by a number of mechanisms. Pritchard *et al* demonstrated that n-LDL can increase O_2^- generation by cultured HUVEC[182]. Incidentally, n-LDL also increases NO generation by the same cells, the combination leading to an increased likelihood of $ONOO^-$ production, which the authors suggest is one of the earliest steps in atherogenesis. Increased O_2^- generation, and NO release, have also been demonstrated in cultured human aortic endothelial cells in response to high glucose in the culture medium, which these authors suggest is a link between diabetes mellitus and early atherogenesis[183]. Holland *et al* showed that bradykinin can increase O_2^- generation by HUVEC and this could be partially inhibited by indomethacin, suggesting that arachadonic acid metabolism is involved[184].

O_2^- generation could be reduced in cultured BAEC by 24 hours exposure to the synthetic oestrogen, ethinylestradiol[185]. The authors do not speculate as to the enzyme(s) involved. This effect of oestrogen may explain the gender difference in O_2^- generation observed by Brandes and Mügge, who found that aortas from male Wistar rats produced 34% more O_2^- than females[186]. They determined that the source of the O_2^- excess was the endothelium and that neither cycloxygenase nor XO were responsible. Gender differences in O_2^- will be further discussed in section 1.3.1.4.

1.2.2 Superoxide dismutases (EC 1.15.1.1)

As described in section 1.2, organisms which depend on oxidative metabolism have evolved a number of enzymes to reduce O_2^-, which is formed as an intermediate. One such family of enzymes are the superoxide dismutases, which catalyse the reaction of O_2^- with an electron and two protons to form H_2O_2 (Figure 5).

Like the family of NOS enzymes, the SODs have been identified throughout evolution. Iron SOD is an enzyme of prokaryotes and some higher plants. Manganese SOD is an enzyme of prokaryotes and eukaryotes. Copper/zinc SOD is an enzyme of eukaryotes and certain prokaryotes[187]. Sequence analysis and comparisons make it clear that at least one member of the family, copper/zinc SOD, arose prior to the divergence of eukaryotes and eubacteria.

Although catalase is also involved in the reduction of H_2O_2 to H_2O (Figure 5), SOD was shown to be a more important antioxidant protector of NO, as inhibition of SOD by diethyldithiocarbamate (DETCA) had a greater effect on biologically active NO than inhibition of catalase by 3-amino-1,2,4-triazole (AT)[188].

1.2.2.1 Copper/zinc superoxide dismutase (Cu/Zn SOD)

This enzyme was the first member of the family to be discovered in mammals in 1969[189]. It is composed of two identical 16kDa subunits, each containing one copper and one zinc atom. It is located in the cytosol and nucleus of all cell types. The enzyme is very sensitive to cyanide, which helps distinguish it from Mn SOD which is relatively resistant.

The human gene for Cu/Zn SOD (*Sod1*) has been localised to the 21q22.1 region of chromosome 21[190]. It consists of 5 exons and 4 introns which interrupt the coding region[191]. The 5' promoter region contains 'TATA' and 'CAAT' boxes. Three other processed pseudogenes have been identified in the haploid human genome, none of which reside on chromosome 21[192].

As *Sod1* resides on human chromosome 21, patients with Down's syndrome (trisomy 21) have an extra copy of the gene, and have been shown to have Cu/Zn SOD activity 50% greater than the normal diploid population in keeping with the gene dosage effect[190]. Transgenic rats containing an extra copy of the human *Sod1* gene display some of the neurological defects characteristic of Down's syndrome, including premature ageing, suggesting that this gene is involved in the pathogenesis of Down's syndrome[193]. Whereas the SOD isoenzymes are normally thought to be protective, it is postulated that increased Cu/Zn SOD activity produces increased amounts of H_2O_2, which become toxic in the presence of normal catalase activity[193]. Therefore, increased Cu/Zn SOD activity may only be beneficial when balanced with increased catalase activity, and induction of one does not lead to induction of the other[194].

The role of O_2^- in hypertension will be discussed in section 1.2.3.2. However, the increased Cu/Zn SOD activity in Down's syndrome may further indicate a role for O_2^- in hypertension. With a higher Cu/Zn SOD activity, Down's syndrome patients will have reduced O_2^- levels. If O_2^- excess is involved in the pathogenesis of hypertension, then one would expect Down's syndrome patients to have lower blood pressure. This was recently found to be the case in a well controlled study[195].

The beneficial effect of increased fluid shear stress on endothelial function has been attributed to increased NO production, which has been described in great detail in section 1.1.1.5.1. However, some of the beneficial effect may also be due to reduced NO scavenging by O_2^- as *Sod1* has been shown to be up-regulated by laminar shear stress in human aortic endothelial cells in culture[93].

1.2.2.2 Manganese superoxide dismutase (Mn SOD)

This was the second mammalian enzyme to be discovered in 1973[196]. Mn SOD is a homotetramer, each 16kDa subunit containing one manganese atom. It is synthesised in the cytoplasm and directed to the mitochondria by a signal peptide, where it is involved in dismutating the O_2^- generated by the respiratory chain of enzymes described in section 1.2.1 above.

The essential role of Mn SOD in maintaining mitochondrial function is demonstrated by the neonatal lethality of mice with targeted disruption of the gene for Mn SOD (*Sod2*)[197]. Such *Sod2* 'knock out' mice die within the first 10 days of life with dilated cardiomyopathy. Another group generated *Sod2* 'knock out' mice and confirmed the dilated cardiomyopathy in -/- homozygotes, but also described several novel pathologic phenotypes including severe anaemia, degeneration of neurons in the basal ganglia and brainstem, and progressive motor disturbances characterised by weakness, rapid fatigue, and circling behaviour[198].

The human *Sod2* gene has been localised to the 6q25 region of chromosome 6[199], and subsequently cloned and characterised[200]. The single copy gene consists of 5 exons and 4 introns. The promoter sequences suggest that *Sod2* is subject to a degree of transcriptional regulation. Indeed, *Sod2* has been shown to be up-regulated in cultured fibroblasts exposed

to excess O_2^-, whereas *Sod3* was down-regulated and *Sod1* was unaltered[201]. In a similar study, H_2O_2 increased *Sod2* expression in cultured tracheobronchial epithelial cells without affecting *Sod1* expression[202]. *Sod2* can also be up-regulated by TNF-α[203] and shear stress[204], all of which are probably via activation of the NF-κB transcription factor.

As stated above, Mn SOD protein depends on a signal sequence to localise it to the mitochondria. Polymorphisms in this signal sequence have been identified in patients with genetic diseases of premature ageing[205]. Such signal sequence polymorphisms could result in diseases of distribution, where Mn SOD is not properly targeted, thereby leading to an absolute or relative deficiency of the enzyme within the mitochondria. This indicates that O_2^- may be involved in the process of ageing, and this will be discussed further in section 1.4.

1.2.2.3 Extra-cellular superoxide dismutase (EC-SOD)

This is the third and currently the last mammalian SOD to be characterised. It was purified from human lung by Marklund in 1982[206]. Most mammalian EC-SOD exists as a homotetramer of molecular weight 130,000 and like Cu/Zn SOD, each 30kDa subunit contains one copper and one zinc atom. Again like Cu/Zn SOD, EC-SOD activity is extremely sensitive to cyanide. EC-SOD is produced in fibroblasts and glial cells[207] and secreted into the extracellular fluid, where it is the principal SOD. The enzyme is a glycoprotein, which binds sulphated polysaccharides, such as heparin and heparan sulphate, via a cluster of six basic amino acids. Therefore, EC-SOD will exist in the vasculature mainly bound to the surface of the endothelial cells and the extracellular matrix, both of which have an abundance of heparan sulphate, although some enzyme activity can be detected in the plasma. Because of its location, EC-SOD has been hailed as the principal

regulator of endothelium derived NO bioavailability[208], although cytosolic Cu/Zn SOD is also thought to be important[188].

The human gene encoding EC-SOD (*Sod3*) has been characterised[209]. Southern blot analysis suggests the existence of a single gene, with no evidence of pseudogenes. Human *Sod3* contains 3 exons and 2 introns spanning ≈5.9kb. The 720-bp coding region is uninterrupted and located within exon 3. No obvious TATA box was identified, but an Alu-J repetitive element was identified 5' to exon 3 and an Alu-Sx repetitive element was identified 3' to the coding exon. Discrepancy between *Sod3* mRNA levels and EC-SOD activity in a variety of tissues would suggest that post-transcriptional or post-translational regulation of the gene occurs.

The important anti-oxidant role of EC-SOD is shown by mice lacking the *Sod3* gene. Such *Sod3* 'knock out' mice have been generated and characterised[210]. When kept under normal laboratory conditions, null mutant mice develop normally and remain healthy until at least 14 months of age, despite no compensatory induction of Cu/Zn SOD or Mn SOD activity. However, when exposed to the oxidative stress of >99% oxygen, the survival time of the homozygous -/- mice is significantly reduced compared to homozygous +/+ mice. The cause of death was fulminant pulmonary oedema, which is in keeping with the fact that the lung is the tissue containing the highest amount of EC-SOD in mice.

1.2.3 Superoxide anion and cardiovascular disease

1.2.3.1 Hypercholesterolaemia

O'Hara *et al* demonstrated increased O_2^- production in vessels from hypercholesterolaemic rabbits and that the endothelium was the source[211]. They showed that O_2^- could be reduced to normocholesterolaemic levels by the non-competitive XO inhibitor oxpurinol, suggesting that XO is the enzyme responsible. White *et al* confirmed that the impaired relaxation to ACh, induced by hypercholesterolaemia in the rabbit aorta, could be reversed by pre-treatment of the aortic rings with allopurinol, another XO inhibitor[212]. They showed that the endothelial function could also be restored by pre-treatment with heparin, which competes with circulating XO for binding to sulphated glycosaminoglycans on the endothelial cells. Plasma XO activity was elevated more than two-fold in hypercholesterolaemic rabbits compared to controls in their study.

Further evidence for the involvement of O_2^- in endothelial dysfunction in hypercholesterolaemia come from Mügge *et al*, who were able to improve endothelium-dependent, but not endothelium-independent, relaxations in aortas from hypercholesterolaemic rabbits by daily injections of polyethylene-glycolated SOD (PEG-SOD)[213]. They demonstrated a two-fold increase in vascular SOD activity using this preparation.

In humans with hypercholesterolaemia, Garcia *et al* confirmed the blunted vasodilator response in the forearm to intrabrachial ACh[214]. They failed to correct this impairment with intrabrachial infusion of SOD, suggesting that increased O_2^- may not be responsible.

However, the preparation of SOD used would not enter the cells, which is the reason Mügge *et al* used PEG-SOD in the rabbit study above[213].

1.2.3.2 Hypertension

Since the landmark study on renovascular hypertension by Goldblatt *et al* in 1934[215], it has become clear that the renin-angiotensin system (RAS) plays a major role in hypertension. The mechanism of RAS-induced hypertension has generally been attributed to the vasoconstrictor effects of angiotensin II and the mineralocorticoid effects of aldosterone. However, recent work has revealed an alternative mechanism. Angiotensin II has been shown to stimulate O_2^- generation by increasing NAD(P)H oxidase activity in cultured rat VSMC[177] and in intact aortas of rats made hypertensive by angiotensin II infusion[179]. This seems to be a fairly specific effect as rats made hypertensive to a similar degree by infusion of noradrenalin showed no increase in NAD(P)H oxidase activity[179]. Blood pressure and vascular reactivity could be restored by exogenous liposome-encapsulated SOD in the angiotensin II hypertensive rats, but not the noradrenalin hypertensive rats, which further implicates O_2^- in hypertension associated with high renin-angiotensin states[216].

O_2^- has been implicated in other models of experimental hypertension. As discussed in section 1.1.2.1, Grunfeld *et al*[114] and Tschudi *et al*[115] demonstrated excess O_2^- in aortas of SHRSP. In the related SHR, Nakazono *et al* were able to lower blood pressure by intravenous injection of a fusion protein of SOD linked to a C-terminal basic domain which has high affinity for heparin-like proteoglycans on vascular endothelial cells[217]. Using immunohistochemistry, they demonstrated that the fusion protein was localised to the endothelium and tunica interna and elastica interna of the aorta and resistance vessels. They also found arterial XO activity and aortic SOD activity to be similar in SHR and WKY. Ito

et al also found increased O_2^-, detected by formazan staining, in the hypertrophied heart of SHR compared to WKY, and in this study reduced SOD activity was found to be the underlying mechanism[218].

Endogenous O_2^- has been shown to affect tone in human vessels[219]. Increased O_2^- generation, albeit by neutrophils, has also been demonstrated in human essential hypertension[220]. Although the mechanism remains unclear, the effect can be reversed by β-adrenoceptor blockade with celiprolol[220]. Red blood cell SOD activity was also found to be reduced in patients with essential hypertension compared to normotensive controls, however the groups were very poorly matched for age[221]. Although not directly measured in this study, the implication is that O_2^- would consequently be increased in the hypertensive group, perhaps contributing to the hypertension.

1.3 Gender differences in cardiovascular disease

Human cardiovascular disease is less common in women of reproductive age compared to age matched males. This was recognised as early as 1955 when Sir George Pickering stated that, "for any given level of arterial pressure, women fare better than men." From early analysis of the now famous Framingham Study, women in the reproductive age group were found to be at a lower risk for developing coronary heart disease, and indeed all cardiovascular events, compared with men of similar age[222]. This was confirmed in a West of Scotland population where women were shown to have lower coronary mortality than men when corrected for age and all other risk factors[223]. In addition, from adolescence onwards, women have lower blood pressure than men[224], and the prevalence of essential hypertension is lower in premenopausal women than age matched men[225].

A similar gender difference exists in animal models. In the SHR, males have a higher 24-hour mean arterial pressure (MAP) than females, and a high salt diet increased 24-hour MAP in males, but had no effect in females[226]. There is also evidence from animal models for delayed and less severe hypertension[227] and atherosclerosis[228] in females compared to males.

There is evidence for gender differences in endothelial function in humans and animal models of genetic hypertension. Chowienczyk *et al* used forearm venous occlusion plethysmography to demonstrate that hypercholesterolaemia produced greater endothelial dysfunction in males compared to aged matched females[229]. Kauser and Rubanyi were the first to demonstrate that significant gender differences exist in endothelial function of thoracic aortas isolated from SHR, as determined by ACh induced relaxation[230]. Gender differences in myogenic tone have also been demonstrated, where females exhibit attenuated

pressure-induced constriction of arterioles, from the coronary[231] or skeletal muscle[232] circulation, compared to males.

These gender differences in endothelial function have been attributed to NO, and indeed reduced basal 'release' of NO was demonstrated in the thoracic aortas of male rabbits compared to females[233]. More recently, Kauser and Rubanyi used a bioassay system to demonstrate reduced basal NO release in aortas from male rats compared to females[234]. In the same study, they also showed that aortas from female rats relaxed to a greater extent after stimulation by ACh, and that the ability of an intact endothelium to suppress serotonin or phenylephrine contractions was greater in females, compared to males.

1.3.1 Sex hormones

It has been suggested that the gender differences in cardiovascular disease, endothelial function and NO are related to sex hormones, though this has never been proven.

1.3.1.1 Oestrogen in cardiovascular disease

There is evidence from animal models of genetic hypertension that sex hormones are important in the development of hypertension. Iams and Wexler found in young male SHR that gonadectomy prevented the rapid development of spontaneous hypertension, and that in young female SHR ovariectomy caused "escape" from the protective effect and led to the development of severe hypertension[235]. They also found that exogenous oestrogen prevented the hypertension in gonad-intact male and ovariectomised female SHR. In another model of experimental hypertension, the [m*Ren*-2]27 transgenic rat, ovariectomy increased mean arterial blood pressure in females and this could be reversed by exogenous

oestrogen[236]. Ovariectomy has also been shown to increase fatty streak lesions in the proximal aorta of apolipoprotein E-deficient mice without affecting plasma cholesterol, and this effect was reversed by exogenous 17β-oestradiol[237]. 17β-oestradiol was also shown to reduce aortic accumulation of cholesterol in ovariectomised, cholesterol-fed rabbits[238].

In human studies a decline in endogenous oestrogen is associated with increased cardiovascular disease. In the Framingham Study, the onset of the menopause was associated with an increased incidence of cardiovascular events[222]. This observation was later confirmed by Colditz *et al* in surgical menopause, where the risk ratio for coronary heart disease was 2.2 (95% confidence interval 1.2 to 4.2), but not in natural menopause, where the risk ratio was only 1.2 (95% confidence interval 0.8 to 1.8)[239].

Exogenous oestrogens in the form of hormone replacement therapy (HRT) have been shown to decrease cardiovascular disease. Eight weeks of HRT can lower systolic and diastolic blood pressure[240]. The 10 year report from the Nurses' Health Study revealed an overall relative risk of major coronary heart disease in women currently taking oestrogen of 0.56 (95% confidence interval, 0.40 to 0.80), after adjustment for age and other risk factors[241]. Around the same time a meta-analysis of all previously published studies revealed a remarkably similar overall relative risk of coronary heart disease of 0.56 (95% confidence interval, 0.50 to 0.61) in oestrogen users[242]. However, a less robust study of pooled data disputes this beneficial effect by calculating an odds ratio of cardiovascular disease of 1.39 (95% confidence interval, 0.48 to 3.95) in women taking HRT[243]. While this increased risk does not achieve statistical significance, there is no suggestion of protection from cardiovascular disease by HRT. This latter study has subsequently been heavily criticised[244] and is the only study to show an increased cardiovascular risk, albeit non-significant, with HRT. The balance of evidence remains in favour of a protective effect of oestrogen,

therefore it was initially surprising that the anti-oestrogen tamoxifen, given as adjuvant therapy for breast cancer, significantly reduced the incidence of fatal myocardial infarction in the Scottish adjuvant tamoxifen trial[245]. The authors attribute this effect to the partial agonist oestrogen-like effect of tamoxifen.

Oestrogen exerts a multitude of effects which are potentially cardioprotective, including improving the lipid profile by increasing high density lipoprotein (HDL) and lowering LDL and fibrinogen levels[246]. However, it is estimated that the improved lipid profile accounts for only 25-50% of the protective effect of oestrogen. Oestrogen is also reported to have a calcium channel blocking action[247] and also appears to possess anti-oxidant properties[248], perhaps related to its phenolic structure, which scavenges hydroxyl radicals. It also alters responses to other vasoactive molecules, but the main beneficial effect of oestrogen is thought to be via its improvement in endothelial function.

1.3.1.2 Oestrogen and endothelial function

The modulation of vascular tone by oestrogens has been recognised since the late 1800s. In 1884, in a paper curiously entitled '*Irritation of the sexual apparatus*', MacKenzie reported that the menstrual cycle and pregnancy could cause changes in the degree of vascularity of mucous membranes in women[249]. Almost 60 years later, Reynolds and Foster demonstrated that oestrogen could induce dilatation of small vessels in the ear of ovariectomised rabbits[250]. In 1966, Ueland and Parer reported a decrease in systemic vascular resistance in ewes during pregnancy[251]. Much work has been done subsequent to these early studies in an attempt to explain the gender differences in cardiovascular disease and the cardioprotective effects of oestrogens.

There have been a multitude of animal studies looking at the effects of oestrogens on endothelial function and these have been comprehensively reviewed recently[252]. There have been a few human studies. Sudhir *et al* used forearm venous occlusion plethysmography to demonstrate that oestrogen given to postmenopausal women in the form of HRT increased basal NO, as determined by the constrictor response to L-NMMA, but not stimulated NO, as determined by the dilator response to ACh[240]. Pinto *et al* took this observation further by examining endothelial function in women before surgical menopause (hysterectomy and bilateral ovariectomy for uterine leiomyoma), afterwards and then again after oestrogen HRT[253]. Before ovariectomy, ACh induced vasodilatation of the forearm was no different from control women matched for age and other risk factors. However, within 4 weeks after surgery, endothelium-dependent vasodilatation was significant impaired in the ovariectomy group compared to pre-surgery. Unlike Sudhir *et al*, they found that 3 months of oestrogen HRT did significantly improve ACh induced vasodilatation back to pre-surgery levels. This group did not assess basal NO, but found no significant effect of ovariectomy or HRT on endothelium-independent vasodilatation.

Some of the human studies, and indeed some of the animal studies, have shown an acute effect of oestrogen. Intrabrachial 17β-oestradiol and ACh produce a significantly greater increase in forearm blood flow than ACh alone[254], and a similar acute effect was demonstrated in the coronary circulation of postmenopausal women, where 17β-oestradiol changed ACh induced constriction to dilatation within 20 minutes[255]. In addition, sublingual 17β-oestradiol has been shown to significantly improve the treadmill exercise capacity of women with coronary heart disease within 40 minutes of administration[256]. These responses are too rapid to be mediated by the conventional genomic actions of oestrogen, but may be attributable to the anti-oxidant or calcium channel blocking effects of the hormone described in section 1.3.1.1.

1.3.1.3 Oestrogen and nitric oxide

There are a number of ways in which oestrogen can increase NO. It can increase basal NO production, as suggested by Sudhir *et al*[240]; increase stimulated NO production, as suggested by Pinto *et al*[253]; or reduce NO scavenging, which will be discussed in section 1.3.1.4. Increased NO production can be achieved by increased expression of the *Nos3* gene, increased activity of the NOS III enzyme, or both.

As discussed in section 1.1.1.5.2, there are theoretical reasons why oestrogens may regulate *Nos3* expression. Oestrogen has been shown to increase *Nos3* mRNA in cultured cells *in* [105] and in skeletal muscle[107] and aorta[108] *in vivo* (Table 2). In only one of these studies was NOS III enzyme activity measured and was found to be increased after 17β-oestradiol[107]. This has been confirmed in HUVEC and BAEC after 8 hours of exposure to 17β-oestradiol[257]. NOS III activity was also increased in foetal pulmonary artery endothelial cells, which could be inhibited by tamoxifen and other oestrogen receptor antagonists, suggesting involvement of the oestrogen receptor[258]. However, this effect was observed within 5 minutes of exposure to 17β-oestradiol, suggesting a non-genomic mechanism. In this study *Nos3* expression was not altered and *Nos2* mRNA was not detected, and the increased NOS III activity was not observed in the absence of extracellular Ca^{2+}. Taken together, these data suggest a novel action of oestrogen whereby it interacts with the oestrogen receptor and very rapidly opens membrane Ca^{2+} channels on endothelial cells, increasing $[Ca^{2+}]_i$, which is a recognised stimulus for increased NOS III activity. This is in contrast to the proposed Ca^{2+} blocking action of oestrogen at the VSMC membrane[247].

17β-oestradiol has also been shown to inhibit interferon and lipopolysaccharide induced *Nos2* expression in cultured macrophages by a receptor mediated mechanism[257]. The

authors speculate that this may be yet another mechanism whereby oestrogen is protective against atherosclerosis.

1.3.1.4 Oestrogen and superoxide anion

Despite all of the above evidence for oestrogen increasing *Nos3* gene expression and/or NOS III enzyme activity, Arnal *et al* produced some evidence to the contrary[185]. In cultured BAEC they found no increase in NOS III activity by the L-arginine/L-citrulline assay, no increase in NOS III protein levels by western blotting, and no increase in *Nos3* mRNA by northern blotting, after stimulation by the synthetic oestrogen ethinylestradiol (EE_2). They did demonstrate increased guanylate cyclase stimulating activity in the supernatant of BAEC treated with EE_2. In the absence of increased NO production, they hypothesised, then demonstrated, that this was due to reduced levels of O_2^-. They conclude that EE_2 reduces O_2^- production, although increased dismutation by SOD remains a possibility.

Some of the conflicting findings of Arnal *et al* may be explained by the synthetic oestrogen used, but there are other reports in the literature of oestrogen affecting O_2^- production and removal. Rajan *et al* showed that 5 days of subcutaneous oestradiol increased NADH oxidase activity in the rat uterus without affecting SOD activity, whereas some synthetic oestrogens dose dependently reduced NADH oxidase and SOD activity[259]. Whiteside *et al* demonstrated that oestrogen increases Cu/Zn SOD in rat mammary tumours[260]. While there are other studies examining the effects of oestrogen on O_2^- in reproductive tissues, the only other study in the cardiovascular system showed no effect of gender, gonadectomy or graded doses of oestradiol on rabbit myocardial SOD activity[261]. It therefore remains unclear to what extent the improvement in endothelial function by oestrogen is attributable to NO or O_2^-, although a combination is likely.

1.4 The effect of age on endothelial function

Cardiovascular disease remains the leading cause of death and a major source of disability in the elderly. While risk factors for atherosclerosis such as hypertension, diabetes, and hyperlipidaemia become more prevalent with ageing, escalation of cardiovascular disease exceeds expected incidence based exclusively on these risks. Ageing itself may fundamentally increase the vulnerability of older adults to cardiovascular disease, although this remains difficult to study in humans.

There is growing evidence to suggest that alterations which occur in the vessel wall in ageing are similar to those seen in hypertension, although in hypertension the changes are accelerated[262]. Many of the functional changes in ageing are also seen in hypertension. In rat mesenteric resistance arteries, ageing is associated with reduced basal and ACh induced NO release[263]. Possibly as a result of this, the constrictor effect of endothelin-1 is potentiated by ageing in the same vessels, whereas contractions to noradrenaline were unaffected[263]. In humans too, endothelial function in the forearm vasculature, as determined by methacholine induced vasorelaxation, declines progressively with age[264]. Similarly, Taddei *et al* found reduced ACh stimulated vasodilatation in the forearm of an elderly group (>60 years), which was remarkably similar to the endothelial dysfunction they found in a young hypertensive group (<30 years)[265]. These studies further suggest that hypertension is an accelerated form of ageing.

Another link between hypertension and ageing is O_2^-. As discussed in section 1.2.3.2, O_2^- appears to be involved in some forms of hypertension, and in section 1.2.2.1, O_2^- has been implicated in premature ageing. Indeed, the most popular current theory of ageing holds that ageing represents an accumulation of free-radical damage to macromolecules such as

proteins, lipids and nucleic acids[266]. One may speculate then, that O_2^- excess, which underlies the endothelial dysfunction of some forms of hypertension, may also underlie the endothelial dysfunction of ageing. Evidence for this has only very recently become available. Tschudi *et al* found calcium-ionophore stimulated NO concentrations in isolated carotid and mesenteric arteries to be significantly reduced in 70 week old WKY rats compared to 15 week old rats[267]. They found increased O_2^- release to account for this age-related reduction in NO concentration, as NOS III protein levels were not reduced in the aged mesenteric artery and even increased in the aged carotid artery.

2 Aims

The aims of the current study were:

1) To determine if there are differences in basal and/or stimulated NO availability between WKY and SHRSP.

2) To determine if there are differences in basal and/or stimulated NO availability between males and females within either strain.

3) To determine if any differences in NO availability are due to differences in NO production, NO destruction, or both.

4) To determine what role, if any, that O_2^- plays in the strain or gender differences in NO availability.

5) To determine what role, if any, 17β-oestradiol plays in gender differences in NO availability.

6) To determine the effect of age on basal NO availability, and to determine the mechanism(s) involved.

3 Methods

Appendix 1 lists the suppliers of all chemicals and reagents used. Appendix 2 details the recipes for all solutions used.

3.1 Experimental animals

Rats were obtained from WKY and SHRSP colonies established in Glasgow by brother sister mating as previously described[268]. These colonies were originally established from rats obtained from the colonies maintained at the University of Michigan, which had obtained their breeding stocks from the National Institute of Health (personal communication, D.F.Bohr, Department of Physiology, University of Michigan). All experiments were approved by the Home Office according to regulations regarding experiments in animals in the United Kingdom.

3.1.1 Measurement of systolic blood pressure and body weight

In the week prior to sacrifice, indirect blood pressure was measured by tail plethysmography in conscious, restrained rats habituated to the procedure as previously described[269]. Rats were pre-warmed to 34°C for 10-15 minutes before measurements. On the day of sacrifice the rats were weighed on bench top scales after anaesthetic and before removal of the thoracic aorta.

3.2 Isometric tension recording

Rats were studied at 16 weeks of age in four groups: WKY females (n=12), WKY males (n=17), SHRSP females (n=14) and SHRSP males (n=14). The rats were killed by halothane overdose. The thoracic aortas were carefully removed, cleaned of adipose tissue and cut into 2-3 mm rings. The rings were suspended under 1g tension in individual 10 mL muscle baths containing physiological saline solution (PSS) of the following composition (mmol/L): NaCl 130, KCl 4.7, $NaHCO_3$ 14.9, KH_2PO_4 1.18, $MgSO_4.7H_2O$ 1.17, $CaCl_2.2H_2O$ 1.6, glucose 5.5 and $CaNa_2EDTA$ 0.03. The PSS was aerated with 95% O_2/5% CO_2. Indomethacin was added to a final bath concentration of 10^{-5} mol/L to inhibit the production of prostanoids, which can affect vascular tone independently of NO. 17β-oestradiol (10^{-5} mol/L) and cocaine HCl (10^{-5} mol/L) were also added to block extraneuronal and neuronal adrenergic uptake respectively. Isometric tension was measured by a force transducer (Grass Medical Instruments, Quincy, USA) and recorded by a multichannel pen recorder (Grass Medical Instruments, Quincy, USA). After a 1 hour equilibration period, four different protocols were followed on rings from each group. These protocols are illustrated in Figures 6 - 9.

3.2.1 Experimental protocols

Potassium chloride (KCl) was used as a receptor independent vascular smooth muscle cell depolarising agent. At 100 mmol/L maximal contraction is obtained. The noradrenaline analogue phenylephrine hydrochloride (PE) was used to constrict the rings via α_1-adrenoceptors. PE has very little β-agonist effect and has no α_2 effects, which is important as the latter may interact with the L-arginine/NO pathway.

3.2.1.1 Carbamylcholine

Carbamylcholine chloride (carbachol), a more stable analogue of acetylcholine[270], was used to relax the rings in an endothelium dependent manner via M2 muscarinic receptors resulting in stimulated NO release[271,272]. As shown in Figure 6, aortic rings from each group were pre-constricted to the EC_{50} for PE. Stimulated endothelial NO release was assessed by measuring the relaxation to carbachol (10^{-8} - 10^{-5} mol/L).

3.2.1.2 Sodium nitroprusside

The NO donor sodium nitroprusside (SNP) was used to relax the rings in an endothelium independent manner. SNP does not release NO until it reaches the vascular smooth muscle cell[273]. As shown in Figure 6, after the concentration response curve was obtained for carbachol the bath was washed out. The rings were then preconstricted to the EC_{50} of PE and a concentration response curve was obtained for SNP (10^{-8} - 10^{-5} mol/L).

3.2.1.3 N^G-nitro-L-arginine methyl ester

The NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) was used to inhibit basal NOS III activity. The resultant contraction is a measure of basal NO availability[125]. L-NAME also appears to be a competitive antagonist of acetylcholine at muscarinic receptors, but competes very poorly with carbachol for the muscarinic receptor[274]. As shown in Figure 7, parallel aortic rings from each group were shown to relax to carbachol indicating that a functioning endothelium was present. After washout the rings were pre-constricted to the EC_{20} for PE. L-NAME was added to a final bath concentration of 100 μmol/L and the

resultant contraction taken as a measure of basal NO availability. If the rings were not preconstricted to the EC_{20} for PE, then they would not contract to L-NAME.

3.2.1.4 Diethyldithiocarbamate

The cell membrane permeable copper chelating agent sodium diethyldithiocarbamate (DETCA) was used to inhibit copper zinc (Cu/Zn) SOD, both intracellular and extracellular[188,275]. This has the effect of increasing levels of O_2^- within the lumen of the vessel and also within the endothelial cell. As shown in Figure 8, the rings were incubated with DETCA (10 mmol/L) for 45 minutes, then concentration response curves were obtained for PE (10^{-8} - 10^{-5} mol/L) and carbachol (10^{-8} - 10^{-5} mol/L). This concentration of DETCA has been shown to completely and irreversibly inhibit Cu/Zn SOD activity[275], therefore it was not necessary to add DETCA after each washout.

3.2.1.5 Superoxide dismutase

Exogenous SOD was added to remove extracellular O_2^-. This form of SOD does not enter cells[213]. As shown in Figure 9, exogenous SOD (45U/mL) was added to the bath and a concentration response curve obtained for PE (10^{-8} - 10^{-5} mol/L). After washout, exogenous SOD (45U/mL) was again added to the bath and a further concentration response curve was obtained for carbachol (10^{-8}-10^{-5} mol/L).

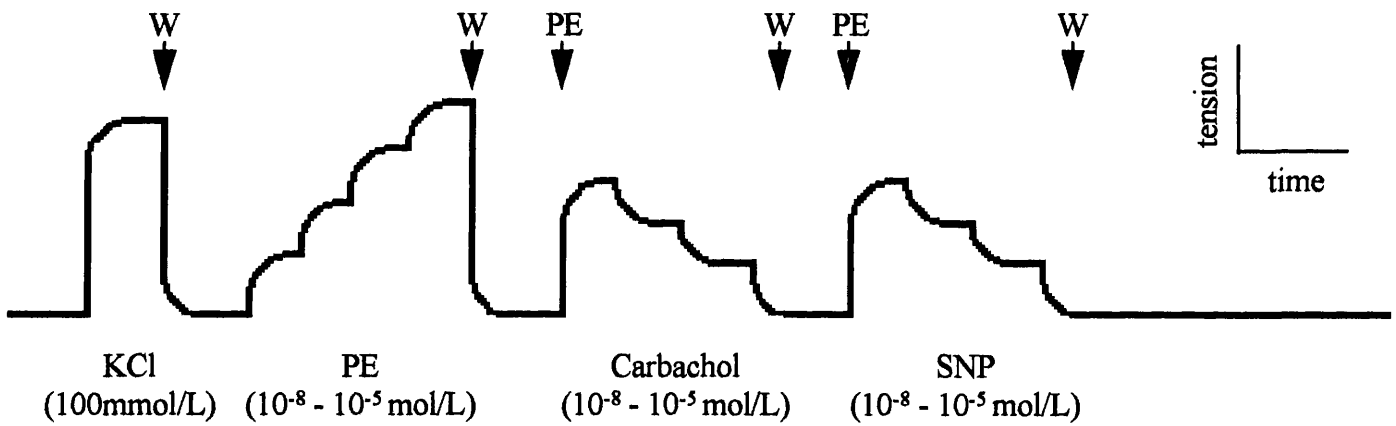

Figure 6 - Responses to carbachol and sodium nitroprusside. Rings were contracted to potassium chloride (KCl, 100 mmol/L). After washout (W), a concentration response curve for phenylephrine (PE, 10^{-8} - 10^{-5} mol/L) was obtained. After washout, the rings were pre-contracted to the EC_{50} for PE and a concentration response curve obtained for carbachol (10^{-8} - 10^{-5} mol/L). After washout, the rings were pre-contracted to the EC_{50} for PE and a concentration response curve obtained for sodium nitroprusside (SNP, 10^{-8} - 10^{-5} mol/L).

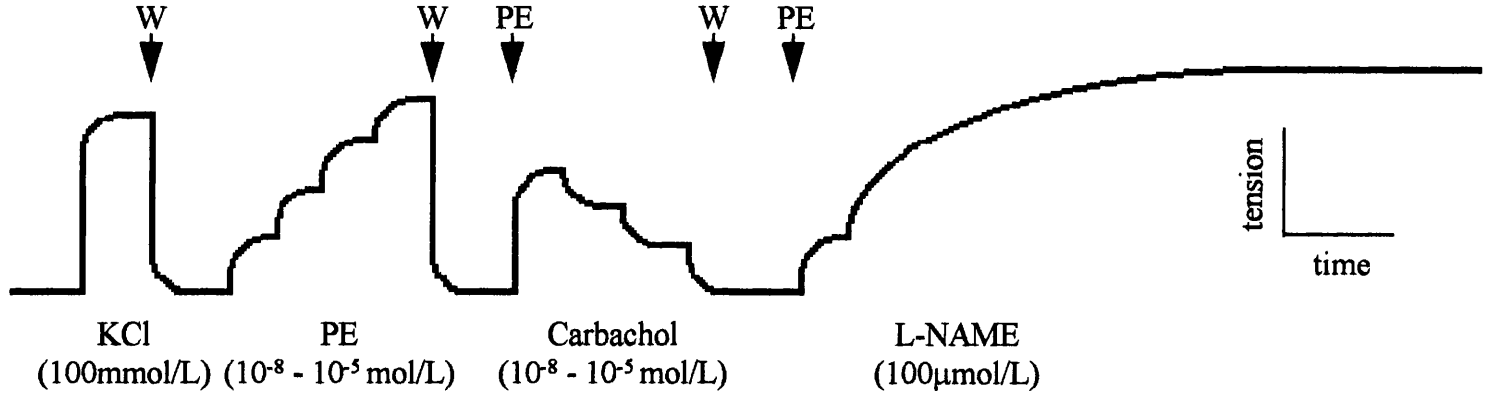

Figure 7 - Response to N^G-nitro-L-arginine methyl ester. Rings were contracted to potassium chloride (KCl, 100 mmol/L). After washout (W), a concentration response curve for phenylephrine (PE, 10^{-8} - 10^{-5} mol/L) was obtained. After washout, the rings were pre-contracted to the EC_{50} for PE and a concentration response curve obtained for carbachol (10^{-8} - 10^{-5} mol/L). After washout, the rings were pre-contracted to the EC_{20} for PE and N^G-nitro-L-arginine methyl ester (L-NAME, 100 µmol/L) added.

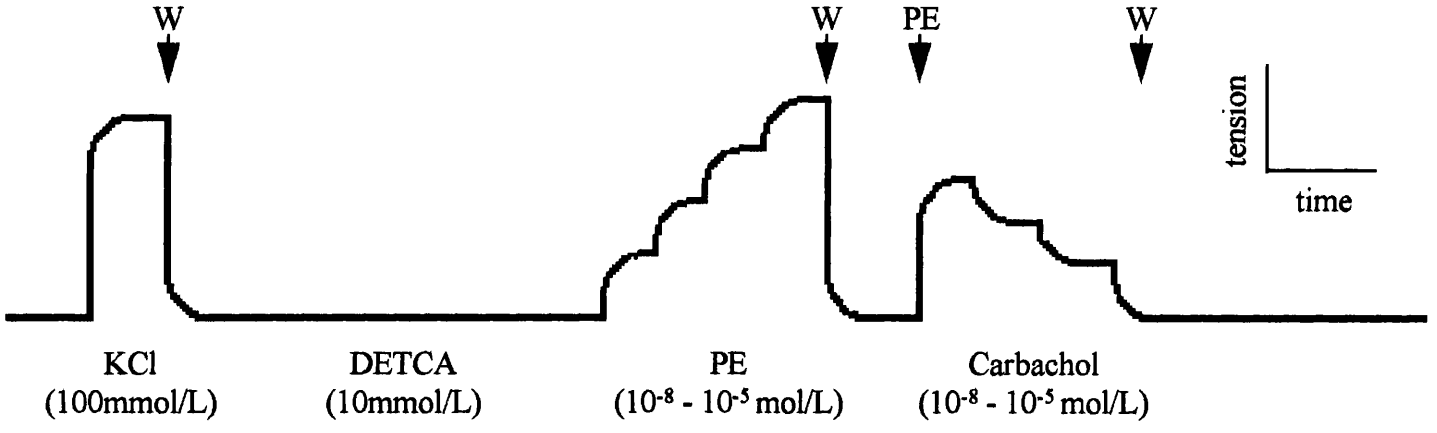

Figure 8 - Effect of diethyldithiocarbamate. Rings were contracted to potassium chloride (KCl, 100 mmol/L). After washout (W), the rings were incubated for 45 minutes with diethyldithiocarbamate (DETCA, 10 mmol/L). A concentration response curve for phenylepherine (PE, 10^{-8} - 10^{-5} mol/L) was obtained. After washout, the rings were pre-contracted to the EC_{50} for PE and a concentration response curve obtained for carbachol (10^{-8} - 10^{-5} mol/L).

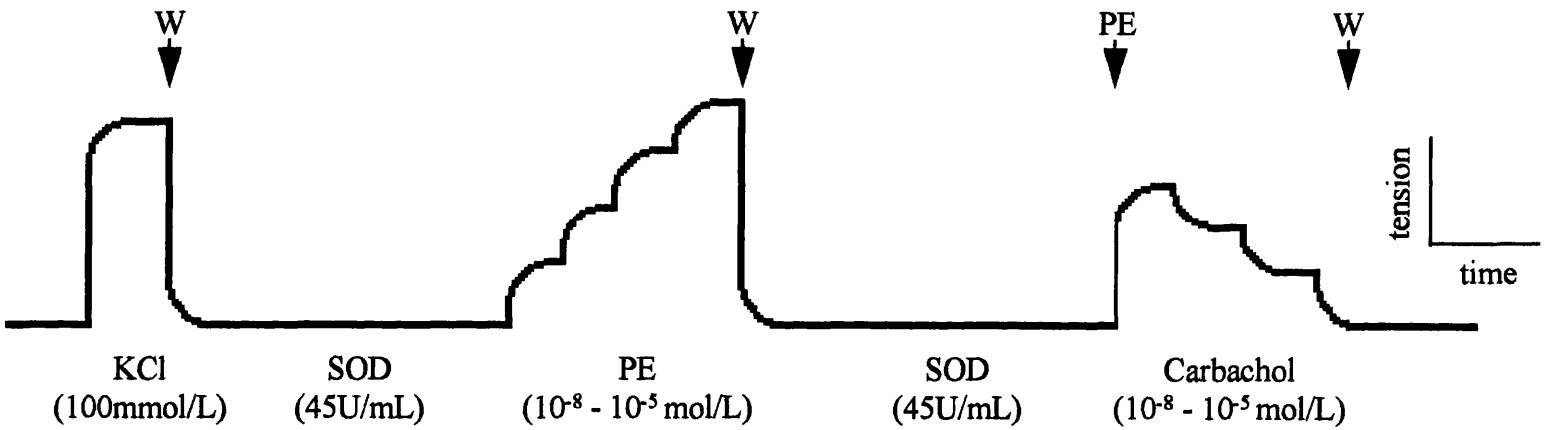

Figure 9 - Effect of superoxide dismutase. Rings were contracted to potassium chloride (KCl, 100 mmol/L). After washout (W), exogenous superoxide dismutase (SOD, 45 U/mL) was added and a concentration response curve for phenylepherine (PE, 10^{-8} - 10^{-5} mol/L) obtained. After washout, SOD (45 U/mL) was again added. The rings were pre-contracted to the EC_{50} for PE and a concentration response curve obtained for carbachol (10^{-8} - 10^{-5} mol/L).

3.3 Endothelial nitric oxide synthase activity measurement

Rats were studied at 16 weeks of age in four groups: WKY females (n=20), WKY males (n=15), SHRSP females (n=20) and SHRSP males (n=20). Measurement of NOS activity was as described[276]. Fresh aortic endothelial cells were obtained by carefully removing and cleaning the thoracic aorta as described above. The aorta was opened along its length and using a scalpel blade the endothelium was removed into homogenising buffer (Tris 50 mmol/L, sucrose 3.2 mmol/L, dithiothreitol 1 mmol/L, leupeptin 10 μg/mL, soybean trypsin inhibitor 10 μg/mL, aprotonin 2 μg/mL, pH 7.4). The aortas from five rats of the same sex and strain were pooled before assaying NOS activity to reduce variability. Endothelial cells in buffer were snap frozen in liquid N_2 and stored at -70°C until ready to assay.

On the day of the assay, the cells were thawed, sonicated 3 times for 3 seconds and centrifuged at 10,000g for 20 minutes. 40μL of the resultant supernatant containing both the soluble and particulate NOS protein was incubated at 37°C with the assay buffer (50 mmol/L KH_2PO_4, 1 mmol/L $MgCl_2$, 0.2 mmol/L $CaCl_2$, 50 mmol/L valine, 20μmol/L L-citrulline, 20μmol/L L-arginine, 1 mmol/L dithiothreitol, 100μmol/L NADPH, 3μmol/L tetrahydrobiopterin, 3μmol/L flavin adenine dinucleotide (FAD), 3μmol/L flavin mononucleotide (FMN) and 0.05μCi≈1μmol/L L-[U-^{14}C]-arginine, pH 7.2). Incubation was repeated in the presence of 3 mmol/L of the calcium chelator EGTA and again in the presence of 1 mmol/L N^{ω}-iminoethyl-L-ornithine (L-NIO), a NOS inhibitor[118]. After 20 minutes the reaction was terminated by the addition of 1:1 (v/v) double distilled water/activated Dowex-AG50W resin (200-400, 8% cross linked, Na^+ form) which removes arginine from the mixture. The resin was allowed to settle for 30 minutes and the amount of labelled citrulline present in the supernatant was quantified in terms of counts per minute

(cpm) by a liquid scintillation counter. The soluble protein content of the supernatant was determined by the Coomassie blue binding method using Bio-Rad Protein Assay with bovine serum albumin as standard. NOS activity was expressed as pmol citrulline produced per minute per mg of protein. Total NOS activity was calculated by subtracting NOS independent citrulline production (i.e. that in the presence of L-NIO) from the overall activity and calcium dependent NOS activity calculated as that which was absent in the presence of EGTA.

3.4 Rat aortic RNA analysis

3.4.1 Strategies for messenger RNA quantification

A number of widely used procedures exist for determining the abundance of a particular messenger RNA (mRNA) in a sample of total RNA. The two methods employed in these studies will be introduced and their relative merits discussed.

3.4.1.1 Northern blotting

Northern analysis remains the standard method for detection and quantification of relatively high abundance mRNA. Northern analysis allows a direct comparison of mRNA abundance between samples on a single blot and is the easiest method of determining transcript size, presence of alternatively spliced transcripts or multiple transcripts generated from a single locus.

Absolute quantification of a message by northern analysis is possible by running a series of different concentrations of an artificial sense strand of target RNA on the gel to construct a

standard curve of concentration *v* density of band. Experimental samples on the same gel can be compared against this standard.

The advantages of northern analysis are that it is fairly straightforward and exceptionally versatile. High specific activity random-primed or nick translated DNA probes, *in vitro* transcribed RNA probes, or oligonucleotide probes can all be used successfully. Additionally, probes with only partial homology, e.g. a complimentary DNA (cDNA) from a different species or a fragment of genomic DNA which might contain an exon(s) may be used.

However, northern analysis is the technique most vulnerable to RNA degradation. If RNA samples are even slightly degraded, the quality of the data and the ability to quantify expression are severely compromised. Thus, ribonuclease (RNase) free technique is essential. In addition, northern analysis is the least sensitive technique, although sensitivity can be improved by using high specific activity antisense RNA probes and charged nylon transfer membranes. Sensitivity can also be improved with oligo deoxythymidine [oligo (dT)] selection for enrichment of poly-adenylated [poly (A)] mRNA, which allows more target mRNA to be run in each lane without loss of resolution or saturation of the transfer membrane. Despite using 20μg of total RNA or 10μg of poly (A) mRNA per lane, rat *Nos3* message was present in too low abundance to be detected by northern analysis. Therefore, competitive reverse transcription polymerase chain reaction was used.

3.4.1.2 Reverse Transcription-Polymerase Chain Reaction

Reverse Transcription, coupled with the Polymerase Chain Reaction (RT-PCR), has revolutionised the study of gene expression. It is now possible to detect the RNA transcript

of any gene, irrespective of the paucity of starting material or relative abundance of the specific mRNA. In RT-PCR, a retroviral reverse transcriptase enzyme, e.g. Avian Myoblastosis Virus (AMV), uses oligo deoxythymidine [oligo $(dT)_{15}$] as a primer hybridising to the poly (A) tail of all mRNA templates to produce cDNA transcripts. As such small quantities of RNA are used, yeast transfer RNA (tRNA) is used in the RT reaction to stabilise the RNA. The target cDNA is then specifically amplified exponentially using sequence specific oligonucleotide primers in the PCR.

Theoretically, the abundance of a transcript can be determined by knowing the amount of total RNA used in the initial cDNA synthesis reaction, the amount of cDNA used in the PCR reaction, and the number of PCR cycles necessary to generate enough product to be detected either by direct visualisation on an ethidium bromide stained agarose gel or by Southern transfer and hybridisation to a radiolabelled probe. In practice, this technique is much more complicated. A major drawback to exponential amplification is that small sample to sample differences in amplification translate to big differences in yield product. Accurate determination of absolute abundance of a specific transcript by RT-PCR requires the use of competitive RT-PCR techniques.

Competitive RT-PCR makes use of an exogenous RNA transcript added during the RT step. This 'competitor' RNA must be accurately quantified and added to replica samples of total RNA in amounts that span the range of target mRNA levels. The competitor mRNA and target mRNA must RT and PCR with equal efficiency and a method must be available to discriminate the products from each other. Therefore, competitor mRNA usually consists of the target mRNA with a small (<100 bp) insertion or deletion in order that they can be resolved on an agarose gel. Alternatively, the target mRNA may be point mutated to insert

or remove a restriction enzyme site, allowing digestion with the enzyme to distinguish between the two products.

This method is technically laborious and costly as multiple RT and PCR reactions are required for each sample. However, competitive RT-PCR remains the accepted approach for quantification of low abundance transcripts, such as rat *Nos3*, which are undetectable by northern analysis.

3.4.2 Rat endothelial nitric oxide mRNA quantification

3.4.2.1 Isolation of total aortic RNA

Solutions used in RNA analysis were treated with 0.01% v/v diethylpyrocarbonate (DEPC) overnight at room temperature, then autoclaved to remove RNases.

Rats were studied at 16 weeks of age in four groups: WKY females (n=3), WKY males (n=3), SHRSP females (n=3) and SHRSP males (n=3). Rats were sacrificed by halothane overdose as described above. The thoracic aortas were quickly removed, cleaned of adipose tissue in ice cold saline and immediately snap frozen in liquid N_2. Frozen aortas were stored at -70°C until extracted for RNA.

On the day of RNA extraction each frozen aorta was dropped into 4 mL of ice cold RNAzol B and homogenised for 10 seconds using a Kinematica polytron homogeniser (Philip Harris Scientific, Aberdeen, UK). The polytron head was washed in 100% ethanol then DEPC treated distilled water (dH_2O) between samples. 400µL of chloroform was added to each

homogenate. The samples were then vortexed for 10 seconds and left on ice for 5 minutes. The samples were centrifuged at 2,500 r.p.m. at 4°C for 5 minutes in a pre-chilled bench top centrifuge (Centra-7R, International Equipment Company). The top layer was carefully removed into a 15 mL Corex tube which had been baked to remove RNases and pre-chilled on ice. An equal volume (≈1.8 mL) of isopropanol was added. Each sample was inverted several times and stored overnight at -20°C.

The following day the samples were centrifuged at 10,000 r.p.m. at 4°C for 15 minutes in a pre-chilled centrifuge (Model J2-21, Beckman). The supernatant was poured off and the RNA pellet washed in 70% ethanol/30% dH_2O (DEPC treated). The tubes were recentrifuged at 10,000 r.p.m. at 4°C for 15 minutes in a pre-chilled centrifuge. The supernatant was removed with a pipette and the pellet allowed to dry on ice for 15 minutes. The pellet was resuspended in 50μL of dH_2O (DEPC treated). Each RNA sample was quantified at least twice by measuring the OD_{260} on a spectrometer (Ultrospec 2000, Pharmacia Biotech, St. Albans, UK). The purity of the RNA was determined by the ratio of $OD_{260/280}$ and its integrity confirmed by running 2μg on an ethidium bromide (EtBr) stained 1% agarose gel. The 28S, 18S and 5S ribosomal RNA bands were identified under U.V. light and the RNA was stored at -70°C until required.

3.4.2.2 Removal of contaminating DNA

Genomic DNA contamination of the RNA would interfere with the RT-PCR quantification as the PCR reaction will amplify templates of genomic DNA as well as target and competitor cDNA. As the amount of contaminating DNA in each sample is unknown and variable, it makes quantification of target and competitor RNA impossible. Therefore, to

remove contaminating genomic DNA 10μg of each RNA was treated with RQ1 deoxyribonuclease (DNase). This enzyme digests double stranded DNA but has no RNase activity therefore leaves the RNA intact. The reaction mixture was as follows:-

$MgCl_2$ (25 mmol/L)	20μL
10X Mg^{2+} free PCR buffer	10μL
RQ1 DNase (1U/μL)	20μL
RNA (200 ng/μL)	50μL
	100μL

The reaction was carried out at 37°C for 1 hour, then terminated by addition of an equal volume of phenol/chloroform, vortexed for 10 seconds and spun at 14,000 r.p.m. at 4°C for 5 minutes in a chilled microcentrifuge (Model 5402, Eppendorf). The aqueous top layer was removed into a clean tube and an equal volume of chloroform was added, vortexed for 10 seconds and spun at 14,000 r.p.m. at 4°C for 5 minutes in a microfuge. The aqueous top layer was again removed into a clean tube and 3 volumes of 100% ethanol and 1/10th volume of DEPC treated sodium acetate (3 mol/L, pH 5.5) were added to precipitate the RNA. Each tube was inverted several times and stored at -20°C for 30 minutes. The tubes were spun at 14,000 r.p.m. at 4°C for 30 minutes in a microcentrifuge and the supernatant poured away. The pellets were washed in 70% ethanol/30% dH_2O (DEPC treated) and spun again at 14,000 r.p.m. at 4°C for 30 minutes in a microcentrifuge. The supernatant was aspirated and the pellet air dried on ice for 15 minutes. The pellet was resuspended in 10μL dH_2O (DEPC treated). Concentration, purity and integrity were determined as above. The RNA was stored at -70°C until required.

3.4.2.3 Reverse Transcription-Polymerase Chain Reaction

3.4.2.3.1 Preparation of competitor RNA

An aliquot of a competitor clone was kindly gifted by F.Soubrier (Paris, France). It was constructed as described[91]. The rat *Nos3* gene has not yet been cloned, therefore the sequence is not known. Oligonucleotide primers were designed from the human *Nos3* gene sequence[28]. The sense primer (5'-TGC,CTG,CCC,CAC,TGC,TCC,TC-3') is located within exon 21 and the antisense primer (5'-TGC,ACG,GTC,TGC,AGG,ACG,TTG,GT-3') is located within exon 25 of the human gene[73]. These primers were used to PCR amplify a fragment of rat cDNA from rat aortic RNA which had been reverse transcribed. This amplicon was gel purified and subcloned into the expression vector pBluescript to create the plasmid pReNOS1, which was sequenced to allow the design of rat *Nos3* specific PCR primers.

The sense primer designed was 5'-TTC,CGG,CTG,CCA,CCT,GAT,CCT,AA-3' and the antisense primer designed was 5'-AAC,ATG,TGT,CCT,TGC,TCG,AGG,CA-3'. These primers PCR amplify a 340 bp fragment from reverse transcribed *Nos3* cDNA. The competitor clone pReNIS5 was constructed by inserting a 64 bp fragment of polylinker between the above two primers, therefore the same primers will amplify a 404 bp fragment from reverse transcribed competitor RNA. One hundred ng of the aliquot of pReNIS5 plasmid was used to transform competent cells.

3.4.2.3.2 Preparation of competent cells

Escherichia coli (DH5α) cells (Cambridge Bioscience, Cambridge,UK) were made competent by a modification of the 'Miller' one-step competent cell procedure[277]. A 40μL aliquot of DH5α cells was inoculated into 2 mL of supplemented LB broth. This was incubated in a shaking incubator overnight at 225 r.p.m., 37°C. One mL of the fresh overnight culture was inoculated into 100 mL of supplemented LB broth and the OD_{600} measured on a spectrometer . This was incubated in a shaking incubator at 225 r.p.m., 37°C until the OD_{600} was 0.2-0.3. The culture was cooled by rotating the flask in ice/water and kept on ice thereafter to maintain maximum competence. The culture was transferred into a sterile, pre-chilled 250 mL centrifuge bottle and centrifuged at 1000g for 10 minutes at 4°C, without braking. The supernatant was decanted and the pellet resuspended in 10 mL of ice-cold 'Miller' transformation solution by swirling gently. The now competent cells were split into 100μL aliquots in 0.5 mL microtubes on dry ice, snap frozen in liquid N_2 and stored at -70°C until required.

3.4.2.3.3 Transformation of competent DH5α cells with pReNIS5 plasmid

A 100μL aliquot of competent DH5α cells was slowly thawed on ice and added to 1μL (100 ng) of pReNIS5 plasmid DNA in a sterile 1.5 mL microtube and the mixture kept on ice for 20 minutes. 900μL of supplemented LB broth, pre-heated to 37°C, was added and the mixture incubated in a shaking incubator at 225 r.p.m., 37°C for 90 minutes. The tube was spun at 14,000 r.p.m. for 1 minute in a microcentrifuge. A 700μL aliquot of supernatant was removed and the pellet resuspended in the remaining 300μL, which was then spread onto an L-amp agar plate. The plate was incubated upright at 37°C for 1 hour

to dry, then inverted and incubated at 37°C overnight. DH5α cells are sensitive to ampicillin, therefore should not grow. Only those which have been transformed with pReNIS5, which confers ampicillin resistance, should grow.

Competent DH5α cells were also transformed with 1μL of dH_2O as a negative control and to 1μL (50 ng) of another plasmid which confers ampicillin resistance as a positive control. Resulting colonies were processed similarly to those derived from the pReNIS5 transfections..

The following day, colonies were present on the pReNIS5 plate and the positive control plate, but not the negative control plate. The cells were confirmed to contain pReNIS5 plasmid by small-scale preparation of plasmid DNA and digestion by Eco RI and Hind III restriction enzymes.

3.4.2.3.4 Small scale preparation of pReNIS5 plasmid DNA

Small scale preparation of pReNIS5 plasmid DNA was performed by alkali lysis as described[278].

Using a sterile pipette tip, 4 colonies were picked from the plate of pReNIS5 transformed cells onto a 'master' L-amp agar plate and the tip dropped into 2 mL of LB-amp medium in 25 mL tubes. These were incubated in a shaking incubator at 225 r.p.m., 37°C overnight. The master plate was incubated in a 37°C oven overnight.

The following day, the master plate was stored at 4°C. A 1.5 mL aliquot of each overnight culture was transferred to 1.5 mL microtubes and centrifuged at 14,000 r.p.m. at 4°C for 30 seconds in a microcentrifuge. The supernatants were then removed by aspiration leaving the pellets as dry as possible. The bacterial pellets were resuspended in 100μL of ice-cold Solution I by vigorous vortexing and 200μL of freshly prepared Solution II was added. The contents were mixed by inverting 5 times. The tubes were stored on ice for 5 minutes and 150μL of ice-cold Solution III was added. The contents were mixed by vortexing in an inverted position for 10 seconds. The tubes were stored on ice for 5 minutes, then centrifuged at 14,000 r.p.m. at 4°C for 5 minutes in a microcentrifuge and the supernatant transferred to a fresh tube. Double stranded plasmid DNA was precipitated by adding 2 volumes of ethanol at room temperature, then vortexing and allowing the mixture to stand for 2 minutes at room temperature. The tubes were centrifuged at 14,000 r.p.m. at 4°C for 5 minutes. The supernatants were removed by aspiration and the pellets dried in an inverted position. The pellets were washed in 1 mL of 70% ethanol at 4°C. The centrifugation, aspiration and drying steps were repeated and the pellets resuspended in 50μL of TE (pH 8.0, 10 mmol/L Tris.Cl, 1 mmol/L EDTA) containing DNase-free RNase (20μg/mL). The concentration of plasmid DNA was calculated from the OD_{260} measured on a spectrometer and the quality assessed by running 2μg on a 1% (w/v) agarose gel.

In theory all of the clones picked should contain pReNIS5 plasmid DNA, but to test this all of the plasmid DNA samples were screened by digesting 10μg of DNA with the restriction enzymes Eco RI and Hind III as follows:-

DNA (2μg/μL)	5μL
10X Buffer B	1μL
Eco RI (10U/μL)	1μL
Hind III (10U/μL,)	1μL
dH_2O	2μL
	10μL

The reaction mixture was incubated at 37°C for 3 hours in a heating block and the products of digestion run on a 1% agarose gel with the undigested plasmid in an adjacent lane. All 4 of the clones screened dropped out a ≈600bp fragment as expected, confirming that they had indeed taken up the pReNIS5 plasmid.

3.4.2.3.5 Large scale preparation of pReNIS5 plasmid DNA

Having established that the colonies contained pReNIS5 plasmid, a large quantity of the transformed cells was grown up and plasmid isolated by alkali lysis as described[279].

Using a sterile pipette tip, one of the colonies was picked from the master plate and dropped into 2 mL LB-amp medium in a 25 mL tube. The culture was incubated in a shaking incubator at 225 r.p.m., 37°C overnight.

The following day, 500 mL of LB-amp medium in a 1L flask was inoculated with the 2 mL overnight culture and again incubated in a shaking incubator at 225 r.p.m., 37°C overnight. The following day the culture was split into 2 x 250 mL bottles and centrifuged at 4000 r.p.m. for 15 minutes at 4°C. The supernatants were poured away and the pellets resuspended in 100 mL of ice-cold STE. These suspensions were again centrifuged at 4000 r.p.m. for 15 minutes at 4°C and the supernatants poured away.

The washed pellets were resuspended in 9 mL of Solution I. 1 mL of a freshly prepared solution of lysozyme was added to each bottle, followed by 20 mL of freshly prepared Solution II. The contents were mixed thoroughly by inversion and stored at room temperature for 10 minutes. Ten mL of ice-cold Solution III was added and the mixture shaken vigorously. The bottles were kept on ice for 10 minutes allowing a flocculent white precipitate of chromosomal DNA, high molecular weight RNA and protein/membrane complexes to form.

The bacterial lysates were centrifuged at 4000 r.p.m. for 15 minutes at 4°C and the rotor allowed to stop without braking. The supernatants were filtered through gauze swabs into 2 clean 250 mL bottles and 0.6 volumes of isopropanol was added, mixed well and kept at room temperature for 10 minutes. Plasmid DNA was recovered by centrifugation at 5000 r.p.m. for 15 minutes at room temperature, then washed in 5 mL of 70% ethanol and dried by aspiration. The dried pellets were resuspended in 1.5 mL of TE and pooled. This volume was made up to exactly 5.0 mL in preparation for purification by equilibrium centrifugation in a caesium chloride (CsCl)-ethidium bromide gradient.

3.4.2.3.6 Purification of pReNIS5 plasmid DNA by CsCl gradient

The pReNIS5 plasmid obtained from the above large scale preparation was purified by equilibrium centrifugation in a CsCl-ethidium bromide gradient as described[280].

Exactly 5.0g of CsCl was added to the 5.0 mL of plasmid DNA and the solution warmed to 37°C for 30 minutes to facilitate the dissolution of the salt. 0.4 mL of an aqueous solution of ethidium bromide (10 mg/mL) was added and mixed thoroughly. Using a disposable syringe fitted with a large-gauge needle, the clear, red solution was transferred to a Quick-

Seal Ultra tube (Beckman, High Wycombe, UK), topped up with mineral oil and heat-sealed. The density gradients were spun on an ultracentrifuge (Beckman L8-55) at 49,000 r.p.m. for 16 hours at 20°C with the brake off.

Under ultraviolet light, 3 bands were visible in the tube; an upper band of protein, a middle band of nicked circular or linearised DNA and a lower band of closed circular DNA. RNA formed a pellet at the bottom. The tube was punctured at the top by a 21G hypodermic needle to break the seal and just below the closed circular DNA band with another 21G hypodermic needle attached to a disposable syringe. The closed circular DNA was 'pulled' into the syringe taking care not to include any nicked circular or linearised DNA.

The DNA solution was added to an equal volume of water saturated butanol and the two phases mixed by vortexing. When the two phases had settled, the upper pink organic phase was discarded. This was repeated several times until the aqueous and organic phases were completely clear.

The DNA was precipitated by aliquotting into 1.5 mL microtubes, adding 2 volumes of ethanol and storing at 4°C for 15 minutes. The DNA was pelleted by spinning at 14,000 r.p.m. for 30 minutes at 4°C in a microcentifuge. The supernatants were removed and the pellets washed in 70% ethanol before respinning at 14,000 r.p.m. for 5 minutes at 4°C. The supernatants were again aspirated and the pellets resuspended in 100μL of TE and pooled.

The DNA was quantified by measuring the OD_{260} on a spectrometer . The quality of the DNA was assessed and absence of RNA confirmed by running 2μg on a 1% agarose gel. The DNA was stored in aliquots at -20°C.

3.4.2.3.7 *In vitro* transcription of pReNIS5 RNA

20μg of pReNIS5 plasmid DNA from the above large scale preparation was linearised with Eco RI restriction enzyme as follows:-

pReNIS5 DNA (1μg/μL)	20μL
Eco RI (10U/μL)	4μL
10X Buffer H	4μL
dH_2O	12μL
	40μL

The reaction was incubated at 37°C. After 4 hours, 2μL of the digest mix was run on a 1% agarose gel with 2μL of uncut plasmid in an adjacent lane to ensure that the digest was complete.

When the Eco RI digest was complete, 2μL of RNase-free Proteinase K (1 mg/mL) was added to remove the restriction enzyme and any RNases. The mixture was incubated for a further 30 minutes at 37°C. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added, vortexed for 10 seconds, spun for at 14,000 r.p.m. for 5 minutes at 4°C in a microcentrifuge; and the aqueous top layer removed into a fresh 1.5 mL microtube. This was repeated, then the mixture was extracted with 100% chloroform in the same way. Three volumes of ethanol and 1/10th volume of sodium acetate (3 mol/L, pH 5.5) were added to the purified, linearised DNA. This was mixed and stored at -20°C overnight.

The DNA was recovered by spinning at 14,000 r.p.m. for 30 minutes at 4°C in a microcentrifuge, washing in 70% ethanol/30% dH_2O (DEPC treated) and respinning at 14,000 r.p.m. for 30 minutes at 4°C. The supernatant was aspirated and the DNA was dried

and resuspended in 20μL of RNase-free TE and quantified by measuring the OD_{260} on a spectrometer.

The mCAP mRNA Capping Kit was used to *in vitro* transcribe pReNIS5. The 5' cap structure of *in vitro* mRNA transcripts increases stability of the synthesised RNA[281]. T3 RNA transcripts, synthesised *in vitro* with the mCAP Capping Kit, are initiated with the 7MeGpppG 5' analogue similar to that present in eukaryotic mRNA. The cap structure is incorporated at the 5' end of 90 - 95% of the resulting RNA transcripts. RNA transcription was initiated at the T3 RNA polymerase site on the linearised pReNIS5 plasmid DNA as follows:-

5X Transcription Buffer	5.0μL
pReNIS5 DNA template (0.5μg/μL)	2.0μL
rNTP mix	1.0μL
mCAP analogue	2.5μL
Dithiothreitol (DTT, 0.75 mol/L)	1.0μL
T3 RNA polymerase (5U/μL)	2.0μL
dH_2O (DEPC treated)	11.5μL
	25.0μL

The reaction was incubated at 37°C. After 30 minutes, 1μL (10U) of RNase-free DNase was added and the incubation continued for a further 5 minutes to remove the template DNA. One hundred μL of dH_2O (DEPC treated) was added and the RNA was phenol/chloroform extracted as described above. The RNA was precipitated in 3 volumes of ethanol and 1/10th volume of DEPC treated sodium acetate (3 mol/L, pH 5.5) and stored at -20°C for 30 minutes. The RNA was recovered by spinning at 14,000 r.p.m. for 30 minutes at 4°C in a microcentrifuge, washing in 70% ethanol/30% dH_2O (DEPC treated) and respinning at 14,000 r.p.m. for 30 minutes at 4°C. The pellet was dried and the RNA was resuspended in 25μL of RNase-free TE and quantified on a spectrometer. A single, intact

transcript was confirmed by running 2μL on a 1% agarose gel. It has been shown that unincorporated nucleotides contribute 20% of the OD_{260}[91], therefore the measured concentration was adjusted accordingly. From the adjusted concentration, the pReNIS5 RNA was serially diluted to a range of concentrations between 40-280 fg/μL for use as competitor RNA in the RT-PCR reaction.

3.4.2.3.8 Reverse transcription

For each sample of aortic RNA, 10 RT reactions were set up as follows:

	Aortic RNA (ng)	Competitor RNA (fg)	RT
1)	100	0	+
2)	100	40	+
3)	100	80	+
4)	100	120	+
5)	100	160	+
6)	100	200	+
7)	100	240	+
8)	100	280	+
9)	100	280	-
10)	dH_2O (DEPC treated)		+

Each reaction mixture consisted of the following:-

$MgCl_2$ (25 mmol/L)	4.0μL
10X Mg^{2+} free PCR buffer	2.0μL
dNTP's (10 mmol/L)	2.0μL
rRNAsin (40 U/μL)	0.6μL
AMV RT (10 U/μL)	0.6μL
Oligo$(dT)_{15}$ primer (0.5 μg/μL)	1.0μL
Yeast tRNA (1 μg/μL)	1.0μL
Target RNA (100ng/μL)	1.0μL
Competitor RNA (0-280fg/μL)	1.0μL
dH_2O (DEPC treated)	6.8μL
	20.0μL

The reaction was left at room temperature for 10 minutes to allow the oligo$(dT)_{15}$ primer to anneal to the mRNA, incubated at 42°C for 45 minutes, then transferred to ice. A 3μL aliquot was used for the PCR reaction and the remainder stored at -20°C.

3.4.2.3.9 Polymerase chain reaction

For each sample of aortic RNA, 10 PCR reactions were set up; 1 for each of the above RT reactions. Note that the aliquots of cDNA from each RT reaction contained 5 mmol/L Mg^{2+}, therefore the amount of $MgCl_2$ added to the PCR was proportionately less to give a final reaction concentration of 1.5 mmol/L. Each reaction therefore consisted of the following:-

$MgCl_2$ (25 mmol/L)	0.7μL
10X Mg^{2+} free PCR buffer	2.0μL
dNTP's (1.25 mmol/L)	3.2μL
Forward primer (10 μmol/L)	1.0μL
Reverse primer (10 μmol/L)	1.0μL
Taq DNA polymerase (5 U/μL)	0.2μL
cDNA	3.0μL
dH_2O	8.9μL
	20.0μL

The reaction was subjected to an initial denaturation at 94°C for 5 minutes; then 28 cycles of 30 seconds at 94°C, 30 seconds annealing at 62°C and 1 minute elongation at 72°C; then completion of ongoing reactions at 62°C for 1 minute and 72°C for 10 minutes in a Hybaid Omnigene PCR machine (Life Sciences International, Basingstoke, UK). The expected PCR product sizes were 340 bp for the target cDNA and 404 bp for the competitor cDNA.

3.4.2.3.10 Visualisation of PCR products

The products of all 10 PCR reactions were separated on a 2% agarose gel at 100V for 2 hours. The unincorporated primers, which run ahead of the PCR products, were cut off and the gel blotted onto Hybond N+ nylon membrane by the alkali transfer method.

3.4.2.3.11 Southern blotting of DNA by alkali transfer

The gel was rinsed in dH_2O then completely covered in denaturing solution and left for 30 minutes at room temperature with shaking. Excess liquid was blotted from the gel then it was equilibrated for 10 minutes in alkali transfer buffer.

A tray was filled with alkali transfer buffer. A platform was made and covered with a wick made from 3 sheets of Whatman 3MM filter paper, saturated with alkali transfer buffer. Both ends of the wick sat in the buffer underneath. The gel was inverted onto the wick. Care was taken to avoid trapping air bubbles beneath it. The gel was surrounded by Parafilm to prevent the alkali transfer buffer from being absorbed directly into the paper towels above. A sheet of Hybond N+ nylon membrane was cut to the exact size of the gel and placed on top of the inverted gel. Air bubbles were removed by carefully rolling a sterile pipette over the membrane. 3 sheets of Whatman 3MM filter paper were cut to the exact size of the gel, wetted in alkali transfer buffer and placed on top of the membrane one at a time. Air bubbles were again removed by rolling with a sterile pipette. A stack of absorbent tissues was placed on top of the 3MM paper. A glass plate was placed on top of the absorbent tissue and a 0.5 Kg weight (500 mL of water) put on top. After 1 hour the apparatus was dismantled and any wet tissues replaced with dry ones. The apparatus was reassembled and the transfer allowed to proceed overnight.

The following day the apparatus was dismantled. The membrane was air dried for 15 minutes, baked at 80°C for 30 minutes, then UV cross-linked (2 x auto setting, Stratalinker 2000, Stratagene Ltd, Cambridge, UK). The membrane was pre-hybridised in the following solution:

20X SSPE	6.25mL (5X)
100X Denhardt's solution	1.25mL (5X)
SDS (10% w/v)	1.25mL (0.5%)
dH_2O	16.25mL
	25.00mL

Prior to adding the membrane, 500µL of sheared salmon sperm DNA (1 mg/mL) was denatured at 100°C for 5 minutes, chilled on ice, then added to the pre-hybridisation mix. The membrane was pre-hybridised for 1 hour at 65°C in a rotating flask in preparation for hybridisation with an end labelled primer probe.

3.4.2.3.12 Making an end labelled primer probe

The forward primer used in the PCR reaction was 3'-end labelled with [α^{32}P]-dCTP using the enzyme terminal deoxynucleotidyl transferase (TdT) as follows:-

5X TdT buffer	4.0µL
Forward primer (10 µmol/L)	0.7µL
TdT (15-30 U/µL)	0.5µL
[α^{32}P]-dCTP (0.37 mCi/µL)	1.0µL
dH_2O	13.8µL
	20µL

The reaction was incubated at 37°C for 30 minutes, then stopped by heating to 68°C for 10 minutes. The probe was added to the membrane in the pre-hybridisation mix and hybridised overnight at 65°C.

The following day the membrane was washed to remove non-hybridised probe as follows:

2X SSPE/0.1% SDS at room temperature for 10 minutes

2X SSPE/0.1% SDS at room temperature for 10 minutes

1X SSPE/0.1% SDS at 65°C for 15 minutes

0.1X SSPE/0.1% SDS at 65°C for 10 minutes

The membrane was wrapped in 'cling-film' and exposed to film (Hyperfilm-MP) in a Hypercassette (Amersham International plc, Amersham, UK) with two intensifying screens overnight at -70°C. The film was developed in a KODAK X-OMAT ME-3 Processor. Two lines of bands were noted (Figure 10a). The top line of bands of increasing intensity corresponds to the competitor, while the lower line corresponds to the target.

3.4.2.3.13 Calculation of target mRNA concentration

A scanned image of the autorad was created by a densitometer (Model GS 670, Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). A typical image is shown in Figure 10a. The total density of each individual band was measured using Molecular Analyst 2.1 software (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). A plot was drawn of log competitor RNA concentration (x-axis) against log ratio of competitor density/target density (y-axis). Such a plot is illustrated in Figure 10b. When the log ratio of competitor density/target density = 0, then competitor and target concentrations are equal. The competitor concentration at this point can be read from x-axis, which therefore corresponds to the target mRNA concentration in fg per 100ng total aortic RNA.

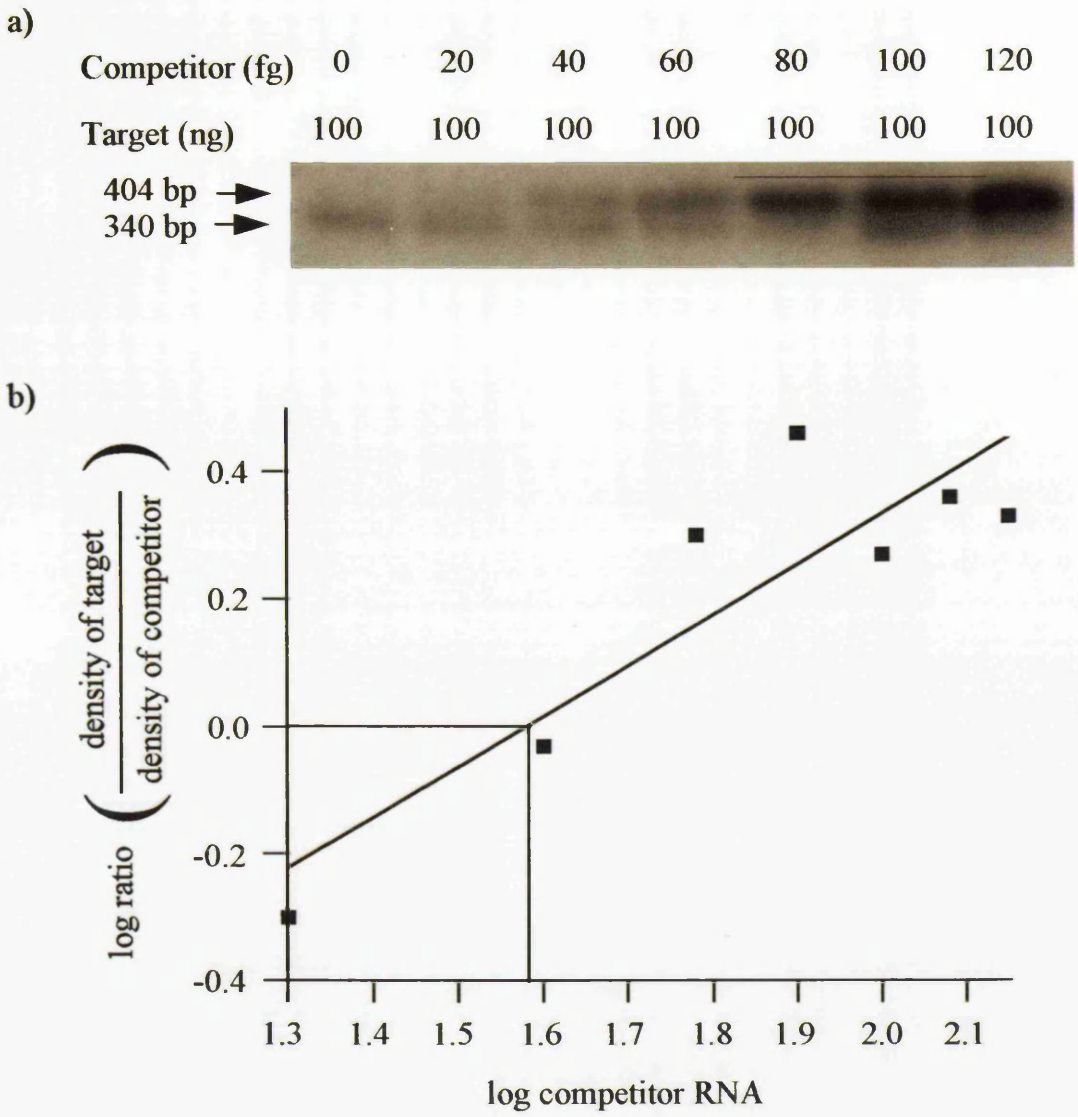

Figure 10 - Quantification of *Nos3* mRNA by competitive RT-PCR. A constant amount of total RNA from rat aorta (target) was mixed with increasing amounts of internal standard mRNA (competitor), reverse transcribed and amplified by PCR with *Nos3* specific primers. The PCR products were run on a 2% (w/v) agarose gel, blotted onto nylon membrane, hybridised with an end labelled primer as a probe and exposed to photographic film. The density of each band was measured and the logarithm of the ratio (density of target/density of competitor) plotted against the logarithm of the known amount of competitor mRNA in each reaction. At the competition equivalence (log ratio = 0), the amount of target mRNA (unknown) corresponds to the amount of competitor mRNA (known). In this example, 100ng of total aortic RNA from a WKY female contained 39fg of *Nos3* mRNA.

3.4.3 Quantification of rat manganese and copper/zinc superoxide dismutase mRNA by northern blotting

3.4.3.1 Isolation of total aortic RNA

Total aortic RNA was isolated by RNAzol B and quantified as described in section 3.4.2.1.

3.4.3.2 Size fractionation by agarose gel electrophoresis

Ten μg samples of total aortic RNA from 4 WKY females and 4 SHRSP females and 2 x 10μL aliquots of λ Hind III DNA size markers were precipitated by adding 3 volumes of ethanol and 1/5th volume of DEPC treated sodium acetate (3 mol/L, pH 5.5), mixing and storing at -20°C overnight. Ten μg samples of total aortic RNA from 4 WKY males and 4 SHRSP males and 2 x 10μL aliquots of λ Hind III DNA size markers were prepared in an identical fashion for running on a subsequent gel.

The following day, samples were spun at 14,000 r.p.m. for 15 minutes at 4°C in a microcentrifuge. The supernatants were removed and the pellets washed with 180μL of 70% ethanol/30% dH_2O (DEPC treated), then respun at 14,000 r.p.m. for 5 minutes at 4°C in a microcentrifuge. The supernatant was removed and the pellet air dried for 5 minutes.

Each pellet was resolubilised in 3.7μL of dH_2O (DEPC treated) and 12.3μL of freshly prepared glyoxal mix added to each, taking care that the RNA was properly redissolved. The samples were glyoxalated at 50°C for 60 minutes.

While the samples were glyoxalating, a 1.2% (w/v) agarose gel was made by adding 1.8g of agarose to 120 mL of dH_2O (DEPC treated) and dissolving by microwaving. When the agarose had cooled to hand warm, 13.4 mL of sodium phosphate buffer (0.1 mol/L, pH 7.0) was added, mixed and poured into a 120 x 140 mm gel tray and allowed to set with a 12-tooth comb at one end. The tray, comb and tank were reserved exclusively for RNA gels. RNases were removed on the day prior to running the gel by soaking in 3% (v/v) hydrogen peroxide for 15 minutes, rinsing with dH_2O then leaving in an inverted position overnight to dry.

When the denaturation was completed, the samples were cooled on ice and 4μL of 5X RNA loading buffer added to each. They were immediately loaded into the wells with the DNA size markers in lanes 1 and 12 and the RNA samples in lanes 3-10. The gel was run submerged in sodium phosphate buffer (10 mmol/L, pH 7.0) at 100V for 4 hours. Glyoxal dissociates from RNA at pH > 8.0, therefore a Hybaid 'Buffer Puffer' tank (Life Sciences International, Basingstoke, UK) was used to circulate the buffer, thus preventing the pH at either electrode reaching 8.0. When the run was completed, the RNA was transferred to nylon membrane.

3.4.3.3 Transfer to nylon membrane

The transfer apparatus was very similar to that used for transfer of the RT-PCR products described in section 3.4.2.3.11.

A tray was filled with 20X SSC (DEPC treated). A platform was made and covered with a wick made from 3 sheets of Whatman 3MM filter paper, saturated with 20X SSC (DEPC treated). Both ends of the wick sat in the buffer underneath. The gel was slid onto the wick.

Care was taken to avoid trapping air bubbles beneath it. The gel was surrounded by Parafilm to prevent the 20X SSC from being absorbed directly into the paper towels above. A sheet of Hybond N+ nylon membrane was cut to the exact size of the gel and placed on top of the gel. Air bubbles were removed by carefully rolling a sterile pipette over the membrane. 6 sheets of Whatman 3MM filter paper were cut to the exact size of the gel, wetted in 2X SSC (1:10 dilution of 20X SSC) and placed on top of the membrane one at a time. Air bubbles were again removed by rolling with a sterile pipette. A stack of absorbent tissues was placed on top of the 3MM paper. A glass plate was placed on top of the paper towels and a 0.5 Kg weight (500 mL of water) put on top. After 1 hour the apparatus was dismantled and any wet tissues replaced with dry ones. The apparatus was reassembled and the transfer allowed to proceed overnight.

The following day the apparatus was dismantled. The membrane was air dried for 30 minutes, baked at 80°C for 30 minutes to deglyoxalate the RNA, then UV cross-linked (2X auto setting, Stratalinker 2000, Stratagene Ltd, Cambridge, UK). Resolution and transfer of the samples were confirmed by cutting off the DNA size marker tracks and staining for 5 minutes in methylene blue (0.4% w/v) in sodium acetate (0.5 mol/L, pH 5.5), followed by destaining for 20 minutes in dH_2O.

The membrane was pre-hybridised in the following solution:

Deionised formamide	20.0mL (50%)
20X SSPE	10.0mL (5X)
100X Denhardt's solution	2.0mL (5X)
SDS (10% w/v)	2.0mL (0.5%)
EDTA (0.4 mol/L, pH 8.0)	0.1mL (1mmol/L)
dH_2O (DEPC treated)	5.9mL
	40.0mL

This was heated to 65°C to ensure complete dissolution of the components, then 20 mL was added to the membrane in a hybridisation tube and pre-hybridised at 42°C for at least 4 hours. The remaining 20 mL was kept at 42°C.

3.4.3.4 Preparation of radiolabelled probes

3.4.3.4.1 Glyceraldehyde phosphate dehydrogenase

An aliquot of the plasmid pRP-GAP was kindly gifted by MJ Brosnan within the department. This plasmid consists of a 216 bp fragment from the 5' end of the murine glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA inserted into the pGEM1 vector which confers ampicillin resistance. Competent DH5α cells were transformed with 100ng of pRP-GAP as described for pReNIS5 in section 3.4.2.3.3. Small scale preparation, large scale preparation and CsCl gradient purification were carried out for pReNIS5 in sections 3.4.2.3.4-6.

Fifty μg of CsCl purified pRP-GAP was digested with Eco RI and Hind III restriction enzymes to drop out the 248 bp GAP fragment (including 32 bp of plasmid polylinker) as follows:-

pRP-GAP DNA (1μg/μL)	50μL
10X Buffer B	10μL
Eco RI (10U/μL)	10μL
Hind III (10U/μL)	10μL
dH_2O	20μL
	100μL

The reaction mixture was incubated at 37°C for 3 hours, then a 2μL aliquot was run on an ethidium bromide stained 1% w/v agarose gel with undigested plasmid in an adjacent lane. When complete digestion was confirmed, the remainder of the digest was run on a 1% w/v agarose gel to separate the GAPDH fragment from the remainder of the plasmid. The band corresponding to the GAPDH fragment was cut out using a scalpel blade and stored in a microfuge tube, wrapped in tin-foil at 4°C.

Ten cm lengths of dialysis tubing were prepared by boiling in $NaHCO_3$ (2% w/v) and EDTA (1 mmol/L, pH 8.0) for 10 minutes; rinsing thoroughly in distilled water; then boiling for 10 minutes in EDTA (1 mmol/L, pH 8.0). They were allowed to cool and stored in EDTA (1 mmol/L, pH 8.0) at 4°C.

A 10 cm length of dialysis tubing was washed in distilled water and sealed with a clip at one end. The gel containing the GAPDH fragment was placed in the dialysis tubing along with 500μL of 1X TAE buffer. The other end was sealed with a clip and the 'bag' placed transversely in a gel tank containing 1X TAE buffer. The GAPDH fragment DNA was electroeluted from the gel onto the inside of the dialysis tubing. After 1 hour, the 'bag' was examined on a UV light box to ensure that all of the ethidium bromide stained DNA had left the gel and was adherent to the wall of the tubing. One of the clips was carefully removed and the gel discarded. The DNA was resuspended in the 500μL of buffer, transferred to a microfuge tube and precipitated with 2 volumes of ethanol and 1/10th volume of sodium acetate (3 mol/L, pH 5.5). The pellet was washed in 70% ethanol, dried and resuspended in 50μL of TE (pH 8.0).

The concentration of the GAPDH fragment DNA was calculated from the OD_{260} and 2μg run on a 2% w/v agarose gel to confirm that a single band of ≈250 bp was present. The GAPDH fragment was diluted to 50 ng/μL and stored at -20°C.

3.4.3.4.2 Rat manganese superoxide dismutase

The plasmid pSP65-RMS was kindly gifted by Y-S Ho (Detroit, USA). This plasmid consists of the entire 1.4kb rat MnSOD cDNA cloned into the Eco RI site of the pSP65 vector which confers ampicillin resistance. Competent DH5α cells were transformed with 100ng of pSP65-RMS as described for pReNIS5 in section 3.4.2.3.3. Small scale preparation, large scale preparation and CsCl gradient purification were carried out for pReNIS5 in sections 3.4.2.3.4-6.

Fifty μg of CsCl purified pSP65-RMS was digested with Eco RI restriction enzyme to drop out the 1.4kb Mn SOD fragment as follows:-

pSP65-RMS DNA (1μg/μL)	50μL
10X Buffer B	10μL
Eco RI (10U/μL)	10μL
dH_2O	30μL
	100μL

The reaction mixture was incubated at 37°C for 3 hours, then a 2μL aliquot was run on an ethidium bromide stained 1% w/v agarose gel with undigested plasmid in an adjacent lane. When complete digestion was confirmed, the remainder of the digest was run on a 1% w/v agarose gel to separate the Mn SOD fragment from the remainder of the plasmid. The band corresponding to the Mn SOD fragment was cut out using a scalpel blade. The MnSOD DNA was electroeluted, ethanol precipitated, washed, resuspended in 50μL TE, quantified,

diluted to 50 ng/μL and stored at -20°C as described for the GAPDH fragment in section 3.4.3.4.1.

3.4.3.4.3 Rat copper/zinc superoxide dismutase

The plasmid pUC13-RCS was also kindly gifted by Y-S Ho (Detroit, USA). This plasmid consists of the entire 0.6kb of rat Cu/Zn SOD cDNA inserted into the Eco RI site of the vector pUC13 which confers ampicillin resistance. Competent DH5α cells were transformed with 100ng of pUC13-RCS as described for pReNIS5 in section 3.4.2.3.3. Small scale preparation, large scale preparation and CsCl gradient purification were carried out for pReNIS5 in sections 3.4.2.3.4-6.

Fifty μg of CsCl purified pUC13-RCS was digested with Eco RI restriction enzyme to drop out the 0.6kb Cu/Zn SOD fragment as described for the MnSOD fragment in section 3.4.3.4.2. The Cu/Zn SOD DNA was separated, electroeluted, ethanol precipitated, washed, resuspended in 50μL TE, quantified, diluted to 50 ng/μL and stored at -20°C as described for the GAPDH fragment in section 3.4.3.4.1.

3.4.3.4.4 Random primer radiolabelling

A random primers DNA labelling kit was used to radiolabel the cDNA probes with [α^{32}P]-dCTP. Fifty ng of template cDNA (GAP, MnSOD or Cu/Zn SOD) was labelled in each reaction as follows:

Template cDNA (50 ng/μL)	1μL
dH_2O	22μL

This was heated to 100°C for 5 minutes then cooled on ice.

dATP	2μL
dGTP	2μL
dTTP	2μL
Random Primers Buffer	15μL
[α^{32}P]-dCTP (0.37 mCi/μL)	5μL
Klenow fragment DNA polymerase	1μL
	50μL

This was incubated at room temperature for at least 1 hour. The incorporation of radiolabel into the probe was calculated by aliquotting 1μL of the reaction mix onto a small filter disc and counting the disintegrations per minute (d.p.m.) in a scintillation counter (TRI-CARB 2100TR, Canberra Packard). Unincorporated label was removed by washing the filter disc five times in Na_2HPO_4 (0.5 mol/L), twice in dH_2O and twice in 100% ethanol. The filter disc was air dried and the d.p.m. counted again. The post-wash d.p.m. expressed as a percentage of the pre-wash d.p.m. was taken as a measure of incorporation. 75-85% incorporation was usually achieved.

3.4.3.5 Hybridising membrane with radiolabelled probes

When the membrane had been pre-hybridising for at least 4 hours (section 3.4.3.3), the pre-hybridisation solution was discarded and replaced with the remaining 20 mL of hybridisation solution at 42°C. The radiolabelled probe was denatured at 100°C for 5 minutes, cooled on ice for 5 minutes, then added to the hybridisation solution. The membrane was hybridised at 42°C overnight.

The following day, non-hybridised probe was removed by washing the membrane as follows:-

2X SSPE/0.1% SDS,	room temperature,	10 minutes
2X SSPE/0.1% SDS,	room temperature,	10 minutes
2X SSPE/0.1% SDS,	65°C,	15 minutes
2X SSPE/0.1% SDS,	65°C,	15 minutes
1X SSPE/0.1% SDS,	65°C,	10 minutes
1X SSPE/0.1% SDS ,	65°C,	10 minutes
1X SSPE/0.1% SDS,	65°C,	30 minutes
1X SSPE/0.1% SDS,	65°C,	30 minutes

The membrane was wrapped in 'cling-film' and exposed to film (Hyperfilm-MP) in a Hypercassette (Amersham International plc, Amersham, UK) with two intensifying screens overnight at -70°C. The film was developed in a KODAK X-OMAT ME-3 Processor. The membrane was exposed to film for a further 2-7 days depending on the darkness of the bands on the autorad.

3.4.3.6 Quantification of band density

A scanned image of the autorad was created by a densitometer (Model GS 670, Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) and the density of each band measured using Molecular Analyst 2.1 software (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). A background density was measured above each band and subtracted from the corresponding band density to produce a net density for each band corrected for any background changes

across the autorad. The net density of each band was expressed as a ratio to GAPDH for that lane to correct for unequal loading of the lanes.

3.5 Effect of age on endothelial function

In addition to the 4 groups of rats studied at 4 months of age in 3.2.2, basal NO availability was also assessed in a further 8 groups of rats at 6 and 12 months of age to give a total of 12 groups as follows:-

WKY females	4 months (n=15)	WKY males	4 months (n=19)
	6 months (n=7)		6 months (n=8)
	12 months (n=11)		12 months (n=7)
SHRSP females	4 months (n=16)	SHRSP males	4 months (n=16)
	6 months (n=11)		6 months (n=7)
	12 months (n=9)		12 months (n=7)

The rats were killed by halothane overdose. The thoracic aortas were carefully removed, cleaned of adipose tissue and cut into 2-3 mm rings. The rings were suspended under 1g tension in individual 10 mL muscle baths as described in section 3.2. Basal NO availability was assessed by observing the contraction in response to L-NAME as described in section 3.2.2.

3.6 The effect of 17β-oestradiol *in vivo*

3.6.1 17β-oestradiol administration

Five female WKY rats at 12 months of age were treated with 17β-oestradiol, 20μg in 200μL arachis oil per day intraperitoneally for 14 days. A further group of 5 female WKY rats at 12 months of age were given arachis oil, 200μL per day, intraperitoneally for 14 days as controls.

3.6.2 Isometric tension recording

The rats were killed by halothane overdose. The thoracic aortas were carefully removed from 17β-oestradiol treated and control rats, cleaned of adipose tissue and cut in half. One half of each aorta was cut into 2-3 mm rings and the other half snap frozen in liquid N_2 for RNA analysis (see section 3.6.3). The rings were suspended under 1g tension in individual 10 mL muscle baths as described in section 3.2. After a 1 hour equilibration period, stimulated NO release was assessed by obtaining a concentration response curve for carbachol (10^{-8} - 10^{-5} mol/L) as described in section 3.2.1. Basal NO availability was then assessed by observing the contraction in response to L-NAME (100 mmol/L) as described in section 3.2.2.

3.6.3 The effect of 17β-oestradiol *in vivo* on mRNA expression

3.6.3.1 Isolation of total aortic RNA

RNA was extracted from the frozen half aortas by RNAzol B as described in section 3.4.2.1.

3.6.3.2 Endothelial nitric oxide synthase mRNA quantification

Endothelial NOS mRNA was quantified in aortas from 17β-oestradiol treated and control rats by RT-PCR as described in sections 3.4.2.2-3.

3.7 Statistical analysis

3.7.1 Phenylephrine, carbachol, sodium nitroprusside and L-NAME

The concentration response curves to PE, carbachol and SNP were characterised by the maximum effect (E_{max}) and the concentration which produced 50% maximum effect (EC_{50}). These parameters were calculated from the raw data for each pharmacological intervention for each aorta using Microsoft Excel®. The response to L-NAME for each aorta was taken as the maximal contraction expressed as a percentage of the contraction to EC_{20} of PE.

The means and standard errors of the mean (SEM) for each group were calculated and the following comparisons made by two way ANOVA followed by unpaired two-tailed t-tests with Bonferroni correction for three comparisons: all SHRSP (males and females) *v* all

WKY (males and females), WKY females *v* WKY males, SHRSP females *v* SHRSP males. Therefore, $p<0.017$ was considered to be statistically significant.

3.7.2 Endothelial NOS activity

The mean NOS III activity for each group (±SEM) was calculated and groups compared as above by two way ANOVA followed by unpaired two-tailed t-tests with Bonferroni correction for three comparisons. Again $p<0.017$ was considered to be statistically significant.

3.7.3 DETCA and SOD

As one might expect from the doses used, the effect of DETCA or exogenous SOD was invariably statistically significant as determined by a paired t-test $p<0.05$ (before *v* after DETCA; with *v* without SOD). However, of greater interest was whether or not DETCA and/or SOD had a greater effect in one group compared to another. Therefore, the percentage change in E_{max} and EC_{50} after DETCA or in the presence of SOD was calculated for each ring as follows:

$$after - \beta \quad / \beta \quad \times 100$$

The mean percentage change (±SEM) was calculated for each group and groups compared by two way ANOVA followed by unpaired t-tests as above, with $p<0.017$ for the difference in percentage change being considered statistically significant.

3.7.4 Endothelial NOS and SOD mRNA

The mean (±SEM) quantity of *Nos3* message and SOD/GAPDH ratio was calculated for each group and groups compared by two way ANOVA followed by unpaired t-tests as above, with $p<0.017$ being considered statistically significant.

3.7.5 17β-oestradiol

The effect of 17β-oestradiol was analysed by comparing the mean (±SEM) response to L-NAME for the treated group with the control group using an unpaired two-tailed t-test. A p value <0.05 was considered statistically significant. The mean (±SEM) carbachol E_{max} and EC_{50} for each group were compared in the same way. The mean (±SEM) quantity of *Nos3* message was calculated for each group and groups compared by an unpaired t-test. The mean for the control group at one year was also compared to the mean for WKY females at 4 months of age. Therefore, with Bonferroni correction for double comparisons, $p<0.025$ was considered statistically significant.

4 Results

4.1 Systolic blood pressure and body weight

Four groups of rats were studied at 16 weeks of age. Systolic blood pressure in the week prior to sacrifice and body weight on the day of sacrifice were measured in rats to be sacrificed for isometric tension recording and NOS III enzyme activity. Mean systolic blood pressure, measured by tail cuff plethysmography, and mean body weight at sacrifice for each of the four groups are shown in Table 3 and Figure 11.

Systolic blood pressure (mmHg ± SEM) was significantly higher in SHRSP (172 ± 4.4, n = 67) compared to WKY (128 ± 2.0, n = 69) (95% CI: 34.2, 53.4; $p<0.0001$). Within the SHRSP strain males had a significantly higher systolic blood pressure (196 ± 5.5, n = 33) than females (148 ± 3.7, n = 34) (95% CI: 34.8, 61.3; $p<0.0001$). However, within the WKY strain there was no significant difference in systolic blood pressure between males (130 ± 1.5, n = 37) and females (125 ± 4.0, n = 32) (95% CI: -3.4, 14.0; $p=0.22$).

Body weight (g ± SEM) was significantly higher in WKY (265 ± 8.5, n = 69) compared to SHRSP (215 ± 5.9, n = 67) (95% CI: 29.4, 70.4; $p<0.0001$) and in WKY males (318 ± 8.8, n = 37) compared to WKY females (205 ± 4.6, n = 32) (95% CI: 93.5, 133.2; $p<0.0001$). Similarly, body weight was significantly higher in SHRSP males (255 ± 6.6, n = 33) compared to SHRSP females (176 ± 2.2, n = 34) (95% CI: 64.3, 92.5; $p<0.0001$).

These data confirm the increased blood pressure in SHRSP compared to WKY and in males compared to females in SHRSP. However, in the this study there is only a small difference,

which is not statistically significant, between WKY males and WKY females. The lack of a gender difference in blood pressure within the WKY strain may be related to the lack of gender differences in some of the other parameters described later.

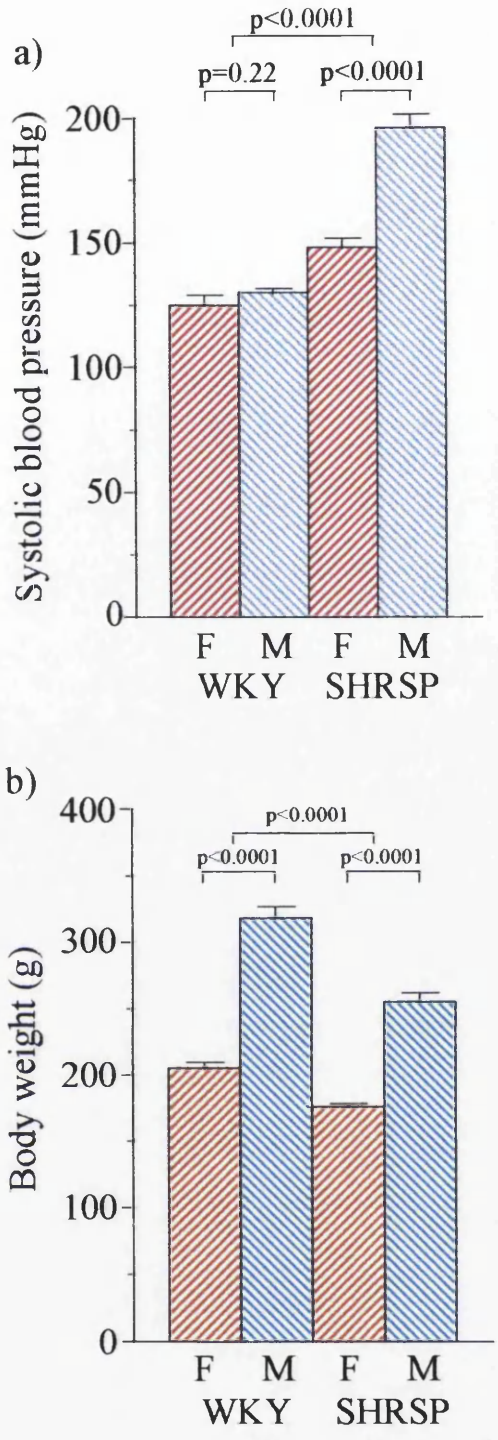

Figure 11 - Mean systolic blood pressure and body weight. **a)** Systolic blood pressure (mmHg) was measured by tail cuff plethysmography in the week prior to sacrifice. **b)** Body weight (g) was measured on bench top scales immediately prior to sacrifice. The mean (± SEM) was calculated for each of the four groups: WKY females (F) (n = 32), WKY males (M) (n = 37), SHRSP females (n = 34) and SHRSP males (n = 33).

$p<0.017$ is statistically significant.

Table 3: Mean Systolic Blood Pressure (SBP) and body weight for each group

		SBP (mmHg)			Weight (g)		
	n	(±SEM)	p	95% CI	(±SEM)	p	95% CI
WKY	69	128±2.0			265±8.5		
SHRSP	67	172±4.4	<0.0001	34.2, 53.4	215±5.9	<0.0001	29.4, 70.4
WKY females	32	125±4.0			205±4.6		
WKY males	37	130±1.5	0.22	-3.4, 14.0	318±8.8	<0.0001	93.5, 133.2
SHRSP females	34	148±3.7			176±2.2		
SHRSP males	33	196±5.5	<0.0001	34.8, 61.3	255±6.6	<0.0001	64.3, 92.5

WKY: Wistar-Kyoto rats: SHRSP: Stroke prone spontaneously hypertensive rats; SEM: standard error of the mean; 95% CI: 95% confidence interval.

$p<0.017$ is statistically significant.

4.2 Isometric tension recording

Fifty-seven rats were studied at 16 weeks of age in four groups: WKY females (n = 12), WKY males (n = 17), SHRSP females (n = 14) and SHRSP males (n = 14). Thoracic aortas were removed, cut into 2-3 mm rings, suspended in standard 10 mL organ baths and equilibrated under 1g resting tension.

4.2.1 Effect of L-NAME

A concentration response curve to PE (10^{-8} - 10^{-5} mol/L) was obtained. When L-NAME (10^{-4} mol/L) was added to the rings at the EC_{20} of PE, the resultant contraction, relative to the initial PE EC_{20} contraction (% of PE ± SEM), was significantly greater in WKY (379 ± 31, n = 29) compared to SHRSP (282 ± 23, n = 28) (95% CI: 20, 174; p=0.015). Further, the contraction in rings from WKY females (519 ± 37, n = 12) was significantly greater than WKY males (280 ± 26, n = 17) (95% CI: 143, 333; p=0.00004). Similarly, the contraction in rings from SHRSP females (356±29, n = 14) was significantly greater than SHRSP males (209 ± 24, n = 14) (95% CI: 70, 224; p=0.0006) (Figure 12).

These data suggest that basal NO availability is greater in WKY compared to SHRSP and in females compared to males in both strains.

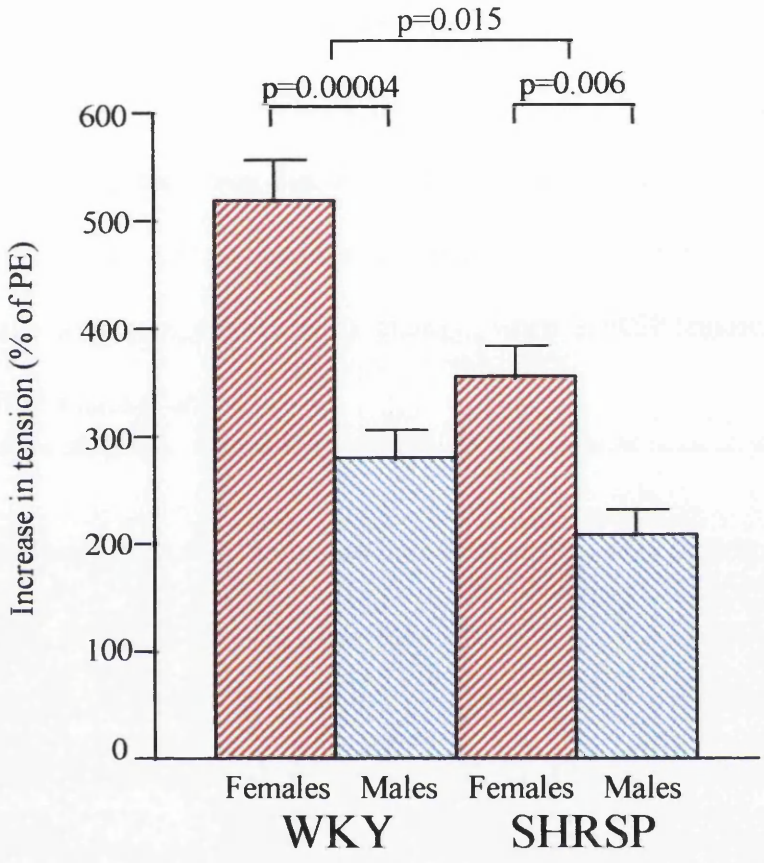

Figure 12 - Basal NO availability. The effect of adding N^G-nitro-L-arginine methyl ester (L-NAME, 10^{-4} mol/L) after the dose of phenylepherine (PE) producing 20% of maximum contraction (EC_{20}). The resultant increase in tension for each ring is expressed as a percentage of the PE EC_{20} and the mean increase (± SEM) for each group calculated. WKY females, n = 12. WKY males, n = 17. SHRSP females, n = 14. SHRSP males, n = 14. $p<0.017$ is statistically significant.

4.2.2 Effect of carbachol

After the concentration response curve to PE (10^{-8} - 10^{-5} mol/L) was obtained, the rings were pre-contracted to the EC_{50} of PE and concentration response curves obtained for carbachol (10^{-8} - 10^{-5} mol/L). Figure 13 shows the concentration response curves for all WKY *v* all SHRSP (3a), WKY females *v* WKY males (3b), and SHRSP females *v* SHRSP males (3c). The E_{max} for carbachol relaxation (% of PE ± SEM) of WKY rings (94.4 ± 1.6, n = 29) was significantly greater than that of SHRSP rings (86.3 ± 2.7, n = 27) (95% CI: 1.7, 14.4; p=0.015). There was no significant difference in the mean maximum relaxation when WKY females were compared to WKY males or when SHRSP females were compared to SHRSP males (Table 4).

There was no significant difference in the log transformed EC_{50} for carbachol relaxation (log mol/L ± SEM) when the mean concentration response curve for WKY rings was compared to that of SHRSP rings, or when WKY females were compared to WKY males (Table 4). However, within the SHRSP strain, there was a significant difference in the mean log transformed EC_{50} when females (-0.39 ± 0.14, n = 14) were compared to males (0.29 ± 0.15, n = 13) (95% CI: -1.1, -0.2; p=0.003) (Figure 13c, inset).

These data confirm that a functioning endothelium is present in each case. They also suggest that stimulated NO availability is greater in WKY compared to SHRSP and in females compared to males within the SHRSP strain. However, there was no significant difference in either the E_{max} or EC_{50} when the carbachol concentration response curve of WKY females was compared to WKY males, which may be related to the lack of a blood pressure difference between these two groups.

Table 4: Mean E_{max} and EC_{50} for carbachol response for each group

		Emax (% of PE)			EC_{50} (log mol/L)		
	n	(±SEM)	p	95% CI	(±SEM)	p	95% CI
WKY	29	94.4 ± 1.6			0.07 ± 0.11		
SHRSP	27	86.3 ± 2.7	0.015	1.7, 14.4	-0.06 ± 0.12	0.45	-0.2, 0.4
WKY females	12	93.4 ± 3.1			0.01 ± 0.17		
WKY males	17	95.0 ± 1.6	0.65	-9.1, 5.8	0.10 ± 0.14	0.67	-0.6, 0.4
SHRSP females	14	89.2 ± 3.7			-0.39 ± 0.14		
SHRSP males	13	83.2 ± 4.0	0.28	-5.3, 17.3	0.29 ± 0.15	0.003	-1.1, -0.2

Definitions are as in Table 3; E_{max}: mean maximum carbachol effect expressed as a percentage of initial phenylepherine contraction; EC_{50}: mean carbachol concentration producing 50% E_{max} (log transformed). $p<0.017$ is statistically significant.

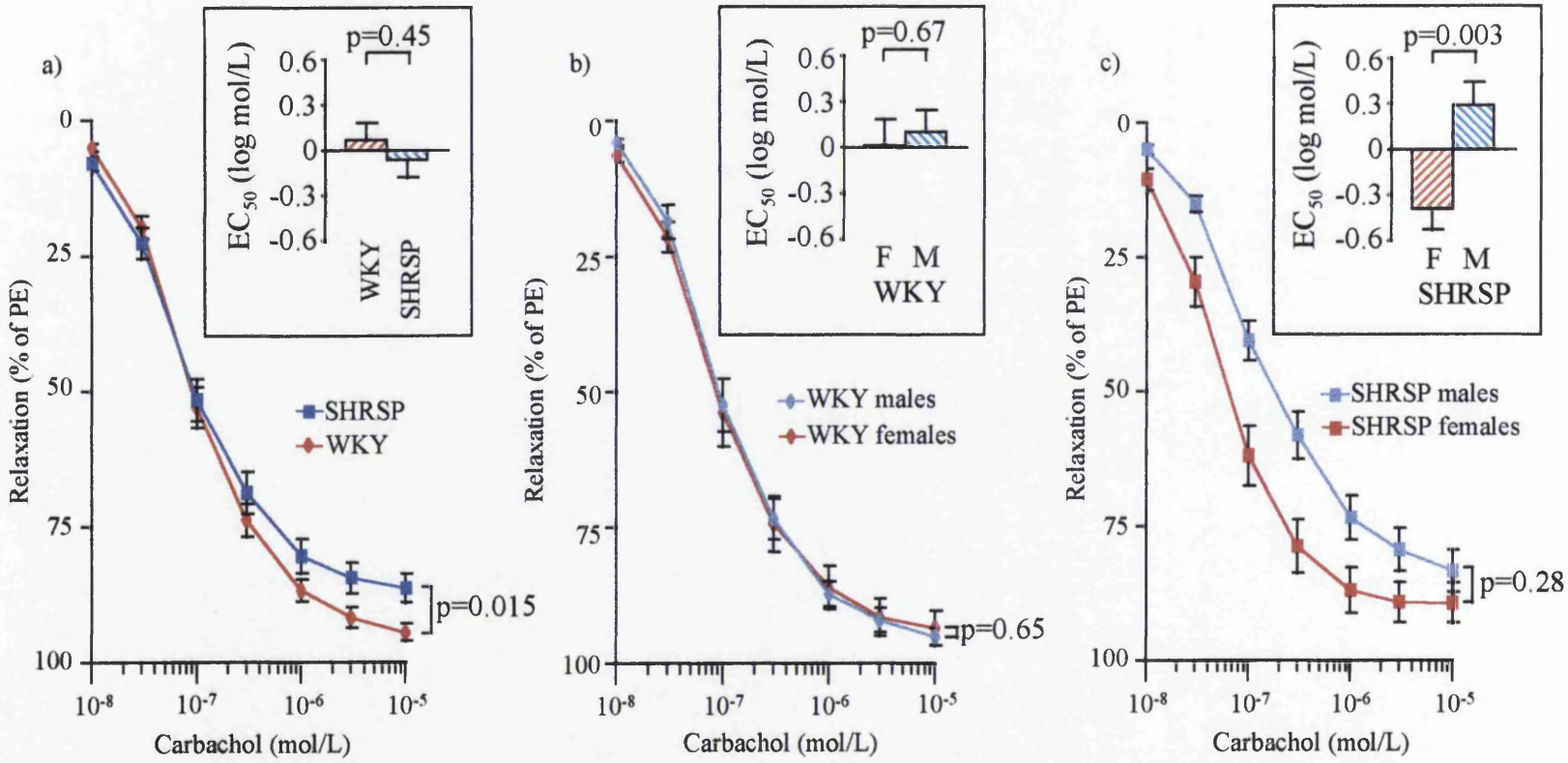

Figure 13 - Stimulated NO release. The concentration response curves to carbachol (10^{-8} - 10^{-5} mol/L) for each group after the dose of phenylepherine (PE) producing 50% of maximum contraction (EC_{50}) expressed as a percent of the PE EC_{50} (±SEM). **a)** All WKY (n = 29) *v* all SHRSP (n = 27). **b)** WKY males (n = 17) *v* females (n = 12). **c)** SHRSP males (n = 13) *v* females (n = 14). Insets show comparisons of the log transformed EC_{50} (log mol/L) for WKY *v* SHRSP, and females (F) *v* males (M) within each strain. $p<0.017$ is statistically significant.

4.2.3 Effect of sodium nitroprusside

After the concentration response curve to PE (10^{-8} - 10^{-5} mol/L) was obtained, the rings were pre-contracted to the EC_{50} of PE and concentration response curves obtained for sodium nitroprusside (SNP, 10^{-8} - 10^{-5} mol/L). As shown in Figure 14, there was no significant difference in the mean E_{max} for SNP relaxation (% of PE±SEM) when WKY rings (100 ± 1.7, n = 11) were compared to SHRSP rings (100 ± 1.9, n = 9) (95% CI: -5.7, 5.4; p=0.95). Similarly when WKY females (99.3 ± 3.4, n = 5) were compared to WKY males (100 ± 1.3, n = 6) (95% CI: -9.6, 6.6; p=0.69) there was no significant difference or when SHRSP females (100 ± 0.5, n = 4) were compared to SHRSP males (100 ± 4.1, n = 5) (95% CI: -9.6, 10.6; p=0.91).

There was no significant difference in the mean log transformed EC_{50} for SNP relaxation (log mol/L ± SEM) when WKY rings (1.59 ± 0.45, n = 11) were compared to SHRSP rings (2.40 ± 0.54, n = 9) (95% CI: -2.3, 0.7; p=0.27); when WKY females (1.17 ± 0.8, n = 5) were compared to WKY males (1.93 ± 0.6, n = 6) (95% CI: -3.0, 1.5; p=0.44) or when SHRSP females (2.72 ± 0.3, n = 4) were compared to SHRSP males (2.14 ± 1.0, n = 5) (95% CI: -2.3, 3.5; p=0.60).

These data suggest that there is no significant difference in the sensitivity of the vascular smooth muscle to NO between WKY and SHRSP, or between males and females in either strain.

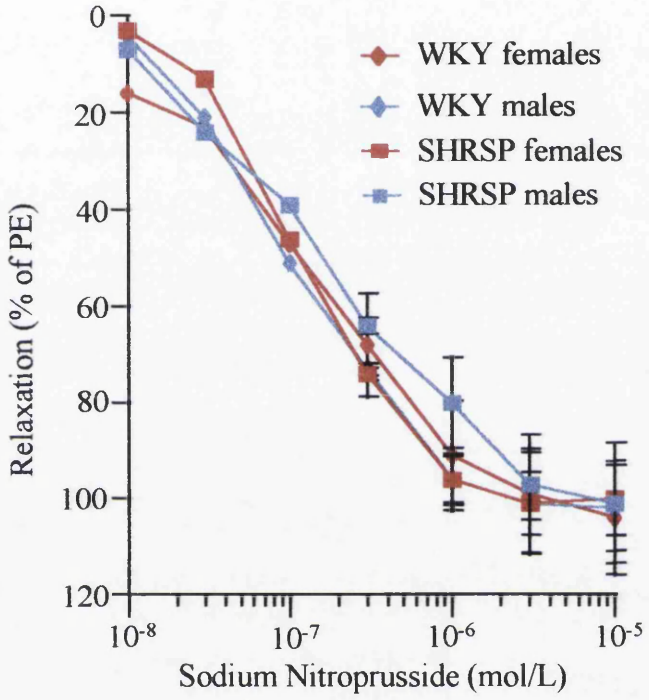

Figure 14 - Endothelium-independent relaxation. The concentration response curves to sodium nitroprusside (SNP, 10^{-8} - 10^{-5} mol/L) for each group after the dose of phenylepherine (PE) producing 50% of maximum contraction (EC_{50}) expressed as a percent of the PE EC_{50} (± SEM). WKY females, n = 11. WKY males, n = 9. SHRSP females, n = 4. SHRSP males, n = 5.

4.2.4 Effect of DETCA on relaxation to carbachol

Carbachol concentration response curves (10^{-8} - 10^{-5} mol/L) were obtained for each ring pre- and post-incubation with the SOD inhibitor DETCA (10 mmol/L) or exogenous SOD (45U/mL). The mean changes in E_{max} and EC_{50} (± SEM) between pre- and post-incubation (expressed as a percentage of pre-incubation) were calculated for each group: WKY females (n = 7), WKY males (n = 8), SHRSP females (n = 4), and SHRSP males (n = 6).

Figure 15 shows that DETCA attenuated the relaxation to carbachol in all WKY and all SHRSP. As shown in Figure 16a, DETCA attenuated the relaxation (% change in E_{max} ± SEM) to carbachol of SHRSP (59 ± 5.1, n = 10) (Figure 15b) significantly more than WKY (29 ± 6.0, n = 15) (95% CI: 14.3, 47.0; p=0.0008) (Figure 15a). Figure 16a also shows that the % change in E_{max} by DETCA on rings from WKY females did not differ significantly from WKY males. Similarly, the attenuation in rings from SHRSP females did not differ significantly from SHRSP males after DETCA pre-incubation.

Figure 16c shows that DETCA pre-incubation tended to increase the EC_{50} ($x10^{-7}$ mol/L) for carbachol relaxation. However, there was no significant difference in this increase (% change in EC_{50} ± SEM) between strains or between genders within each strain.

4.2.5 Effect of exogenous SOD on relaxation to carbachol

Figure 16b shows the effect of co-administration of exogenous SOD to the rings on their response to carbachol. Exogenous SOD tended to improve the relaxation to carbachol, especially in SHRSP. However, the improvement (% change in E_{max} ± SEM) did not differ significantly between strains or between genders within each strain.

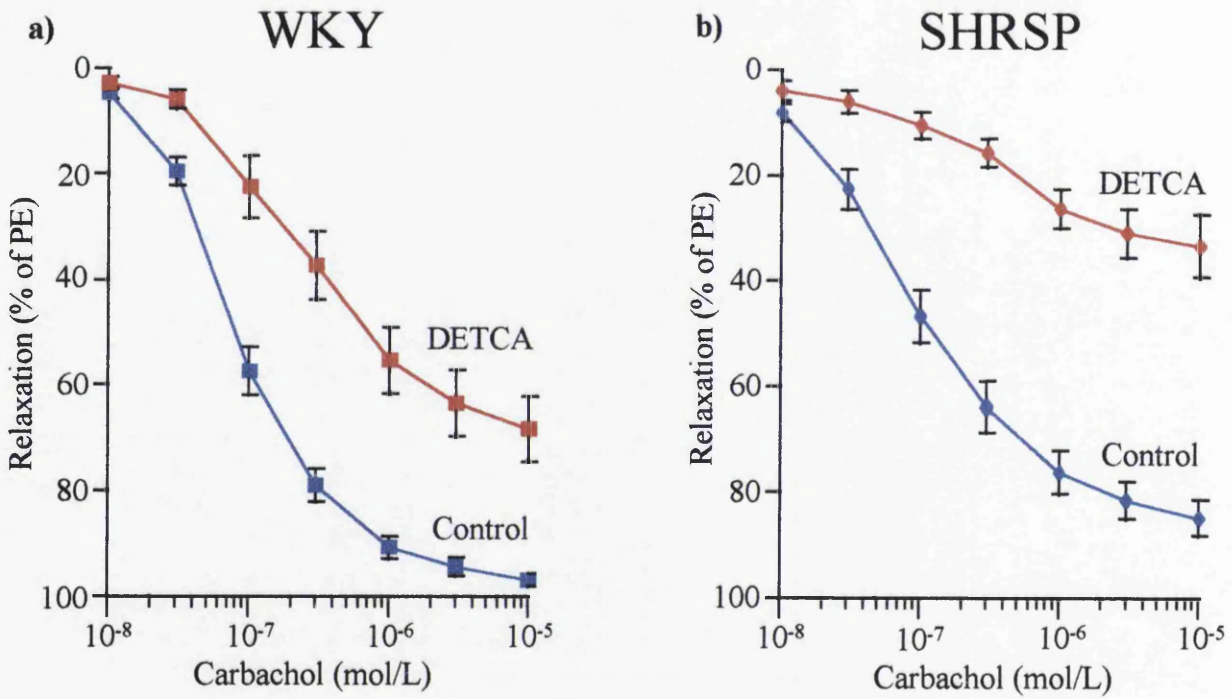

Figure 15 - Inhibition of superoxide dismutase. The effect of pre-incubating rings with diethyldithiocarbamate (DETCA, 10 mmol/L) on the carbachol concentration response curves for **a)** WKY (n = 15) and **b)** SHRSP (n = 10).

Figure 16d shows that exogenous SOD tended to reduce the EC_{50} ($x10^{-7}$ mol/L) of the carbachol response curves. However, when the % change in EC_{50} was compared between strains or between genders within each strain, there was no significant difference.

4.2.6 Effect of DETCA on contraction to PE

Phenylephrine concentration response curves (10^{-8} - 10^{-5} mol/L) were obtained for each ring pre- and post-incubation with the SOD inhibitor DETCA (10 mmol/L) or exogenous SOD (45U/mL). The mean changes in E_{max} and EC_{50} (± SEM) between pre- and post-incubation (expressed as a percentage of pre-incubation) were calculated for each group: WKY females (n = 8), WKY males (n = 8), SHRSP females (n = 6), and SHRSP males (n = 9).

The effect of pre-incubation of the rings with DETCA on the contraction to PE is shown in Figure 17. Pre-incubating the rings with DETCA increased the mean E_{max} and EC_{50} to PE (relative to KCl) in all groups. However, there was no significant difference in the % change when strains or genders within each strain were compared (Figures 17a and 17c).

4.2.7 Effect of exogenous SOD on contraction to PE

Figure 17 also shows the effect of co-administration of exogenous SOD to the rings on their response to PE. Exogenous SOD reduced the maximum contraction to PE and increased the EC_{50} in all groups. This effect (% change ± SEM) did not differ significantly between strains or between genders within each strain (Figures 17b and 17d).

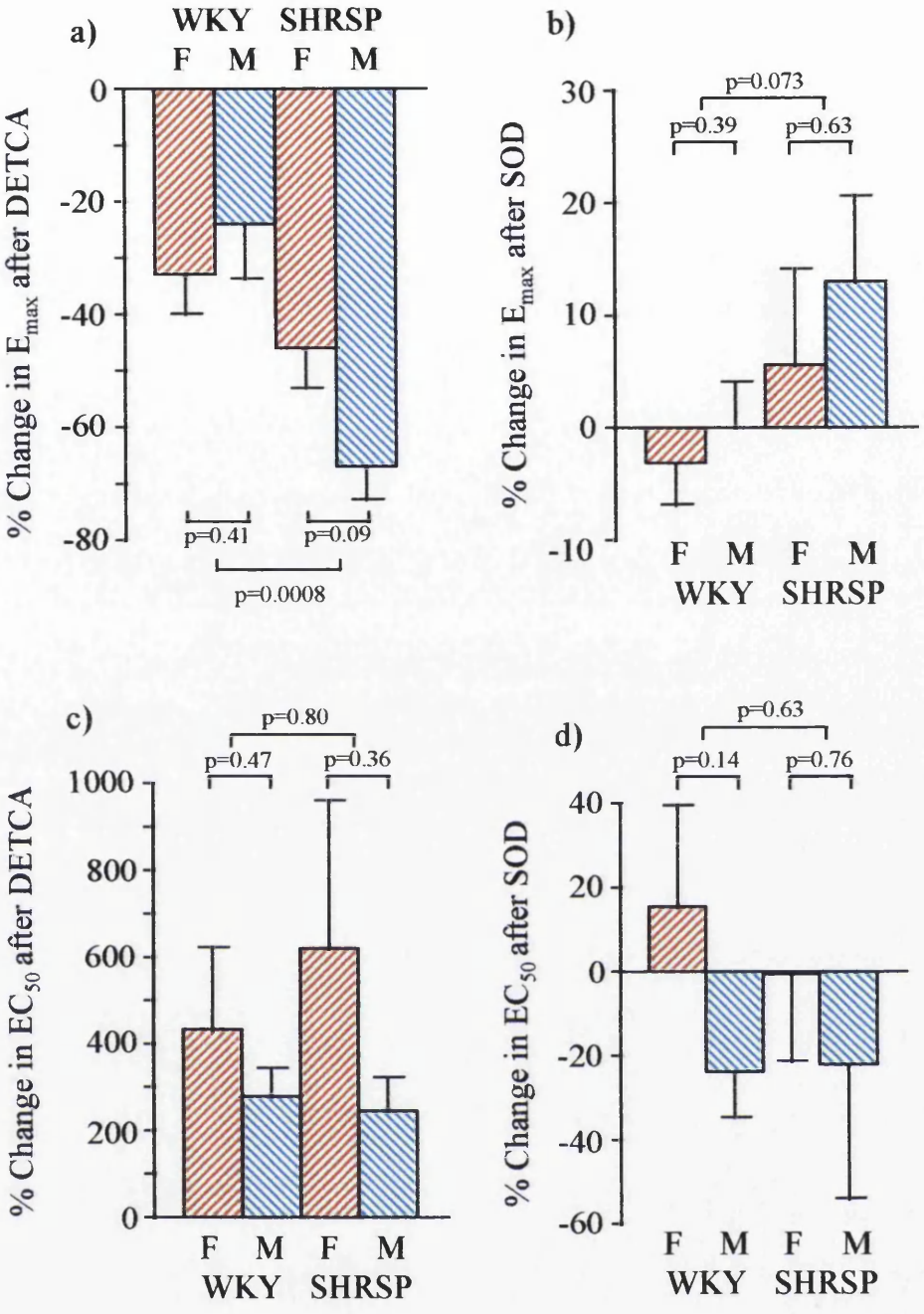

Figure 16 - Effect of DETCA and SOD on carbachol. Carbachol concentration response curves (10^{-8} - 10^{-5} mol/L) were obtained for each ring pre- and post-incubation with DETCA (10 mmol/L) or SOD (45U/mL). The mean changes in E_{max} and EC_{50} (± SEM) between pre- and post-incubation (expressed as a percentage of pre-incubation) were calculated for each group.

a) E_{max} pre- and post-DETCA. **b)** E_{max} pre- and post-SOD. **c)** EC_{50} pre- and post-DETCA. **d)** EC_{50} pre- and post-SOD. WKY females (F) n = 7, WKY males (M) n = 8, SHRSP females n = 4, SHRSP males n = 6. $p<0.017$ is statistically significant.

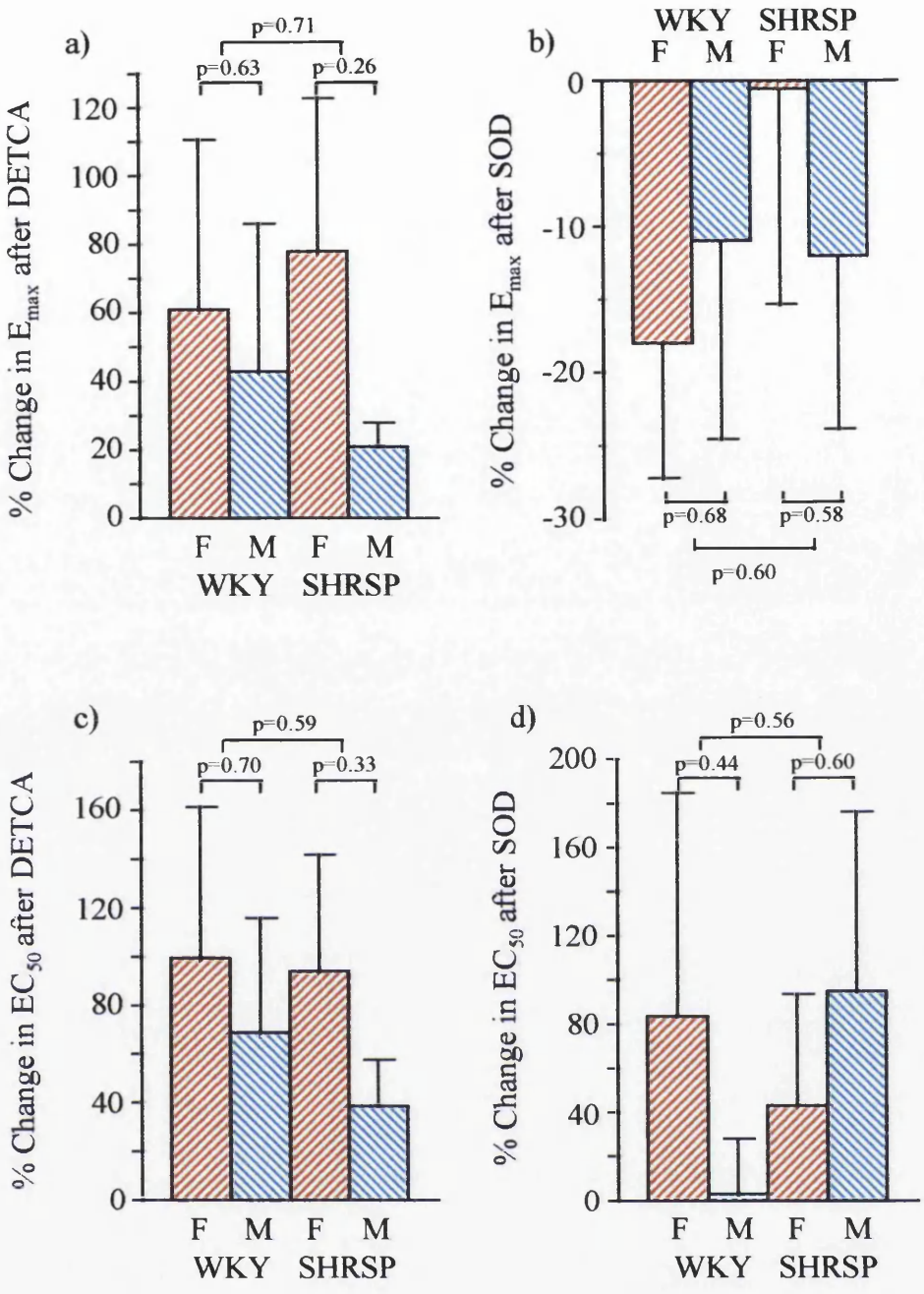

Figure 17 - Effect of DETCA and SOD on phenylephrine (PE). PE concentration response curves (10^{-8} - 10^{-5} mol/L) were obtained for each ring pre- and post-incubation with DETCA (10 mmol/L) or SOD (45U/mL). The mean changes in E_{max} and EC_{50} (± SEM) between pre- and post-incubation (expressed as a percentage of pre-incubation) were calculated for each group.

a) E_{max} pre- and post-DETCA. **b)** E_{max} pre- and post-SOD. **c)** EC_{50} pre- and post-DETCA. **d)** EC_{50} pre- and post-SOD. WKY females (F) n = 8, WKY males (M) n = 8, SHRSP females n = 6, SHRSP males n = 9. $p<0.017$ is statistically significant.

The only significant effect of these crude manipulations of the O_2^-/SOD system was that DETCA attenuated the relaxation to carbachol in SHRSP significantly more than WKY. This may suggest that SOD is more active in SHRSP, or that when SOD is inhibited by DETCA, O_2^- accumulates faster in SHRSP, or perhaps a combination of both. The manipulations of the O_2^-/SOD system produced the predicted effects, but significant strain or gender differences may have been masked by the large variation in the measurements.

4.3 NOS III activity

To investigate the possibility that the strain and gender differences in NO availability are due to differences in NO production, NOS III activity was measured in endothelial cells removed from aortas of the same four groups of animals at 16 weeks of age: WKY females (n -= 20), WKY males (n = 15), SHRSP females (n = 20) and SHRSP males (n = 20). NOS III activity was quantified by the radiolabelled L-arginine to L-citrulline assay and corrected for the amount of protein in each assay.

NOS III activity (pmol/min/mg protein ± SEM) was significantly greater in the aortas from SHRSP (24.6 ± 3.1, n = 40) compared to WKY (14.6 ± 1.3, n = 35) (95% CI: 2.3, 17.6; p=0.016). NOS III activity tended to be greater in WKY males compared to WKY females and similarly in SHRSP males compared to SHRSP females, but these differences did not achieve statistical significance (Figure 18).

These data suggest that reduced NO availability in SHRSP compared to WKY is not due to reduced NOS III activity, which is significantly higher in SHRSP. The gender differences in NO availability are also unlikely to be due to differences in NOS III activity, which tends to be higher in males compared to females.

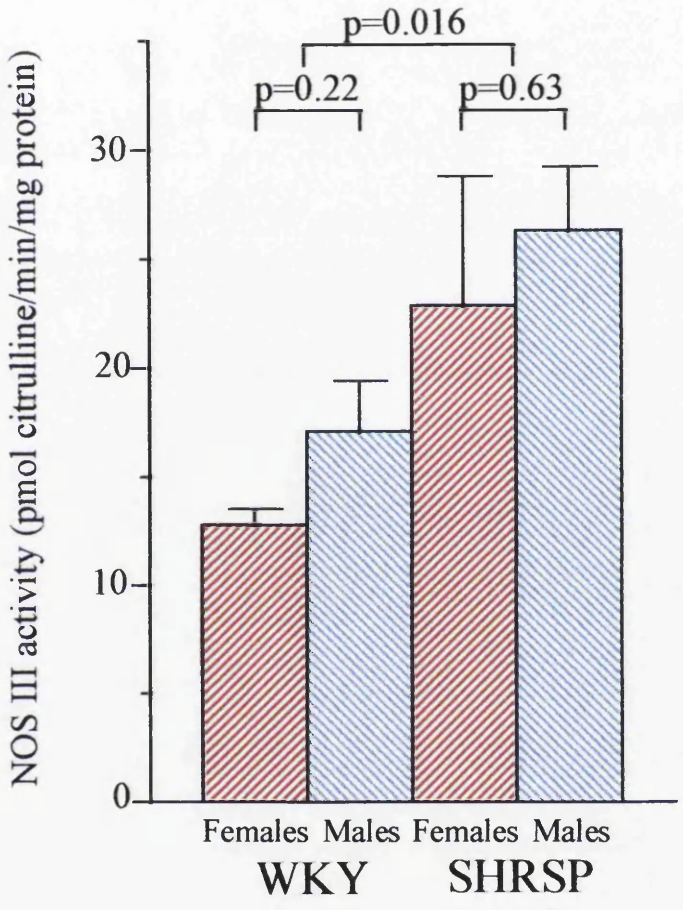

Figure 18 - Activity of endothelial nitric oxide synthase (NOS III) (pmol citrulline/min/mg protein ± SEM) in endothelial cells obtained from aortas of WKY and SHRSP males and females (see methods). WKY females, n = 20. WKY males, n = 15. SHRSP females, n = 20. SHRSP males, n = 20. $p<0.017$ is statistically significant.

4.4 *Nos3* mRNA expression

To determine if strain and/or gender differences in NOS III activity were related to gene expression, *Nos3* mRNA was quantified in total RNA isolated from thoracic aortas. Due to the low abundance of *Nos3* mRNA in rat tissue, northern blotting was not sensitive enough to quantify the gene expression, even when 10μg of poly (A) mRNA was used. Instead a reverse transcription-polymerase chain reaction (RT-PCR) system was developed to quantify *Nos3* mRNA levels.

As shown in Figure 19, *Nos3* mRNA expression (fg/100ng total aortic RNA ± SEM) is greater in SHRSP (302 ± 69, n = 6) compared to WKY (71.7 ± 15, n = 6) (95% CI: 50, 412; p=0.02), which just fails to reach statistical significance when corrected for triple comparisons. Although there is a tendency for *Nos3* mRNA expression to be greater in males compared to females, in neither strain did this achieve statistical significance. WKY males (95.0 ± 22, n = 3), WKY females (48.3 ± 5.2, n = 3) (95% CI: -144, 51; p= 0.18). SHRSP males (435 ± 76, n = 3), SHRSP females (170 ± 17, n = 3) (95% CI: -600, 68; p=0.08).

The trend in these data between groups matches that of NOS III activity (Figure 18) providing further evidence that strain and gender differences in NO availability are not due to differences in NO production. These data would also suggest that NOS III activity is largely related to *Nos3* gene expression without much in the way of post-transcriptional or post-translational regulation.

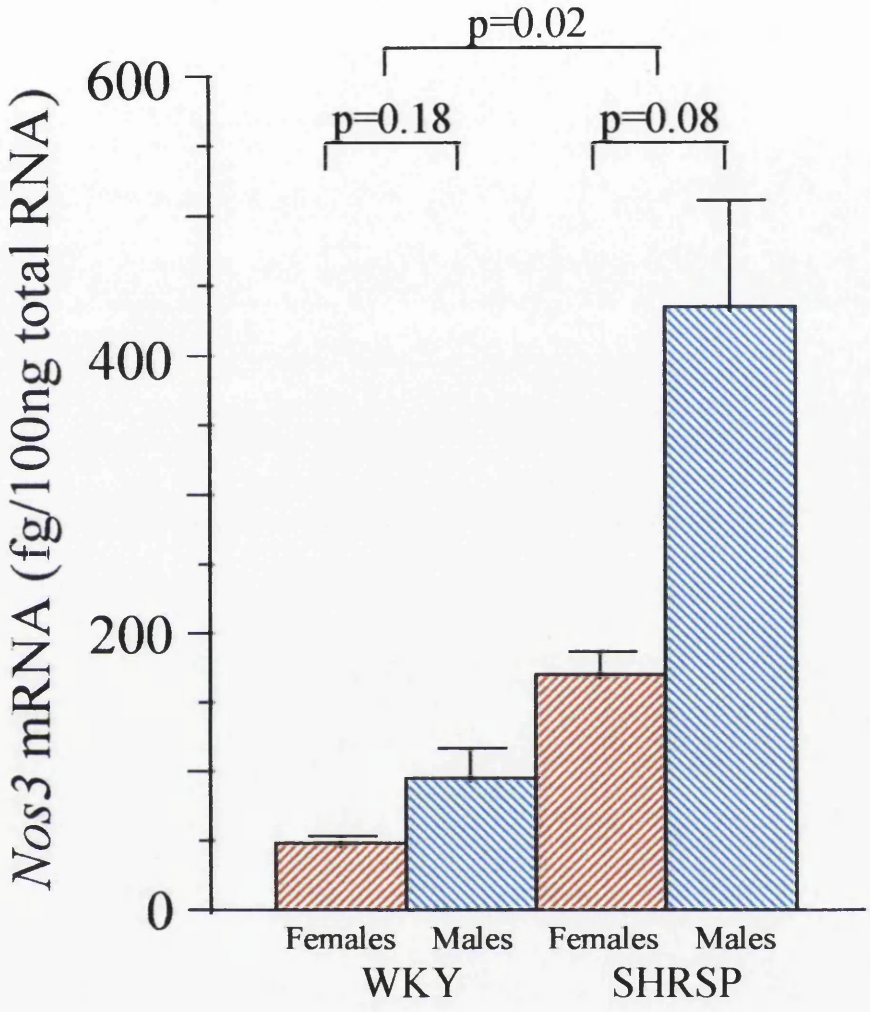

Figure 19 - Endothelial nitric oxide synthase (*Nos3*) mRNA expression (fg/100ng total RNA ± SEM) measured by reverse transcription-polymerase chain reaction in total aortic RNA from WKY and SHRSP males and females. n=3 in each group. $p<0.017$ is statistically significant.

4.5 SOD mRNA expression

As the DETCA data suggested that there may be differences in SOD activity between SHRSP and WKY, SOD gene expression was quantified as an indirect measure of SOD activity. SOD mRNA is abundant enough in the rat aorta to be quantified by northern blotting. cDNA probes were available for rat manganese SOD (Mn SOD), rat copper/zinc SOD (CuZn SOD) and murine house-keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as an internal standard to correct for slight variations in total RNA in each sample. Figure 20 shows representative northern blots of a membrane hybridised with the above probes. Bands on the autoradiographs corresponding to MnSOD and CuZn SOD were quantified by densitometry and expressed as a ratio to GAPDH.

4.5.1 Manganese SOD (*Sod2*) mRNA expression

As shown in Figure 21b, *Sod2* mRNA expression (ratio to GAPDH ± SEM) is significantly greater in SHRSP males (0.68 ± 0.05, n = 3) compared to WKY males (0.32 ± 0.01, n = 4) (95% CI: 0.15 0.57; p=0.02). There was no significant difference in *Sod2* mRNA expression between SHRSP females (0.38 ± 0.12, n = 4) and WKY females (0.44 ± 0.12, n = 3) (95% CI: -0.67, 0.78; p=0.82) (Figure 21a).

4.5.2 Copper/Zinc SOD (*Sod1*) mRNA expression

Figures 21c and 21d show that there is no significant difference in *Sod1* mRNA expression (ratio to GAPDH ± SEM) between WKY females (0.46 ± 0.14, n = 4) and SHRSP females

(0.74 ± 0.05, n = 4) (95% CI: -0.74, 0.18; p=0.14) or between WKY males (2.04 ± 0.59, n = 4) and SHRSP males (1.12 ± 0.08, n = 3) (95% CI: -0.98, 2.81; p=0.22).

The fact that *Sod2* mRNA expression is significantly greater in SHRSP males compared to WKY males provides some evidence that MnSOD activity may be greater in the former group, although this was not directly measured. This would explain the greater effect of the SOD inhibitor DETCA on SHRSP carbachol relaxation compared to WKY reported in section 4.2.4. *Sod2* gene expression may be up-regulated in SHRSP males to compensate for increased oxidative stress or some other blood pressure raising effect.

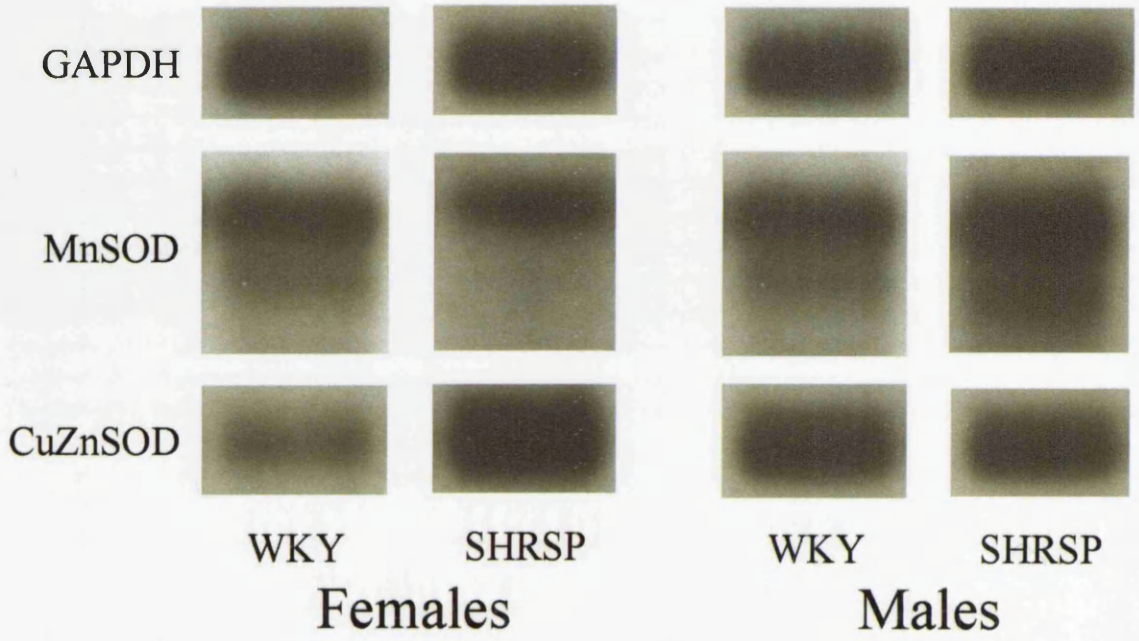

Figure 20 - Representative northern blots. Ten μg of total aortic RNA from each group was size fractionated on an agarose gel, blotted on to a nylon membrane and hybridised with probes specific for Manganese superoxide dismutase (MnSOD), Copper/zinc superoxide dismutase (CuZn SOD) and glyceraldehyde phosphate dehydrogenase (GAPDH) as an internal standard.

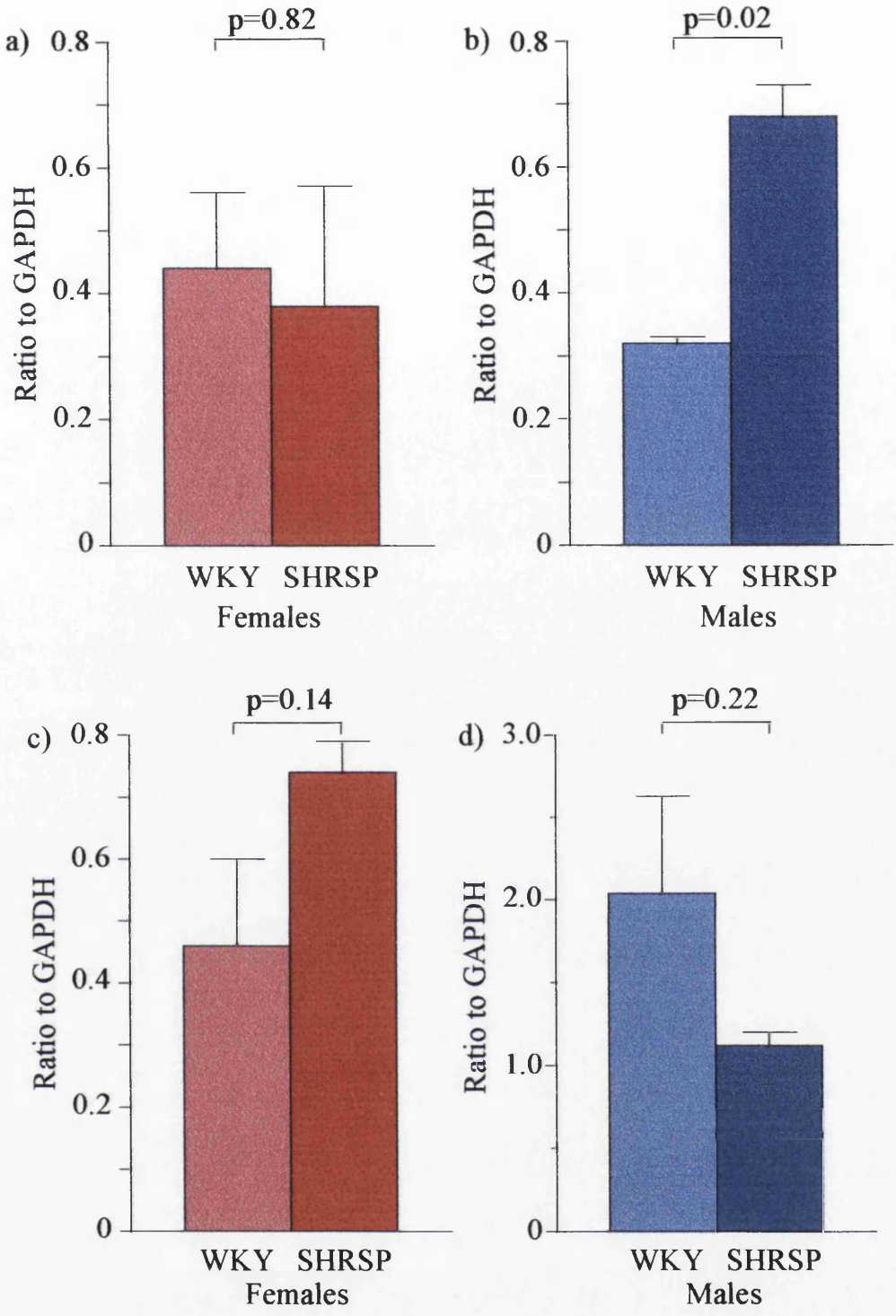

Figure 21 - Expression of superoxide dismutase (SOD) mRNA. SOD mRNA was quantified by northern blotting and expressed as a ratio to glyceraldehyde phosphate dehydrogenase (GAPDH). **a)** Manganese SOD in females (WKY n = 3, SHRSP n = 4). **b)** Manganese SOD in males (WKY n = 4, SHRSP n = 3). **c)** Copper/Zinc SOD in females (WKY n = 4, SHRSP n = 4). **d)** Copper/Zinc SOD in males (WKY n = 4, SHRSP n = 3). $p<0.05$ is statistically significant.

4.6 Effect of age on L-NAME response

To investigate changes in endothelial function with advancing age, changes in isometric tension were used to quantify basal NO in four groups of rats at 4 months of age (WKY females n = 15, WKY males n = 19, SHRSP females n = 16 and SHRSP males n = 16), 6 months of age (WKY females n = 7, WKY males n = 8, SHRSP females n = 11 and SHRSP males n = 7), and 12 months of age (WKY females n = 11, WKY males n = 7, SHRSP females n = 8 and SHRSP males n = 7). As before, a PE concentration response curve was obtained (10^{-8} - 10^{-5} mol/L), then rings were pre-contracted to the PE EC_{20} and L-NAME added to a final bath concentration of 10^{-4} mol/L.

As shown in Figures 22a and 22b, there is progressive reduction in the response to L-NAME from 4 months to 12 months in WKY females and SHRSP females respectively. In the WKY females the response to L-NAME (% of PE ± SEM) is significantly lower at 12 months (296 ± 30, n = 11) than 4 months (523 ± 51, n = 15) (95% CI: 104, 349; p=0.0009). In the SHRSP females the response to L-NAME is also significantly lower at 12 months (220 ± 28, n = 8) compared to 4 months (341 ± 26, n = 16) (95% CI: 41, 201; p=0.0054).

In the males there is a tendency for the response to L-NAME to increase with age between 4 months and 12 months (Figures 22c and 22d). However, in WKY males there is no significant difference in the response to L-NAME (% of PE ± SEM) at 12 months (408 ± 64, n = 7) compared to 4 months (269 ± 25, n = 19) (95% CI: -300, 22; p=0.081). The same is true of SHRSP males, where there is no significant difference in L-NAME response

at 12 months (270 ± 26, n = 7)) compared to 4 months (227 ± 24, n = 16) (95% CI: -118, 33; p=0.25).

These data suggest that basal NO availability declines with age in females of both strains, but not in males of either strain, which if anything tends to increase. As before, this could be due to reduced NO production in older females, or increased scavenging by say O_2^-, or a combination of the two.

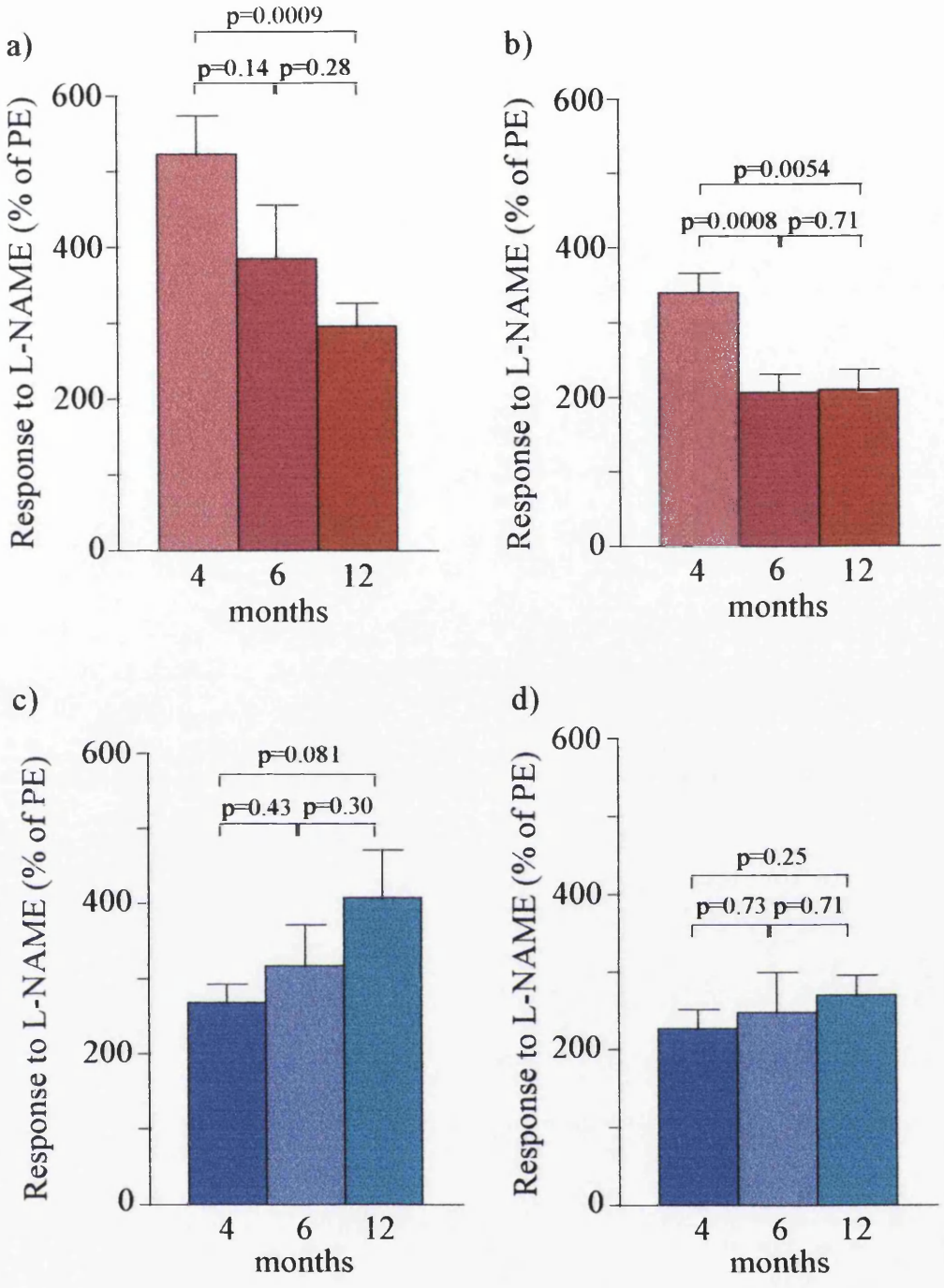

Figure 22 - Effect of age on basal NO. L-NAME, 10^{-4} mol/L was added at the PE EC_{20}. The resultant increase in tension for each ring is expressed as a percentage of the PE EC_{20} and the mean increase (± SEM) calculated for each group at 4, 6 and 12 months of age. **a)** WKY females (4 months n = 15, 6 months n = 7, 12 months n = 11). **b)** SHRSP females (4 months n = 16, 6 months n = 11, 12 months n = 8). **c)** WKY males (4 months n = 19, 6 months n = 8, 12 months n = 7). **d)** SHRSP males (4 months n = 16, 6 months n = 7, 12 months n = 7). $p<0.017$ is statistically significant.

4.7 Effect of 17β-oestradiol

Although not measured in this study due to technical difficulties, circulating 17β-oestradiol is likely to be reduced in the 12 month old females compared to the 4 month old females. This, together with the absence of an age related decline in male animals, may lead one to hypothesise the level of circulating 17β-oestradiol determines NO availability in the vasculature.

4.7.1 Effect of 17β-oestradiol on basal NO

To test this hypothesis, changes in isometric tension in response to L-NAME (10^{-4} mol/L) were used to quantify basal NO in aortas removed from one year old WKY females (n = 5) treated with intraperitoneal 17β-oestradiol (20μg per day) for 14 days compared to age matched WKY females (n = 5) treated with intraperitoneal vehicle for the same time period.

The response to L-NAME (% of PE ± SEM) was greater in the group treated with 17β-oestradiol (207 ± 25, n = 5) compared to the group treated with vehicle (146 ± 16, n = 5). This difference just achieves statistical significance (95% CI: 0, 128; p=0.05) (Figure 23a).

4.7.2 Effect of 17β-oestradiol on carbachol stimulated NO

Concentration response curves to carbachol were used to measure stimulated NO in aortic rings from the same 17β-oestradiol or vehicle treated WKY females.

As shown in Figure 23b, there was no significant difference in the carbachol E_{max} (% of PE ± SEM) in rings from 17β-oestradiol treated WKY females (80.9 ± 3.9, n = 5) compared to vehicle treated age matched WKY females (84.4 ± 2.8, n = 5) (95% CI: -13.4, 6.4; p=0.48). Similarly, there was no significant difference in the EC_{50} ($x10^{-7}$ mol/L ± SEM) between 17β-oestradiol treated WKY females (1.22 ± 0.29, n = 5) and vehicle treated age matched WKY females (1.01 ± 0.13, n = 5) (95% CI: -0.45, 0.86; p=0.51).

These data provide further evidence that 17β-oestradiol can increase basal NO availability with apparently having little effect on stimulated NO availability. It is not possible from these data to tell whether this is due to increased NO production, reduced NO scavenging, or both. This could be determined by measuring NOS III activity in both groups of animals. Quantifying *Nos3* gene expression in both groups may give some indication of NOS III enzyme activity.

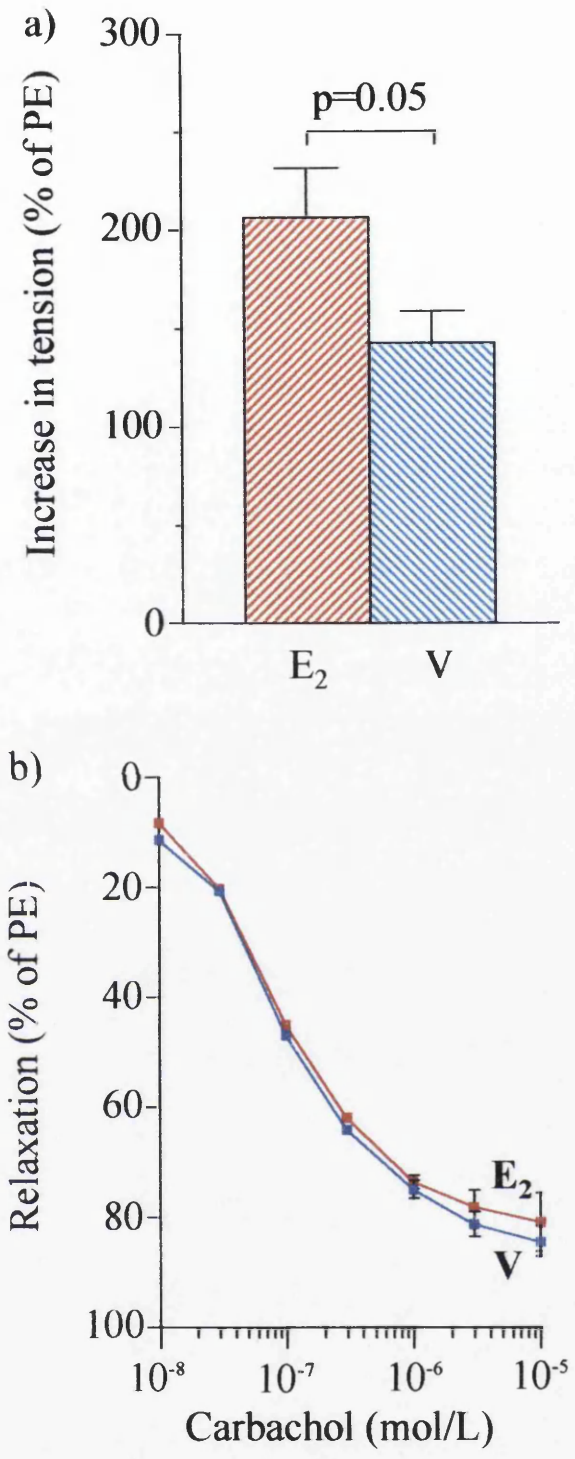

Figure 23 - Effect of 17β-oestradiol on basal and stimulated NO. 17β-oestradiol (E_2, n = 5) or vehicle (V, n = 5) were administered subcutaneously to 1 year old WKY females for 2 weeks. **a)** Basal NO availability was assessed in rings of thoracic aorta by measuring the increase in tension after addition of L-NAME (10^{-4} mol/L) after the PE EC_{20}. **b)** Stimulated NO was assessed by the concentration response curve for carbachol (10^{-8} to 10^{-5} mol/L). $p<0.05$ is statistically significant.

4.8 Effect of age and 17β-oestradiol on *Nos3* mRNA expression

As before, RT-PCR was used to quantify *Nos3* mRNA in total aortic RNA isolated from the same vehicle treated WKY females (n = 4) and 17β-oestradiol treated WKY females (n = 4) at one year of age. Comparisons could be made to *Nos3* mRNA levels quantified in aortas from 4 month old WKY females (n = 3) reported in section 4.4.

Nos3 mRNA expression (fg/100ng total RNA ± SEM) was significantly greater in aortas from WKY females at 12 months of age (487 ± 75, n = 4) compared to aortas from WKY females at 4 months of age (48.3 ± 5.2, n = 3) (95% CI: 200, 677; p=0.01). Treatment of the WKY females at 12 months of age with 17β-oestradiol for two weeks did not significantly affect Nos3 mRNA expression (342 ± 101, n = 4) compared to vehicle treated age matched WKY females (487 ± 75, n = 4) (95% CI: -469, 178; p=0.30) (Figure 24).

These data suggest that the reduced basal NO availability in females at 12 months of age compared to 4 months of age is not due to reduced NO production, as *Nos3* gene expression is increased 10-fold in aortas from the older females. Increased NO production does not appear to be responsible for the increased basal NO availability in 17β-oestradiol treated one year old females as there is no significant change in *Nos3* gene expression in 17β-oestradiol treated animals compared to vehicle treated controls. The decline in basal NO availability with age is more likely to be related to increased scavenging and the increased basal NO availability when the older females are treated with 17β-oestradiol is likely to be due to a reduction in this scavenging.

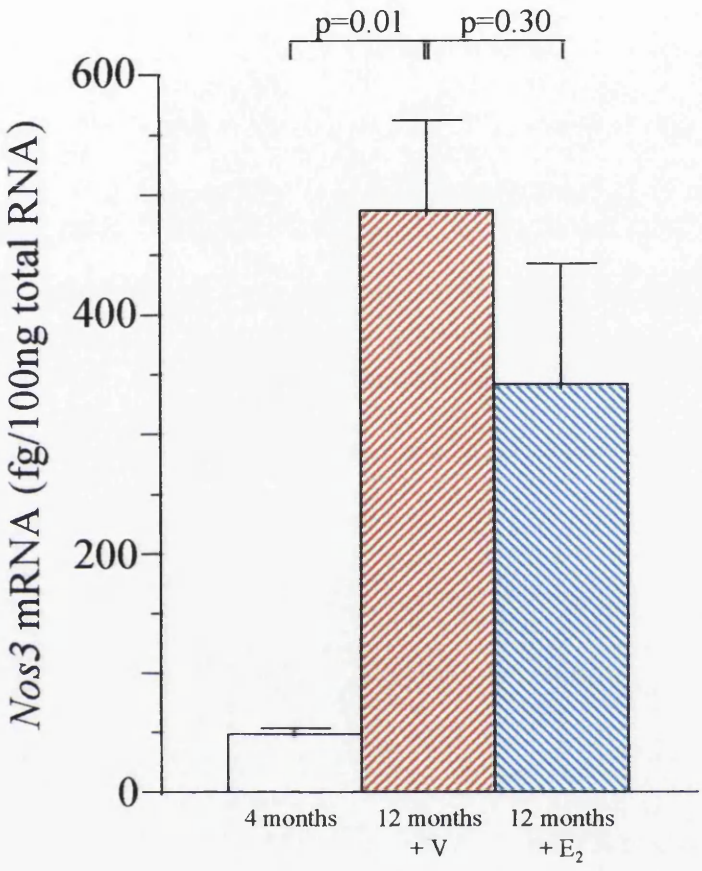

Figure 24 - Effect of age and 17β-oestradiol on *Nos3* expression. *Nos3* mRNA expression was quantified (fg/100ng total RNA ± SEM) using reverse transcription-polymerase chain reaction on total RNA extracted from aortas of WKY females at 4 months of age (n = 3), WKY females at 12 months of age treated with vehicle (V, n = 4) and WKY females at 12 months of age treated with 17β-oestradiol (E_2, n = 4). $p<0.025$ is statistically significant.

5 Discussion

5.1 NO availability

These studies demonstrate reduced basal NO availability in aortas of SHRSP compared to WKY at 16 weeks of age by examining the response of aortic rings to the NOS inhibitor L-NAME *in vitro*. This is in accordance with Malinski *et al*[282] who found stimulated NO release in response to bradykinin in cultured aortic endothelial cells isolated from SHRSP to be significantly reduced compared to WKY. In addition, these studies demonstrate reduced basal NO availability in males compared to females in both strains. Hayashi *et al* have previously shown reduced basal NO 'release' in aortic rings from male rabbits compared to females[233], and others have demonstrated similar gender differences in myogenic tone in rats as an alternative measure of basal NO availability[231,232]. In addition, Kauser and Rubanyi used a bioassay system to demonstrate reduced basal NO 'release' in aortas from male rats compared to females[234].

Stimulated NO release in response to the ACh analogue carbachol was also examined in aortic rings *in vitro*. A strain difference in carbachol E_{max}, but not EC_{50} was found as SHRSP rings relaxed to a significantly lesser extent than WKY rings. This has been demonstrated previously by some groups, but not others (see ref. [113] for review). A gender difference was found within the SHRSP strain. Whereas there was no difference in the E_{max}, males were less sensitive to carbachol having a significantly greater carbachol EC_{50} than females. Such a gender difference in stimulated NO was first described by Kauser and Rubanyi, who demonstrated significantly greater ACh induced relaxation in aortas from female SHR compared to males[230]. Despite the gender difference in basal NO in WKY, no such gender difference was detected in the carbachol E_{max} or EC_{50} within the WKY strain.

This suggests a different mechanism for regulation of basal and stimulated NO availability. Such a difference has been reported by Kauser and Rubanyi[234] and Mian and Martin[116]. The latter authors speculate that O_2^- may be responsible for this difference by scavenging the small amounts of basal NO, but being unable to scavenge the relatively large amounts of stimulated NO[116]. The presence of a gender effect within the SHRSP strain, but not within the WKY strain is contrary to Kauser and Rubanyi who found a gender difference in both SHR and WKY[230], but may be related to the large gender difference in blood pressure in SHRSP, while WKY show only a small gender difference.

The question of whether the endothelial dysfunction is primary or secondary to hypertension is frequently raised. This question is impossible to answer, but the fact that endothelial dysfunction is found in secondary forms of hypertension[127], and can be improved by treatment of hypertension with captopril[283] or other antihypertensive agents[284], would suggest that, at least in some cases, it is a result of hypertension rather than a cause. However, this may not always be the case as Taddei *et al* have recently shown a defective L-arginine-nitric oxide pathway in normotensive offspring of essential hypertensive patients compared to matched normotensive offspring of normotensive parents[285]. The authors conclude from this that endothelial dysfunction is primary and pre-dates the onset of hypertension. The offspring in this study are in their 20's, so it would be interesting to determine which subjects develop hypertension in later life.

No strain or gender differences were detected in the response of aortic rings to SNP *in* , suggesting that the responsiveness of the vascular smooth muscle to NO is not altered. The absence of a gender difference in the response of aortic rings to SNP has been described before[230]. One would not expect differences in luminal or endothelial O_2^- to affect

the response to SNP, which is an endothelium independent vasodilator and does not give up its NO until it reaches the vascular smooth muscle[273].

In summary, there have been several reports of blood pressure or gender differences in basal or stimulated NO, but there are no previous reports describing the effects of blood pressure and gender on basal and stimulated nitric oxide availability in the same model. These results examine only the functional levels of basal and stimulated NO that are available to the underlying vascular smooth muscle. The reduced availability of basal and stimulated NO in SHRSP may be due to reduced production, increased scavenging or both. Therefore, subsequent experiments were designed to address this issue.

5.2 NO production

NOS III activity was measured directly by the L-arginine/L-citrulline method in endothelial cells isolated from the aortas of SHRSP and WKY rats. NOS III activity was increased in SHRSP compared to WKY, suggesting NO production is increased rather than decreased in the hypertensive animals. This is mirrored in *Nos3* gene expression, quantified by reverse transcription-polymerase chain reaction (RT-PCR), where *Nos3* mRNA was more abundant in the aortas of SHRSP compared to WKY. The difference in *Nos3* gene expression just fails to achieve statistical significance when corrected for triple comparisons, possibly because of the small numbers studied. The increased NOS III activity (and *Nos3* expression) may represent a compensatory mechanism to some other blood pressure increasing factor(s). Schini *et al* also found impaired endothelium-dependent vasorelaxation in aortas of rats with genetic hypertension which was not due to reduced NOS III activity[286]. It therefore seems likely that the reduced NO availability in SHRSP is not due to reduced NO production, but instead due to increased scavenging.

In addition, a small reduction in NOS III activity was demonstrated in females compared to males of both strains. Although this gender difference does not reach statistical significance, it has not been demonstrated before. Again the pattern of NOS III activity is mirrored in the levels of *Nos3* gene expression. The gender differences in *Nos3* expression, while large in SHRSP, do not reach statistical significance again possibly due to the very small numbers involved. It would therefore also appear that reduced NO availability in males compared to females is not due to reduced NO production, which if anything is greater in males.

The reduced NO availability in males and SHRSP in general, for whatever reason, may lead to increased *Nos3* expression and NOS III activity as a compensatory mechanism, which fails to correct the relative deficiency of NO. Although by no means certain, there is some evidence that NO can reversibly and dose-dependently inhibit NOS III enzyme activity *in* [27]. This may be the mechanism whereby reduced NO availability could increase NOS III activity by removal of the auto-inhibition, although it is less clear how reduced NO could up-regulate *Nos3* expression. Conceivably, the vasoconstriction produced by reduced NO would increase fluid shear stress, which is known to up-regulate *Nos3* gene expression[90,91].

Although differences in *Nos3* expression and NOS III activity cannot explain the strain and gender differences in NO availability, other NOS isoforms may be responsible. 17β-oestradiol has been shown to increase activity of purified NOS I *in vitro*[257]. However, despite having a number of interesting phenotypes, *Nos1* 'knock out' mice have normal blood pressure[36]. In any case, NOS I is unlikely to have an effect on denervated aortic rings *in vitro*.

NOS II has greater potential to contribute to differences in NO availability. If NOS II were at least partially responsible, then it might explain some of the discrepancies between basal

NO detected by L-NAME and stimulated NO detected by ACh. L-NAME would inhibit all isoforms of NOS, but ACh would only stimulate NOS III. In addition, Deng and Rapp found linkage between a QTL for hypertension and the *Nos2* gene on rat chromosome 10 in an F2 population derived from a cross of inbred Dahl salt-sensitive rats with Milan normotensive rats[123]. The *Ace* locus, encoding angiotensin I converting enzyme, also mapped to this region and is another, and possibly more likely candidate. This implicates *Nos2* in hypertension, however, as with *Nos1* described above, *Nos2* 'knock out' mice have normal blood pressure[124]. As far as gender differences are concerned, 17β-oestradiol has also been shown to alter *Nos2* expression after IFN or LPS induction, but the effect is an attenuation of induction[257], which would not explain the increased NO availability in females.

In summary, there is no evidence for reduced NO production by SHRSP compared to WKY. In fact, when NOS III enzyme activity and *Nos3* gene expression are examined, there is evidence that SHRSP may produce more NO. The same is true of males compared to females. There is no evidence, from studies of NOS III enzyme activity or *Nos3* gene expression, of reduced NOS III activity in males compared to females. If anything, NOS III activity may be increased in males. Isoforms of NOS other than NOS III may be responsible for the observed differences in NO availability, but there is little evidence from the literature and no data from these studies that this is the case.

5.3 NO scavenging

It is known that NO can be scavenged by the free radical O_2^- to form peroxynitrite[54]. Although being itself a weak vasodilator[287], the formation of peroxynitrite would reduce the availability of NO. In addition, peroxinitrite (or one of its decomposition products) has been

shown to be directly toxic to the endothelium[172]. There is evidence that the apparent reduced NO availability demonstrated in the SHRSP can be exactly accounted for by increased levels of O_2^{-}[114] and that in SHRSP mesenteric resistance vessels NO production is normal, but scavenging by O_2^{-} is increased[115]. There is also evidence that O_2^{-} can influence the vascular reactivity of human vessels[219] and may contribute to the hypertension in some animal models[217]. Excess of O_2^{-} is therefore a likely candidate for reducing basal and/or stimulated NO availability in SHRSP. To investigate this possibility the effects of exogenous SOD and the SOD inhibitor DETCA on carbachol and PE response curves were studied *in* , together with quantification of *Sod1* and *Sod2* mRNA expression.

As one would expect, by inhibiting endogenous SOD and thereby increasing O_2^{-} levels, DETCA attenuated the carbachol relaxation in WKY and SHRSP and also in males and females within both strains. It has previously been demonstrated in male animals that the attenuation by DETCA is significantly greater in SHRSP than WKY[114]. This is confirmed in the current study when males and females of both strains are combined. The attenuation of the carbachol response by DETCA remains significantly greater in SHRSP compared to WKY. This could mean that SOD is more active or present in higher concentrations in the aorta of SHRSP and therefore inhibiting the enzyme produces a greater effect. When SOD mRNA was quantified by northern blotting this turned out to be the case, at least as far as Mn SOD in males is concerned, where *Sod2* expression in SHRSP aortas was significantly greater than in WKY aortas. No strain difference in *Sod2* expression was detected in females. The reason for this is not clear, but again may reflect the relatively smaller blood pressure difference and/or possibly a smaller difference in oxidative stress.

Alternatively, the DETCA result could mean that when SOD is completely inhibited, O_2^- accumulates faster in SHRSP and thus impairs relaxation to carbachol to a greater extent. There is now evidence from further work by our group[288] and others[186] to suggest that O_2^- does accumulate faster in aortas from SHRSP compared to WKY. It remains unclear why O_2^- production should be greater in the vasculature of SHRSP, although Kerr *et al* provide evidence that the endothelium is responsible[288]. Hypercholesterolaemia has been shown to increase O_2^- generation by endothelial XO[211], although there is no evidence for hypercholesterolaemia in SHRSP. A more likely candidate enzyme is NAD(P)H oxidase, which has been shown to produce more O_2^- in models of angiotensin II dependent hypertension[179]. Angiotensin II has been shown to up-regulate expression of the *p22phox* gene, which encodes NAD(P)H oxidase[289]. However, there is very little evidence for angiotensin II excess in SHRSP, at least at 16 weeks of age before any renal damage has occurred. Other factors, as yet undetermined, may increase *p22phox* expression in SHRSP.

A possible mechanism which may explain both an increased XO activity and NAD(P)H oxidase activity in SHRSP and males is the reduced availability of NO itself. NO has been shown to suppress XO activity *in vitro*, reversibly and dose-dependently[290]. The suppression occurred irrespective of the presence or absence of xanthine; indicating that the reaction product of NO and O_2^-, peroxynitrite, is not responsible for the suppression. Similarly, NO inhibits O_2^- production *in vitro* by NAD(P)H oxidase, albeit isolated from neutrophils and not endothelial cells[291]. These findings may indicate that a vicious cycle exists where increased O_2^- leads to reduced NO, which leads to increased XO and/or NAD(P)H oxidase activity, which leads to increased O_2^- etc.

An alternative source of O_2^- is NOS III itself. HUVEC can be stimulated to produce O_2^- by native LDL (n-LDL), which can be inhibited by L-NAME, suggesting that NOS III is the

enzyme responsible[182]. Again there is no reason to believe that SHRSP have more circulating n-LDL than WKY. More work is required, and indeed is underway, to determine the possible mechanism(s) for O_2^- excess in SHRSP.

Of course, increased O_2^- production and increased SOD activity are not mutually exclusive, and may both occur within the SHRSP vasculature. Increased O_2^- may cause a compensatory up-regulation of *Sod* gene expression. As discussed in section 1.2.2.2, other groups have shown induction of *Sod2* gene expression in response to increased O_2^- and other oxidative stress[201,202]. In these studies *Sod1* gene expression was unaltered[201,202], therefore it is less surprising that no differences were detected in *Sod1* mRNA in males or females in the current study. Although *Sod1* gene expression is thought not to be regulated by oxidative stress, there is evidence that laminar shear stress of 0.6 to 15 dyne/cm^2 can increase *Sod1* mRNA in a time- and dose-dependent manner in human aortic endothelial cells by a 1.6-fold increase in transcriptional activity[93]. Therefore, one might have expected that the increased laminar shear stress of higher blood pressure would have increased *Sod1* expression in SHRSP compared to WKY. In the above study the 1.6-fold increase in mRNA was detected by nuclear run-on assays, so northern blotting used in the current study may not have been sensitive enough to detect such a difference.

Because of its localisation on endothelial cells and the extracellular matrix of the vasculature, EC-SOD has been hailed as the principal regulator of endothelium derived NO bioavailability[208]. There are no data from the current study as to whether or not there are differences in *Sod3* mRNA expression between SHRSP and WKY. Although there is some evidence for post-transcriptional or post-translational regulation of the human *Sod3* gene[209], there is no evidence in the literature for up-regulation in response to oxidative stress or any

other stimulus. If a difference in *Sod3* expression did exist between SHRSP and WKY, then it is likely to be primary and not secondary to differences in O_2^- levels or shear stress.

There were no gender differences in the attenuation of carbachol relaxation by DETCA in the current study. This would suggest that if a difference in O_2^- is responsible for the gender differences in NO availability, then this crude manipulation of SOD/O_2^- was not sensitive enough to detect it. There are data in the literature to suggest gender differences in O_2^- production. Kerr *et al* recently found O_2^- production to be greater in male WKY compared to female WKY, but found no gender difference in SHRSP[288]. Brandes and Mügge also found a gender difference in Wistar rats, with O_2^- production being greater in males[186]. Despite this, these latter authors failed to detect a significant gender difference in the effect of DETCA[186], as in the current study. An alternative explanation is that some other mechanism may be responsible for gender differences in NO availability, but it is not clear what this may be, as NO production does not seem to be greater in females.

DETCA increased the mean E_{max} and mean EC_{50} of the PE concentration response curves in all groups as one would expect from its action of indirectly removing the vasodilator tone of NO. The changes in individual groups were variable and no significant strain or gender effects were detected.

The addition of exogenous SOD to the rings tended to augment the carbachol relaxation. This effect was most marked in SHRSP males who had the poorest relaxation to begin with. Nonetheless, the control relaxations were so good (>85% of PE in most cases) that there was very little room for improvement. The responses to SOD were consequently relatively small and quite variable resulting in no significant strain or gender differences. Again as one might expect, exogenous SOD attenuated the contraction to PE in all groups. As before, the

effects were relatively small and variable resulting in no significant strain or gender differences.

Another reason for the small effects of exogenous SOD, and perhaps even the lack of strain or gender differences, may be that increased SOD activity is thought to be beneficial only when balanced by an increase in catalase activity[194]. If SOD activity is increased on its own then O_2^- will be converted to H_2O_2, which may become toxic to the vessel if not removed by catalase[193].

As mentioned in section 1.2.2.1, an imbalance of SOD and catalase has been proposed as a common mechanism for the premature ageing of the central nervous system found in humans with Down's syndrome and transgenic rats containing an extra copy of the human *Sod1* gene. In both of these situations there is increased Cu/Zn SOD activity in keeping with the gene dosage effect[190,193], but this seems to produce detrimental effects as there is no associated increase in catalase activity. This places some importance on catalase as an antioxidant defence mechanism, but it must not be forgotten that inhibition of SOD by DETCA had a greater detrimental effect on endothelium-dependent relaxations than the catalase inhibitor 3-amino-1,2,4-triazole[188], suggesting that SOD is more important.

Exogenous SOD added to the water-bath is unable to enter the cells and therefore only removes extracellular O_2^-. This may also partly explain the relatively small effect. Others have encountered the same problem, but Mügge *et al* managed to overcome this by employing polyethylene-glycolated SOD to scavenge O_2^- and reduce atherogenesis in hypercholesterolaemic rabbits[213]. Similarly, Nakazono *et al* were able to lower blood pressure in SHR by intravenous injection of a fusion protein of SOD linked to a C-terminal

basic domain, which has high affinity for heparin-like proteoglycans on vascular endothelial cells[217]. However, such compounds are not widely available.

In summary, there is evidence from previous studies[114,115], and more recently from Kerr *et* [288], that the vessels of SHRSP have O_2^- in excess. This is confirmed in the current study and is likely to be responsible for reduced basal NO in SHRSP. The consequent increased oxidative stress appears to upregulate *Sod2* gene expression in male SHRSP, as described by others[201,202]. Both of these differences are likely to contribute to the marked difference in the effect of DETCA on the carbachol response between SHRSP and WKY.

5.4 Effect of age

There was an age related decline in basal NO availability in female rats, as measured by the contraction of aortic rings to L-NAME. Basal NO at 12 months was significantly reduced compared to 4 months in both WKY and SHRSP female aortas. There was a slight tendency for basal NO measurements to increase with age in both WKY and SHRSP males, although basal NO availability at 12 months did not differ significantly from 4 months. As discussed in section 1.4, a decline in endothelial function with age has been reported in rats[263,267] and humans[264,265]. In these studies only males were studied[267] or males and females were analysed together without comparison between genders[265]. The present study agrees with these data, but suggests that the effect is more marked in females.

As with the blood pressure and gender differences, reduced basal NO in the older females may be due to reduced NO production, increased NO scavenging or both. *Nos3* gene expression was quantified by RT-PCR in the aortas from a small number of 12 month old female rats and was found to be significantly increased compared to 4 month old females.

Accepting that increased *Nos3* gene expression does not necessarily equate with increased NOS III activity, this provides some evidence that NO production is not reduced. Instead, NO production may be increased, perhaps to compensate for increased O_2^- which Harman suggests is the underlying mechanism of ageing[266]. Tschudi *et al* provide some evidence that increased O_2^- is indeed responsible for age related reduction in NO in male WKY rats[267]. A recent study by Barton *et al* suggests a possible mechanism for increased O_2^- in older rats[292]. They found reduced plasma SOD activity in older rats, but aortic SOD activity was unaffected by age. As with the current study they found basal NO availability to be reduced in the older animals. However, they also measured *Nos3* gene expression by RT-PCR and found it to be reduced in the aortas from the older animals, which is contrary to the current study.

In summary, there was an age related decline in basal NO availability in females, with an age related increase in *Nos3* gene expression, suggesting that age related increases in O_2^- within the aorta may be responsible for the associated decline in endothelial function.

5.5 Effect of oestrogen

Although circulating 17β-oestradiol was not measured in these animals, it is tempting to speculate, from the gender differences in NO availability and decline with age in females, that 17β-oestradiol alters the NO/O_2^- balance. To address this point basal NO availability was measured by the response of aortic rings to L-NAME as before. Aortic rings from one year old WKY females treated with intraperitoneal 17β-oestradiol for two weeks were compared with rings from one year old WKY females treated with intraperitoneal vehicle. Basal NO availability was greater in the 17β-oestradiol treated group, but the difference only bordered on statistical significance.

As discussed in section 1.1.1.5.2, the *Nos3* gene contains several copies of the oestrogen response element half-palindrome[71,74], which can, in combination, up-regulate transcription of *Nos3* in response to oestrogen[95]. One may speculate, that this is the mechanism whereby *in vivo* 17β-oestradiol augments basal NO availability in the current study. Indeed, as discussed in section 1.3.1.3, there are many reports in the literature where oestrogen has been shown to directly increase *Nos3* mRNA[105-107]. However, when *Nos3* mRNA was quantified by RT-PCR in a small number of aortas from 17β-oestradiol and vehicle treated WKY females at one year of age, there was no significant difference between the two groups. The trend was towards a decrease after 17β-oestradiol treatment, rather than an increase. The reason why this result contradicts other reports is unclear. Perhaps at one year of age *Nos3* gene expression has been maximally stimulated by the oxidative stress of ageing. Therefore, further stimulation would be difficult, if not impossible, with the dose of 17β-oestradiol used. In addition, at one year of age female rats are still fertile, although less so. Therefore, their endogenous oestrogen may also be stimulating *Nos3* gene expression in both groups. Whatever the reason, the lack of a difference in *Nos3* gene expression makes a difference in NO production less likely as the mechanism for the oestrogen effect on NO availability. However, it is still possible that oestrogen increases NOS III activity in the absence of increased *Nos3* expression.

It may therefore be that oestrogen prevents NO scavenging. There are no data from the current study to support this hypothesis, but there is at least one report in the literature by Arnal *et al*[185], who found evidence of increased NO effect (i.e. increased guanylate cyclase stimulation) from BAEC treated with the synthetic oestrogen ethinylestradiol, which they showed was due to reduced O_2^-. They found no effect of ethinylestradiol on *Nos3* gene expression or NOS III enzyme activity. More recently, a couple of studies, including data

from our own group, have demonstrated gender differences in O_2^-, with male rats having more O_2^- compared to females[186,288].

As with NO, the amount of O_2^- present in the aorta at any one time will depend on the rate of production and the rate of dismutation. Neither of the above two studies provides evidence as to which mechanism is responsible for the increased O_2^- in males, although there is evidence for reduced activity of the O_2^- generating enzyme NADH oxidase by ethinylestradiol from another study[259]. The only previous study to look at the effects of oestrogen on SOD activity within the cardiovascular system found no effect of gender, gonadectomy or graded doses of oestradiol on the rabbit myocardium[261]. However, oestrogen has been shown to increase Cu/Zn SOD activity in non-vascular tissue such as rat mammary tumours[260].

As well as by dismutation, O_2^- can be reduced by interaction with anti-oxidant molecules. Oestrogen appears to possess anti-oxidant properties[248], perhaps related to its phenolic structure, and may also reduce O_2^- by this means. It therefore seems possible, if not likely, that oestrogen can reduce O_2^- by reducing production and increasing removal. This would explain the increased NO availability in the current study after 17β-oestradiol treatment, with no apparent increase in *Nos3* expression. As O_2^- is thought to be important in atherogenesis, some of the cardioprotective effects of oestrogen in animals[237,238] and humans[241,242] may be due to a reduction in O_2^-, in addition to increasing NO production.

There was no difference it the carbachol concentration response curves between the 17β-oestradiol treated group and the vehicle treated group. This is further evidence for a difference in regulation between basal and stimulated NO availability, as discussed in section

5.1. As O_2^- is thought to underlie the difference in availability of basal and stimulated NO availability, then this is further evidence for oestrogen affecting O_2^-.

Circulating oestrogen is not the only difference between males and females. Progestogens may also be important. There is much debate about whether progestogens diminish the beneficial effect of oestrogen. This is an important issue as most women, certainly those with an intact uterus, are given a progestogen in conjunction with an oestrogen as conventional HRT. The Postmenopausal Estrogen/Progestin Intervention (PEPI) Trial showed that cyclical medroxyprogesterone acetate marginally attenuated the improved lipid profile obtained from conjugated equine oestrogen, although the combination was still highly significant compared to placebo[246]. However, a recent report from the Nurses' Health Study[293] show a marked decrease in the risk of major coronary heart disease among women who took oestrogens with progestogens, as compared with the risk among women who did not use hormones (multivariate adjusted relative risk, 0.39; 95 percent confidence interval, 0.19 to 0.78) or oestrogens alone (relative risk, 0.60; 95 percent confidence interval, 0.43 to 0.83). Some of the cardiovascular benefit may be obtained by inhibition of VSMC proliferation, which has been demonstrated for oestrogen and progesterone independently, without synergistic or additive effects of the two steroids[294].

What about vascular reactivity? Jiang *et al* showed that progesterone on its own, albeit at micromolar concentrations, caused relaxation of rabbit coronary artery and rat aorta *in*

[295]. However, this effect was endothelium-independent and not inhibited by haemoglobin or methylene blue, suggesting that neither NO nor guanylyl cyclase are involved. Administration of more conventional HRT doses of medroxyprogesterone acetate to cynomolgus monkeys, either cyclically or continuously, attenuated the improvement in endothelium-dependent dilatation, at coronary angiography, elicited by conjugated equine

oestrogen[296]. It has been speculated that part of the difference between the progestogen effect in these two studies is due to structural differences between progesterone and medroxyprogesterone acetate. However, Miller and Vanhoutte had previously shown that progesterone *in vivo* reversed the beneficial effects of oestrogen on endothelium-dependent relaxations to ACh in canine coronary arteries *in vitro*[297].

It would appear that in terms of endothelial function, an oestrogen/progestogen combination is less beneficial than oestrogen alone. However, the improved lipid profile of the combination may still outweigh this and confer an overall benefit in cardiovascular disease prevention.

An additional difference between males and females is the much higher levels of testosterone in males. Relatively little attention has been paid to the role of androgens in the sexual dimorphism in cardiovascular disease. The Rancho Bernardo population study of 1132 men aged between 30 and 79 years found that those with systolic blood pressure greater than 160mmHg and/or diastolic blood pressure greater than 95mmHg had significantly lower testosterone than the normotensive population[298]. Indeed, systolic and diastolic blood pressure were inversely correlated with testosterone levels in the whole cohort, even after adjusting for age and body mass index. This inverse correlation between testosterone levels and blood pressure was confirmed in a 12 year prospective population-based study of 1,009 normotensive white men, although no significant correlation was detected between testosterone levels and cardiovascular disease[299]. The lack of correlation between testosterone and cardiovascular disease is especially surprising since testosterone has now been shown to increase cardioprotective high density lipoprotein (HDL) cholesterol and lower atherogenic very low density lipoprotein (VLDL)[300]. Nonetheless, these studies were the first indication that androgens may affect vascular reactivity.

The role of androgens in blood pressure regulation was subsequently studied in animals. Ganten *et al* used the SHR and SHRSP as models of hypertension to investigate the effect of androgens and found the opposite effect to the human studies[301]. They found in male rats at nine weeks of age that castration, or treatment with one of two different testosterone receptor antagonists, reduced blood pressure in both models. These interventions failed to reduce blood pressure at 25 weeks of age when hypertension was established. It is not clear why the rat studies are in conflict with the human studies, but perhaps high levels of testosterone during adolescence may be a better predictor of developing hypertension, rather than looking at older men in whom hypertension has or has not been established.

The studies which have examined the direct effects of testosterone on vascular reactivity seem to favour a dilator response in keeping with a hypotensive action. The first such *in*

study was carried out by Yue *et al*, who found that testosterone added to the organ bath directly and immediately relaxed rings of rabbit aorta and coronary artery[302]. This effect was similar if the vessels were removed from males or females, and was not attenuated by removal of the endothelium or inhibition of NOS. The mechanism of this relaxation therefore remains unclear, although the investigators provide some evidence that K^+ channels, but not ATP-sensitive K^+ channels, may be partly responsible. This *in vitro* observation was confirmed *in vivo* in the coronary circulation of anaesthetised dogs[303]. In this study, the vasodilatation in response to testosterone was again independent of sex, but was attenuated by the NOS inhibitor L-NAME. The involvement of endothelium derived NO was also suggested by another *in vitro* study of Sprague-Dawley rat thoracic aortic rings[304]. Again these authors report an acute, dose dependent vasodilator effect of testosterone on endothelium intact rings, which was attenuated, but not completely abolished, by L-NAME. Interestingly, they observed the same vasodilator effect in endothelium intact aortic rings from male testicular-feminised rats, which do not possess a

functional testosterone receptor, suggesting that the classical receptor is not involved in this effect.

Therefore, although the precise mechanism remains unclear, testosterone seems to have a vasodilator, and consequent blood pressure lowering effect, which together with its beneficial effect on lipid profile should make it cardioprotective. There are no data from the current study on the role of testosterone on the gender differences in blood pressure and NO availability, but such a role cannot be excluded. It may be that testosterone does improve endothelial function, but not as much as oestrogen, resulting in the observed gender difference. Alternatively, other differences between males and females, such as the Y chromosome, may contribute to the sexual dimorphism in blood pressure and cardiovascular disease.

The earliest experimental evidence that the Y chromosome may influence blood pressure came from Ely and Turner in 1990[305]. They crossed SHR and WKY rats and found that blood pressure was significantly higher from 12 to 20 weeks in the male offspring derived from WKY mothers and SHR fathers as compared with male offspring derived from SHR mothers and WKY fathers. The blood pressure in the female offspring was not related to the strain of the male progenitor, further supporting Y chromosome linkage and not parental imprinting. This observation was confirmed in a cross between the closely related SHRSP x WKY[306].

Again no attempt was made in the current study to assess the role the Y chromosome in gender differences in blood pressure or NO availability. The precise mechanism of the Y chromosome effect on blood pressure remains unclear, but there is no evidence it affects the NO/O_2^- balance directly. The androgen receptor is thought to be involved as the effect is not

apparent in a cross between SHR fathers and mothers heterozygous for the gene for testicular feminisation, where some male offspring will have no functional androgen receptor[307]. However, the increases in blood pressure may contribute to secondary gender differences in endothelial function.

In summary, oestrogen increases basal NO availability, without apparently increasing *Nos3* gene expression. It is reasonable to speculate that oestrogen reduces NO scavenging by O_2^-, although it is not clear whether the mechanism is increased O_2^- removal, reduced O_2^- production or both. The contributions of progesterone, testosterone and the Y chromosome were not investigated, but are likely to be significant, considering what is known from the literature and previous work performed in our laboratory.

6 Conclusion

The main conclusion from these studies is that the NO/O_2^- balance seems to be more important than the absolute amount of either molecule alone. Data presented in this thesis, and that of others, support the idea that O_2^- excess is responsible for the reduced basal, if not stimulated, NO availability and hence endothelial dysfunction and cardiovascular disease in SHRSP. *Nos3* and *Sod2* gene expression appear to be up-regulated to compensate, but are unable to correct the NO/O_2^- imbalance. It remains unclear whether O_2^- excess results from the primary genetic defect in SHRSP, or if it is simply a consequence of the hypertension.

Neither differences in *Nos3* gene expression, nor differences in NOS III enzyme activity can explain the gender differences in basal NO availability. Therefore, although not supported by these data, one may speculate that scavenging by O_2^-, at least in part, accounts for gender differences in NO availability. However, the effect of oestrogen on NO production may still be important. One may further speculate that both of these effects contribute to the gender differences in blood pressure and cardiovascular disease and the beneficial effects of postmenopausal hormone replacement therapy.

Figure 25 shows a schematic representation of the factors and enzymes thought to be involved in controlling NO availability within the vasculature.

Further work is required to fully elucidate the enzyme(s) responsible for the O_2^- excess in SHRSP, including EC-SOD. Such enzyme dysfunction may underlie the pathogenesis of some forms of human essential hypertension. The assumption that enzyme activity equates with gene expression needs to be confirmed by further functional studies, especially of the

SOD isoenzymes. One can speculate that males are in a state of O_2^- excess combined with a relative NO deficiency; a vascular status which predisposes to cardiovascular disease.

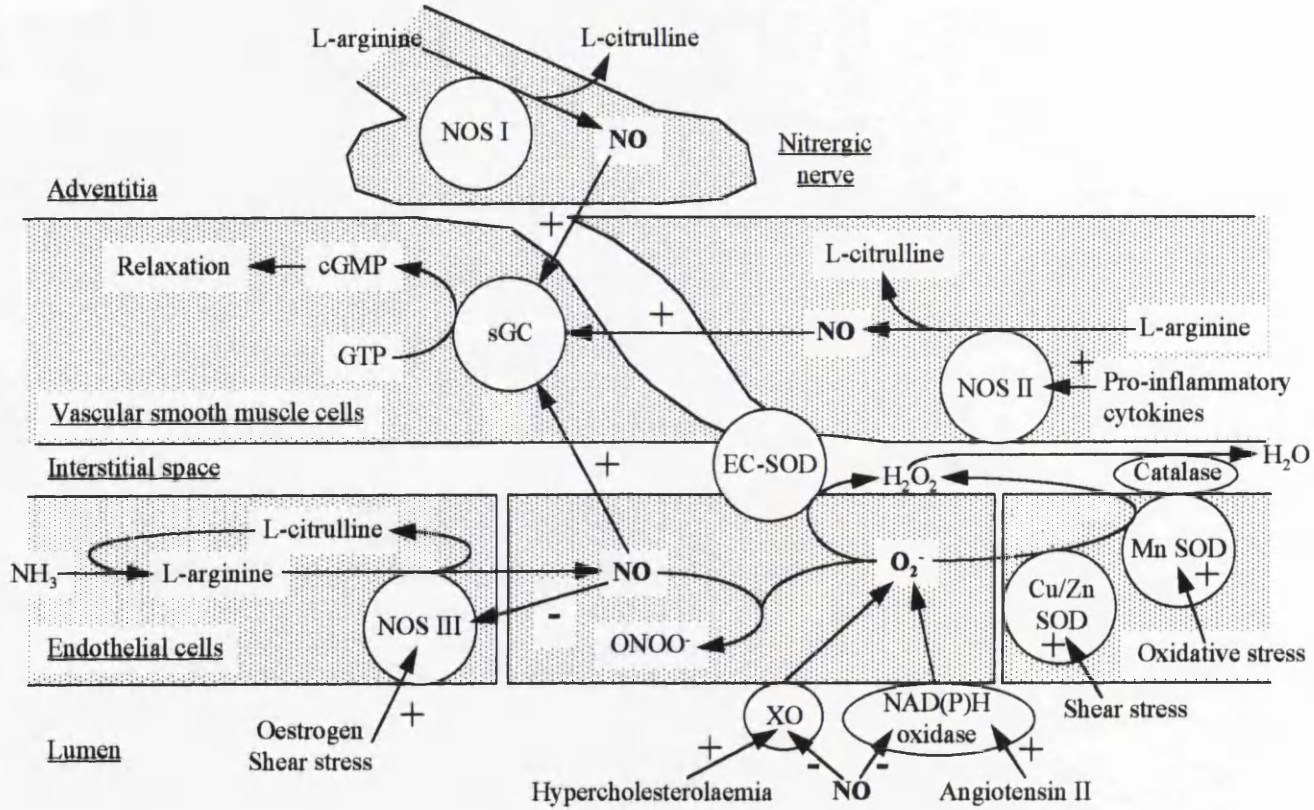

Figure 25 - Factors involved in regulating nitric oxide (**NO**) within the vasculature. **NOS I:** neuronal NOS, **NOS II:** inducible NOS, **NOS III:** endothelial NOS, **EC-SOD:** extracellular SOD, **MnSOD:** manganese SOD, **Cu/Zn SOD:** copper zinc SOD, **sGC:** soluble guanylate cyclase, **XO:** xanthine oxidase, **$ONOO^-$:** peroxynitrite, **H_2O_2:** hydrogen peroxide, **GTP:** guanosine 5'-triphosphate, **cGMP:** guanosine-3',5'-cyclic monophosphate.

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Appendices

Appendix 1 - List of suppliers

Amersham International plc, Amersham, UK

- [α^{32}P]-dCTP
- Hybond N+ nylon membrane
- Hyperfilm-MP

Biogenesis, Poole, UK

- RNAzol B

Bio-Rad Laboratories Ltd, Hemel Hempstead, UK

- AG 501-X8 mixed bed resin
- Bio-Rad protein assay
- Dowex-AG50W resin (200-400, 8% cross linked, Na^+ form)
- Sodium dodecyl sulphate (SDS)

Boehringer Mannheim, Lewes, UK

- DNase free RNase
- Eco RI restriction enzyme
- Hind III restriction enzyme
- Lysozyme
- Restriction enzyme buffer B
- Restriction enzyme buffer H

Fluka Chemicals, Gillingham, UK

Dimethylsulphoxide (DMSO)

Gibco BRL, Paisley, UK

Agarose

Yeast tRNA

Random primers DNA labelling kit consisting of:-

dATP, dGTP, dTTP

Klenow fragment DNA polymerase

Random primers buffer

ICN Biomedicals Ltd, Thame, UK

L-[U-^{14}C]-arginine,

Promega, Southampton, UK

AMV reverse transcriptase

dNTP's

Magnesium chloride ($MgCl_2$) for PCR

Oligo$(dT)_{15}$ primer

10X PCR buffer (Mg^{2+} free)

RQ1 DNase

rRNAsin

Taq DNA polymerase

Terminal deoxynucleotidyl tranferase (TdT)

5X TdT buffer

Sigma-Aldrich, Poole, UK

A: Aprotonin, L-arginine

C: Caesium chloride (CsCl), Calcium chloride ($CaCl_2.2H_2O$), Calcium disodium ethylenediaminetetra-acetate ($CaNa_2EDTA$), Carbamylcholine chloride (carbachol), L-citrulline, Cocaine HCl

D: Diethylpyrocarbonate (DEPC), Disodium phosphate (Na_2HPO_4), Dithiothreitol (DTT)

E: Ethidium bromide (EtBr), Ethylene glycol-bis(β-aminoethyl ether)-N,N,N', N'-tetra-acetic acid (EGTA)

F: Flavin adenine dinucleotide (FAD), Flavin mononucleotide (FMN)

G: Glucose

H: Hydrogen peroxide

I: N^{ω}-iminoethyl-L-ornithine (L-NIO), Indomethacin

O: 17β-oestradiol

L: λ Hind III DNA size markers, Leupeptin

M: Magnesium chloride ($MgCl_2$), Magnesium sulphate ($MgSO_4.7H_2O$), Methylene blue, Monopotassium phosphate (KH_2PO_4)

N: Nicotinamide adenine dinucleotide phosphate reduced form (NADPH), N^{G}-nitro-L-arginine methyl ester (L-NAME)

P: Potassium chloride (KCl), Phenylephrine (PE)

S: Sheared salmon sperm DNA, Sodium acetate, Sodium bicarbonate ($NaHCO_3$), Sodium chloride (NaCl), Sodium diethyldithiocarbamate (DETCA), Sodium nitroprusside (SNP), Soybean trypsin inhibitor (SBTI), Sucrose, Superoxide dismutase (SOD)

T: Tetrahydrobiopterin, Tris

V: Valine

Stratagene Ltd, Cambridge, UK

mCAP mRNA Capping Kit consisting of:-

Dithiothreitol

mCAP analogue

Proteinase K

RNase free DNase

RNase free TE

rNTP mix

T3 RNA polymerase

5X Transcription buffer

Zeneca, Macclesfield, UK

Halothane

Appendix 2 - Recipes for solutions

All chemicals were obtained from Sigma-Aldrich Co. Ltd, Poole, UK, unless otherwise stated in appendix 1.

Alkali Lysis Solution I

Glucose (1mol/L)	5mL
Tris.Cl (0.1mol/L, pH 8.0)	25mL
EDTA (0.1mol/L, pH 8.0)	10mL
dH_2O	60mL
	100mL

This was autoclaved, allowed to cool and stored at 4°C.

Alkali Lysis Solution II

NaOH (5mol/L)	0.2mL
SDS (10% w/v)	0.5mL
dH_2O	4.3mL
	5.0mL

This was freshly prepared on the day of use.

Alkali Lysis Solution III

Potassium acetate (5mol/L)	60mL
Glacial acetic acid	11.5mL
dH_2O	28.5mL
	100mL

This was prepared and stored at 4°C.

Alkali transfer buffer

NaCl (5mol/L)	150mL
NaOH (5mol/L)	25mL
dH_2O	325mL
	500mL

Deionised formamide

Formamide	200mL
AG501-X8 mixed bed resin	10g

This was stirred for 1 hour, filtered, split into 20mL aliquots, gassed in O_2-free N_2 and stored at -70°C.

Deionised glyoxal (Ethanedial)

Glyoxal (40% w/v)	20mL
AG 501-X8 mixed bed resin	20g

This was mixed by stirring until pH > 5.0, filtered, split into 30µL aliquots, gassed in O_2-free N_2 and stored at -70°C.

Denaturation solution

NaCl (5mol/L)	150mL
NaOH (5mol/L)	50mL
dH_2O	300mL
	500mL

100X Denhardt's Solution (2% BSA, 2% Ficoll, 2% PVP)

Bovine serum albumine (BSA, Fraction V)	2g
Ficoll (Type 400)	2g
Polyvinylpyrollidone (PVP, M_w 360,000)	2g
dH_2O	100mL

This was split into 1-2mL aliquots and stored at -70°C.

Dimethylsulphoxide (DMSO)

A fresh bottle of spectroscopy grade DMSO was separated into 100µL aliquots, gassed in O_2-free N_2 and stored at -70°C.

Gloxal mix

Spectroscopy grade DMSO	160µL
Sodium phosphate buffer (0.1mol/L, pH 7.0)	32µL
Deionised glyoxal (40% w/v)	54µL
	246µL

This was freshly prepared on the day of use.

L-amp agar plates

LB agar tablets	16.8g (10 tabs)
dH_2O	500mL

This was autoclaved and allowed to cool to hand warm.

Ampicillin sodium (25mg/mL stock)	2mL
	502mL

This was poured into sterile 100mm plates, allowed to set at room temperature and stored at 4°C.

LB-amp medium

LB-broth	500ml
Ampicillin sodium (25mg/mL stock)	2mL
	502mL

This was prepared and stored at 4°C.

LB broth

LB broth tablets	11g (10 tabs)
dH_2O	500mL

This was autoclaved, allowed to cool and stored at 4°C.

Lysozyme (10mg/mL in 10mmol/L Tris.Cl, pH 8.0)

Lysozyme	50mg
Tris·Cl (0.1mol/L, pH 8.0)	0.5mL
dH_2O	4.5mL
	5.0mL

This was freshly prepared on the day of use.

'Miller' transformation solution

Polyethylene glycol (Mw 8000, 50% w/v)	100mL
$MgSO_4$ (1mol/L)	25mL
Dimethyl sulphoxide (DMSO)	25mL
dH_2O + 10 LB broth tablets	350mL
	500mL

This was autoclaved, allowed to cool and stored at 4°C.

5X RNA loading buffer

Bromophenol blue	160mg
Glycerol	20mL
Sodium phosphate buffer (0.1mol/L, pH 7.0)	4mL
DEPC H_2O	16mL
	40mL

This was incubated at 37°C in a shaking incubator for 60 minutes to dissolve and mix the components, treated with DEPC (0.01% v/v) overnight, autoclaved, then stored at -20°C in small aliquots.

Sodium Dodecyl Sulphate (SDS, 10% w/v)

Wearing a face mask, 100g of electrophoresis grade SDS was added to 900mL of dH_2O and heated to 68°C to facilitate dissolution. The pH was adjusted to 7.2 with concentrated HCl and the volume made up to 1000mL.

Sodium phosphate buffer (0.1mol/L, pH 7.0)

NaH_2PO_4 (0.5mol/L)	78mL
Na_2HPO_4 (0.5mol/L)	122mL
DEPC H_2O	800mL
	1000mL

This was treated overnight with DEPC (0.01% v/v) and then autoclaved.

20X SSC

NaCl	175.3g
Na3Citrate	88.2g
dH_20	800mL

This was adjusted to pH 7.0 with HCl (1mol/L), made up to 1000mL and autoclaved.

20X SSPE

NaCl	175.3g
NaH_2PO_4	27.6g
$Na_2EDTA.2H_2O$	7.4g
dH_2O	900mL

This was adjusted to pH 7.4 with 1N NaOH, made up to 1000mL and autoclaved.

2X SSPE/0.1% SDS

20X SSPE	50mL
SDS (10% w/v)	5mL
dH_2O	445mL
	500mL

1X SSPE/0.1% SDS

20X SSPE	25mL
SDS (10% w/v)	5mL
dH_2O	470mL
	500mL

0.1X SSPE/0.1% SDS

20X SSPE	2.5mL
SDS (10% w/v)	5.0mL
dH_2O	492.5mL
	500mL

STE

NaCl (5mol/L)	10mL
Tris·Cl (0.1mol/L, pH 8.0)	50mL
EDTA (0.1mol/L, pH 8.0)	5mL
dH_2O	435mL
	500mL

Supplemented LB broth (10mmol/L $MgSO_4$, 0.2% w/v glucose)

Sterile LB broth	98mL
Sterile $MgSO_4$ (1mol/L)	1mL
Filter sterilised glucose (20% w/v)	1mL
	100mL