
**TARGETING THE NO/sGC/cGMP SIGNALING
PATHWAY IN HEALTH AND DISEASE**

A THESIS SUBMITTED TO
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DOCTOR OF PHILOSOPHY

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DECLARATION

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

In the case of Chapters 4, 6 and 7, my contribution to the work involved the following:

Chapter 4: All rat and mouse experiments were carried out by myself and rabbit experiments were carried out by Dr Johannes-Peter Stasch (Bayer HealthCare, Germany; Figure 2B-C). UV/VIS spectroscopy studies were carried out by Dr Peter Schmidt (Monash University, Australia; Figure 4). I wrote the chapter with intellectual advice and editorial assistance from my supervisors and collaborators.

Chapter 6: All experiments were carried out by myself, except mean arterial blood pressure (MABP) experiments and heart weight measurements which were carried out by Dr Emma Jones (Monash University, Australia; Figure 1-2) and $\cdot\text{O}_2^-$ detection experiments which were carried out by Dr Alyson Miller (Monash University, Australia; Figure 3). I wrote the chapter with intellectual advice and editorial assistance from my supervisors and collaborators.

Chapter 7: All experiments were carried out by myself, except those in Figure 1 which were performed by Dr Barbara Kemp-Harper (Monash, University, Australia). I wrote the chapter with intellectual advice and editorial assistance from my supervisors.

Signed:.....

Date:.....

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SUMMARY

The therapeutic utility of the nitric oxide (NO)/soluble guanylyl cyclase (sGC)/cyclic guanosine 3'5'-monophosphate (cGMP) pathway is well-recognised, with NO donors used in the treatment of diseases such as heart failure, acute hypertensive crisis and pulmonary hypertension. However, traditional nitrovasodilator therapy is somewhat limited due to the development of tolerance following prolonged administration. Furthermore, in vascular disease states such as hypertension, the NO/sGC/cGMP pathway appears to be dysfunctional. Such dysfunction is thought to arise as a consequence of oxidative stress and the associated increase in vascular superoxide anion radical ($\cdot\text{O}_2^-$) levels, leading to enhanced scavenging of NO to form the oxidant, peroxynitrite (ONOO^-). Indeed, ONOO^- is able to oxidise the reduced (Fe^{2+}) heme group of sGC, converting it into its NO-insensitive ferric (Fe^{3+}) or heme-free forms. Under such conditions, the efficacy of NO donors/nitrovasodilators are compromised, due to their inability to target these altered states of sGC, underlining the need for novel non-NO based compounds. As such, this thesis examines the vaso-protective actions and therapeutic potential of the redox sibling of NO, nitroxyl (HNO), as well as NO-independent sGC stimulators (BAY 41-2272) and activators (BAY 58-2667) in a number of cardiovascular disease states.

Chapter 3 describes experiments in isolated common carotid arteries from C57BL/6J and *Nox2*^{-/-} mice, which explored the potential $\cdot\text{O}_2^-$ suppressing and vaso-protective effects of HNO in the vasculature. Using a model of short-term oxidative stress, this chapter firstly demonstrated that 24-hour treatment with angiotensin II (Ang II) significantly elevated NADPH-driven $\cdot\text{O}_2^-$ production in the carotid arteries of mice, an effect which was dependent on an increase in *Nox2* activity. Short-term (24 hr) treatment with the HNO donors, Angeli's salt and isopropylamine NONOate (IPA/NO) reversed this Ang II-induced increase in $\cdot\text{O}_2^-$ generation. Importantly, we demonstrated that HNO mediates its $\cdot\text{O}_2^-$ limiting effects in the vasculature via a cGMP-independent mechanism. These results are, therefore, indicative of a direct modulation of *Nox2*-

containing NADPH oxidase itself by HNO, and given its highly thiophilic nature, it may point to its possible interaction with thiol groups on one or more of the regulatory subunits of NADPH oxidase.

Furthermore, the NO-independent sGC stimulator, BAY 41-2272 and activator, BAY 58-2667 also show promise in the treatment of cardiovascular disease. Excitingly, vasorelaxant responses to the sGC activator, BAY 58-2667, which preferentially targets the oxidised/heme-free form of sGC are not only preserved but markedly enhanced under pathophysiological conditions.

Chapter 4 describes *in vitro* experiments performed in isolated aortae (Wistar-Kyoto, WKY, and spontaneously hypertensive, SHR, rats; C57BL/6J and eNOS^{-/-} mice; New Zealand rabbits), mesenteric arteries (Sprague-Dawley rats, SD) and saphenous arteries (New Zealand rabbits) in order to investigate the role of endogenous NO in modulating the response to BAY 58-2667. The main findings of this chapter revealed that endogenous NO may serve to attenuate BAY 58-2667-mediated relaxation, an effect which appeared to be species-specific. Collectively this chapter suggests that endothelial-derived NO may play a protective role in the vasculature by limiting the accumulation of oxidised/heme-free sGC. The mechanisms by which this occurs are currently unknown. However, we speculate that NO activates an endogenous NADPH-dependent flavoprotein-containing reductase, which serves to preserve the heme of sGC in its reduced state, a concept which remains to be explored.

While we have shown that endogenous NO may limit the response to BAY 58-2667, previous studies have reported that the sGC stimulator, BAY 41-2272 elicits its effects in a synergistic manner with NO. Thus, **Chapter 5** describes *in vitro* experiments performed in isolated endothelium-intact and -denuded aortae from WKY and SHR rats in order to ascertain the contribution of the endothelium in regulating the vasorelaxant responses to BAY 41-2272 in hypertension. Despite a reduction in NO bioavailability in hypertension, BAY 41-2272-induced

relaxation was unchanged in the aortae of SHR versus WKY rats. The main findings of this study revealed the existence of an unidentified NO- and COX-independent endothelial-derived factor, which is up-regulated in the setting of hypertension and serves to preserve the vasorelaxant response to BAY 41-2272. While the identity and nature of this factor is yet to be confirmed, we proposed that its protective properties may be related to a potential ability to up-regulate the antioxidant capacity of the vasculature in the SHR.

Additionally, long-term treatment with both BAY 41-2272 and BAY 58-2667 have been reported to be protective in various models of hypertension. Thus, **Chapter 6** describes experiments performed in instrumented conscious aged rats (20-22 months: WKY and SHR) administered BAY 41-2272 (1 mg/kg/day) or BAY 58-2667 (0.3 or 1 mg/kg/day) intraperitoneally for a 4-week period, in order to investigate the potential cardio-protective and vaso-protective effects of chronic sGC stimulation and activation. This study revealed that while both BAY 41-2272 and BAY 58-2667 transiently reduced mean arterial blood pressure (MABP), this effect was not sustained and subsequently lost by the end of the treatment period, indicative of *in vivo* hemodynamic tolerance development. However, despite the absence of a maintained hypotensive effect, both compounds reversed cardiac hypertrophy while the higher dose of BAY 58-2667 (1 mg/kg/day) suppressed $\cdot\text{O}_2^-$ production in aged SHR aortae. At the level of the vasculature, both BAY 41-2272 and BAY 58-2667 restored 8-bromo-cGMP-mediated relaxation, presumably in a synergistic manner to elevate cGMP levels. Importantly, this study has shown that both BAY 41-2272 and BAY 58-2667 can exert cardio-protective effects (i.e. limit hypertrophy) following chronic administration independently of a decrease in blood pressure.

Indeed, we and others have shown that the vasorelaxant potency to BAY 58-2667 is enhanced in disease states and it has been assumed that an increase in vascular $\cdot\text{O}_2^-$ leads to augmented pools

of oxidised/heme-free sGC. This concept, however, has not been tested directly. The studies in **Chapter 7** sought to determine if NADPH oxidase was capable of modulating the vasorelaxant responses to BAY 58-2667 in a model of short-term oxidative stress (24 hour Ang II-treatment), and during chronic disease (ApoE^{-/-} mice on high fat diet). The study showed that an increase in NADPH-driven $\cdot\text{O}_2^-$ production correlated with a potentiated vasorelaxant response to BAY 58-2667, an effect which was Nox2-dependent. Interestingly, BAY 58-2667-induced relaxation was equipotent in the arteries of all mice fed the high fat diet (C57BL/6J, ApoE^{-/-} and Nox2^{-/-}/ApoE^{-/-}), irrespective of differences in $\cdot\text{O}_2^-$ levels detected. Thus, these results revealed that both NADPH oxidase and the Western-style high fat diet were capable of independently modulating the response to BAY 58-2667, presumably by augmenting the pools of oxidised/heme-free sGC. Whilst the exact mechanism(s) pertaining to this diet-induced effect remains unclear, it may relate to the increased plasma levels of low-density lipoproteins (LDL) and total cholesterol present in the animals receiving the high fat diet.

From a clinical perspective, NO-independent compounds such as HNO donors and/or stimulators and activators of sGC may offer considerable therapeutic advantages over traditional nitrovasodilator therapies due to their well-documented resistance to $\cdot\text{O}_2^-$ scavenging and vascular tolerance development, as well as the ability to target oxidised/heme-free sGC (i.e. BAY 58-2667) and the potential to mediate their effects via cGMP-independent mechanisms of action. Such compounds may also offer the basis from which future therapies, for the treatment of cardiovascular diseases, are designed.

PUBLICATIONS

MANUSCRIPTS

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ABSTRACTS

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ABBREVIATIONS

ACE	angiotensin-converting enzyme
ACh	acetylcholine
ALDH-2	aldehyde dehydrogenase 2
Ang II	angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe)
ANOVA	analysis of variance
ANP	atrial natriuretic peptide
Apo	apolipoprotein
ApoE ^{-/-}	apolipoprotein E-deficient
AS	Angeli's salt; sodium trioxodinitrate
AT ₁ R	angiotensin type-1 receptor
ATP	adenosine triphosphate
BAY 41-2272	(5-cyclopropyl-2-[1-(2-fluorobenzyl)-1H-pyrazolo[3,4-b]pyridin-3-yl]pyridin-4-ylamine)
BAY 58-2667	(4-[[[(4-carboxybutyl){2-[(4-phenethyl-benzyl)oxy]phenethyl}amino]methyl]benzoic]acid)
	Cinaciguat
BNP	B-type natriuretic peptide
BH ₄	(6R)-5,6,7,8-tetrahydrobiopterin
Ca ²⁺	calcium ion
CaCl ₂	calcium chloride
cAMP	cyclic adenosine 3',5'-monophosphate
Carboxy-PTIO	[2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazole-1-oxo-3-oxide]
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine 3'5'-monophosphate
CGRP	calcitonin gene-related peptide

CNP	C-type natriuretic peptide
CO ₂	carbon dioxide
CVD	cardiovascular disease
DEA/NO	diethylamine/NONOate
DMEM	Dulbecco' modified eagles medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EDHF	endothelial-derived hyperpolarising factor
EDRF	endothelial-derived relaxing factor
EDTA	ethylenediaminetetraacetic acid
eNOS	endothelial nitric oxide synthase
EtOH	ethanol
FAD	flavin adenine dinucleotide
Fe	iron
Fe ²⁺	ferrous iron
Fe ³⁺	ferric iron
FMN	flavin mononucleotide
GTP	guanosine 5'-triphosphate
H ₂ O	water
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFD	high fat diet
HMR 1766	Ataciguat
HNO	nitroxyl (protonated state)
HO-1	heme-oxygenase 1
HR	heart rate
H-NOX	Heme-Nitric oxide and Oxygen

iNO	inhaled NO
i.p.	intraperitoneal
Indomethacin	1-[p-Chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid
iNOS	inducible nitric oxide synthase
IPA/NO	isopropylamine NONOate
ISO	isoprenaline
K ⁺	potassium ion
K _{ATP}	ATP-sensitive potassium channel
K _{Ca}	calcium-dependent potassium channel
KCl	potassium chloride
KH ₂ PO ₄	potassium dihydrogen orthophosphate
K _v	voltage-dependent potassium channel
LDL	low density lipoprotein
LKM	levcromakalim
L-NAME	N ^G -nitro-L-arginine methyl ester
MAP	mean arterial pressure
MgSO ₄ •7H ₂ O	magnesium sulphate dihydrate
mmHg	millimetre of mercury
mRNA	messenger ribonucleic acid
Na ⁺	sodium ion
Na ₂ CO ₃	sodium carbonate
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NaHCO ₃	sodium hydrogen carbonate
NaOH	sodium hydroxide
nNOS	neuronal nitric oxide synthase

NO	nitric oxide
NO ⁻	nitroxyl anion
NO ⁺	nitrosonium cation
NO [•]	uncharged form of NO
NOS 1	neuronal nitric oxide synthase
NOS 2	inducible nitric oxide synthase
NOS 3	endothelial nitric oxide synthase
NOS	nitric oxide synthase
Nox1	nicotinamide adenine dinucleotide phosphate oxidase 1
Nox2	nicotinamide adenine dinucleotide phosphate oxidase 2
Nox2 ^{-/-}	nicotinamide adenine dinucleotide phosphate oxidase 2 deficient
NPs	natriuretic peptides
O ₂	oxygen
•O ₂ ⁻	superoxide anion radical
ODQ	1H-[1,2,4]oxodiazolo[4,3-a]quinoxaline-1-one
ONOO ⁻	peroxynitrite
PAS	Period clock protein/ARNT protein/simple minded protein
PDE	phosphodiesterase
pGC	particulate guanylyl cyclase
PKG	cGMP-dependent protein kinase G
RAS	renin-angiotensin system
ROS	reactive oxygen species
RSNOH	S-nitrosothiols
RyR2	cardiac ryanodine receptor 2
SBP	systolic blood pressure
SD	Sprague-Dawley (rat)

SEM	standard error of the mean
sGC	soluble guanylyl cyclase
SHR	spontaneously hypertensive rat
SNP	sodium nitroprusside
SOD	superoxide dismutase
U46619	9,11-dideoxy-11 alpha, 9 alpha-epoxy methano-prostaglandin F2 alpha
UV/VIS	ultra violet/visible
VSM	vascular smooth muscle
WKY	Wistar-Kyoto (rat)
Zn-PPIX	zinc protoporphyrin IX



CHAPTER 1

General Introduction



1. GENERAL INTRODUCTION

1.1 General Background

The endogenous signaling molecule, nitric oxide (NO) plays an integral role in the control of numerous physiological processes, including vascular homeostasis, platelet aggregation, cellular proliferation and neurotransmission (Fernhoff *et al.*, 2009; Gao, 2009; Munzel *et al.*, 2003). Of particular relevance to this thesis is the ability of endothelial-derived NO to induce vasodilatation and thereby regulate blood flow and pressure. These actions of NO are mediated predominantly by its receptor, soluble guanylyl cyclase (sGC) and the subsequent increase in the second messenger cyclic guanosine-3',5'-monophosphate (cGMP).

Indeed, the therapeutic utility of the NO/sGC/cGMP pathway has been well recognized for over a century (Horowitz, 2000) with NO donors and nitrovasodilators being at the forefront, providing the foundation for the diverse range of pharmacotherapeutics in cardiovascular medicine (Ignarro *et al.*, 2002). However, there are a number of limitations associated with the current clinical use of traditional nitrovasodilators such as tolerance development, reduced efficacy and potential cytotoxic effects (Ignarro *et al.*, 2002). To date, mounting evidence has suggested a correlation between the pathogenesis of several vascular diseases such as hypertension (Ruetten *et al.*, 1999) and atherosclerosis (Napoli & Ignarro, 2001) with an impairment in the generation or action of the NO/sGC/cGMP pathway. Thus, impaired endothelium-dependent relaxation, decreased responsiveness to NO donors/nitrovasodilators and/or reduced NO bioavailability due to its scavenging by reactive oxygen species (ROS), such as superoxide anion radical ($\cdot\text{O}_2^-$) have been reported (Cai & Harrison, 2000; Napoli & Ignarro, 2001; Ruetten *et al.*, 1999). Additionally, dysfunction at the level of sGC is also apparent in cardiovascular diseases such that ROS can lead to the oxidative modification of the heme-containing subunit of sGC, converting it into the ferric (Fe^{3+}) or heme-free forms which are

insensitive to NO stimulation (Stasch *et al.*, 2006). Thus, under such conditions the efficacy of NO donors are compromised, highlighting the need for new NO-independent sGC ligands.

Excitingly, nitroxyl (HNO) donors and NO-independent sGC stimulators and activators such as BAY 41-2272 and BAY 58-2667, respectively represent novel and alternate therapeutic avenues in the treatment of vascular diseases. HNO, the one electron reduced and protonated congener of NO[•], has unique pharmacological properties as compared to its redox sibling (Irvine *et al.*, 2008). Thus, unlike NO[•], HNO has been shown to interact directly with thiol-containing proteins to increase myocardial contractility (Dai *et al.*, 2007; Paolocci *et al.*, 2003; Tochetti *et al.*, 2007), increase plasma levels of calcitonin gene-related peptide (CGRP) (Paolocci *et al.*, 2001), as well as being resistant to tolerance development (Irvine *et al.*, 2007; Irvine *et al.*, 2011) and scavenging by [•]O₂⁻ (Li *et al.*, 1999; Miranda *et al.*, 2003a; Miranda *et al.*, 2002). Furthermore, HNO also exhibits clear vasoprotective properties, inhibiting platelet aggregation (Bermejo *et al.*, 2005) and serving as a potent vasodilator in both resistance (Irvine *et al.*, 2003) and conduit arteries (Ellis *et al.*, 2000). Together, these properties confer advantages to HNO over NO[•], inferring that HNO donors have potential in cardiovascular medicine, particularly in the treatment of heart failure (Paolocci *et al.*, 2003; Paolocci *et al.*, 2001; Tochetti *et al.*, 2007).

Moreover, the limitations of traditional NO donors can be overcome with the use of novel NO-independent sGC stimulators (BAY 41-2272, BAY 41-8543) and/or activators (BAY 58-2667, HMR 1766) which target sGC in the reduced (Fe²⁺) and oxidized (Fe³⁺)/heme-free states, respectively (Evgenov *et al.*, 2006; Schindler *et al.*, 2005; Schmidt *et al.*, 2004). Similar to NO[•], sGC stimulators and activators induce vasodilation and inhibit platelet aggregation yet lack the development of tolerance or susceptibility to [•]O₂⁻ scavenging (Evgenov *et al.*, 2006; Stasch *et al.*, 2006; Stasch *et al.*, 2001). In addition, the unique ability of sGC activators to target oxidized/heme-free sGC allows these compounds to preferentially dilate the diseased versus non-

diseased vasculature (Stasch *et al.*, 2006). Thus, HNO donors and NO-independent sGC stimulators and activators exhibit properties favourable for the treatment of cardiovascular disease, demonstrating clear advantages over traditionally used nitrovasodilators.

1.2 Nitric Oxide (NO)

Since the 1870s, nitroglycerin has been used clinically for the treatment of angina pectoris despite the lack of understanding behind its mechanisms of action (Murad, 2003b). In 1977, Murad and co-workers demonstrated that the vasodilatory effects of nitroglycerin and related nitrates were mediated by their conversion to NO and the subsequent activation of guanylyl cyclase (Murad, 2003b; Murad *et al.*, 1978). In 1980, Furchgott and Zawadski identified an unknown vasodilator substance which was released by endothelial cells and diffused across to the smooth muscle to elicit vasorelaxation (Furchgott & Zawadski, 1980). This factor was named endothelium-derived relaxing factor (EDRF) and subsequently identified as NO (Palmer *et al.*, 1987). To date, NO has been identified as a ubiquitous signaling molecule present in all vertebrates and invertebrates, and is responsible for a number of important physiological processes, including the maintenance of vasodilator tone, inhibition of platelet aggregation and adhesion, modulation of smooth muscle proliferation as well as cellular defence (Denninger & Marletta, 1999; Jacklet, 1997; Moncada & Higgs, 2006).

1.2.1 Nitric Oxide Synthases (NOS)

A gas with the half-life of several seconds, NO is synthesized from the guanadino nitrogen molecules of the amino acid L-arginine by three NO synthases (NOS): neuronal NOS (nNOS; NOS1), inducible NOS (iNOS; NOS2) and endothelial NOS (eNOS; NOS3) (Barbato & Tzeng, 2004; Dudsinski *et al.*, 2006; Moncada *et al.*, 1997). Table 1 below provides a brief overview of the physiological roles of NO produced by all three NOS isoforms as well as their locations.

Table 1. Brief overview of the location and function of the three isoforms of NOS.

Name	NOS 1	NOS 2	NOS 3	References
<i>Alternate Names</i>	nNOS neuronal NOS NOS I	iNOS inducible NOS NOS II	eNOS endothelial NOS NOS III	(Murad, 2006) (Alderton <i>et al.</i> , 2001)
<i>Tissues</i>	Central & peripheral Nervous system Skeletal muscle Pancreatic islets Epithelial cells of lung, uterus & stomach	Macrophages Heart Liver Smooth muscle	Endothelium Brain Epithelium Platelets Heart	(Forstermann, 2000) (Murad, 2006) (Shaul, 2002) (Michel & Feron, 1997)
<i>Subcellular localisation</i>	Predominantly cytosolic	Both cytosolic & particulate	Predominantly particulate, caveolae	(Dudsinski <i>et al.</i> , 2006)
<i>Function</i>	Neurotransmission (NANC)	Host defence Cytotoxic Inflammation	Vascular relaxation Anti-aggregatory Angiogenesis	(Moncada, 1999) (Bhagat & Vallance, 1996)

Furthermore, despite being products of distinct genes, the mammalian NOSs share approximately 50-60% sequence homology suggesting the existence of a common NOS ancestral gene (Alderton *et al.*, 2001; Dudsinski *et al.*, 2006). As illustrated in Figure 1 below, NOSs exists as functional dimers, exhibiting a bidomain structure in which the N-terminal oxygenase domain is comprised of the binding sites for the heme group (iron protoporphyrin IX), the co-factor (6*R*)-5,6,7,8-tetrahydrobiopterin (BH₄) and L-arginine, whilst the reductase domain contains the binding sites for flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and β-nicotinamide adenine dinucleotide phosphate (NADPH) (Alderton *et al.*, 2001). Both domains are connected by a linker containing a Ca²⁺/calmodulin (CaM) binding site which is believed to be crucial in facilitating the flow of electrons from the reductase domain to the

oxygenase domain in addition to being responsible for electron flow from NADPH to FAD and subsequently to FMN (Dudsinski *et al.*, 2006; Sagami *et al.*, 2002).

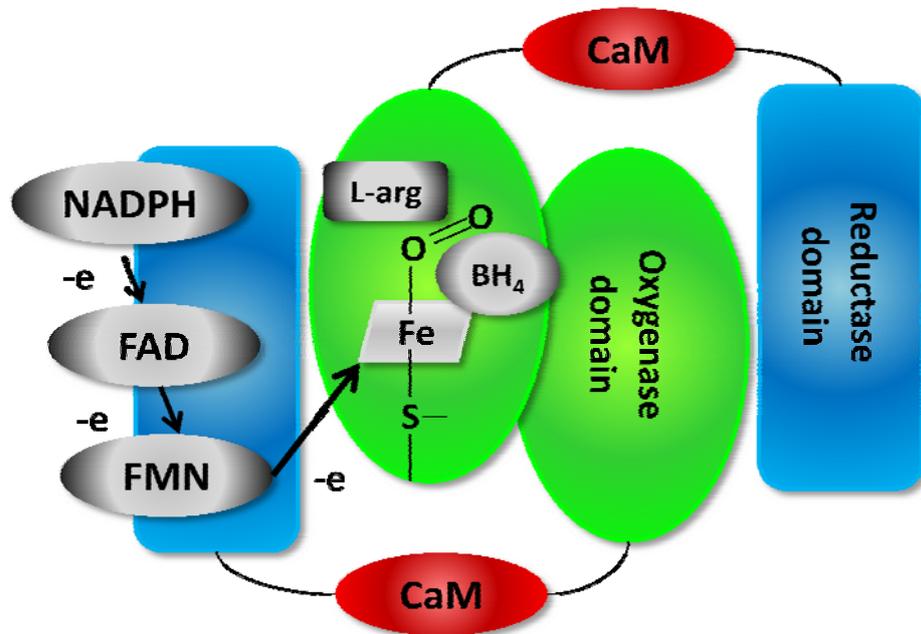


Figure 1. Structure of NOS. All NOS catalytic reactions involve the flow of electrons in the reductase domain from NADPH to FAD and finally to FMN, after which electron flow proceeds to the heme in the oxygenase domain facilitating the conversion of O₂ and L-arginine into NO and L-citrulline. The co-factor BH₄ is required for the efficient generation of NO (adapted from Sagami *et al.*, 2002).

The synthesis of NO by the constitutive NOS isoforms (eNOS and nNOS) is highly regulated at both the transcriptional (expression and abundance) and post-transcriptional (activity and function) levels, and this regulation is dependent on the binding of Ca²⁺ to CaM (Michel & Feron, 1997; Schulz *et al.*, 2005). Conversely, iNOS is almost exclusively regulated transcriptionally and is not influenced by changes in intracellular calcium, but instead is controlled by external factors such as lipopolysaccharides and cytokines namely, interleukins (e.g. IL-1), interferons (e.g. IFN- γ) and tumour necrosis factor- α (TNF- α) (Dudsinski *et al.*, 2006; Michel & Feron, 1997; Sagami *et al.*, 2002). Thus, while eNOS and nNOS only produce

low amounts of NO (picomolar to nanomolar concentrations), iNOS is able to generate large quantities of NO (micromolar concentrations) and plays a central role in host defence (Felley-Bosco *et al.*, 2002; Fostermann *et al.*, 1995). The synthesis of NO from any of the NOS isoforms, however, only occurs in the presence of sufficient quantities of the co-factor, BH₄, which binds to the immediate vicinity of the heme at the dimer interface (Werner *et al.*, 2003).

BH₄ is essential for the catalytic function of all three NOS isoforms and acts by stabilizing the NOS enzymes, enhancing the substrate binding affinity of NOS as well as participating in the electron transfer process (Moens & Kass, 2006; Sagami *et al.*, 2002; Werner *et al.*, 2003). Indeed, a decline in BH₄ bioavailability has been shown to contribute to pathophysiological conditions such as hypertension, where coupling between the reductase domain and L-arginine is lost resulting in the ‘uncoupling’ of NOS and the formation of superoxide (O_2^-) and/or hydrogen peroxide (H₂O₂) (Moens & Kass, 2006). Moreover, it has also been suggested that NO⁻ (HNO) rather than NO[•] is produced by NOS under conditions of oxidative stress, when there is an absence or depletion of BH₄ (Adak *et al.*, 2000; Rusche *et al.*, 1998). Thus, there is no doubt that BH₄ is an absolute requirement for the catalytic function of NOS and the subsequent synthesis of NO (Sagami *et al.*, 2002).

The synthesis of NO from L-arginine involves a two-step oxidation process which incorporates the initial hydroxylation of L-arginine to the intermediate N^G-hydroxy-L-arginine (NHA) followed by its own oxidation using a single electron from NADPH to generate L-citrulline and NO as the final products (Andrew & Mayer, 1999). The overall stoichiometry of this process consumes 1 mole of L-arginine, 1.5 mole of NADPH and 2 moles of molecular oxygen (O₂) to generate 1 mole of NO, 1 mole of L-citrulline and 2 moles of water (H₂O) as depicted in Figure 2 (Dudsinski *et al.*, 2006).

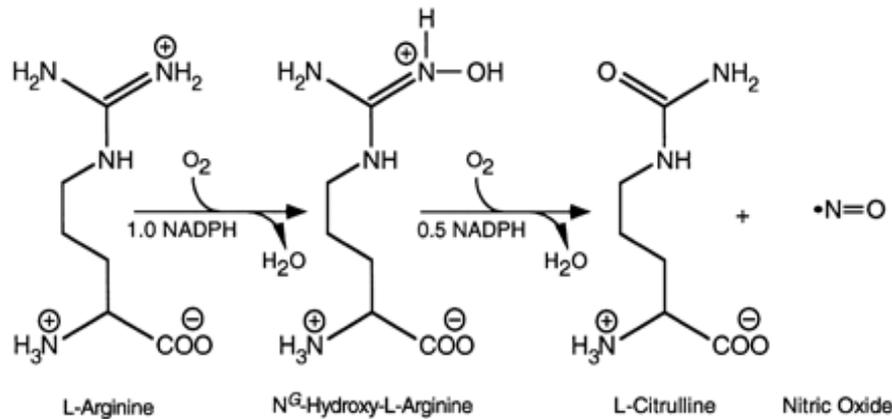


Figure 2. NOS-catalysed synthesis of NO from L-arginine (Sagami *et al.*, 2002).

NO synthesis and signaling commonly occurs in a paracrine manner, whereby NO produced can rapidly diffuse across the endothelial cell membrane into the adjacent smooth muscle cell, where it binds to the heme group of its intracellular receptor, soluble guanylyl cyclase (sGC) (Ballou *et al.*, 2002; Mateo & De Artinano, 2000). Upon activation by NO, sGC facilitates the conversion of guanosine 5'-triphosphate (GTP) into the second messenger cyclic guanosine-3',5'-monophosphate (cGMP) (Perkins, 2006). The resultant accumulation of cGMP plays an integral role in the regulation of vascular function (Kemp-Harper & Schmidt, 2009).

1.3 Guanylyl cyclases (GC)

In the vasculature, cGMP can be generated via two main guanylyl cyclases: the cytosolic soluble guanylyl cyclase (sGC) and the membrane-bound particulate guanylyl cyclase (pGC) (Kemp-Harper & Schmidt, 2009). Guanylyl cyclases are expressed in virtually all cell types and have evolved to synthesise cGMP in response to various signals namely NO, carbon monoxide (CO) and peptide ligands (i.e. atrial (ANP), B-type (BNP) and C-type (CNP) natriuretic peptides) (Lucas *et al.*, 2000). Specifically, sGC responds to NO and CO, while pGC is activated by natriuretic peptides such as ANP, BNP and CNP (Lucas *et al.*, 2000).

1.3.1 Structure and function of soluble guanylyl cyclase (sGC)

As alluded to previously, sGC is a member of the guanylyl cyclase family which catalyses the enzymatic conversion of GTP to cGMP and the only conclusively proven receptor for NO (Denninger & Marletta, 1999; Lucas *et al.*, 2000). Originally isolated from bovine lung, sGC was initially thought to be expressed entirely in the cytosolic fraction of vascular smooth muscle cells, and while this is mostly the case, recent evidence also suggests that sGC can be associated with synaptosomal membranes of neurons and the plasma membranes of endothelial cells and platelets (Zhao *et al.*, 2004). sGC is a heterodimeric heme protein consisting of a large 80-kDa α and a smaller 70-kDa heme-containing β subunit. In mammals, there exists two isoforms of each subunit (α_1 and α_2 ; β_1 and β_2) with the heme group bound to the N-terminal of the β -subunit (Fernhoff *et al.*, 2009).

Theoretically, the association of α and β subunits could result in at least four different isoforms, however, only the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ forms have reported to be active (Nimmegeers *et al.*, 2007; Russwurm *et al.*, 1998). The predominant and most abundant isoform of sGC is the $\alpha_1\beta_1$, which is ubiquitously distributed in most mammalian tissues and is highly expressed in the brain, lung, kidney, spleen and muscle (Pyriochou & Papapetropoulos, 2005). Despite both the α and β subunits containing their own catalytic domains, co-expression or heterodimerisation of both subunits is essential for sGC activity (Pyriochou & Papapetropoulos, 2005).

Furthermore, the subunits of sGC can also be divided into four modular domains: an N-terminal domain, a central Per/Arnt/Sim (PAS)-like domain and putative amphipathic helix and a C-terminal catalytic domain as illustrated in Figure 3 (Derbyshire & Marletta, 2009). The heme group on the β_1 subunit is essential for conferring NO sensitivity to sGC and is bound to the subunit (Heme nitric oxide and oxygen (H-NOX) domain) via the axial ligand histidine₁₀₅ (His₁₀₅), and the heme binding motif tyrosine₁₃₉ (Tyr₁₃₉), serine₁₃₇ (Ser₁₃₇) and Arginine₁₃₉

1.3.1.1 Activation of sGC by NO

The activation of sGC by NO comprises a two-step process. Thus, studies employing purified sGC and spectroscopic methods have shown that NO initially binds to the reduced (Fe^{2+}) heme iron (Soret peak 431nm) of sGC to form a hexa-coordinate complex (Soret peak 420nm) (Ballou *et al.*, 2002; Poulos, 2006; Russwurm & Koesling, 2004). Subsequently, the bond between the Fe^{2+} heme and the axial histidine₁₀₅ is broken forming a penta-coordinate Fe^{2+} nitrosyl-heme complex, which is associated with a shift in the Soret peak to 399nm as illustrated in Figure 4. Opening of the histidine-iron bond is believed to cause a conformational change resulting in the activation of sGC. Additionally, CO is an endogenous, albeit weaker (~4-fold) activator of sGC compared to NO, and this has been attributed to the differential pivoting and bending in the H-NOX heme in response to these ligands (Ma *et al.*, 2007). Thus, the binding of CO to the heme of sGC results in a stable hexa-coordinated species, where histidine remains bound to the heme making CO a less efficient activator of sGC (Ma *et al.*, 2007).

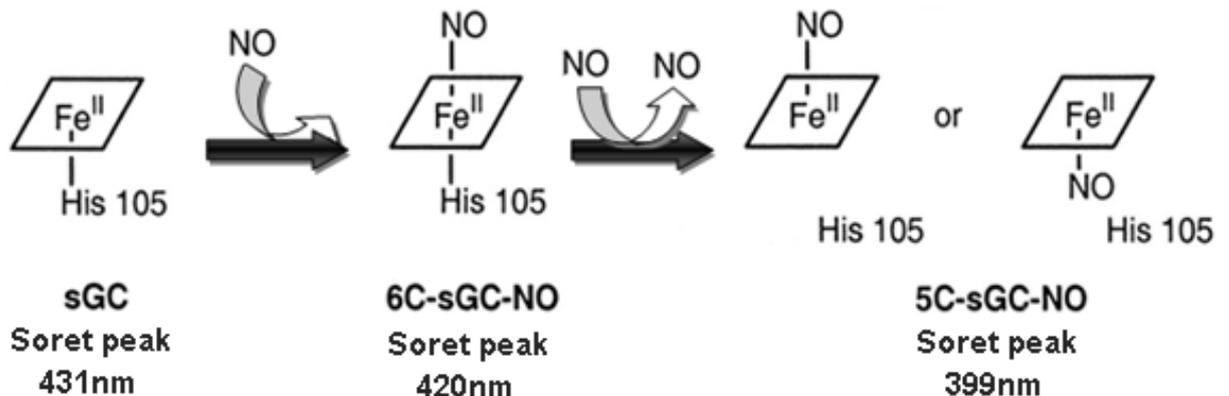


Figure 4. Simplified two-step model for activation of sGC (Adapted from Ballou *et al.*, 2002).

Indeed, NO binds to the heme of sGC with picomolar affinity (Fernhoff *et al.*, 2009; Zhao *et al.*, 1999), inducing a large increase in enzyme activity (~400-fold) which results in the subsequent accumulation of cGMP (Stone & Marletta, 1996). However, it has recently been reported that the

binding of NO to the heme group alone is not sufficient to fully activate the enzyme, rather excess NO binding to non-heme sites on sGC may be required for maximal activation (Cary *et al.*, 2005; Fernhoff *et al.*, 2009; Russwurm & Koesling, 2004). In consideration of this concept, a non-heme site model was recently proposed.

This non-heme site model involves two spectrally indistinguishable forms of penta-coordinate Fe^{2+} -NO complexes, one which exhibits low activity (~10-fold) and is formed at stoichiometric amounts of NO, whilst the other is fully activated (~200-fold) and formed in the presence of excess NO (Figure 5) (Derbyshire & Marletta, 2009; Fernhoff *et al.*, 2009; Poulos, 2006). Recently, Fernhoff and co-workers have proposed that this non-heme sGC activation by excess NO is mediated by cysteines at a thiol-containing site of sGC, leading to full activation of sGC via mechanisms which remain to be fully elucidated (Fernhoff *et al.*, 2009). Importantly, the heme co-factor is crucial for non-heme NO activation as a loss of this heme group is concomitant with a loss of NO activation, suggesting that the conformational changes induced by the initial NO and heme interaction may be essential for non-heme binding in the presence of excess NO (Derbyshire & Marletta, 2007).

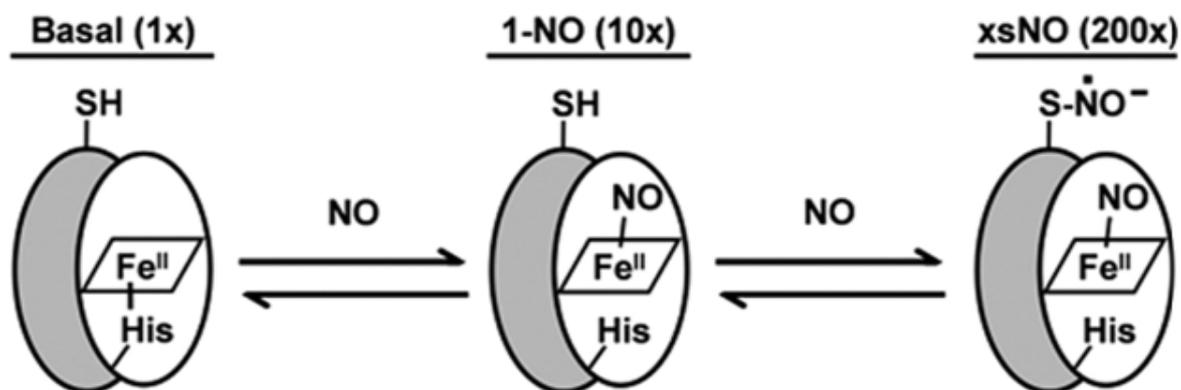


Figure 5. Graphical representation of Fernhoff's proposed non-heme site model depicting the role of cysteines in the mechanisms of sGC activation. Initially, NO binds to the heme of sGC, activating the enzyme by ~10-fold (1-NO, low activity). However, in the presence of excess NO (xsNO, high activity), NO binds to a thiol-containing site on sGC forming a thiol-NO adduct which activates the enzyme by ~200-fold above basal level (Adapted from Fernhoff *et al.*, 2009).

It should be noted, however, that other investigators have shown that S-nitrosylation of the cysteine residues, Cys₂₄₃ and Cys₁₂₂ on the α_1 and β_1 subunits of sGC, respectively, results in a reduction in NO-stimulated sGC activity (Sayed *et al.*, 2007) and may contribute to mechanisms of NO tolerance (Sayed *et al.*, 2007; Sayed *et al.*, 2008). Thus, taken together, the mechanisms involved in the activation of sGC by NO is far more complex than simple coordination to the heme of sGC as initially believed, and the molecular details regarding this aspect of sGC activation remain to be fully resolved (Derbyshire & Marletta, 2007).

1.3.2 Redox regulation of sGC

Under conditions of oxidative stress, the heme group of sGC may undergo oxidation, converting it from its reduced (Fe²⁺) to its oxidised/ferric (Fe³⁺) state, rendering it insensitive to NO stimulation (Evgenov *et al.*, 2006; Stasch *et al.*, 2006). Thus, removal of the heme or its oxidation by sGC inhibitors such as 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ),

ferricyanide (FeCN), 1H-(1,2,4)-oxdiazolo-(4,3-a)-6-bromo-quinoxazin-1-one (NS2028) or methylene blue promotes the formation of an NO-insensitive (Fe³⁺/heme-free) form of sGC (Dierks & Burstyn, 1998; Gupte *et al.*, 1999; Olesen *et al.*, 1998; Schrammel *et al.*, 1996; Zhao *et al.*, 2000). Indeed, spectroscopic studies have shown that heme oxidation by ODQ results in a shift of the Soret peak from 431nm to 392nm (Schmidt *et al.*, 2004). Importantly, peroxynitrite (ONOO⁻), the product of NO scavenging by ⁻O₂, has also been shown to be involved in the redox modification of sGC, oxidising the heme group such that there is a loss in NO-stimulated sGC activity (Stasch *et al.*, 2006, Figure 6). Furthermore, oxidation is thought to weaken the binding of the heme moiety by destabilising the bonds between the heme and sGC, thereby facilitating the loss of the heme group and making heme-free sGC susceptible to ubiquitin-dependent degradation (Hobbs, 2000; Meurer *et al.*, 2009; Schmidt *et al.*, 2004).

While it is well established that the redox state of the heme of sGC regulates the sensitivity of vascular tissue to NO, there may exist a number of endogenous regulators which serve to counteract the aforementioned oxidative influences, thereby preserving the heme of sGC in its reduced (Fe²⁺) state. Indeed, evidence exists for a cytosolic NADPH-dependent flavoprotein-containing methemoprotein reductase system with the ability to restore sGC sensitivity to NO following the oxidation of its heme moiety by heme oxidants, ODQ and ferricyanide (Gupte *et al.*, 1999). Furthermore, the presence of the flavoprotein inhibitor, diphenyliodonium (DPI) has been shown to attenuate NO stimulation of sGC activity, independent of cGMP-mediated vascular function. As DPI itself does not appear to act as a direct heme oxidant, these results suggest that a flavoprotein-dependent process is responsible for maintaining the heme of sGC in its reduced form (Iesaki *et al.*, 1999).

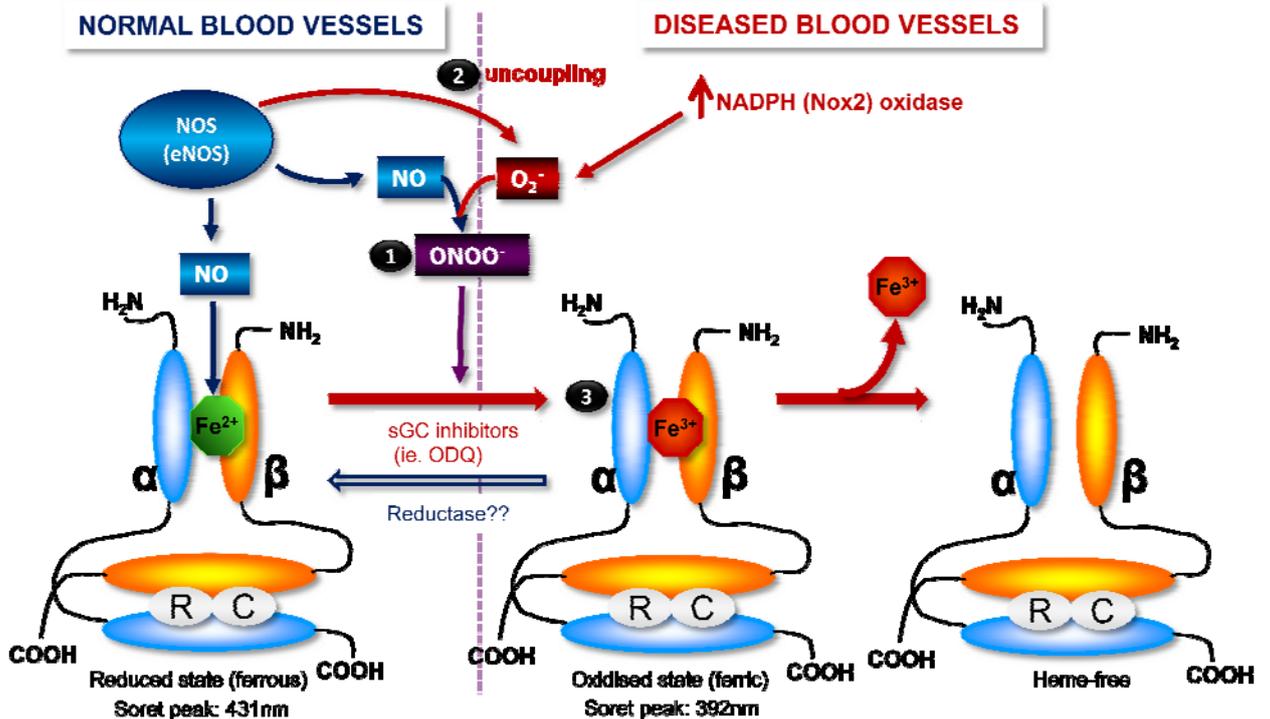


Figure 6. Schematic summarizing the proposed mechanism of oxidative modification of NO/sGC/cGMP signaling by ROS in the vasculature. A disease-associated increase in NADPH oxidase activity leads to the generation of O_2^- which scavenges NO to form ONOO⁻, thereby decreasing NO bioavailability. Subsequently, ONOO⁻ (1) can also cause eNOS uncoupling, exacerbating O_2^- formation by eNOS (2) and/or induce the oxidative modification of the heme group of sGC, rendering the enzyme insensitive to NO stimulation (3). The oxidised state of the heme is weakly bound and over time the heme is lost resulting in a heme-free form of sGC (Adapted from Hobbs, 2000).

1.4 Mechanisms of cGMP signaling

It is well established that in the cardiovascular system, cGMP signaling is important for the regulation of vascular tone and primary homeostasis. Following its generation from either the sGC or pGC pathway, the effects of cGMP in the vasculature are in turn mediated via a series of effectors namely the cGMP-dependent protein kinases (cGKs or PKG), cGMP-regulated phosphodiesterases (PDEs) and cGMP-gated cation channels (CNGs) (Kemp-Harper & Schmidt, 2009; Tsai & Kass, 2009).

PKG belongs to a family of serine/threonine kinases and exists in three different forms, PKGI α , PKGI β and PKGII, with PKGI representing the major form present in vascular smooth muscle cells (VSMCs), platelets and cardiomyocytes (Hofmann *et al.*, 2006). In the cardiovascular system, PKG induces vasorelaxation by phosphorylating a number of biologically important target proteins, subsequently lowering levels of intracellular Ca²⁺ and thereby, counteracting the contractile mechanisms (Hofmann *et al.*, 2006). Such targets include Ca²⁺-activated potassium channels (BK_{Ca}), myosin light chain phosphatase (MLCP), Rho kinase, the sarcoplasmic reticulum (SR) protein, phospholamban and G-protein signaling proteins (Lincoln *et al.*, 2011). In addition, PKG also catalyses the phosphorylation of the vasodilator-stimulated phosphoprotein (VASP), regulating the binding of VASP to actin filaments. While the role of VASP in smooth muscle contraction is yet to be defined, VASP may act as an *in vitro* substrate and marker for PKG activity (Butt *et al.*, 1994; Lincoln *et al.*, 2011).

PDEs, of which there are 11 families/isoforms, are responsible for the degradation of cGMP and/or cyclic adenosine 3',5'-monophosphate (cAMP). In the vasculature, PDE5 is believed to be the primary isoform involved in cGMP degradation (Baliga *et al.*, 2008). Indeed, PKG can also phosphorylate PDE5 to increase its activity, thereby serving as a form of autoregulation (Rybalkin *et al.*, 2002). Thus, PKGs and PDEs are the predominant effectors of cGMP in the vasculature and whilst CNGs have been identified in endothelial cells (Wu *et al.*, 2000; Yao *et al.*, 1999), their role remains to be elucidated.

1.5 Therapeutic utility of the NO/sGC/cGMP signaling system

In the vasculature, the sGC/cGMP signaling pathway mediates the majority of the vasoprotective actions of NO including vasodilation, inhibition of platelet aggregation as well as VSMC proliferation and migration (Feletou & Vanhoutte, 2006b; Russwurm & Koesling, 2004). With such an array of vasoprotective actions, there can be no doubt as to the clear therapeutic potential

of NO. Indeed, the therapeutic importance of the NO/sGC/cGMP signaling pathway has been well recognised for over a century, with NO donors such as glyceryl trinitrate (GTN) and sodium nitroprusside (SNP) being used for the treatment of cardiovascular disorders such as angina and acute hypertensive crises (Murad, 2003a; Padden, 1999). More recently, inhaled NO (iNO) has been used successfully in the treatment of pulmonary hypertension in newborns/infants (Clark *et al.*, 2000; Davidson *et al.*, 1998) and adults (Krasuski *et al.*, 2000). However, such NO-based therapies have several limitations. Thus, the effects of iNO are short-lived, necessitating continuous therapy (Bloch *et al.*, 2007). In addition, organic nitrates such as GTN are susceptible to the development of tolerance, in which an attenuation or loss of hemodynamic, anti-ischemic and vasodilatory effects during continuous nitrate medication have been reported (Bertel, 1988; Warnholtz *et al.*, 2002).

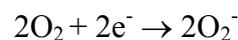
Nitrate tolerance is indeed a complex phenomenon with the mechanisms underlying such an event likely to be multifactorial and may involve: (i) neurohormonal counter-regulatory mechanisms, otherwise referred to as pseudotolerance (Munzel *et al.*, 2005), (ii) increase in the activity of the cGMP degrading phosphodiesterase 1A1 (Kim *et al.*, 2001), (iii) desensitisation of sGC (Artz *et al.*, 2002), (iv) elevated production of ROS (Munzel *et al.*, 2005; Munzel *et al.*, 1995) and/or (v) a decrease in mitochondrial aldehyde dehydrogenase (ALDH-2) activity which catalyses the biotransformation of GTN (Sydow *et al.*, 2004). Furthermore, another phenomenon induced by prolonged nitrovasodilator therapy is the development of cross-tolerance to other nitrovasodilators and to endothelium-dependent vasodilators such as acetylcholine (ACh) (Munzel *et al.*, 2000; Munzel *et al.*, 1995).

Moreover, a disease associated increase in ROS compromises the NO/sGC/cGMP signaling pathway and further limits the clinical applications of NO donors. Indeed, in disease states such as hypertension and atherosclerosis, impaired endothelium-dependent relaxation and a decrease

in NO bioavailability is observed (Ruetten *et al.*, 1999; Selemidis *et al.*, 2008). In addition, a loss of efficacy of NO donors has been reported, due to increased scavenging by ROS such as $\cdot\text{O}_2^-$, an increase in PDE activity (Mullershausen *et al.*, 2003), and/or dysfunction at the level of sGC itself (Kloß *et al.*, 2000; Melichar *et al.*, 2004; Ruetten *et al.*, 1999; Stasch *et al.*, 2006).

1.6 Modulation of NO/cGMP signaling by reactive oxygen species (ROS)

The term ROS refers to a family of oxygen-derived small molecules which incorporates oxygen radicals such as superoxide anion ($\cdot\text{O}_2^-$) and hydroxyl ($\text{OH}\cdot$) radicals in addition to certain non-radicals that are either oxidising agents or are able to be easily converted to radicals such as hypochlorous acid (HOCl) and hydrogen peroxide (H_2O_2) (Bedard & Krause, 2007). ROS production is generally a cascade of reactions which begins with the one electron reduction of molecular oxygen to generate $\cdot\text{O}_2^-$ (Bedard & Krause, 2007; Selemidis *et al.*, 2008):



The parent species, $\cdot\text{O}_2^-$ can then rapidly dismutate to H_2O_2 either spontaneously or via a catalytic reaction involving superoxide dismutases (SODs), or generate the powerful oxidant, peroxynitrite (ONOO^-) upon reacting with NO (Bedard & Krause, 2007), thereby serving as a key source for numerous other downstream ROS. Indeed, vascular homeostasis is achieved when there is a balance between the generation of ROS and NO, where NO aids in protecting against ROS-induced damage to macromolecules, and ROS in turn limits the effects of NO (Wink *et al.*, 2003). While low levels of ROS play an important role in the regulation of cellular signaling and function (Kamata & Hirata, 1999), they are tightly regulated by endogenous antioxidant defences. However, in disease states the excess generation of ROS overwhelms such defences (Pacher *et al.*, 2007). This imbalance between oxidants and antioxidants, where the generation of the former is favoured, is traditionally termed ‘oxidative stress’. Indeed, increased oxidative stress as a result of a chronic elevation in ROS, is a prominent feature of several vascular disease

states including atherosclerosis, diabetes and hypertension (Paravicini & Touyz, 2006; Selemidis *et al.*, 2008; Thomas *et al.*, 2008).

1.6.1 Nature and sources of ROS

There are a multitude of enzymes and mechanisms involved in the production of ROS, the most-well documented sources being NADPH oxidase, mitochondria, xanthine oxidase, cyclooxygenase, lipoxygenase, peroxidases and uncoupled NOS (Griendling *et al.*, 2000a; Ogita & Liao, 2004). Although several enzyme systems are capable of generating $\cdot\text{O}_2^-$, for the majority, $\cdot\text{O}_2^-$ is a by-product of another reaction which represents the main function of the enzyme. On the other hand, the sole function of NADPH oxidases appears to be the generation of ROS. Thus, the excessive generation of $\cdot\text{O}_2^-$ by NADPH oxidases is thought to play a key role in the development of oxidative stress in cardiovascular disease (Drummond *et al.*, 2011; Griendling *et al.*, 2000a; Selemidis *et al.*, 2008).

1.6.1.1 NADPH oxidase

NADPH oxidase was first described in phagocytes (i.e. neutrophils, eosinophils and macrophages) of the innate immune system, where it is responsible for a large burst of $\cdot\text{O}_2^-$ during the process of phagocytosis (Cave *et al.*, 2006; Lambeth *et al.*, 2007; Vignais, 2002). NADPH oxidases are a family of multi-component enzyme complexes with each isoform comprised of a membrane-bound cytochrome *b558* reductase domain, up to three cytoplasmic subunits (p47^{phox}/NoxO1 and p67^{phox}/NoxA1 and p40^{phox}), as well as a small G-protein (Rac1/Rac2) (Drummond *et al.*, 2011; Selemidis *et al.*, 2008; Thomas *et al.*, 2008). The cytochrome *b558* domain is made up of the catalytic subunit Nox (β -subunit) and the small integral membrane protein, p22^{phox} (α -subunit), which appears to act by stabilising the Nox protein with various cellular membranes (Opitz *et al.*, 2007; Selemidis *et al.*, 2008; Thomas *et al.*, 2008). The Nox family consists of seven catalytic homologues, four of which (Nox1, Nox2,

Nox4 and Nox5, Figure 7) have been identified in the vasculature, as well as under different states of physiology and pathophysiology (Drummond *et al.*, 2011; Lassegue & Griendling, 2010; Thomas *et al.*, 2008).

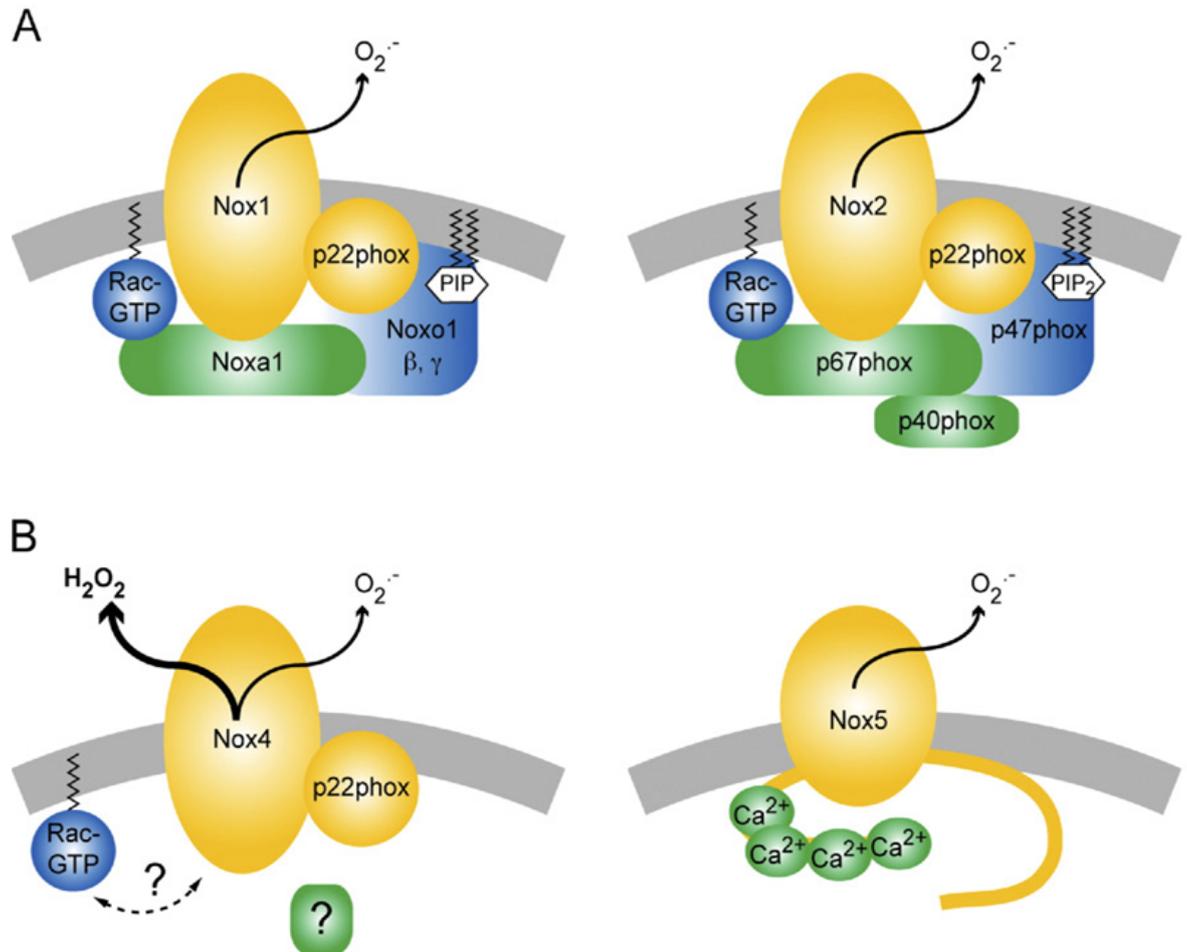
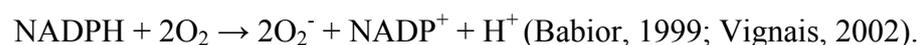


Figure 7. Models of the proposed complexes for Nox1, Nox2, Nox4 and Nox5. Nox1 consists of the Nox organiser 1 (NoxO1) and the Nox activator 1 (NoxA1) protein, while the activity of Nox2 (gp91^{phox}) depends on p47^{phox}, p67^{phox} and p40^{phox} with both isoforms also requiring the association of p22^{phox} and GTPase Rac. Conversely, Nox4 is not regulated by any of the known regulatory subunits and appears to be capable of inducing full activation in the presence of p22^{phox} alone. Furthermore, Nox4 is believed to mainly release H₂O₂. Finally Nox5, the evolutionary most ancient isoform, is thought to be activated by Ca²⁺ binding to its EF-hand motifs (Adapted from Opitz *et al.*, 2007).

In contrast to Nox4, which only requires the association of p22^{phox} to achieve full activation, both Nox1 and Nox2 require the additional translocation of cytosolic ‘organiser’ proteins p47^{phox} (and/or NoxO1 for Nox1) and ‘activator’ proteins p67^{phox} (and/or NoxA1 for Nox1) to the Nox catalytic subunit, a process dependent upon the phosphorylation of p47^{phox} by protein kinase C (PKC) (Opitz *et al.*, 2007; Selemidis *et al.*, 2008; Thomas *et al.*, 2008). Indeed, activation of NADPH oxidases (Nox2-containing) generally begins with the translocation of a ternary complex which constitutes p47^{phox}, p67^{phox} and p40^{phox}, of which p47^{phox} is responsible for chaperoning the entire complex to the Nox2 catalytic subunit, which acts as a docking site (Selemidis *et al.*, 2008). Phosphorylation of serine groups on p47^{phox} leads to a conformational change exposing its internal SH3 domains, which facilitates the binding of p47^{phox} to the proline-rich region (PRR) on p22^{phox} (Figure 8) (Cave *et al.*, 2006; Vignais, 2002). Previous studies have also shown that p47^{phox} is able to bind to several sites on Nox2, an interaction which is important for the assembly of the oxidase (DeLeo *et al.*, 1995). While the third cytosolic subunit, p40^{phox} may also be associated with Nox2-containing NADPH oxidases, its precise role in the enzyme complex is yet to be elucidated (Matute *et al.*, 2005; Thomas *et al.*, 2008). Finally, Rac is recruited to the membrane independently of the cytosolic ternary complex where it binds to the N-terminal region of p67^{phox} (Opitz *et al.*, 2007; Selemidis *et al.*, 2008). The fully assembled NADPH oxidase complex then causes a transient burst of $\cdot\text{O}_2^-$ by transferring electrons from its substrate NADPH to molecular oxygen via its Nox subunit (Griendling *et al.*, 2000b; Lassegue & Clempus, 2003), according to the following equation:



Finally, while the Nox5 isoform builds on the basic structure of Nox2, it possesses an additional calmodulin-like EF-hand domain which is comprised of four Ca^{2+} binding sites (Figure 7), and therefore its activation is elicited by elevating cytosolic Ca^{2+} (Cave *et al.*, 2006). Thus, from these isoforms, only the Nox1 and Nox2 isoforms appear to exhibit some similarities in their mechanisms of activation.

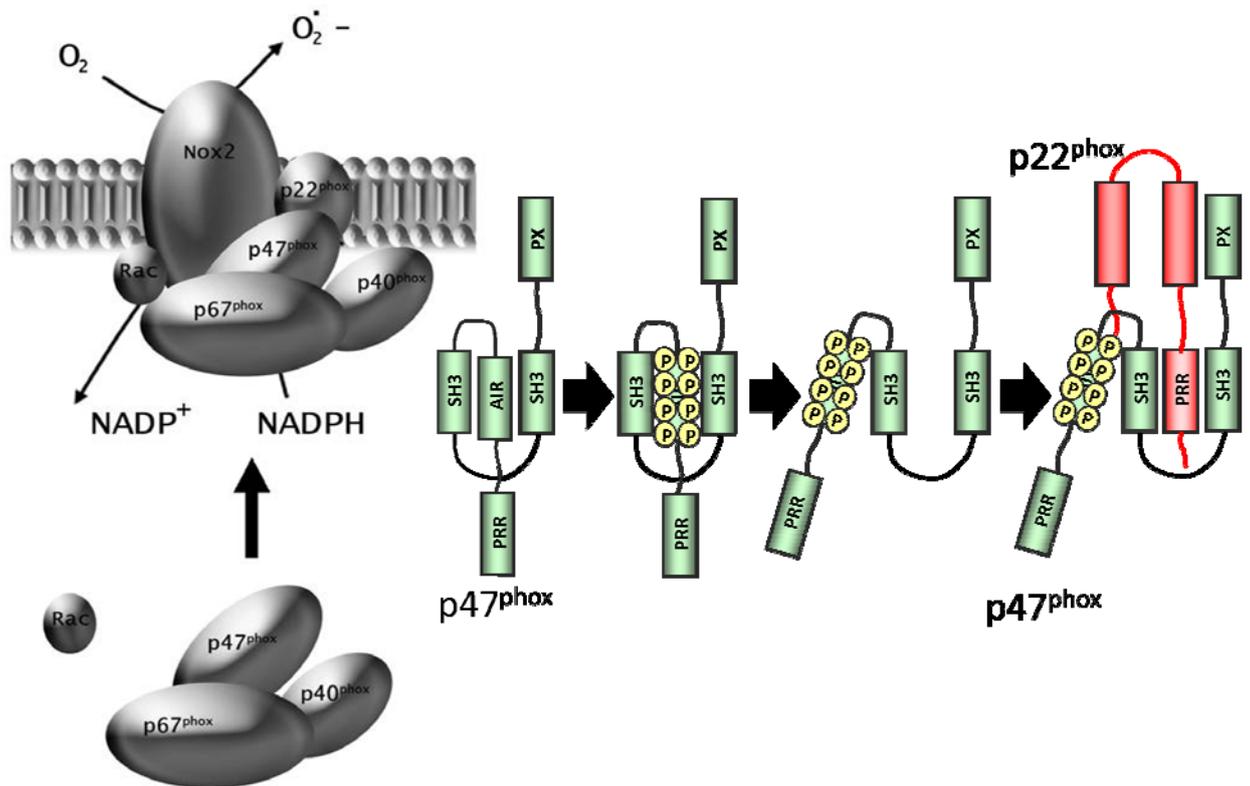


Figure 8. Schematic diagram of the structure of classical Nox2-containing NADPH oxidase in its activated state (left) and the phosphorylation of p47^{phox} and its subsequent binding to the PRR of p22^{phox} (right). Oxidase activation involves the translocation of the cytosolic subunits p47^{phox}, p67^{phox} and p40^{phox} as well as the GTP-bound Rac to the Nox2-containing membrane bound cytochrome b₅₅₈ and p22^{phox} (left panel). Separately, the phosphorylation of serine residues on p47^{phox} results in a conformational change, allowing the SH3 domains of p47^{phox} to bind to the PRR of p22^{phox} which is crucial for oxidase assembly (right panel). AIR represents the autoinhibitory region of p47^{phox}, where phosphorylation initially occurs (Adapted from Cave *et al.*, 2006; schematic on the right courtesy of Janahan Dharmarajah and Dr Grant Drummond, Department of Pharmacology, Monash University).

Originally identified in the kidneys, Nox4 was also shown to be expressed in various other tissues including endothelial cells, VSMCs and fibroblasts. While Nox4 is expressed at high levels under physiological conditions, and does not require the translocation of additional cytoplasmic subunits for activation, both Nox1 (VSMCs) and Nox2 (endothelial cells, fibroblasts and inflammatory cells) are expressed at much lower levels in the vasculature. However, their expression and activity appear to be up-regulated in cardiovascular disease states (Selemidis *et al.*, 2008; Touyz & Briones, 2011). Furthermore, both Nox4 and Nox2 expression are perinuclear, localised to the nuclear as well as the endoplasmic reticulum membranes of endothelial cells, where Nox4 is thought to play a homeostatic role while Nox2 appears to be involved in endothelial cell proliferation and angiogenesis (Lassegue & Griendling, 2010; Thomas *et al.*, 2008). Currently, no studies have yet characterised the subcellular distribution of Nox1 (Bedard & Krause, 2007). To date, Nox5 has only been identified in humans and in the absence of an appropriate animal model, experiments are limited to cultured cells and isolated tissue (Lassegue & Griendling, 2010).

Nox2 (gp91^{phox}) was the first isoform to be identified and subsequently is the best understood and when fully assembled, the amounts of $\cdot\text{O}_2^-$ generated from this Nox2 complex far exceeds that of any other known NADPH oxidases (Babior, 1999; Opitz *et al.*, 2007). Indeed, Nox2-containing NADPH oxidases have been implicated as having a causal role in various pathophysiological conditions such as atherosclerosis (Miller *et al.*, 2010) and hypertension (Cai *et al.*, 2003), whilst other studies have also suggested that endothelial Nox2 plays a specific and critical role in vascular oxidative stress as well as mediating the hemodynamic response to angiotensin II (Ang II) (Bendall *et al.*, 2007). One of the major stimuli of Nox2-containing NADPH oxidase is Ang II, the primary effector of the renin-angiotensin system (RAS) system which has been implicated in the pathogenesis of many cardiovascular diseases (Armitage *et al.*, 2009; Griendling *et al.*, 2000b).

1.6.1.2 Nox2-containing NADPH oxidases and Angiotensin II (Ang II)

As mentioned previously, Ang II is a major effector of the RAS, acting primarily via two different receptors: Ang II type 1 (AT₁) and Ang II type 2 (AT₂) receptors (Lemarie & Schiffrin, 2009). The AT₁ receptor is responsible for the majority of the well known effects of Ang II including vasoconstriction, cell growth, vascular and cardiac hypertrophy as well as the generation of oxidative stress and inflammation (Lemarie & Schiffrin, 2009). Moreover, stimulation of the AT₁ receptor by Ang II leads to PKC-dependent phosphorylation of serine residues on p47^{phox} and the subsequent activation of NADPH oxidase and production of $\cdot\text{O}_2^-$ (Hoyal *et al.*, 2003; Lambeth, 2004).

A study by Mollnau and co-workers reported that a chronic 7 day infusion of Ang II (1 mg/kg/day) markedly increased the expression of Nox2 (~3-fold), p22^{phox} (~3-fold) and Nox1 (~7-fold) in the vasculature, actions which were attributed, at least in part, to a PKC-dependent mechanism (Mollnau *et al.*, 2002). Thus, Nox1 is also thought to be involved in Ang II signaling and is potentially critical in vascular diseases (Lassegue *et al.*, 2001). Furthermore, the Nox2 subunit has been demonstrated to be essential for Ang II-induced vascular hypertrophy and oxidative stress (Rey *et al.*, 2001; Wang *et al.*, 2001). Moreover, the central role of Nox2-containing NADPH oxidase in Ang II signaling is highlighted by studies utilising mice lacking either the Nox2 or p47^{phox} subunit (Griendling, 2004).

Indeed, 24 hour Ang II-induced $\cdot\text{O}_2^-$ production was reversed to levels comparable with control in the carotid arteries of Nox2^{-/-} mice, further highlighting the vital role Nox2 plays in Ang II-mediated $\cdot\text{O}_2^-$ generation (Scrader *et al.*, 2007). As highlighted previously, p47^{phox} binds at multiple sites with Nox2, an interaction which is essential to the assembly and subsequent function of this oxidase. Therefore, it has been suggested that by interfering with the interaction between Nox2 and p47^{phox}, one is able to inhibit the activity of NADPH oxidase. In fact, *in vivo*

administration of Ang II (0.7mg/kg/day for 7 days) increased systolic blood pressure by approximately 40 mmHg and elevated vascular $\cdot\text{O}_2^-$ production 2- to 3-fold in wildtype mice, effects which were reduced and blunted respectively in $\text{p47}^{\text{phox-/-}}$ mice (Landmesser *et al.*, 2002). In addition, the ability of Ang II to elevate ROS production is severely compromised in endothelial cells (Landmesser *et al.*, 2002) and VSMCs (Lavigne *et al.*, 2001) derived from p47^{phox} deficient mice, the crucial subunit for the activation of Nox2. As such, much of the vascular dysfunction associated with Ang II generation (i.e. impairment of endogenous NO, increase contractility) in disease may arise as a consequence of its ability to increase NADPH oxidase-derived $\cdot\text{O}_2^-$ and subsequently attenuate NO/sGC signaling (Chrissobolis *et al.*, 2008; Didion *et al.*, 2005; Mollnau *et al.*, 2002).

1.6.2 Mechanisms underlying ROS-mediated impairment in NO/sGC/cGMP signaling

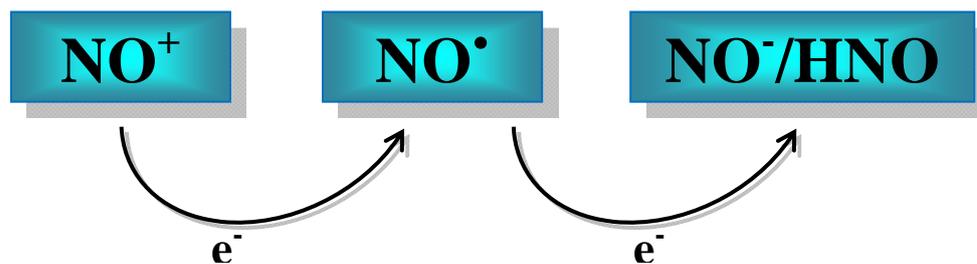
ROS can compromise the NO/sGC/cGMP signaling cascade at a number of levels causing a decrease in NO bioavailability and dysfunction at the level of sGC itself. First, a decrease in the bioavailability of endogenously-derived NO through its scavenging by excess $\cdot\text{O}_2^-$ results in the subsequent formation of the powerful oxidant, ONOO⁻ (Didion *et al.*, 2005; Munzel *et al.*, 2003; Pacher *et al.*, 2007). ONOO⁻ is essentially generated when there is an overproduction of ROS, in particular $\cdot\text{O}_2^-$, which is at a level too high to be metabolised by superoxide dismutase (SOD). The reaction rate between NO and $\cdot\text{O}_2^-$ occurs at a near-diffusion rate of $6.7 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$, where almost every collision results in the irreversible formation of ONOO⁻ (Beckman & Koppenol, 1996; Mateo & De Artinano, 2000). Indeed, elevated levels of ONOO⁻ have been implicated in several clinical conditions such as hypercholesterolemia, diabetes and coronary artery disease (Greenacre & Ischiropoulos, 2001). Second, ONOO⁻ has been reported to instigate the uncoupling of eNOS via the oxidation of its crucial co-factor, BH₄ into its ineffective form, dihydrobiopterin. This leads to eNOS uncoupling, a reduction in NO production and further generation of $\cdot\text{O}_2^-$ (Kuzkaya *et al.*, 2003; Laursen *et al.*, 2001). Furthermore, ROS such as

ONOO⁻ can also impair the function of sGC itself by oxidising the heme group and rendering it insensitive to NO (Evgenov *et al.*, 2006).

Thus, there is clearly interplay between ROS generation and NO/sCG/cGMP signaling. This confers limitations on the use of traditional NO donors, highlighting the need for NO mimetics which are resistant to the detrimental effects of ROS and can potentially target the NO-insensitive, oxidised form of sGC. Excitingly, nitroxyl donors and NO-independent sGC stimulators and activators represent novel drug classes which have the potential to overcome the limitations posed by conventional NO donors and exhibit preserved and/or enhanced efficacy under disease conditions associated with oxidative stress.

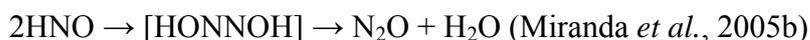
1.7 Alternate redox forms of NO: potential therapy in pathophysiology

It is a traditional assumption that 'NO' accounts for all the biological activity of nitrogen monoxide when in fact this molecule can exist in three different redox states; the uncharged free radical (NO[•]), the reduced form known as nitroxyl (HNO) and finally the oxidized form known as the nitrosonium cation (NO⁺) as outlined in the diagram below. In order to fully account for the biological activity of NO, all three species must be considered as interconversion between the species can occur under cellular conditions (Hughes, 1999). It should however be noted that as NO⁺ is only found in aqueous solutions of very high acidity (Hughes, 1999), its role under physiological conditions seems improbable. In contrast, the one-electron reduced and protonated congener, HNO may serve as an endogenous signaling molecule as it can exist at neutral pH (Hughes, 1999) and a number of biochemical pathways for its synthesis have been identified (Irvine *et al.*, 2008). Moreover, HNO exhibits quite distinct pharmacology compared to NO[•] (Irvine *et al.*, 2008; Paolocci *et al.*, 2007), and thus may confer therapeutic advantages.



1.8 Nitroxyl (HNO)

At physiological pH, the nitroxyl anion (NO^-) exists in its protonated form as HNO (pKa 11.6) and in such a state is able to readily cross cell membranes (Bartberger *et al.*, 2001; Favalaro & Kemp-Harper, 2007). In physiological buffers, HNO undergoes rapid dimerisation and dehydration to nitrous oxide (N_2O) and water in an irreversible process according to the equation:



Consequently, this aspect of HNO chemistry results in an inability to store HNO, and thus necessitates the use of HNO donor compounds (Miranda *et al.*, 2005b).

To date, the endogenous generation of HNO has yet to be conclusively proven due to the lack of direct detection methods, although numerous biochemical pathways have been proposed for its generation under physiological conditions (Fukuto *et al.*, 2005a). One such pathway is depicted in Figure 9, where nitroxyl, rather than NO^\bullet , is formed from either the intermediate N^ω -hydroxy-L-arginine due to an incomplete oxidation of L-arginine by the NOS enzyme (Donzelli *et al.*, 2006; Fukuto *et al.*, 1992; Pufahl *et al.*, 1995) or produced directly from L-arginine itself via the NOS enzyme under conditions of oxidative stress when an absence or depletion of the NOS co-factor, BH_4 occurs (Adak *et al.*, 2000; Hobbs *et al.*, 1994; Irvine *et al.*, 2008; Rusche *et al.*, 1998).

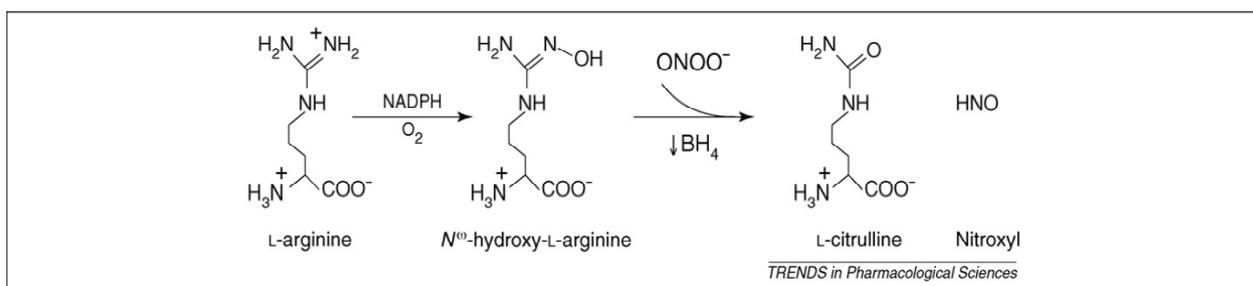


Figure 9. Biosynthetic pathway of HNO. HNO can be generated from N^o-hydroxy-L-arginine, the nitric oxide synthase (NOS) intermediate, following its oxidation by peroxynitrite (ONOO⁻) and in the absence of the co-factor tetrahydrobiopterin (BH₄) (Irvine *et al.*, 2008).

A multitude of other biosynthetic pathways for endogenous HNO generation have also been postulated. For example Mn and Fe-type SODs (Niketic *et al.*, 1999), mitochondrial cytochrome c (Sharpe & Cooper, 1998), xanthine oxidase (Saleem & Ohshima, 2004), ubiquinol (Poderoso *et al.*, 1999), and haemoglobin (Gow & Stamler, 1998) have been reported to yield HNO via the reduction of NO[•]. The decomposition of ONOO⁻ has also been shown to result in HNO formation and an electronically excited singlet molecule of O₂ (Khan *et al.*, 2000). Additionally, HNO has been reported to be formed via the decomposition of S-nitrosothiols (RSNOH) by low molecular weight thiols such as GSH and thiol proteins, under both physiological and nitrosative stress conditions (Arnelle & Stamler, 1995; Donzelli *et al.*, 2006; Wong *et al.*, 1998). Furthermore, pharmacological studies have provided evidence to suggest that HNO may serve, at least in part, as an EDRF in the vasculature (Andrews *et al.*, 2009; Ellis *et al.*, 2000) as well as a nitrenergic transmitter in rat anococcygeus muscle (Li *et al.*, 1999).

1.8.1 HNO signaling

HNO can serve as a nucleophile or electrophile, and therefore readily interacts with thiols and metalloproteins (Irvine *et al.*, 2008). Thus, HNO can reduce metals such as iron (Fe) (Miranda *et al.*, 2003b), copper (Cu) (Nelli *et al.*, 2000) and manganese (Mn) (Miranda *et al.*, 2003a).

Specifically, HNO reacts with a ferric (Fe^{3+}) heme to generate a stable ferrous (Fe^{2+})-nitrosyl complex via a process referred to as reductive nitrosylation (Eq. 1) (Mancardi *et al.*, 2004):



In contrast, NO^{\bullet} preferentially interacts with Fe^{2+} heme to form the same stable Fe^{2+} -nitrosyl complex (Eq. 2).



One of the main heme-containing proteins HNO targets is sGC (Kemp-Harper, 2011). Thus, HNO elicits vasorelaxation and inhibits platelet aggregation predominantly via the sGC/cGMP pathway (Bullen *et al.*, 2011). However, it remains controversial as to whether HNO itself can directly activate sGC or if it first requires oxidation to NO^{\bullet} , or if HNO is able to target the oxidised (Fe^{3+}) state of sGC. Initial biochemical studies indicated that NO^{\bullet} was the only nitrogen oxide that could stimulate sGC (Dierks & Burstyn, 1996). These studies, however, were performed in the presence of high concentrations of thiols which may scavenge HNO.

This concept was recently re-examined by Miller and co-workers, who demonstrated an ability of two HNO donor compounds, Angeli's salt and 1-Nitrosocyclohexyl trifluoroacetate (NCTFA), to stimulate purified bovine sGC in the absence of extracellular thiols and independently of NO^{\bullet} generation (Miller *et al.*, 2009). In contrast, Zeller and co-workers found that Angeli's salt had no significant effect on sGC in the absence of superoxide dismutase (SOD), which is known to convert HNO to NO^{\bullet} (Zeller *et al.*, 2009). Indeed, there are a multitude of biological oxidants which can facilitate this conversion (Paolocci *et al.*, 2007) such as (i) flavins (Fukuto *et al.*, 1993), (ii) cytochrome P450 (Nelli *et al.*, 2001), (iii) copper containing enzymes (Nelli *et al.*, 2000) and (iv) quinones and ferricytochrome c (Buyukafsar *et al.*, 2001). However, it still remains unclear if HNO itself can stimulate sGC. Moreover, given

the preference of HNO for Fe³⁺ over Fe²⁺ heme groups, it may have been predicted that HNO would target the oxidised/heme-free form of sGC. Recent studies, however, have shown that HNO activates the Fe²⁺ rather than the Fe³⁺ form of sGC (Miller *et al.*, 2009). Whether this holds true in the intact cell remains to be determined.

1.8.2 Discriminating between NO and HNO, and HNO detection

Accumulating evidence has attributed a substantial component of the biological activity of HNO to its highly thiophilic nature (Bartberger *et al.*, 2001). The initial reaction of thiols with HNO results in the product, N-hydroxysulfenamide, which in turn has two possible fates. Thus, N-hydroxysulfenamide can i) further react with another thiol to generate the corresponding disulfide and hydroxylamine (NH₂OH), via a biologically reversible process (Figure 10, pathway a) or ii) undergo rearrangement to produce sulfinamide via an irreversible process (Figure 10, pathway b) (Fukuto *et al.*, 2009; Fukuto *et al.*, 2005b). Whilst both products have been observed in biological systems, the chemical factors involved in promoting one pathway over the other has not been thoroughly examined (Fukuto *et al.*, 2009; Shen & English, 2005).

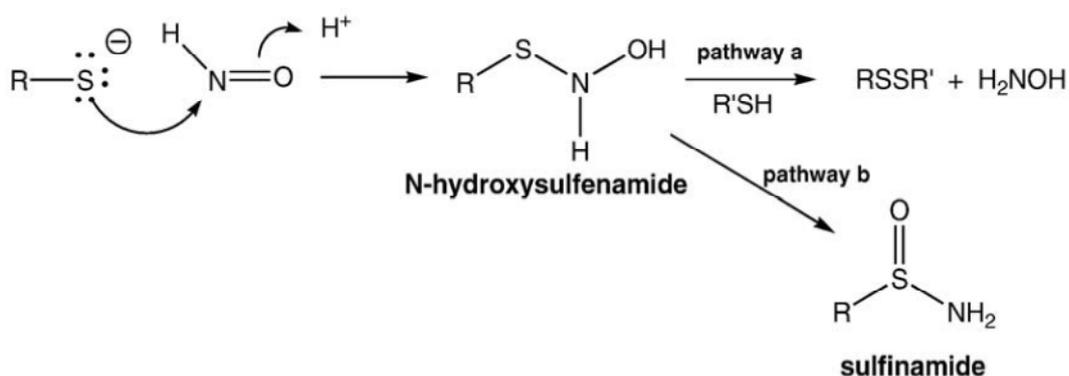


Figure 10. Possible pathways for the reaction between HNO and thiols (Fukuto *et al.*, 2009).

Furthermore, several studies have identified HNO as a potent inhibitor of thiol-containing enzymes such as aldehyde dehydrogenase (ALDH) (DeMaster *et al.*, 1998; Nagasawa *et al.*,

1990), the yeast transcription factor Ace 1 (Cook *et al.*, 2003) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Lopez *et al.*, 2005), to name a few. Despite targeting GAPDH, cellular glutathione (GSH) levels were unchanged in the presence of HNO indicating the capacity for HNO to selectively modulate certain thiols without affecting the levels of others (Lopez *et al.*, 2005). Furthermore, HNO modifies thiol groups on receptors and ion channels, including the regulation of skeletal (RyR1) and cardiac (RyR2) ryanodine receptors, sarcoplasmic reticulum (SR) Ca²⁺ ATPase (SERCA) as well as contractile proteins to modulate cardiac function (Irvine *et al.*, 2008; Tochetti *et al.*, 2007). Similarly, HNO may target critical thiol groups on Ca²⁺ channels to modulate their function (Switzer *et al.*, 2009). Indeed, HNO's highly thiophilic nature suggests that other enzymes such as caspases, fatty acid acyl transferase and ubiquitin ligases, which incorporate thiols in their respective catalytic processes, may also pose as potential targets for HNO activity (Switzer *et al.*, 2009).

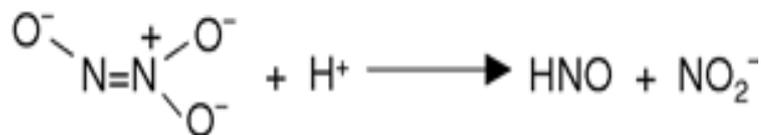
It is this thiol reactivity of HNO that confers many of the distinct pharmacological actions of this redox form of NO compared to NO[•], such as its ability to increase myocardial contractility, as will be discussed in later sections. Thus, while HNO is able to interact directly with thiols leading to their oxidation, NO[•] must firstly undergo conversion to a reactive nitrogen oxide species (RNOS) like dinitrogen trioxide (N₂O₃) (Irvine *et al.*, 2008; Wink *et al.*, 1994). Moreover, this reactivity of HNO with thiols is exploited to distinguish the actions of HNO versus NO[•]. Indeed, high concentrations of thiols such as L-cysteine, N-acetyl-L-cysteine (NAC) and dithiothreitol (DTT) will attenuate the actions of HNO, but not those of NO[•] both *in vitro* and *in vivo* (Dai *et al.*, 2007; Irvine *et al.*, 2007; Irvine *et al.*, 2003; Irvine *et al.*, 2008; Paolocci *et al.*, 2001; Tochetti *et al.*, 2007). Conversely, NO[•] scavengers such as 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxy-3-oxide (carboxy-PTIO) and hydroxocobalamin reduce the response to NO[•] but not HNO (Ellis *et al.*, 2001; Irvine *et al.*, 2007; Irvine *et al.*, 2003; Irvine

et al., 2008; Wanstall *et al.*, 2001). Due to the lack of direct detection methods for HNO, thiols represent the main strategy to distinguish between these two redox forms of NO.

Additionally, the measurement of the dimerisation product of HNO, N₂O is often used as an indirect marker for HNO production (Fukuto *et al.*, 1992). However, the measurement of modified thiols is expected to be a more convenient and accurate method of HNO detection compared to the detection of N₂O (Donzelli *et al.*, 2006). Thus, it has been proposed that the detection of sulfinamide ([GS(O)NH₂]), the unique product of the reaction between HNO and thiols, may provide a new alternative method in examining the intriguing pharmacology, chemistry and biology of HNO (Donzelli *et al.*, 2006). Similarly in the vasculature at least, K_v channel blockade using the inhibitor, 4-aminopyridine (4-AP) attenuates HNO-mediated vasorelaxation yet has no effect on the response to the established NO donor, DEA/NO and therefore represents an additional tool which is able to distinguish between the effects of HNO versus NO^{*} (Andrews *et al.*, 2009). Excitingly, the Tripodal copper (II) BODIPY complex (Cu^{II}[BOT1]), a membrane permeable fluorescent molecular probe, has been recently identified for the selective detection of HNO in living cells (Rosenthal & Lippard, 2010). Indeed, such a detection technique will be important in providing conclusive evidence for the endogenous generation of HNO.

1.8.3 HNO donors

Due to the rapid dimerisation of HNO, donors are required to study the biological activity of this nitrogen oxide. Angeli's salt has been the mainstay donor of the field, and both this donor and isopropylamine NONOate (IPA/NO) are briefly reviewed.

1.8.3.1 Angeli's salt (AS, $\text{Na}_2\text{N}_2\text{O}_3$, sodium trioxodinitrate)

Angeli's salt is a member of a class of NO donors called diazeniumdiolate or NONOates, however, in contrast to the other members of its class, Angeli's salt is referred to as an oxide diazeniumdiolate due to the replacement of the amine function with an oxygen atom (Paolocci *et al.*, 2007). At $\text{pH} > 7$, Angeli's salt spontaneously dissociates to generate HNO and nitrite (NO_2^-) (Miranda *et al.*, 2005b; Paolocci *et al.*, 2007) with a half-life of 2.3-2.8 minutes at 37°C (Maragos *et al.*, 1991).

Whilst Angeli's salt is also capable of generating low amounts of NO^\bullet , this is only apparent at a $\text{pH} < 4$ (Dutton *et al.*, 2004; Switzer *et al.*, 2009) and at concentrations of the donor $> 10 \mu\text{mol/L}$ (Irvine *et al.*, 2003). Although Angeli's salt also decomposes to generate NO_2^- , which itself can modulate cardiovascular function (Bryan *et al.*, 2005; Gladwin *et al.*, 2005), studies have indicated that NO_2^- plays little role in the action of Angeli's salt (Irvine *et al.*, 2003; Paolocci *et al.*, 2007).

1.8.3.2 Isopropylamine NONOate (IPA/NO)



Interestingly, diazeniumdiolates synthesized using primary amines may also release HNO under physiological conditions (Miranda *et al.*, 2005a; Paolocci *et al.*, 2007). One such primary amine is isopropylamine NONOate (IPA/NO) which releases HNO with a half life of 2.3 minutes at 37°C (Maragos *et al.*, 1991; Miranda *et al.*, 2005a). IPA/NO acts as a mixed donor, donating

HNO in the pH range of 5-13 whilst donating NO[•] at pH<7 (Dutton *et al.*, 2006). It also decomposes to form the by-product, nitrosamine, a species associated with potential toxicity (Irvine *et al.*, 2008; Paolocci *et al.*, 2007). Despite this, IPA/NO exhibits all the markers for HNO production, including reductive nitrosylation, reactivity with thiols and positive cardiac inotropic effects *in vivo* (Miranda *et al.*, 2005a). Thus, it is expected that at physiological pH, IPA/NO will release HNO rather than NO[•] (Miranda *et al.*, 2005a).

Indeed, it is important to note that both Angeli's salt and IPA/NO have limitations specifically relating to their short half-lives and the concomitant release of by-products (i.e. NO₂⁻ or nitrosamine, respectively). Thus, the field awaits the development of new, pure longer acting HNO donors. Nevertheless, Angeli's salt has, to date, provided important and valuable information regarding the actions of HNO in the cardiovascular system.

1.8.4 HNO and vasoprotection (sGC/cGMP-dependent and -independent signaling)

Since the early 1990s, accumulating evidence has revealed that HNO donors such as Angeli's salt have the capacity to act as powerful vasorelaxants (Fukuto *et al.*, 1992). Indeed the vasodilator ability of HNO is well documented in both isolated arteries and in the intact circulation. More specifically, exogenous HNO mediates (i) vasorelaxation in large conduit (Ellis *et al.*, 2000; Fukuto *et al.*, 1992; Irvine *et al.*, 2007) and small resistance arteries (Irvine *et al.*, 2003), (ii) a concentration-dependent decrease in coronary perfusion pressure in isolated perfused rat hearts (Favaloro & Kemp-Harper, 2007), (iii) dose-related decreases in arterial lobar pressure in the intact pulmonary vascular beds of cats (De Witt *et al.*, 2001) as well as (iv) a reduction in the mean arterial pressure in anaesthetized rabbits (Ma *et al.*, 1999) and conscious rats (Irvine *et al.*, 2011). Like NO[•], HNO mediates vasorelaxation predominantly via the activation of sGC and a subsequent increase in cGMP (Ellis *et al.*, 2000; Favaloro & Kemp-Harper, 2007; Irvine *et al.*, 2007; Irvine *et al.*, 2003; Paolocci *et al.*, 2007; Wanstall *et al.*, 2001).

Additionally, studies in rat (Ellis *et al.*, 2000) and mouse aortae (Wanstall *et al.*, 2001) and sheep urethra (Costa *et al.*, 2001) have demonstrated that Angeli's salt was more susceptible to inhibition by ODC than authentic NO gas, suggesting that perhaps HNO has a direct interaction with sGC. Indeed, mounting evidence, as highlighted previously, have suggested that HNO like NO[•] targets Fe²⁺ sGC (Miller *et al.*, 2009).

In addition to activating sGC, HNO can also target other signaling pathways to evoke vasorelaxation. Thus, in the coronary vasculature, the neuropeptide calcitonin gene related peptide (CGRP) appears to contribute, at least in part, to HNO-induced vasorelaxation (Favaloro & Kemp-Harper, 2007). Furthermore, HNO can target K_{ATP} and K_v channels in coronary and small mesenteric resistance arteries, respectively resulting in vascular smooth muscle hyperpolarisation (Favaloro & Kemp-Harper, 2007; Irvine *et al.*, 2003). Such actions are distinct from those of NO[•], which directly targets K_{Ca} channels in rat small mesenteric arteries (Mistry & Garland, 1998).

Thus, the vasoprotective effects and distinct pharmacology of HNO may confer therapeutic advantages over traditional NO[•] donors. In addition, unlike nitrovasodilators such as GTN, HNO does not develop tolerance with continued use (Irvine *et al.*, 2007; Irvine *et al.*, 2011) and is resistant to scavenging by [•]O₂⁻ (Li *et al.*, 1999; Miranda *et al.*, 2002). Indeed, NO[•] has been shown to interact rapidly with [•]O₂⁻ to produce the powerful oxidant, ONOO⁻, while the anionic nature of HNO impedes its possible interaction with [•]O₂⁻ anions. Moreover, the [•]O₂⁻ generator, pyrogallol (100 μmol/L) was shown to reduce responses to NO[•], yet had no effect on responses to Angeli's salt in rat anococcygeus muscle (Li *et al.*, 1999). Whilst HNO has the capacity to serve as an antioxidant via the donation of its hydrogen atom (H⁺), it has only been reported to donate its H⁺ atom to high energy radicals such as a lipid radical (Lopez *et al.*, 2007). There is therefore no evidence to date of an interaction between HNO and [•]O₂⁻. Thus, the clinical efficacy

of HNO donors may be sustained under conditions of oxidative stress. These properties, together with an ability of HNO to increase myocardial contractility and unload the heart, confer potential in the treatment of heart failure (Dai *et al.*, 2007; Paolocci *et al.*, 2003; Tochetti *et al.*, 2007).

1.8.5 HNO and cardioprotection

Perhaps one of the more exciting discoveries with regard to the biology and chemistry of HNO was earlier unveiled by Paolocci and co-workers in canine failing hearts. Here they reported a unique ability of HNO to increase myocardial contractility while concomitantly lowering cardiac preload and afterload without altering plasma cGMP levels (Paolocci *et al.*, 2003). These positive inotropic (force of muscle contraction) and lusitropic (relaxation of cardiac muscle) actions of HNO were independent of, but additive to, β -adrenergic stimulation and associated with increased plasma levels of CGRP. Conversely, traditional NO[•] donors such as DEA/NO and GTN have negligible inotropic actions and blunt β -adrenergic signaling (Paolocci *et al.*, 2003; Paolocci *et al.*, 2001).

It was originally thought that CGRP mediated the ability of HNO to increase myocardial contractility, however, this concept has now been refuted. Rather, the ability of HNO to serve as a positive cardiac inotrope is related to its ability to interact with thiol proteins involved in SR Ca²⁺ cycling. Thus, HNO activates ryanodine receptors (RyR2) on the SR (Tochetti *et al.*, 2007), as well as targeting critical cysteine residues (cysteine 674) on SERCA via S-glutathionylation (Lancel *et al.*, 2009) to increase SR Ca²⁺ uptake and release, leading to improved myocardial contractility. Additionally, HNO may also target thiol groups on phospholamban, the regulatory partner of SERCA to modulate its activity (Froelich *et al.*, 2008). Furthermore, HNO is also capable of enhancing the responsiveness of myofilaments to Ca²⁺, subsequently augmenting myocardial contractility in rat isolated cardiac trabeculae (Dai *et al.*, 2007). This unique action of HNO is being explored clinically with a novel HNO donor, CXL-1020, which is currently in

Phase I/IIa clinical trials as a promising therapeutic avenue in the treatment of acute decompensated heart failure (ADHF) (Du, 2009).

1.8.6 $\cdot\text{O}_2^-$ limiting effects: antioxidant properties of HNO

Similar to its redox sibling $\text{NO}\cdot$, HNO has the potential to serve as either a prooxidant or an antioxidant depending on its concentration and environment (Lopez *et al.*, 2007). However, some of the chemical properties of HNO allude to the possibility of it serving primarily as an antioxidant. Thus, HNO may serve as a 1-electron reductant via the donation of its H^+ atom, and this property, coupled with the ability of HNO to cross favourably into hydrophobic environments, such as membranes, suggests that HNO may be able to protect membranes against oxidative damage (Lopez *et al.*, 2007). Recent findings by Lopez and co-workers reported that Angeli's salt protected against both toxicity and lipid peroxidation in the yeast *Saccharomyces cerevisiae* model of oxidative stress by quenching ROS signaling (Lopez *et al.*, 2007). In addition, in non-vascular cells (rat cardiac cells), HNO has been shown to induce an increase in the expression of the cytoprotective enzyme, heme-oxygenase 1 (HO-1), which plays an important role in the defence against oxidative damage (Naughton *et al.*, 2002). While $\text{NO}\cdot$ has also been reported to elevate HO-1 expression (Foresti *et al.*, 1997; Motterlini *et al.*, 2000), this antioxidant property was shown to be associated with a detectable reduction in cell metabolism, an occurrence not observed with HNO (Naughton *et al.*, 2002).

Indeed, we have preliminary evidence in rat neonatal cardiomyocytes that HNO suppresses the activity of the Nox2-containing isoform of NADPH oxidase to limit $\cdot\text{O}_2^-$ generation (Ritchie *et al.*, 2007). Thus, HNO limits cardiac hypertrophy, $\cdot\text{O}_2^-$ generation and the increase in Nox2 expression in response to Ang II in cardiomyocytes. Moreover, such $\cdot\text{O}_2^-$ limiting actions of HNO appear to be cGMP-dependent as they were reversed by the sGC and PKG inhibitors, ODQ and KT5823 respectively (Ritchie *et al.*, 2007). Based upon these observations, it is anticipated

that HNO may also suppress NADPH oxidase activity in the vasculature. Interestingly, a study by Selemidis and co-workers have shown that NO[•] (DETA/NONOate) decreased [•]O₂⁻ production in human cultured endothelial cells (HMEC-1), albeit via a cGMP-independent mechanism, involving S-nitrosylation of p47^{phox} (Selemidis *et al.*, 2007). ***Therefore, it remains to be determined if HNO has a similar effect in the vasculature and if such an action is sGC-dependent or -independent.*** Given NADPH oxidase is a major contributor to oxidative stress in the vasculature, an ability of HNO to suppress vascular [•]O₂⁻ generation would be of significant therapeutic benefit.

Like HNO donors, the NO-independent stimulators and activators of sGC, created initially by Bayer HealthCare in Germany, represent another promising alternative therapy devoid of the limitations posed by the current clinically used NO[•] based compounds.

1.9 Novel NO-independent sGC stimulators and activators

1.9.1 NO-independent and heme-dependent sGC stimulators

The novel class of NO-independent, heme-dependent sGC stimulators is comprised of the compounds YC-1, BAY 41-2272, BAY 41-8543, CMF 1571 and A-350619, so defined based on several shared characteristics including NO-independent activation of sGC, crucial dependency of the reduced (Fe²⁺) prosthetic heme moiety of sGC and strong synergism with NO (Evgenov *et al.*, 2006).

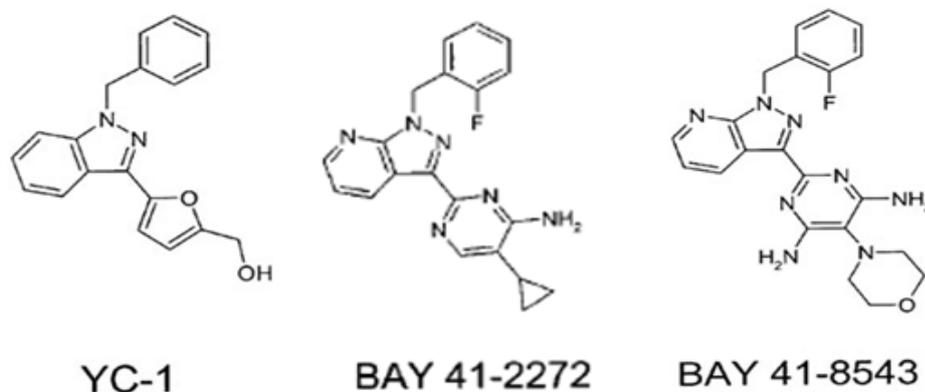


Figure 11. Structure of the NO-independent and heme-dependent stimulators of sGC (Hobbs, 2002).

1.9.1.1 YC-1

In 1994, the first NO-independent and heme-dependent stimulator of purified sGC, the indazole derivative YC-1 was developed. YC-1 activates sGC in an NO-independent manner (Mulsch *et al.*, 1997; Wu *et al.*, 1995), whereby its effects are dependent on the presence of the reduced (Fe^{2+}) heme moiety of sGC such that its oxidation and/or removal abolishes YC-1-induced relaxation (Evgenov *et al.*, 2006).

The precise mechanism(s) via which YC-1 elicits an effect on sGC is still ambiguous, but is believed to be related to its ability to stabilize sGC in its active configuration (Becker *et al.*, 2001; Hobbs, 1997). Additionally, YC-1 has been shown to exhibit potent anti-aggregatory activity via the combined activation of sGC and the inhibition of cGMP degradation by PDEs in platelets (Friebe *et al.*, 1999), properties which are in contrast to that observed with organic nitrate therapy. Despite being a highly effective vasodilator of its time, the search began for the generation of compounds which were more potent and more specific for sGC activation. This search resulted in the identification of BAY 41-2272 and BAY 41-8543, which proved to be

more potent direct stimulators of sGC both *in vitro* and *ex vivo* (Evgenov *et al.*, 2006; Ghofrani & Grimminger, 2009).

1.9.1.2 BAY 41-2272 and BAY 41-8543

Using YC-1 as a chemical lead structure, Bayer HealthCare developed a series of novel NO-independent and heme-dependent sGC stimulators, two of which are BAY 41-2272 and BAY 41-8543. Both these compounds are orally active, resistant to tolerance development and two orders of magnitude more potent than YC-1 (Stasch *et al.*, 2002). BAY 41-2272 (5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1Hpyrazolo[3,4-b]pyridin-3-yl]-pyrimidin-4-ylamine) is a non-NO-based pyrazolopyridine and using photoaffinity labelling has been shown to stimulate sGC in the cysteine 238 and cysteine 243 spanning region of the α -subunit of the enzyme (Stasch *et al.*, 2001). Furthermore, the addition of BAY 41-2272 to purified sGC did not alter the Soret peak of either the non-stimulated (431nm) or the NO-stimulated (399nm) enzyme. Thus, taken together, the authors concluded that it was unlikely that BAY 41-2272 activates sGC by binding directly to the heme moiety yet removal of the heme, using a non-ionic detergent Tween 20, abolished the ability of BAY 41-2272 to activate the enzyme (Stasch *et al.*, 2001). Indeed, it has been suggested that, in the absence of a crystal structure of sGC, it is possible that the cysteine 238 and cysteine 243 residues may actually be situated in the interface between the sGC subunits, thereby allowing for the labelling of the α subunit even if the compound is bound to the corresponding β subunit (Evgenov *et al.*, 2006). Either way, this spanning region, in addition to the heme, appears to play a crucial role in the BAY 41-2272-mediated response.

Moreover, these compounds act in a synergistic manner with NO thereby enhancing the sensitivity of the reduced (Fe^{2+}) heme of sGC to low levels of bioavailable NO (Evgenov *et al.*, 2006; Stasch *et al.*, 2002c). The synergy between sGC stimulators and NO was first reported in assays utilising purified sGC, where BAY 41-2272 was shown to synergise with NO donors over

a wide range of concentrations in order to stimulate sGC activity (Stasch *et al.*, 2001). Thus, these NO-independent and heme-dependent compounds offer a new therapeutic principle which directly stimulates sGC and increases the sensitivity of the enzyme to NO, subsequently resulting in the elevation of cGMP levels (Evgenov *et al.*, 2006).

Such synergism is particularly evident when considering the vasorelaxant activity of BAY 41-2272 and BAY 41-8543. Thus, these NO-independent sGC stimulators induce vasorelaxation in the isolated vasculature (Priviero *et al.*, 2009; Stasch *et al.*, 2002b) and intact circulation (Dumitrascu *et al.*, 2006; Stasch *et al.*, 2002a) via a sGC/cGMP-dependent mechanism. However, following endothelial removal or NOS inhibition, vasorelaxation to BAY 41-2272 has been reported to be attenuated (Priviero *et al.*, 2005; Priviero *et al.*, 2009; Teixeira *et al.*, 2006). This synergy with NO was further demonstrated in lambs with acute pulmonary hypertension where the magnitude and duration of pulmonary vasodilation induced by inhaled NO (iNO) was augmented following the inhalation of microparticles encapsulated with either BAY 41-2272 or BAY 41-8543 (Evgenov *et al.*, 2007). Indeed, the importance of the cGMP-dependent pathway in the response to both BAY 41-2272 and BAY 41-8543 is further highlighted by the finding that inhibition of cGMP degradation by PDE inhibitors potentiates the relaxant responses to these sGC stimulators (Evgenov *et al.*, 2007; Priviero *et al.*, 2005). Moreover, there is evidence which argues both for (Mullershausen *et al.*, 2004) and against (Stasch *et al.*, 2001) an ability of BAY 41-2272 itself to inhibit PDE5 activity.

Furthermore, BAY 41-2272 has also been shown to activate sGC/cGMP-independent mechanisms such that vasorelaxant responses to BAY 41-2272 were not completely abolished by the sGC inhibitor, ODQ (Teixeira *et al.*, 2006). Indeed, it appears that BAY 41-2272 may directly target Ca²⁺ channels to limit Ca²⁺ influx. This ability of BAY 41-2272 to mediate its effects via a cGMP-independent manner was further verified in the corpus cavernosum of

humans and rabbits (Baracat *et al.*, 2003) as well as the mesenteric arteries of SHR rats (Priviero *et al.*, 2009). In addition to their vasodilatory capacity, BAY 41-2272 and BAY 41-8543 also exhibit anti-proliferative properties in smooth muscle and anti-aggregatory effects in platelets (Evgenov *et al.*, 2006; Stasch *et al.*, 2001; Stasch *et al.*, 2002a). Importantly, these compounds remain active in tissues made tolerant to GTN (Hobbs, 2002; Stasch *et al.*, 2002a; Stasch *et al.*, 2002c). Moreover, preclinical studies have highlighted the therapeutic potential of BAY 41-2272 in several animal models of pulmonary hypertension (Deruelle *et al.*, 2006; Deruelle *et al.*, 2005a; Deruelle *et al.*, 2005b; Dumitrascu *et al.*, 2006; Evgenov *et al.*, 2004) and heart failure (Boerrigter *et al.*, 2003).

Taken together, however, the synergism of these compounds with NO may result in attenuated efficacy under oxidative stress conditions where NO[•] is scavenged. In addition, their reliance on the reduced heme of sGC (Evgenov *et al.*, 2006) may lead to potential limitations with the use of these compounds. Indeed, in a hypertensive rat model (SHR), both cGMP-dependent and -independent relaxation induced by BAY 41-2272 was impaired in mesenteric arteries, yet responses were improved by limiting oxidative stress with the [•]O₂⁻ scavenger, SOD and the NADPH oxidase inhibitor, apocynin (Priviero *et al.*, 2009). Given the elevation in ROS generation and dysfunction in the NO/sGC signalling pathway in disease, there is a need for NO-independent compounds which target the oxidised/heme-free form of sGC.

1.9.2 NO- and heme-independent sGC activators

In 2002, BAY 58-2667 (Cinaciguat) was selected from a series of approximately 800 analogues and described as the first NO- and heme-independent sGC activator. Remarkably, in contrast to any of the previously described heme-dependent sGC stimulators, the activation of sGC by BAY 58-2667 was more pronounced following the oxidation or the removal of the heme moiety of sGC (Stasch *et al.*, 2002c). Since then, HMR 1766 (ataciguat) has been described, demonstrating

similar characteristics as that observed with BAY 58-2667 despite the two chemical structures exhibiting no similarities (Schindler *et al.*, 2006). These two compounds founded the novel class of NO- and heme-independent activators of sGC (Evgenov *et al.*, 2006). However, in terms of this thesis, only BAY 58-2667 is discussed. Furthermore, despite not being classed as a member of the NO- and heme-independent activators, the biosynthetic precursor to heme, protoporphyrin IX (PPIX) is also an activator of heme-free sGC and may compete with the heme group for binding to the enzyme (Mingone *et al.*, 2006).

1.9.2.1 BAY 58-2667 (Cinaciguat)

BAY 58-2667 was first identified via high throughput screening as an amino dicarboxylic acid able to activate sGC with an EC₅₀ and K_d value in the low nanomolar range, making this compound the most potent NO-independent sGC activator to date (Evgenov *et al.*, 2006; Schmidt *et al.*, 2003; Stasch *et al.*, 2002c). In contrast to the sGC stimulators, BAY 58-2667 functions in an additive, not synergistic, manner when combined with NO and is the first NO-independent compound with a hemodynamic profile similar to that of organic nitrates, inducing significant *in vivo* relaxation in both arterial and venous blood vessels (Evgenov *et al.*, 2006). Comparatively, the pharmacokinetic profile of BAY 58-2667 is different to that of GTN, having a lower clearance and longer half life, thus resulting in a longer lasting effect than that observed with GTN (Stasch *et al.*, 2002c). Strikingly, in contrast to NO and sGC stimulators, BAY 58-2667-induced sGC activation is potentiated upon the oxidation (Fe³⁺) or removal (heme-free) of the prosthetic heme group of sGC (Stasch *et al.*, 2002c). While BAY 58-2667 is able to stimulate reduced purified sGC to a low level, oxidation of the heme with ODQ resulted in up to a 187-fold increase in sGC activity (Stasch *et al.*, 2002c). Furthermore, BAY 58-2667 activated heme-free sGC in a concentration-dependent manner, inducing up to a 190-fold increase in enzyme activity (Stasch *et al.*, 2002c), an effect which was not further potentiated by the presence of ODQ.

This unique and preferential targeting of oxidised/heme-free sGC can be explained by the mechanistic way in which BAY 58-2667 replaces the heme moiety resulting in enzyme activation. Thus, BAY 58-2667 is able to mimic the spatial structure of the heme and involves the competition between the carboxylic groups of this compound and heme propionic groups for binding to the unique sGC heme binding motif, Y-x-S-x-R (tyrosine₁₃₉, serine₁₃₇ and arginine₁₃₉, Figure 12) (Evgenov *et al.*, 2006; Schmidt *et al.*, 2005; Schmidt *et al.*, 2004). It was originally proposed that following oxidation of the heme, the bond between sGC and the prosthetic heme group is weakened, thus allowing for BAY 58-2667 to easily displace the ferric (Fe^{3+}) heme and activate sGC (Schmidt *et al.*, 2004). Indeed, this concept was supported by direct spectroscopic observations which demonstrated that BAY 58-2667 was capable of displacing the heme, even in the reduced (Fe^{2+}) tightly bound state, when concentrations exceeded 30 $\mu\text{mol/L}$ (Schmidt *et al.*, 2004).

A more recent study by Roy and colleagues however, have challenged this view, demonstrating in their study that BAY 58-2667 is unable to distinguish between the reduced (Fe^{2+}) or the oxidised (Fe^{3+}) heme species but will effectively only activate the heme-free entity (Roy *et al.*, 2008). The authors thus propose that the activity of BAY 58-2667 observed in the reduced and/or ODQ-treated sGC populations therefore reflects the presence of heme-free enzymes in all these preparations and not the active displacement of the weakly or tightly bound heme by BAY 58-2667 (Roy *et al.*, 2008). Therefore, while BAY 58-2667 activates heme-free sGC, further investigation is required to determine if this compound is also capable of targeting the ferric heme. Indeed, oxidised sGC appears to be prone to accelerated degradation via ubiquitination, and in addition to activating oxidised/heme-free sGC, BAY 58-2667 is also able to stabilise both the α and β subunits of the heterodimer, preventing ubiquitination and proteosomal degradation (Meurer *et al.*, 2009; Stasch *et al.*, 2006).

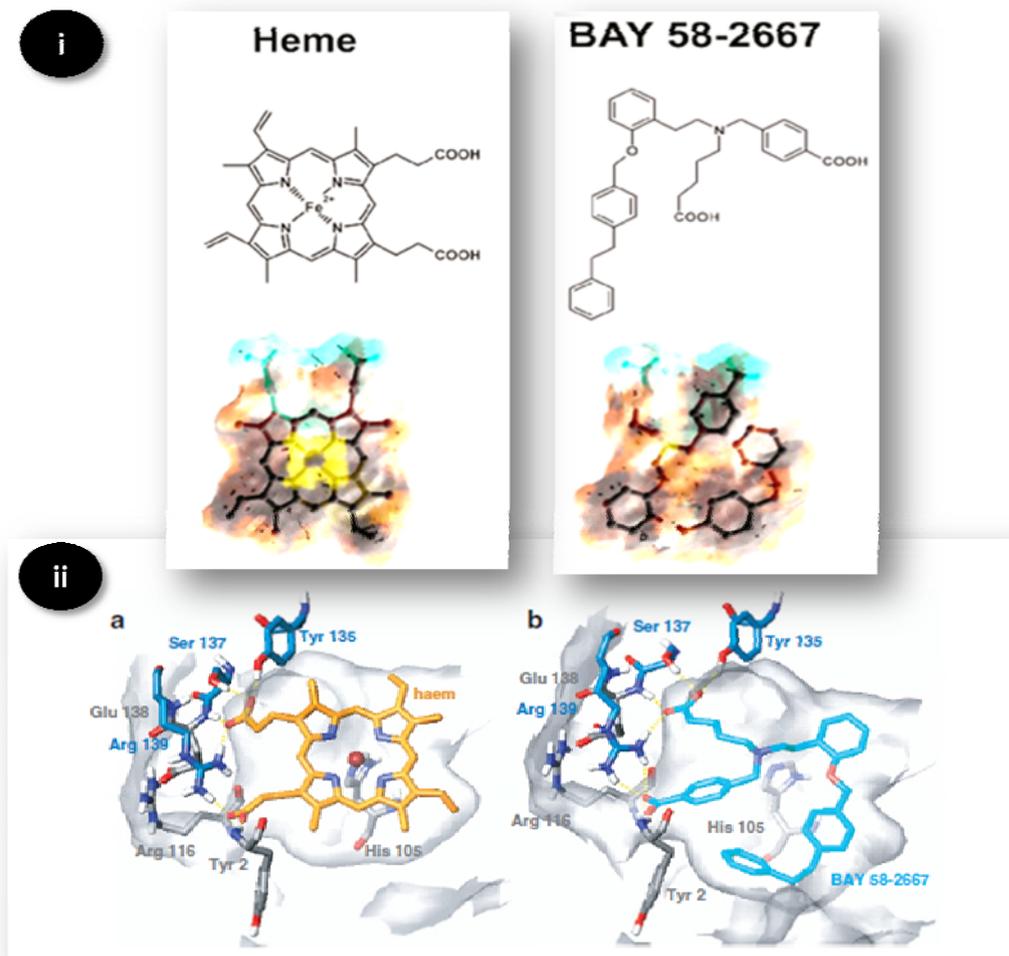


Figure 12. (i) Graphical representation of the chemical structure and spatial arrangement of heme and BAY 58-2667. Note that despite substantial differences in the chemical structure, the spatial arrangements are similar, allowing BAY 58-2667 to easily replace the heme component of sGC (Adapted from Schmidt *et al.*, 2004). (ii) Model of the heme binding domain of rat sGC. (a) represents the heme binding motif, Y-x-S-x-R, to which the heme is bound and (b) represents the proposed mode of binding of the heme mimetic, BAY 58-2667 to sGC (Adapted from Schmidt *et al.*, 2009).

Interestingly, BAY 58-2667 is a potent vasodilator in the non-diseased vasculature, being up to 160-fold more potent than BAY 41-2272 and >1000-fold more potent than the nitrovasodilators, SNP and 3-morpholinosydnonimine (Stasch *et al.*, 2002c). Given the preference of BAY 58-2667 for oxidised/heme-free sGC, these findings indicate that a significant pool of oxidised/heme-free sGC exists under physiological conditions. Indeed, Stasch and co-workers

demonstrated that the vasorelaxant effects of BAY 58-2667 were markedly augmented in three different animal models of disease, namely spontaneously hypertensive rats (SHR), hyperlipidaemic rabbits and apolipoprotein E deficient (ApoE^{-/-}) mice as well as in patients with type II diabetes, compared to their respective healthy controls (Stasch *et al.*, 2006). It is likely that the preferential targeting of diseased vessels by BAY 58-2667 is related to the elevated oxidative stress associated with these pathologies (i.e. increase $\cdot\text{O}_2^-$, ONOO⁻) and subsequent oxidation of sGC. Indeed, acute treatment of isolated rat thoracic aortae with ONOO⁻ leads to improved vasorelaxation to BAY 58-2667 but diminished responsiveness to the NO donor, DEA/NO (Stasch *et al.*, 2006). Thus, BAY 58-2667 may also serve as a novel tool to probe the intracellular redox shifts of sGC (Stasch *et al.*, 2006). To date, it has not been possible to quantify the pool of oxidized or heme-free sGC in living cells with sufficient sensitivity, given the vast amounts of cellularly expressed heme proteins (Ingi *et al.*, 1996) and the susceptibility of sGC to oxidation during extraction (Craven *et al.*, 1978; Gerber *et al.*, 1981; Wolin *et al.*, 1982). Such technical limitations can conceivably be circumvented by BAY 58-2667, with its unusual ability to preferentially target the heme-oxidized/heme-free sGC (Stasch *et al.*, 2006; Stasch *et al.*, 2001). **Currently, however, a direct link between an increase in vascular $\cdot\text{O}_2^-$ generation and improved BAY 58-2667 responsiveness has not been demonstrated.**

In addition to its vasodilator activity, BAY 58-2667 inhibits platelet aggregation and its actions are sustained in arteries rendered tolerant to GTN (Evgenov *et al.*, 2006; Stasch *et al.*, 2002c). These vasoprotective actions of BAY 58-2667 and its enhanced efficacy in disease may confer potential for this class of sGC activator in the treatment of cardiovascular disorders. Indeed, in rodent models of pulmonary hypertension, chronic treatment with BAY 58-2667 reduced systolic blood pressure in addition to reversing right ventricular hypertrophy and structural remodelling of the lung vasculature in chronically hypoxic mice and monocrotaline-treated rats (Dumitrascu *et al.*, 2006). In lambs with acute pulmonary hypertension, the administration of

BAY 58-2667 via inhalation (BAY 58-2667-encapsulated microparticle) has been shown to significantly elevate transpulmonary cGMP as well as limiting adverse systemic arterial hypotension (Evgenov *et al.*, 2007).

Moreover, a recent study conducted by Boerrigter and colleagues reported potent systemic and renal vasodilation following intravenous administration of BAY 58-2667 in male mongrel dogs with severe congestive heart failure (CHF). In this model of severe CHF, BAY 58-2667 also elicited a substantial decrease in right atrial pressure as well as a decrease in pulmonary capillary wedge pressure without tolerance development following prolonged administration (Boerrigter *et al.*, 2007). Recently in a Phase I clinical trial, the safety, tolerability, pharmacokinetics and pharmacodynamics of increasing concentrations of BAY 58-2667 as administered intravenously were examined in 76 healthy males volunteers (Frey *et al.*, 2008). The results from this study indicated that BAY 58-2667 exhibited a favourable safety profile and was well tolerated in the healthy male subjects warranting further investigations of this compound in the treatment of acute decompensated heart failure (ADHF) (Lapp *et al.*, 2009). The first clinical results examining the effects of BAY 58-2667 in patients with ADHF revealed that BAY 58-2667 exhibited promising cardiac effects resulting in potent unloading of the heart (Lapp *et al.*, 2009). These properties may be therapeutically useful when overcoming tolerance issues experienced with sustained nitrate therapy. Overall, BAY 58-2667 exhibits a novel mechanism for modulating the NO/sGC/cGMP pathway in the treatment of cardiovascular diseases, with the ability to counteract dysfunction without increasing vascular $\cdot\text{O}_2^-$ production and/or tolerance development (Evgenov *et al.*, 2006). Moreover, BAY 58-2667 may also serve as a sensitive diagnostic tool to identify blood vessels in which NO/sGC signaling is dysfunctional.

In light of the altered redox states of sGC, sGC activity studies have revealed that protoporphyrin IX (PPIX) and various metalloporphyrins (Zn-PPIX, Mn-PPIX) are also capable of competing with BAY 58-2667 for the heme binding motif (Stasch *et al.*, 2006).

1.9.2.2 Protoporphyrin IX (PPIX)

Protoporphyrin IX (PPIX) is a naturally occurring intracellular metabolite in mammalian cells and is a potent activator of purified sGC (Ignarro *et al.*, 1982; Wolin *et al.*, 1982). PPIX is synthesised from δ -aminolevulinic acid (ALA) and serves as the iron-free biosynthetic precursor to heme (Figure 13) (Ignarro *et al.*, 1982). Under physiological conditions, ferrochelatase catalyses the insertion of Fe into PPIX, efficiently converting PPIX into heme (Perkins, 2006). However, in the presence of excess levels of ALA and/or reduced Fe bioavailability, PPIX rather than heme accumulates (Mingone *et al.*, 2006; Perkins, 2006).

PPIX is able to mimic the conformational complex of heme and thus, serve as a partial agonist at heme-depleted sGC (Stasch *et al.*, 2006). Heme itself serves as a competitive inhibitor of PPIX-activated sGC and the detergent, Tween-20 augments PPIX-induced sGC activation, providing further evidence that PPIX targets sGC in its heme-free state (Koesling & Friebe, 1999).

1.10 Vascular dysfunction in disease

As previously highlighted, there are numerous ways in which an impairment of the NO/sGC/cGMP pathway can occur in disease: (1) decline in NO generation as a consequence of a decrease in NOS activity (Boerrigter & Burnett Jr, 2007); (2) NO scavenging by ROS under conditions of oxidative stress, hence reducing NO bioavailability (Tomasz *et al.*, 2002), (3) decreased expression of sGC (Boerrigter & Burnett Jr, 2002), (4) oxidation or subsequent removal of the heme moiety of sGC rendering the enzyme insensitive to NO (Hobbs, 1997; Stasch *et al.*, 2006) and/or (5) cGMP degradation accelerated by a rise in PDE activity (Boerrigter & Burnett Jr, 2007; Melichar *et al.*, 2004; Sobey & Quan, 1999).

1.10.1 Endothelial dysfunction

Situated in direct contact with the blood, the vascular endothelium is a layer of squamous epithelial cells which respond to various autocrine and paracrine stimuli as well as mechanical factors such as shear stress and thus plays a central and vital role in the regulation of vascular homeostasis (Thuillez & Richard, 2005; Vanhoutte *et al.*, 1986). (Feletou & Vanhoutte, 2006a). Among its numerous essential roles in the control of homeostasis is the production and release of NO which diffuses to the surrounding tissues and cells to maintain the balance between vasodilatation and vasoconstriction, regulate vascular smooth muscle proliferation and migration as well as limit platelet adhesion and aggregation (Feletou *et al.*, 2009; Vanhoutte *et al.*, 1986). Under disease conditions the endothelium undergoes functional and structural alterations such that it is transformed from a protective organ to a source of vasoconstrictors (i.e. endothelial-derived contracting factors; EDCFs) and pro-aggregatory and pro-mitogenic factors (Vanhoutte, 1989; Versari *et al.*, 2009). Under such conditions, the ability of the endothelium to generate the vasoprotective signaling molecule, NO is also reduced (Versari *et al.*, 2009). Such ‘endothelial dysfunction’ is a hallmark of many cardiovascular diseases and may serve as an initiating factor in the development of hypertension, diabetes and atherosclerosis. Indeed, evidence of endothelial

dysfunction has been reported in the resistance arteries and conduit vessels of several hypertensive rat models including the SHR (Sekiguchi *et al.*, 2002; Yang *et al.*, 2004), the two-kidney one-clip models (Lee *et al.*, 1995; Stankevicius *et al.*, 2002), deoxycorticosterone acetate salt-treated (Cordellini, 1999) and the Dahl-salt sensitive hypertensive rats (Zhou *et al.*, 2001; Zhou *et al.*, 1999). Thus, endothelial dysfunction is not only a collateral feature of established risk factors but may also present as a possible pathogenic mechanism for their onset (Versari *et al.*, 2009)

1.10.2 Hypertension

Established hypertension is characterised by an elevation of arterial blood pressure believed to be driven by a rise in peripheral resistance rather than an increase in cardiac output (Pries *et al.*, 1999). Hypertension is commonly associated with altered vascular tone and impaired endothelium-dependent vasodilation (Touyz, 2002; Zalba *et al.*, 2001). Importantly, vascular oxidative stress is associated with hypertension, with elevated $\cdot\text{O}_2^-$ generation from NADPH oxidase and subsequently uncoupled eNOS appearing to be major contributors (Lassegue & Griending, 2004; Paravicini & Touyz, 2006; Ray & Shah, 2005; Vanhoutte, 2006). Indeed, evidence indicates NO bioavailability is attenuated in hypertensive rats due to scavenging of NO by $\cdot\text{O}_2^-$ (Cuzzocrea *et al.*, 2004). Moreover, reduced sGC expression and attenuated vasorelaxant responses to NO donors has been observed in aged SHR (Kloß *et al.*, 2000).

Thus, hypertension appears to be associated with oxidative stress in the vascular wall and a combination of: (i) decreased NO synthesis and bioavailability, (ii) reduced sGC subunit expression, and (iii) elevation of cGMP degradation, consequently resulting in impaired NO/sGC/cGMP signaling (Gupte *et al.*, 1999; Kloss *et al.*, 2000; Ruetten *et al.*, 1999). In addition, it has also been proposed that the increase in ROS generation in hypertension may lead to impairment in the NO signaling pathway via oxidation of the heme group of sGC from its

ferrous (Fe^{2+}) to its ferric (Fe^{3+}) and thereby NO insensitive form (Gupte *et al.*, 1999; Stasch *et al.*, 2006).

1.10.3 Aging and hypertension

Advanced age and chronic hypertension are both associated with endothelial dysfunction (Kojda & Harrison, 1999; Vanhoutte, 2002). In aging, like hypertension, alterations in endothelial structure and function are apparent, there is an increased release of vasoconstrictor prostanoids and a reduction in NO bioavailability (Vanhoutte, 2002). Thus, treatment with cyclo-oxygenase (COX) inhibitors and antioxidants, such as vitamin C has been reported to prevent the age-related decrease in endothelium-dependent vasodilation in humans, implicating the up-regulation of endothelium-derived contractile factors and ROS in aging (Taddei *et al.*, 2001). The coexistence of hypertension with age accelerates the onset of such dysfunction (Taddei *et al.*, 1997). There is no doubt that the alterations in vascular function, whether brought about by hypertension or aging, is associated with an impairment in the NO/sGC/cGMP signaling pathway. Therefore, compounds which are able to act in an NO-independent manner or via an alternate pathway such as the HNO donors or sGC stimulators (BAY 41-2272) and activators (BAY 58-2667) provide an alternative and perhaps more beneficial, therapeutic avenue when treating cardiovascular complications associated with hypertension and/or aging.

1.10.3.1 The Spontaneously Hypertensive Rat (SHR) as a model of essential hypertension

Generated in Japan in 1963 in the laboratory of Professor Kozo Okamoto (Department of Pathology Kyoto University Japan), the spontaneously hypertensive rat (SHR) was initially bred from the Wistar strain by selectively mating animals which displayed slightly elevated blood pressures. This method of selective mating was continued until 100% of the obtained progeny inherited naturally occurring hypertension (Okamoto & Aoki, 1963; Udenfriend & Spector, 1972). Conveniently, the Wistar Kyoto strain, from which these hypertensive animals were

initially bred, was then developed as a subsequent model of normotension. This SHR strain displayed many similarities to human essential hypertension such as the elevation in arterial pressure and was associated with a slow and progressive increase in total peripheral resistance (Udenfriend & Spector, 1972). Additionally, studies utilizing this model have revealed that the SHR is associated with endothelial dysfunction (Bauersachs *et al.*, 1998; Kojda *et al.*, 1998), enhanced NADPH induced $\cdot\text{O}_2^-$ production (Zalba *et al.*, 2000) as well as a reduction in the expression of the sGC subunits (Kloß *et al.*, 2000; Ruetten *et al.*, 1999).

Thus, the SHR offers a convenient and relatively inexpensive method of studying the etiology of hypertension in a laboratory animal with a short enough life span to be able to evaluate the entire duration of the disease (Trippodo & Frohlich, 1981). Furthermore, their general availability allows for the comparison of results from investigators from different labs (Udenfriend & Spector, 1972). As the SHR appears to be a sensitive experimental model for the evaluation of antihypertensive drugs (Trippodo & Frohlich, 1981), it is well suited for investigating the potential therapeutic advantages of NO-independent acting compounds in a model of chronic disease.

Interestingly, the SHR is characterised by Mendelian type inheritance, which despite its genetic aspects are not commonly encountered in human essential hypertension (Pinto *et al.*, 1998). Therefore, it is important to note that essential hypertension is a complex and multifactorial disease and although the SHR does exhibit several common features, it may only reflect a small subtype of the human population. Furthermore, as different rodent models exhibit varying etiologies, careful consideration should be given to the choice of model as it may influence the outcome of the experiment (Pinto *et al.*, 1998). Thus, while rodent models of genetic hypertension such as the SHR are undoubtedly valuable, it is also important to acknowledge the potential limitations associated with their use.

1.10.4 Atherosclerosis

Atherosclerosis is a multifocal immunoinflammatory disease of medium and large arteries, representing the major source of morbidity and mortality in the developed world (Falk, 2006; Keaney Jr, 2000). While atherosclerosis alone is rarely fatal, it is undoubtedly the most frequent underlying cause of coronary and carotid artery disease as well as peripheral arterial disease (Falk, 2006). Indeed, it is the associated complications of atherosclerosis such as thrombosis and plaque rupture which precipitates clinical events such as myocardial infarction and stroke, representing one of the most common causes of death in western society (Falk, 2006; Glass & Witztum, 2001). Atherosclerosis is a progressive disease characterised by lesions within the vessel wall, which begin as ‘fatty streaks’ underlying the endothelium (Glass & Witztum, 2001; Lusis, 2000). Fatty streak formation generally evolves from the recruitment of macrophages and their subsequent uptake of low density lipoprotein (LDL)-derived cholesterol (Glass & Witztum, 2001), which can usually be found in the aorta of humans within the first decade of life (Lusis, 2000). Indeed, LDLs have been shown to be a major pro-inflammatory factor in the development of atherosclerosis, with the oxidative modification of LDL (oxLDL) being implicated as key mediator in the progression of this disease (Heinloth *et al.*, 2000; Witztum, 1993). Atherosclerosis is accompanied by morphological and pathological alterations including the replacement of fatty streaks by fibrous plaques (Melichar *et al.*, 2004), which can result in clinical complications in the event of a rupture or erosion of these lesions (Lusis, 2000).

Given that the endothelium plays an integral role in the control of homeostasis, endothelial dysfunction represents one of the primary causes of the initiation of atherosclerosis (Chatterjee & Catravas, 2008). Indeed, the endothelium is major source of NO, which exhibits important anti-atherosclerotic properties. Such properties include the anti-proliferative and vasodilatory effects on the vasculature and anti-aggregatory effects on platelets as well as preventing the oxidative modification of LDL (Chatterjee & Catravas, 2008; Rubbo *et al.*, 2002). A decline in NO

bioavailability in atherosclerosis may be a consequence of a decrease in the expression and/or activity of eNOS, due to lack of substrate or co-factors such as BH₄, or due to an increased scavenging by ROS, such as $\cdot\text{O}_2^-$, derived from NADPH oxidase, xanthine oxidase or uncoupled NOS (Melichar *et al.*, 2004). Indeed, eNOS deficiency in apolipoprotein E-deficient (eNOS^{-/-}/ApoE^{-/-}) mice has been shown to augment atherosclerotic lesion formation, further supporting the protective role of both eNOS and NO in atherosclerosis (Knowles *et al.*, 2000; Kuhlencordt *et al.*, 2001). Interestingly, one study has shown that an over-expression of eNOS in ApoE^{-/-} mice accelerated rather than inhibited atherogenesis, suggesting that impaired NO bioactivity rather than eNOS expression was more important in the initiation of atherosclerosis. Nevertheless, a reduction in NO bioavailability certainly contributes to both the initiation and progression of atherosclerosis, while there is no doubt that alterations in the NO/cGMP signaling pathway is associated with this disease (Melichar *et al.*, 2004).

1.10.4.1 Apolipoprotein E-Deficient (ApoE^{-/-}) mouse as a model of atherosclerosis

First developed in 1992 by two separate laboratories, apolipoprotein E-deficient (ApoE^{-/-}) mice were generated as a mouse model of spontaneous atherosclerosis, associated with hypercholesterolemia as well as exhibiting severe atherosclerotic lesion development (Plump *et al.*, 1992; Zhang *et al.*, 1992). ApoE is a glycoprotein synthesised mainly in the liver and brain which acts as a ligand for receptors that clear remnants of lipids, mainly in the form of chylomicrons and very low density lipoproteins (VLDL) (Breslow, 1996; Potteaux *et al.*, 2007). Additionally, within vessels, ApoE is synthesised by monocytes and macrophages, where it is thought to have local effects on cholesterol homeostasis (Curtiss & Boisvert, 2000; Meir & Leiterdorf, 2004). Furthermore, it may also function in regulating dietary absorption and biliary excretion of cholesterol (Sehayek *et al.*, 2000). Thus, due to the impaired clearance of cholesterol-enriched lipoproteins, ApoE^{-/-} mice display elevated levels of plasma cholesterol when compared to wild-type mice (Curtiss, 2000). Indeed, mice lacking this gene exhibited five

times the normal plasma cholesterol level as well as develop foam cell-rich depositions in their proximal aortas by three months of age (Zhang *et al.*, 1992). The initial sites of lesion development appear to be the aortic root, aortic arch and its principle branches, branches of the superior mesenteric arteries as well as in the renal and pulmonary arteries, whilst in older mice, lesions were also detected in the descending and abdominal aorta, proximal coronary, common iliac and femoral arteries (Nakashima *et al.*, 1994). Furthermore, there is evidence that the Western-style high fat diet exacerbates the process of atherogenesis in ApoE^{-/-} mice, such that lesion development is more advanced, occurs much earlier and contains more lipid compared to those on a normal chow diet (Nakashima *et al.*, 1994). Such lesions also appear to be wider, medial destruction more severe and associated with complications like calcification (Nakashima *et al.*, 1994). Additionally, the distribution and size of the lesions also appear to increase with age (Reddick *et al.*, 1994).

Indeed, atherosclerotic lesion development in the ApoE^{-/-} mouse model resembles that observed in the progressive phases of human atherosclerosis, while dietary manipulations are able to accelerate the development of lesions, thereby shortening the period of time required to achieve advanced atherosclerosis (Nakashima *et al.*, 1994; Potteaux *et al.*, 2007). Such features have lead to the extensive use of this model for the study of the cellular and molecular mechanisms of atherosclerosis (Potteaux *et al.*, 2007). As with any animal model, it should also be noted that the extrapolation from rodent to human outcomes is problematic due to several factors including variations in cholesterol metabolism, lipid profile, cardiovascular physiology as well as plaque pathology (Meir & Leitersdorf, 2004). However, despite its imperfections, the ApoE^{-/-} is still the most convenient and popular model for investigating atherosclerosis.

1.10.4.2 Nox2 deficient mice (*Nox2*^{-/-})

Nox2 deficient mice (*Nox2*^{-/-}, also known as gp91^{phox}^{-/-} mice) were originally generated in the laboratory of Professor Mary Dinauer to aid with the development of new treatments for the recessive disorder, chronic granulomatous disease (CGD) in addition to examining the role of phagocyte-derived oxidants in inflammation (Pollock *et al.*, 1995). All *Nox2*^{-/-} mice bred are fully backcrossed to the wildtype C57BL/6J background (Judkins *et al.*, 2009).

Nox2 appears to be the most widely distributed among the Nox isoforms (Bedard & Krause, 2007) and the best characterised at the molecular level (Cave *et al.*, 2006). Importantly, Nox2 appears to be up-regulated in the presence of cardiovascular risk factors, generating high levels of ROS and therefore presenting as a strong candidate as a therapeutic target in disease states (Drummond *et al.*, 2011; Selemidis *et al.*, 2008). Indeed, *Nox2*^{-/-} mice are associated with a profound reduction in $\cdot\text{O}_2^-$ production, improvement in NO bioavailability and in the ApoE^{-/-} mice, Nox2 deletion exhibits strikingly less atherosclerotic plaque burden along the length of the aorta (Judkins *et al.*, 2009; Scradler *et al.*, 2007). Furthermore, a deficiency in Nox2 also reduces vascular inflammation, cellular proliferation and neointimal thickening following experimental angioplasty (Chen *et al.*, 2004).

Previous studies examining the effects of Ang II in *Nox2*^{-/-} mice demonstrated that Nox2 deletion suppressed Ang II-induced vascular and cardiac hypertrophy as well as $\cdot\text{O}_2^-$ production (Bendall *et al.*, 2002; Wang *et al.*, 2001). Thus, Nox2 deletion appears to be in essence removing the link between the risk factor and vascular disease, thereby also providing an indication as to the relative role Nox2 plays in the pathogenesis of various disease states (Selemidis *et al.*, 2008).

Aims of the thesis

Considering **(i)** the potent vasodilator ability of HNO in both conduit and resistance vessels, **(ii)** its therapeutic potential in heart failure, **(iii)** its resistance to tolerance development and $\cdot\text{O}_2^-$ scavenging, **(iv)** the distinct pharmacology of HNO presenting advantages over traditional nitrovasodilator therapy and **(v)** its anti-hypertrophic and $\cdot\text{O}_2^-$ limiting effects in cardiac myocytes, the aim of the first chapter was to:

- I) Explore the ability of HNO to limit ROS generation in the vasculature by examining the ability of HNO donors (Angeli's salt, IPA/NO; 24-hour treatment) to limit NADPH oxidase-derived $\cdot\text{O}_2^-$ production utilising an Ang II-induced model of oxidative stress in mouse isolated carotid arteries (Chapter 3).

Furthermore, given that NO-independent stimulators (ie BAY 41-2272) and activators (ie BAY 58-2667) demonstrate therapeutic advantages over traditional nitrovasodilators (ie GTN) and pure NO donors, this thesis aimed to further investigate their vasoprotective actions under conditions of health and disease. Thus, the aim of subsequent chapters were to:

- I) Determine the potential protective role of endothelial-derived NO in limiting the formation and accumulation of oxidized/heme-free sGC by examining the responsiveness of the NO- and heme-independent sGC activator, BAY 58-2667 under both physiological and disease conditions (Chapter 4).
- II) Examine the role of the endothelium in regulating the vasorelaxant responses to sGC stimulator, BAY 41-2272 in hypertension (Chapter 5).

- III) Examine the cardio- and vaso-protective effects of chronic BAY 58-2667 and BAY 41-2272 treatment in an advanced model of hypertensive heart disease. Additionally, the potential for either compound to elicit self tolerance and/or cross tolerance following chronic administration was also assessed (Chapter 6).

- IV) Elucidate the contribution of Nox2-containing NADPH oxidase in influencing the pools of oxidised/heme-free sGC and thereby modulating vasorelaxant responses to BAY 58-2667 in a model of short-term *in vitro* oxidative stress and chronic disease (Chapter 7).



CHAPTER 2

General Methods



2. GENERAL METHODS

2.1 Animals

All experimental procedures performed in these studies were conducted in accordance with the Pharmacology Animal Ethics Committee, Monash University, Australia.

2.1.1 Mice

2.1.1.1 C57BL/6J mice

Adult male C57BL/6J mice (10-14 weeks of age, n = 136) were obtained from the Monash Animal Services (MAS), Monash University and housed in the School of Biomedical Sciences (SOBS) Rodent Facility or Animal Research Laboratory (ARL), Monash University. Mice were maintained in conventional cages on a 12 hour day/night cycle where standard chow and water was available *ad libitum*. In order to examine the effects of chronic NO synthase (NOS) inhibition, a subgroup of these C57BL/6J mice were treated chronically with the NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME, 100 mg/kg/day for 28 days, n = 4). L-NAME was dissolved in and administered via the drinking water, which was changed twice weekly, for a period of 28 days. Vehicle-treated animals (n = 4) were permitted access to normal drinking water throughout the 28 day period. Throughout the duration of the treatment period, C57BL/6J control and L-NAME-treated mice were housed in the SOBS Rodent Facility under the aforementioned conditions. All procedures conducted utilizing C57BL/6J mice were approved by the Pharmacology Animal Ethics Committee, Monash University, Australia in accordance with approval PHAR2005/25 and SOBSB/PHAR/2007/44 (Chapters 3 and 7). Additionally, all experimental procedures conducted with the L-NAME-treated C57BL/6J mice and their corresponding C57BL/6J controls were in accordance with approval SOBSB/2006/36 (Chapter 4).

2.1.1.2 Knockout mice colonies (*eNOS*^{-/-}, *Nox2*^{-/-} and *ApoE*^{-/-})

Male C57BL/6J (10-14 weeks of age, n = 5) and their aged-matched genetically engineered *eNOS*^{-/-} mice (n = 6) were obtained from MAS and Mouseworks, Monash University respectively. All experimental procedures conducted utilizing these animals were in accordance with approvals SOBSB/2006/36 (Chapter 4).

Male mice deficient in Nox2-containing NADPH oxidase (*Nox2*^{-/-}, 10-12 weeks of age, n = 12) were obtained from the Cybb/*ApoE* breeding colony at Mouseworks, Monash University. Similarly, male B6 apolipoprotein E deficient (*ApoE*^{-/-}; 12 weeks of age, n = 11), *Nox2*^{-/-}/*ApoE*^{-/-} (12 weeks of age, n = 10) and their age-matched C57BL/6J (n = 9) animals were also obtained from the same colony, however these mice were weaned at 3 weeks of age and at such time were given access to standard rodent chow and water. From 5 weeks of age, this diet was substituted with a Western-style high fat diet (HFD: 21% fat, 0.15% cholesterol, SFOO-219, Speciality Feeds, Australia) for a period of 7 weeks (Chapter 7). All procedures conducted utilizing these animals were approved by the Pharmacology Animal Ethics Committee, Monash University, Australia in accordance with approvals SOBSB/PHAR/2008/22 (Chapter 3 and 7).

All mice were bred and housed in conventional mouse cages on a 12 hour day/night cycle and, where a speciality diet was not part of the treatment, standard rodent chow and water was available *ad libitum*. Upon use, all mice were killed humanely with isoflurane (2-4% in O₂, Isorrane, Baxter HealthCare Pty Ltd) followed by exsanguination.

2.1.2 Rats

Adult male normotensive Wistar-Kyoto (WKY; 15-17 weeks of age, n = 87) and spontaneously hypertensive (SHR; 15-17 weeks of age, n = 57) in addition to aged WKY (20-22 months of age, n = 10) and SHR (20-22 months of age, n = 40) rats were obtained from the Animals Resources Centre (ARC), Western Australia. Adult male Sprague-Dawley rats (SD; 12-16 weeks of age, n = 7) were obtained from MAS. All rats were housed in the Department of Pharmacology Animal House, Monash University and maintained on a 12 hour day/night cycle where standard chow and water was available *ad libitum*. All animals were used with the approval of the Pharmacology Animal Ethics Committee, Monash University, Australia in accordance with approvals SOBSB2005/24 and SOBS2005/25 (Chapters 4-6).

Rats were predominantly used for functional blood vessel studies with adult WKY and SHR rats used in Chapters 4 and 5, whilst adult SD rats were utilized in studies conducted in Chapter 4 only. Aged animals (WKY and SHR) were employed in Chapter 6, however, were first chronically treated according to the protocol outlined in section 2.3. Rats were killed humanely via CO₂ inhalation (95% CO₂, 5% O₂) followed promptly by decapitation via a guillotine.

2.1.3 Rabbits

Adult male New Zealand rabbits (Crl:KBL NZW; 1.5 years of age, n = 18) were obtained from Charles River Laboratories, Sulzfeld, Germany and were housed at Bayer Pharma, Wuppertal. All experimental procedures conducted using rabbits conformed to the Guide for the Care and Use of Laboratory Animals published by the national legislation (Deutsches Tierschutzgesetz, May 18th, 2006) and EU directives (86/609), and were approved by the competent regional authority (LANUV NRW, Landesamt für Natur-, Umwelt- und Verbraucherschutz, 45610 Recklinghausen).

Rabbits were utilized in functional blood vessel studies conducted in Chapter 4. Upon use, all rabbits were killed humanely via an overdose with thiopental (100 mg/kg), which was administered intravenously through the ear vein, after which the thoracic aortae and/or saphenous arteries were excised.

2.2 Model of short-term *in vitro* oxidative stress: Ang II-stimulated carotid arteries (Chapter 3 and 7)

The organoid culture technique was utilized to establish a short-term model of oxidative stress in the carotid arteries of mice. The following protocol is relevant to Chapters 3 and 7.

2.2.1 Culture reagents

Dulbecco's Modified Eagle's Medium (500 ml DMEM-low glucose: contains 1000 mg/L glucose, NaHCO₃ and pyridoxine HCl, without L-glutamine, sodium pyruvate or phenol red, D5921, Sigma-Alrich) was used in the dissection and incubation of all vessels. To this stock, 5 ml of penicillin/streptomycin solution (final concentration 0.1%; 5000 U penicillin, 5 mg/ml streptomycin, Sigma-Alrich) and 50 ml of heat inactivated fetal calf serum (final concentration 10%; FBS Qualified 26140-079, ≤ 50 EU/ml Endotoxin level, ≤ 25 mg/dl hemoglobin level, Invitrogen) were added. The Cu²⁺ chelator, ethylenediaminetetraacetic acid (EDTA, Ajax Chemicals) was made up separately to 20 mg/ml in distilled water and then filter sterilized using 0.2 µm filter syringes (Acrodisc filter syringe with 0.2 µm supor membrane, Pall Corporation) into an autoclaved duran bottle and stored at 4°C. All procedures were conducted in a PS2 laminar flow hood (HWS120 Series, CLYBE-APAC Environmental Products Division) and all culture reagents were stored at 4°C until ready for use. On the experimental day, the required quantity of DMEM (containing 0.1% penicillin/streptomycin and 10% fetal calf serum) was transferred into 50 ml sterile falcon tubes (Becton Dickinson) to which EDTA (20 mg/ml) was added to achieve a final concentration of 0.026 mmol/L.

2.2.2 Equipment preparation

Dissecting equipment, glassware as well as distilled water to be used in the organoid culture procedure were autoclaved/sterilised using a Siltex HC2 Bench Autoclave (Siltex Australia Pty Ltd) before use. This equipment was then dried overnight at 60°C in an incubator (Mettler model 400, Medos Company Pty Ltd) to allow for the evaporation of any residual liquid. On the day prior to experimentation, all dissecting and experimental equipment were cleaned initially with 70% ethanol and then exposed to ultra-violet (UV) light overnight in the PS2 laminar flow hood to maintain sterility. On the experimental day, the dissecting equipment was immersed in 100% ethanol for a period of 15-20 minutes after which the ethanol was discarded and the dissecting tools heat sterilized using a naked flame provided by a general multi-purpose lighter (Scripto, USA).

2.2.3 Tissue Isolation

Mice (C57BL/6J and Nox2^{-/-}) were sacrificed via anaesthetic overdose (Isoflurane Isorane, Baxter HealthCare PTY Ltd), submerged in 70% ethanol to maintain sterility and transferred to a laminar flow hood. Subsequently, the neck region was exposed, and the left and right common carotid arteries excised with the aid of a binocular dissecting microscope (Nikon SMZ645). Arteries were placed in a sterilized cell culture dish (100 mm diameter) comprised of filter sterilized ice-cold low glucose DMEM, where they were cleared of connective tissue and adherent fat and cut into ~2mm long segments. Approximately three segments were obtained from each carotid artery providing a total of six carotid segments per mouse. For superoxide anion radical ($\cdot\text{O}_2^-$) detection experiments (outlined in section 2.4), all treatments were carried out in duplicate thus allowing for three different treatments per animal. The abdominal aortae from some mice were also used in preliminary organoid culture and $\cdot\text{O}_2^-$ detection experiments (Supplementary Figure 1, Chapter 3). Each ~2 mm arterial segment was placed in an individual well of a 12-well cell culture plate (Becton Dickinson) containing 1ml DMEM with either the

vehicle (50 nmol/L glacial acetic acid) or angiotensin II (Ang II, 10 nmol/L) added. Details of the treatment groups employed are described in section 2.2.4 below. All drugs were filter sterilized using a 0.2 µm filter syringe prior to addition to the wells. The 12-well cell culture plates containing the carotid vessels were then placed in a CO₂ water-jacketed incubator (Forma Scientific) for a period of 24 hours (37°C, 5% CO₂ and 85% humidity). Following this treatment period, carotid arteries were used for either $\cdot\text{O}_2^-$ detection (refer to section 2.4) or for functional vasorelaxation studies (refer to section 2.6).

2.2.4 Vessel Treatments

2.2.4.1 Effect of Ang II treatment upon vascular $\cdot\text{O}_2^-$ levels and function, and the role of Nox2 in this response (Chapter 3 and 7)

To examine the effect of Ang II treatment upon vascular $\cdot\text{O}_2^-$ levels and function, isolated carotid arteries from C57BL/6J and Nox2^{-/-} mice (Chapter 3 and 7) were treated with either vehicle (50 nmol/L glacial acetic acid) or Ang II (10 nmol/L) for 24 hours under culture conditions as described in 2.2.3. Subsequently, $\cdot\text{O}_2^-$ levels, NO bioavailability and vasodilator responses in these vessels were detected via lucigenin-enhanced chemiluminescence (refer to section 2.4.1) or via a functional assay using small vessel wire myography (refer to section 2.6.2), respectively.

2.2.4.2 Effect of HNO donors upon Ang II-stimulated $\cdot\text{O}_2^-$ generation (Chapter 3)

To determine if HNO donors could suppress the Ang II-mediated (10 nmol/L) increase in vascular $\cdot\text{O}_2^-$ production, isolated carotid arteries from C57BL/6J mice were treated with either vehicle or Ang II in the absence and presence of increasing concentrations of Angeli's salt (1 nmol/L - 1 µmol/L) or isopropylamine NONOate (IPA/NO, 1 µmol/L). These HNO donors were initially administered concomitantly with Ang II. From these experiments, the optimal $\cdot\text{O}_2^-$ suppressing concentration of Angeli's salt was chosen (0.3 µmol/L), which was used for all subsequent experiments. Furthermore, due to their relatively short half-life, Angeli's salt and

IPA/NO were administered 3 times over a 24-hour period at approximately 5 hour intervals. Due to an observed interaction between $\cdot\text{O}_2^-$ production and chronic 0.01 mol/L NaOH treatment in organoid culture (Supplementary Figure 3, Chapter 3), Angeli's salt and IPA/NO were constituted and diluted in sterilized saline (Baxter HealthCare Pty Ltd) immediately prior to administration. Additionally, the carotid arteries from some $\text{Nox}2^{-/-}$ mice were also treated with Ang II in the presence of Angeli's salt.

To elucidate the redox form of NO responsible for the $\cdot\text{O}_2^-$ limiting effects of both Angeli's salt and IPA/NO, the HNO scavenger L-cysteine and the $\text{NO}\cdot$ scavenger carboxy-PTIO were employed. Thus, isolated carotid arteries of C57BL/6J mice were treated with Ang II (10 nmol/L) and either AS (0.3 $\mu\text{mol/L}$) or IPA/NO (1 $\mu\text{mol/L}$) in the absence or presence of L-cysteine (3 mmol/L) or carboxy-PTIO (200 $\mu\text{mol/L}$).

To elucidate the contribution of the sGC/cGMP signaling pathway to the $\cdot\text{O}_2^-$ limiting effects of HNO, the sGC inhibitor, 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxaline-1-one (ODQ) and the protein kinase G (PKG) inhibitor, Rp-8-pCPT-cGMPS were employed. Isolated carotid arteries of C57BL/6J mice were treated with Ang II (10 nmol/L) and Angeli's salt (0.3 $\mu\text{mol/L}$) in the absence and presence of either ODQ (10 $\mu\text{mol/L}$) or Rp-8-pCPT-cGMPS (10 $\mu\text{mol/L}$). Refer to Table 1 for a more concise representation of the treatment groups employed in Chapter 3 and 7, as outlined in sections 2.2.4.1 and 2.2.4.2. Refer to Figure 1A and B for a graphical representation of the 12-well plate layout for the treatment of isolated carotid arteries. Note that an Ang II (10 nmol/L) treatment was always present as an internal control for all treatment combinations.

A subset of carotid segments were also treated with Ang II (10 nmol/L) in the presence of either carboxy-PTIO (200 $\mu\text{mol/L}$), ODQ (10 $\mu\text{mol/L}$) or Rp-8-pCPT-cGMPS (10 $\mu\text{mol/L}$) alone to

eliminate the possibility that these scavengers/inhibitors were suppressing $\cdot\text{O}_2^-$ production independently of HNO in the isolated carotid arteries. All scavengers/inhibitors were administered only once at the commencement of the 24-hour treatment period, and where applicable, concomitantly with the initial HNO donor addition. Refer to Table 1 for a representation of the treatment groups used for this purpose.

2.2.4.3 Effects of Ang II treatment upon Nox2 and p47^{phox} mRNA expression and the NO signaling pathway (RT² Profile PCR Array, Chapter 3)

Prior to commencing experiments, all equipment, dissecting instruments and the laminar flow hood were sprayed with RNaseZap (Ambion's RNaseZap®, Applied Biosystems) to avoid RNA contamination. Thirty minutes later, RNase was washed off using sterilized dH₂O. To further ensure high quality RNA yields, a separate stock bottle of DMEM (0.1% penicillin/streptomycin 10% fetal calf serum, 0.026 mmol/L EDTA) was used for these studies. Under aseptic conditions, the left and right carotid arteries were isolated from C57BL/6J mice as described previously in section 2.2.3. Carotid arteries were divided into 3 segments with a total of 6 segments obtained from each mouse. Due to the low levels of mRNA able to be extracted from these vessels, carotid arteries from 2 animals were pooled. Treatment groups were as follows: (i) vehicle (50 nmol/L glacial acetic acid), (ii) Ang II (10 nmol/L), (iii) Ang II (10 nmol/L) and Angeli's salt (0.3 $\mu\text{mol/L}$) for 24 hours. Refer to Figure 2 for the 12-well plate layout.

Table 1. Treatment groups employed in carotid arteries (Chapters 3 and 7) used in organoid culture experiments. HNO donors were administered 3 times over the 24-hour period at approximately 5 hour intervals.

Group	Treatment	HNO donors	Inhibitors/ Scavengers	Animals (Mice)	Assay	Relevant chapter
1	Vehicle (50 nmol/L glacial acetic acid)	—	—	C57BL/6J and Nox2 ^{-/-}	•O ₂ ⁻ detection & functional	Chapter 3 & 7
2	Ang II (10 nmol/L)	—	—	C57BL/6J and Nox2 ^{-/-}	•O ₂ ⁻ detection & functional	Chapter 3 & 7
3	Ang II (10 nmol/L)	Angeli's salt (1 nmol/L-1 μmol/L)	—	C57BL/6J	•O ₂ ⁻ detection	Chapter 3
4	Ang II (10 nmol/L)	Angeli's salt (0.3 μmol/L)	—	C57BL/6J	•O ₂ ⁻ detection & functional	Chapter 3
5	Ang II (10 nmol/L)	IPA/NO (1 μmol/L)	—	C57BL/6J	•O ₂ ⁻ detection	Chapter 3
6	Ang II (10 nmol/L)	Angeli's salt (0.3 μmol/L)	L-cysteine (3 mmol/L) or carboxy-PTIO (200 μmol/L)	C57BL/6J	•O ₂ ⁻ detection	Chapter 3
7	Ang II (10 nmol/L)	IPA/NO (1 μmol/L)	L-cysteine (3 mmol/L) or carboxy-PTIO (200 μmol/L)	C57BL/6J	•O ₂ ⁻ detection	Chapter 3
8	Ang II (10 nmol/L)	Angeli's salt (0.3 μmol/L)	ODQ (10 μmol/L) or Rp-8-pCPT-cGMPS (10 μmol/L)	C57BL/6J	•O ₂ ⁻ detection	Chapter 3

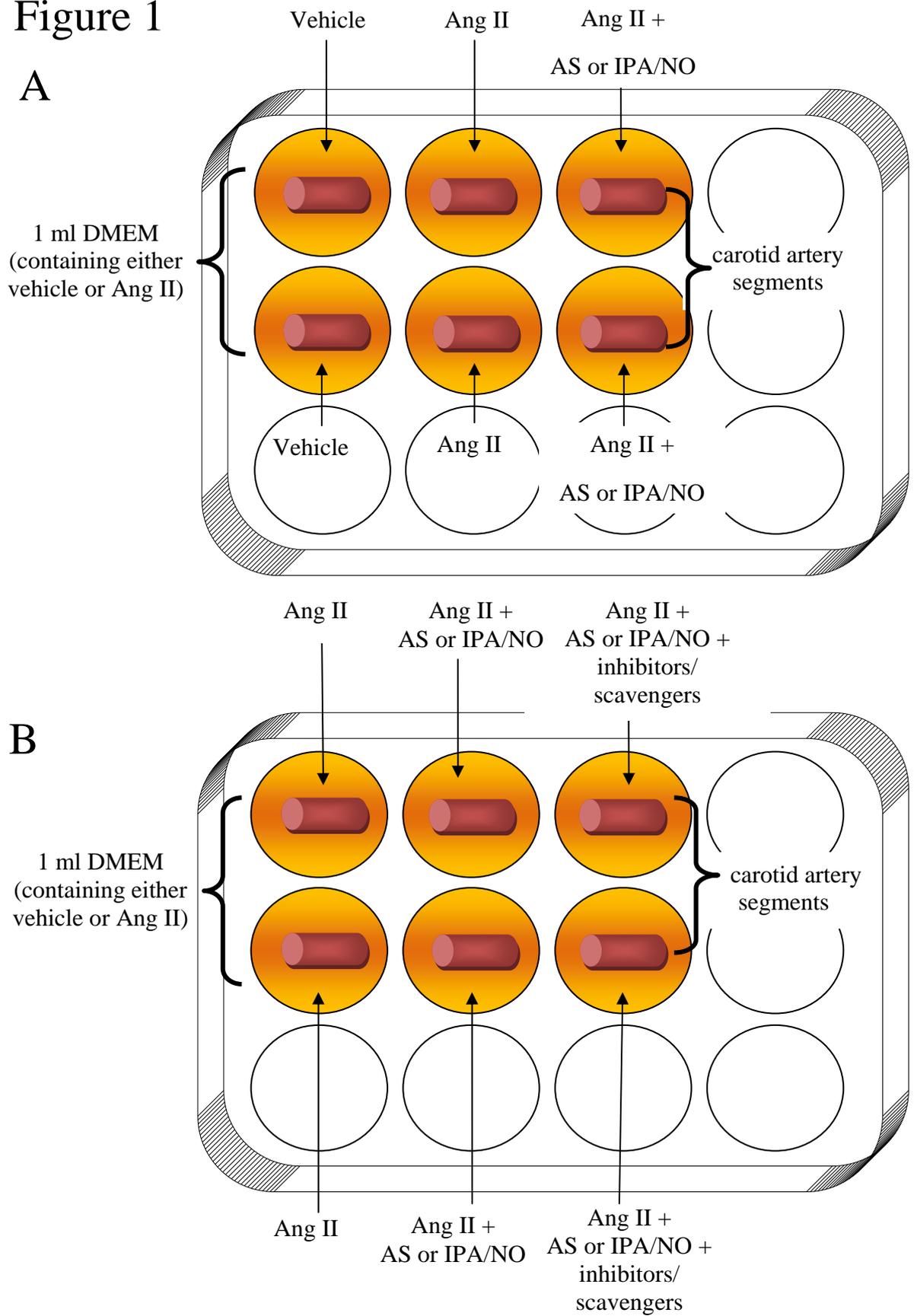
Table 1 continued. Treatment groups employed to ensure that the selected scavengers/inhibitors were not responsible for the depression in $\bullet\text{O}_2^-$ production (Chapter 3)

<i>Group</i>	<i>Treatment</i>	<i>HNO donors</i>	<i>Inhibitors/ Scavengers</i>	<i>Animals (Mice)</i>	<i>Assay</i>	<i>Relevant chapter</i>
1	Ang II (10 nmol/L)	—	carboxy-PTIO (200 $\mu\text{mol/L}$)	C57BL/6J	$\bullet\text{O}_2^-$ detection	Chapter 3
2	Ang II (10 nmol/L)	—	ODQ (10 $\mu\text{mol/L}$)	C57BL/6J	$\bullet\text{O}_2^-$ detection	Chapter 3
3	Ang II (10 nmol/L)	—	Rp-8-pCPT-cGMPS (10 $\mu\text{mol/L}$)	C57BL/6J	$\bullet\text{O}_2^-$ detection	Chapter 3

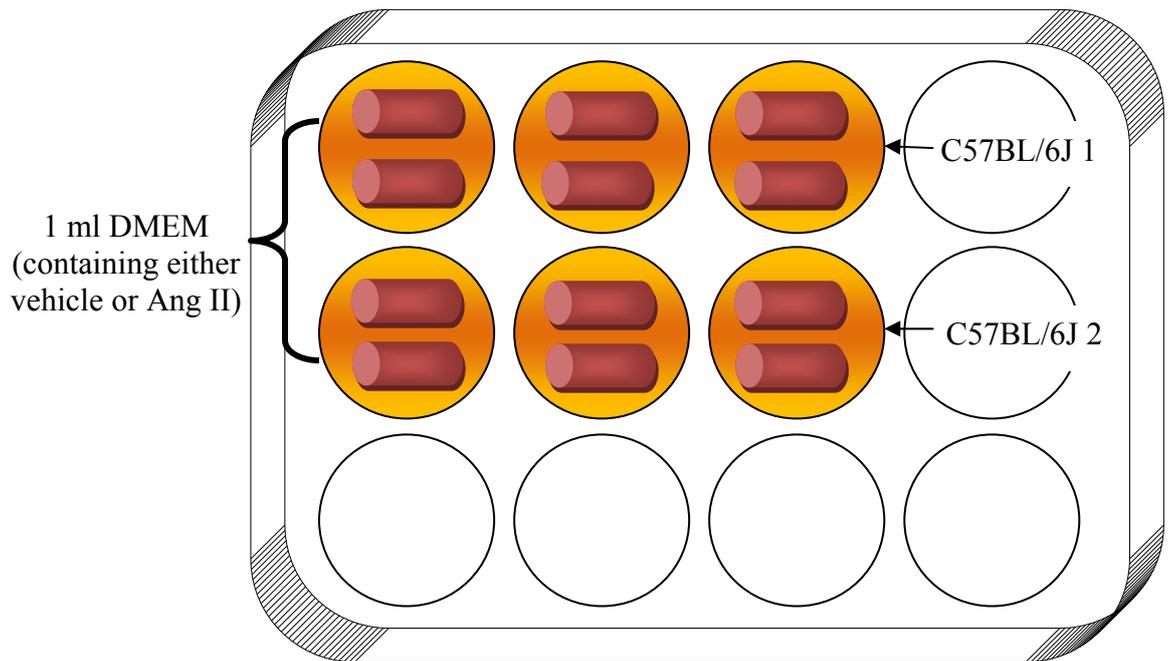
Figure 1. Schematic diagram depicting the 12-well cell culture plate layout for the combinations of treatments employed in Chapters 3 and 7. Note that an Ang II (10 nmol/L) treatment was always performed. Treatment of arteries for $\bullet\text{O}_2^-$ detection (Chapter 3 and 7) and functional studies (Chapter 3) were conducted in duplicates to accommodate for variability whilst one treatment was afforded to each segment for the functional studies conducted in Chapter 7. All treatments were conducted over a 24-hour period, where HNO donors (Angeli's salt, AS; IPA/NO) were replenished x 3 per day at 5 hour intervals. Inhibitors and scavengers were administered only once during that time at the commencement of the 24-hour period and, where applicable, in conjunction with the initial HNO donor addition.

—

Figure 1



(6 x ~2mm long segments obtained per animal, 3 treatment groups)



6 segments per animal, 2 animals pooled for one treatment

Figure 2. Graphical representation of the 12-well cell culture plate layout for one treatment group which incorporates 2 mice for mRNA gene expression experiments (RT² Profile PCR Array). Where appropriate, Angeli's salt was replenished 3 times per day as described previously in section 2.2.4.2.

Following the treatment period, pooled arteries were snap frozen using liquid nitrogen in RNA-free eppendorf tubes after which they underwent a series of RNA extraction methods before finally undergoing gene expression analysis according to the protocols outlined in section 2.7.

2.2.5 Drug dilutions for the organoid culture procedure (Chapter 3 and 7)

Stock solutions of Angiotensin II (Ang II, 10 mmol/L) were made up in 50 mmol/L glacial acetic acid and stored with aliquots of glacial acetic acid (vehicle, 50 mmol/L) alone at -20°C. On the experimental day, both the vehicle and Ang II stocks were thawed and initially diluted 1:1000 in sterilized dH₂O and then subsequently diluted 1:1000 in DMEM (10 µl added to 10ml) to achieve a final vehicle concentration of 50 nmol/L glacial acetic acid and Ang II concentration of

10 nmol/L. DMEM (1 ml) containing either vehicle (50 nmol/L) or Ang II (10 nmol/L) was filter sterilized into the 12-well cell culture plate according to the treatments described in sections 2.2.4.1.-2.2.4.3.

Given Angeli's salt and IPA/NO decompose at physiological pH to liberate HNO, with half-lives of 2-3minutes, they are traditionally constituted and diluted in 0.01 mol/L NaOH to maintain stability. However, due to an observed ability of 0.01 mol/L NaOH alone to suppress Ang II-stimulated $\cdot\text{O}_2^-$ production in carotid arteries (Supplementary Figure 3, Chapter 3), HNO donors were constituted in sterilized saline immediately prior to administration. Experiments were conducted at 37°C at pH 7.

The HNO scavenger, L-cysteine (1 mol/L), the NO \cdot scavenger, carboxy-PTIO (10 mmol/L) and the PKG inhibitor, Rp-8-pCPT-cGMPS (10 mmol/L) were made up in sterilized dH₂O whilst the sGC inhibitor, ODQ (1 mmol/L) was constituted in 100% ethanol. All drugs were filter sterilized using a 0.2 μm filter syringe prior to administration to carotid arteries. Scavengers and inhibitors were administered once only, at the commencement of the 24-hour treatment period.

2.3 Model of chronic oxidative stress: Aged hypertensive animals (Chapter 6)

Male normotensive WKY (20-22 months) and age-matched SHR rats were utilized. Under isoflurane anaesthesia (2-4% in O₂), a midline incision was made, the abdominal aorta exposed and the distal end fitted with a radio telemetry probe (TA11-PAC40, Data Sciences International, St Paul, MN, USA) in order to measure heart rate and blood pressure. Post surgery, the wound site was cleaned and antibiotic powder (cicatracin) applied topically. A single bolus dose of the antibiotic, oxytetracycline (2.5 mg/kg) and the analgesic, buprenorphine (0.1 $\mu\text{g/g}$) were administered intramuscularly. Animals were allowed a 1-week recovery period, during which

time, oxytetracycline was administered via the drinking water. At the completion of this recovery period, animals were placed into one of following five treatment groups (refer to Table 2).

Table 2. Details of treatment, dose and route of administration in aged WKY and SHR rats.

<i>Groups</i>	<i>Animals (Rats)</i>	<i>Treatments</i>	<i>Doses</i>
1	WKY (20-22 months)	Vehicle (PEG400/glycerol/H ₂ O) in 1% DMSO	0.1 ml/100g/day, i.p (Single bolus dose daily)
2	SHR (20-22 months)	Vehicle (PEG400/glycerol/H ₂ O) in 1% DMSO	0.1 ml/100g/day, i.p (Single bolus dose daily)
3	SHR (20-22 months)	Low dose BAY 58-2667	0.3 mg/kg/day, i.p (Single bolus dose daily)
4	SHR (20-22 months)	High dose BAY 58-2667	1 mg/kg/day, i.p (0.5 mg/kg twice daily)
5	SHR (20-22 months)	High dose BAY 41-2272	1 mg/kg/day, i.p (0.5 mg/kg twice daily)

A dose of 1 mg/kg/day BAY 41-2272 and BAY 58-2667 was administered to SHR via twice daily i.p. injections of 0.5 mg/kg, once in the morning and in the afternoon (~8-9 hours between injections) for 4 weeks. Vehicle (0.1 ml/100g) and the low dose BAY 58-2667 (0.3 mg/kg) were administered as a single daily injection in the morning over a 4 week period. Radiotelemetry was utilized to continuously record heart rate and systolic (SBP) and diastolic (DBP) blood pressures throughout both the baseline (1 week recovery period) and the following 4 week treatment period. Sampling was carried out for 10 seconds every 10 minutes, 24 hours a day. Mean arterial blood pressure (MABP) was calculated from SBP and DBP recordings. Daily average values for heart rate, SBP, DBP and MABP were calculated from the entire data set collected over 24 hours for each animal. During both the recovery and treatment period, animals were housed in the Pharmacology Departmental Animal Holding Room, where they were maintained on a standard

chow diet and a 12 hour day/night cycle. At the end of 4 weeks, animals were anaesthetised using isoflurane (2-4% in O₂) and sacrificed via decapitation. The thoracic aortae and heart were removed for subsequent studies of vascular function and ventricular hypertrophy, respectively. Refer to section 2.6 for the general protocol for the functional blood vessel studies. Hearts from treated animals were isolated and weighed and the heart to body weight ratio (HW:BW) expressed as mg/g.

2.3.1 Data analysis for MABP and HW:BW

The effect of drug treatments on MABP over time was assessed by Two-way ANOVA (Graphpad Prism, Version 5) with repeated measures whilst differences in morphometric data between treatment groups were determined using a One-way ANOVA, followed by Bonferroni's multiple comparisons test (Graphpad Prism, Version 5). All results were expressed as mean \pm s.e. mean, with n representing the number of rats. Statistical significance was accepted at the $P < 0.05$ level.

2.4 Superoxide anion radical detection via chemiluminescence probes

2.4.1 Lucigenin-enhanced chemiluminescence (Chapters 3, 4 and 7)

Lucigenin-enhanced chemiluminescence is a widely used technique for measuring $\cdot\text{O}_2^-$ levels in enzymatic systems and vascular preparations (Dikalov *et al.*, 2007). Lucigenin reacts with $\cdot\text{O}_2^-$ to form the lucigenin cation radical via a one electron reduction reaction to yield the unstable intermediate, dioxetane. Dioxetane in turn decomposes to produce 2 molecules of N-methylacridone, one of which in its excited state emits a photon that is then detected by the scintillation counter, thus allowing $\cdot\text{O}_2^-$ levels to be quantified (Dikalov *et al.*, 2007; Faulkner & Fridovich, 1993; Li *et al.*, 1998).

An advantage of lucigenin, over other chemiluminescence probes, is its ability to penetrate the cell, thus allowing it to detect both extracellular and intracellular $\cdot\text{O}_2^-$ (Dikalov *et al.*, 2007). However, it is important to note that lucigenin can undergo redox cycling, potentially resulting in an artificial signal and thus overestimation of $\cdot\text{O}_2^-$ levels (Dikalov *et al.*, 2007; Munzel *et al.*, 2002). Such redox cycling appears to only occur at concentrations of lucigenin $>50 \mu\text{mol/L}$ and limiting the concentration of lucigenin, as employed in this thesis, minimizes such a phenomenon (Dikalov *et al.*, 2007; Li *et al.*, 1998).

2.4.1.1 Lucigenin-enhanced chemiluminescence: Mouse arteries (Chapters 3 and 7)

Common carotid arteries from C57BL/6J and $\text{Nox2}^{-/-}$ mice were excised under sterile conditions and treated using the standard organoid culture technique as outlined in section 2.2. In semi-darkness, carotid arteries (24 hr treatment) were transferred into a 96-well OptiPlate (Packard Bioscience) containing 80 μl of warmed ($\sim 25^\circ\text{C}$) Krebs-HEPES solution (99 mmol/L NaCl, 4.7 mmol/L KCl, 1.0 mmol/L KH_2PO_4 , 1.2 mmol/L CaCl_2 , 25 mmol/L NaHCO_3 , 20 mmol/L Na-HEPES, 11 mmol/L glucose, pH 7.4). Experiments were performed in duplicates. Additionally, 4 separate wells of the 96-well OptiPlate were used as background wells. The 96-well OptiPlate was then placed into the TopCount NXT single photon counter (Perkin Elmer, Wellesly, USA) or Hidex ChameleonTM Luminescence Plate Reader (Hidex Oy, Finland) where an initial equilibration period of 15 cycles at 3 seconds per well at (25°C) was observed with a 2 minute interval between each cycle. Following this equilibration period, the Optiplate was removed from the counter and 10 μl of NADPH (1 mmol/L; final concentration of 100 $\mu\text{mol/L}$) and 10 μl lucigenin (50 $\mu\text{mol/L}$; final concentration of 5 $\mu\text{mol/L}$) were added to each well under semi-darkness. Figure 3 below demonstrates the general 96-well OptiPlate layout for $\cdot\text{O}_2^-$ detection assays for a single animal.

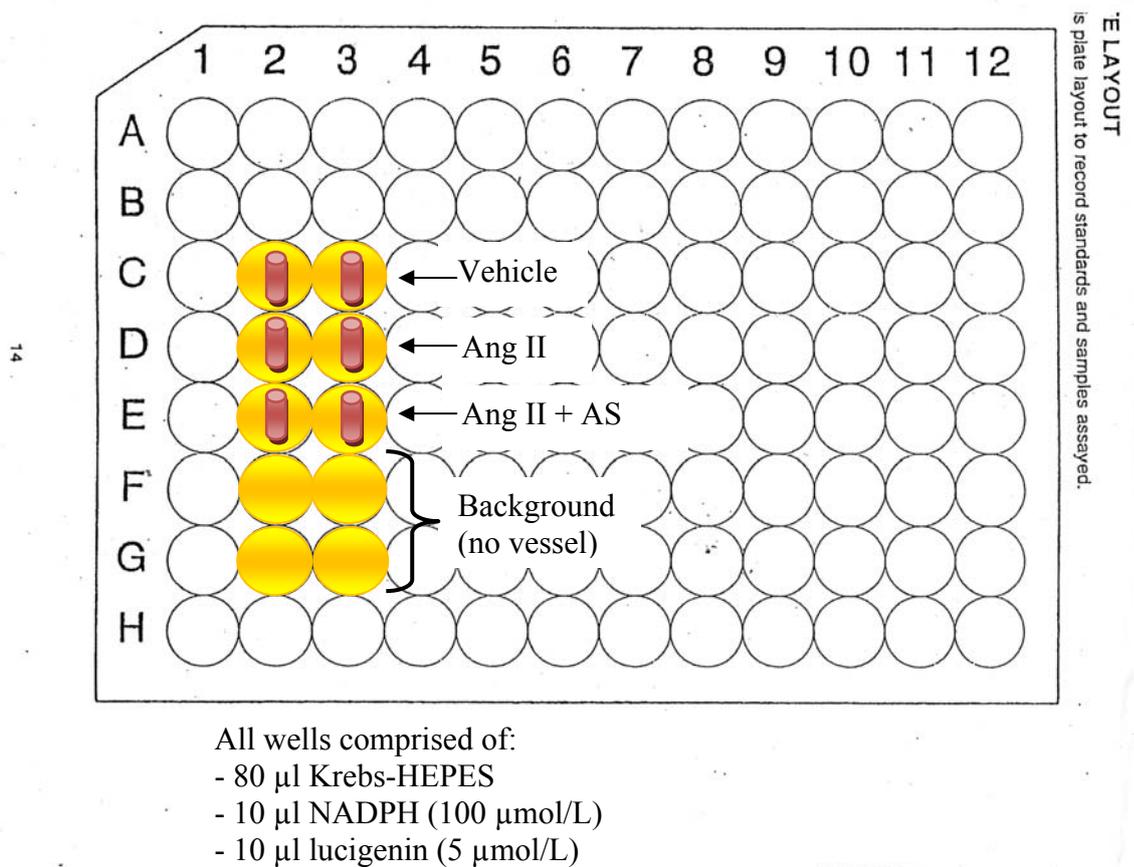


Figure 3. Illustrates an example of the general 96-well OptiPlate setup for each animal used. Treatment groups were constructed in duplicates and the average of 4 x vessel-free wells served as background wells.

The OptiPlate was placed back into the TopCount counter or the Hidex plate reader, where $^{\bullet}\text{O}_2^-$ generation was measured for a further 25 cycles at 3 seconds per well (2 minute interval between each cycle). Additionally, $^{\bullet}\text{O}_2^-$ generation was also measured and compared in freshly isolated carotid arteries from C57BL/6J mice on normal (ND) and high fat diet (HFD, 7 weeks), and ApoE^{-/-} and Nox2^{-/-}/ApoE^{-/-} on HFD (7 weeks) according to the same protocol described above. Due to circumstances beyond our control, $^{\bullet}\text{O}_2^-$ generation in carotid arteries of Nox2^{-/-} (Chapter 3 and 7), C57BL/6J (ND and HFD), ApoE^{-/-} (HFD) and Nox2^{-/-}/ApoE^{-/-} (HFD) mice (Chapter 7) were measured using the Hidex luminescence plate reader. All other $^{\bullet}\text{O}_2^-$ detection experiments in mice were performed using the TopCount single photon counter.

2.4.1.2 Lucigenin-enhanced chemiluminescence: Rat thoracic aortae (Chapter 4)

In order to validate the occurrence of enhanced oxidative stress in the vascular wall of SHR, thoracic aortae were freshly harvested from WKY and SHR rats (sacrificed as described in section 2.1.2) and placed in ice-cold Krebs-HEPES solution. Aortae were cleared of adherent fat and connective tissue and two, 5 mm long ring segments per animal were cut and pre-incubated in Krebs-HEPES buffer solution for 30 minutes at 37°C. Aortic rings were then transferred into a 96-well OptiPlate with each well containing 300 µl of Krebs-HEPES solution containing 5 µmol/L lucigenin. The plate was then placed in the Hidex Chameleon™ Luminescence Plate Reader (Hidex Oy, Finland), where, as described previously in section 2.4.1.1, photon emission per well was recorded.

2.4.2 L-012 enhanced chemiluminescence (Preliminary experiments in Chapter 3, Chapter 6)

L-012 enhanced chemiluminescence is a modified form of luminol which detects vascular $\cdot\text{O}_2^-$ in a similar manner to lucigenin, whereby the luminol reacts with $\cdot\text{O}_2^-$ to form an unstable endoperoxide or dioxetane which in turn decomposes resulting in an electronically excited product (Dikalov *et al.*, 2007; Faulkner & Fridovich, 1993). The light emission produced by L-012, however is reported to be greater than lucigenin and although L-012 has been proposed to be quite specific for extracellular $\cdot\text{O}_2^-$, it may also detect ONOO⁻ as well as other forms of ROS (Dikalov *et al.*, 2007). Furthermore, in contrast to lucigenin, L-012 has not been reported to undergo redox cycling however upon examination of its structure, there are suggestions that it has the potential to do so (Dikalov *et al.*, 2007).

In preliminary experiments to confirm that 24-hour treatment of isolated arteries with Ang II results in increased vascular $\cdot\text{O}_2^-$ generation, L-012 chemiluminescence assay was employed (Chapter 3). Similar to the lucigenin protocol, common carotid arteries and aortae from C57BL/6J mice, following 24-hour vehicle or Ang II treatment, were transferred into a 96-well

OptiPlate containing 90 μl of Krebs-HEPES solution. The same cycle parameters and temperature specifications as described in the lucigenin chemiluminescence protocol, outlined in section 2.4.1.1, were used. All readings using L-012 were conducted using the Hidex ChameleonTM Luminescence Plate Reader and following the initial equilibration period, 10 μl of L-012 (final concentration: 100 $\mu\text{mol/L}$) was added to each well for the detection of $\cdot\text{O}_2^-$. Measurements were made over the ensuing 25 cycles, 3 seconds per well, with a 2 minute interval between each cycle.

The use of L-012 for $\cdot\text{O}_2^-$ detection, however, was discontinued due to an unexpected, non-specific interaction between L-012 and the HNO donors, Angeli's salt and IPA/NO. These HNO donors caused an increase in L-012 chemiluminescence, an effect which was also apparent in a cell-free assay. This phenomenon was not observed with lucigenin. Thus in Chapter 3, L-012 was only utilized in the preliminary experiments after which all subsequent experiments were conducted using lucigenin.

Similarly, vascular $\cdot\text{O}_2^-$ production was measured in the thoracic aortae from vehicle-treated aged WKY and aged SHR treated with either vehicle, BAY 41-2272 (1 mg/kg/day) or BAY 58-2667 (0.3 or 1 mg/kg/day) using L-012-enhanced chemiluminescence, in order to validate the occurrence of enhanced oxidative stress in aged SHR, and the potential $\cdot\text{O}_2^-$ suppressing effects of chronic BAY 41-2272 and BAY 58-2667 treatment (Chapter 6). Utilising the same cycle parameters and temperature specifications as outlined above, aortic rings were placed into a well of a 96-well OptiPlate, each containing 300 μl of Krebs-HEPES which consisted of 100 $\mu\text{mol/L}$ L-012. $\cdot\text{O}_2^-$ production was detected using the Hidex ChameleonTM Luminescence Plate Reader (Hidex Oy, Finland), as described above and previously in section 2.4.1.1,

2.4.3 Data Analysis for chemiluminescence assays

Photon release upon exposure to $\cdot\text{O}_2^-$ was measured using values from the last 5 cycles of the 25 cycle count. In four designated wells with no tissue present, background lucigenin or L-012 chemiluminescence were also measured and averaged over the last 5 cycles. These values were subtracted from the total chemiluminescence generated by the carotid or aortic segments to quantify the $\cdot\text{O}_2^-$ produced by the tissue itself. Following $\cdot\text{O}_2^-$ detection, either via lucigenin or L-012 chemiluminescence, arteries were removed from the 96-well Optiplate and transferred onto pre-cut foil pieces, where they were oven dried in an incubator (Scientific Equipment Manufacturers Pty Ltd; SEM) for 24 hours at 37°C (mice arteries, Chapters 3 and 7) or for 3 days at 65°C (rat thoracic aortae, Chapter 4). Arteries were then weighed following this drying period using a microbalance (Mettler-Toledo International Inc.), and the subsequent results were expressed as 10^3 counts/mg of dry tissue weight. A student's unpaired t-test was used when comparing values between two different treatment groups, whilst a One-way ANOVA followed by a Bonferroni's multiple comparisons test was employed when comparing between three or more different groups (Graphpad Prism, Version 5).

2.4.4 Drug dilutions for chemiluminescence protocol

Lucigenin (Bis-n-methylacridinium nitrate, Sigma) was prepared fresh on the day of the experiment and constituted as a stock solution of 10 mmol/L in Krebs-HEPES, after which it was further diluted in Krebs-HEPES to a working solution of 50 $\mu\text{mol/L}$. NADPH (Nicotinamide Adenine Dinucleotided Phosphate) was also made up fresh on the day of experiment to 10 mmol/L in dH_2O and further diluted in dH_2O to a working solution of 1 mmol/L. When required, 10 μl of the working solution of both lucigenin (50 $\mu\text{mol/L}$) and NADPH (1 mmol/L) was added to 80 μl of Krebs-HEPES (in the 96-well OptiPlate) to achieve a final concentration of 5 $\mu\text{mol/L}$ for lucigenin and 100 $\mu\text{mol/L}$ for NADPH. The outlined drug dilutions above are in relation to the lucigenin experiments conducted in mice. These volumes were adjusted accordingly for the

experiments conducted in rats such that a final concentration of 5 $\mu\text{mol/L}$ of lucigenin was contained in 300 μl of Krebs-HEPES solution.

The L-012 stock (100 mmol/L, Wako Chemicals) was made up in 100% dimethyl sulfoxide (DMSO) and stored as 10-20 μl aliquots at -20°C . On the day of the experiment, an aliquot of L-012 was thawed and diluted in Krebs-HEPES to achieve a working solution of 1 mmol/L. When required, 10 μl of this working solution was added to 90 μl of Krebs-HEPES (in the 96-well OptiPlate) to achieve a final L-012 concentration of 100 $\mu\text{mol/L}$. As all of the aforementioned compounds were light sensitive, all weighing, dilutions and administration of these drugs throughout the experimental period were conducted in semi-darkness.

2.5 Tail-cuff systolic blood pressure measurements (SBP, Chapter 4)

Systolic blood pressures (SBP) were measured and compared between WKY and SHR rats via the tail-cuff method as previously described (Widdop & Li, 1997). Initially, rats were exposed to the tail-cuff method over several days in order to acclimatize the animals to the procedure. Prior to tail cuffing, and throughout the duration of the procedure, rats were externally heated with a 100W light bulb. Rats were restrained in a Perspex holder with an inflatable cuff positioned around the proximal end of the tail. The pressure applied by the inflated cuff was measured via the MacLab-8 data acquisition system through a pressure transducer (Gould P23, Oxnard, CA, USA) connected to a MacLab bridge amp. Furthermore, a piezo-electric transducer (MLT1010, ADInstruments Pty Ltd, Sydney, Australia) was strapped to the ventral surface of the tail, distal to the cuff to detect a pulsatile signal from the caudal artery. Once a pulse was detected, the cuff would inflate subsequently restricting the flow and eliminating the pulse. The pressure cuff was then slowly released until the pulse was detected again. Thus, the corresponding pressure at which the pulse signal first returns following the occlusion period was taken as the SBP. To

compensate for variability, an average of at least 5 recordings were taken from each rat to provide a single SBP value.

2.6 Functional Blood Vessel Studies

2.6.1 Organ baths

2.6.1.1 Organ bath studies: General protocol for rat arteries (Chapters 4-6)

Tissue isolation, general setup and equilibration of vessels for functional organ bath experiments were similar across all studies. Rats were sacrificed as described in section 2.1.2. Following isolation, the thoracic aortae of rats were placed in ice-cold oxygenated Krebs' solution (composition in mmol/L: NaCl 119, KCl 4.7, MgSO₄ 1.17, NaHCO₃ 25, KH₂PO₄ 1.18, CaCl₂ 2.5, glucose 5.5, EDTA 0.026, pH 7.4), cleared of fat and connective tissue and cut into 5 mm long ring segments. Aortic segments were then mounted in 10 ml jacketed organ baths to measure isometric tension which was displayed on a MacLab data acquisition device (AD Instruments, Australia). Vessels were maintained in Krebs' solution at 37°C and bubbled continuously with carbogen (95% O₂, 5% CO₂). The endothelium was deliberately removed from some of the aortic segments by gently rolling the segment around the wire hooks. The absence of the endothelium was confirmed by a loss in response to the endothelium-dependent vasodilator, acetylcholine (ACh, 10 µmol/L).

Vessels were allowed a 30 minute equilibration period before passive baseline tension was set at 2g and left to equilibrate for a further 30 minutes. Following this period, aortae were maximally contracted (F_{max}) using a high potassium solution (KPSS; composition: 123 mmol/L KCl, 1.17 mmol/L MgSO₄, 2.37 mmol/L KH₂PO₄, 2.5 mmol/L CaCl₂, 5.5 mmol/L glucose, 0.026 mmol/L EDTA). Once the KPSS-induced contraction reached a plateau, the arteries were washed and allowed to relax to baseline over a 30-40 minute period.

Arteries were maintained at baseline tension for a 30 minute period, after which they were pre-contracted once more to ~50% F_{\max} with a combination of U46619 (2 nmol/L) and titrated concentrations of cirazoline (10 nmol/L - 1 μ mol/L), as U46619 was found to stabilise the contractions to cirazoline. Once a stable plateau was achieved, cumulative concentration-response curves to selected vasodilators were constructed (refer to sections 2.6.1.3 - 2.6.1.5 for the individual protocols relevant to each chapter). At the completion of each concentration-response curve, maximal relaxation was established by the addition of the endothelium-independent NO donor, sodium nitroprusside (SNP, 10 μ mol/L) followed by the β -adrenoceptor agonist, isoprenaline (1 μ mol/L). All inhibitors/scavengers used were administered for a 30 minute period prior to pre-contraction with U46619 and cirazoline. Refer to Figure 4 for a graphical representation of the protocol.

BAY 58-2667 was found to have a slow time course of action such that approximately 10-15 minutes was required for each concentration to reach a stable plateau, whilst BAY 41-2272 required up to ~5 minutes for each concentration to achieve a stable plateau.

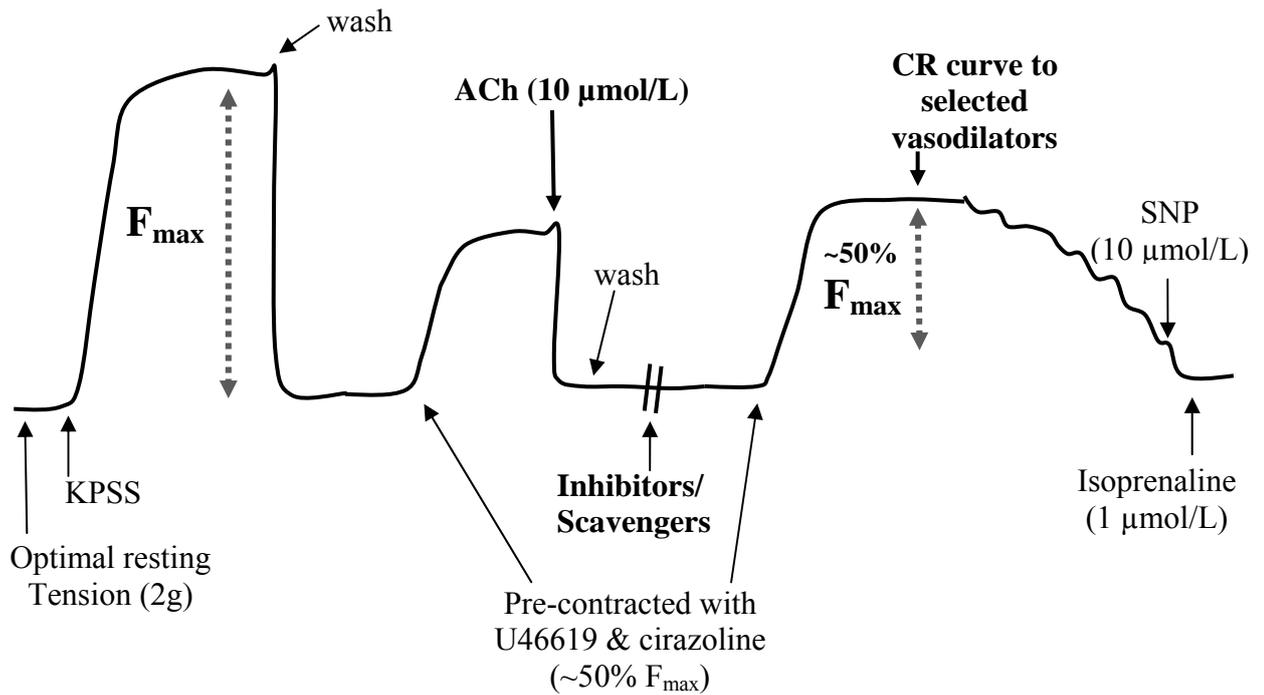


Figure 4. Schematic representation of the standard organ bath protocol employed in functional experiments conducted in chapters 4 - 6 in rat isolated thoracic aortae. A 30 minute equilibration period was observed after each wash and all inhibitors/scavengers were administered 30 minutes prior to pre-contraction.

2.6.1.2 Organ bath studies: General protocol for rabbit arteries (Chapter 4)

Rabbits were sacrificed as described in section 2.1.3. Rabbit thoracic aortae and saphenous arteries were isolated and cleared of adherent fat and sectioned into 3mm wide segments. These arteries were then suspended in 5ml organ baths containing Krebs-Henseleit solution (composition in mmol/L: NaCl 118, KCl 4.7, CaCl₂ 2.5, MgCl₂ 1.2, NaHCO₃ 25, KH₂PO₄ 1.2, glucose 5.5 and EDTA-Ca-salt 25 $\mu\text{mol/L}$ 0.01% BSA, pH 7.4) at 37°C where isometric tension was measured using Statham UC2 strain gauges connected to a DAS1802HC data acquisition board (Keithley Instruments, Germering, Germany). Rings were pre-contracted with a submaximal concentration of phenylephrine or KCl four times with each contraction followed by a series of 16 washing cycles and a resting period of 28 minutes. BAY 58-2667 was administered to the organ baths following the fourth/last pre-contraction, where its concentration was increased by a factor of 10 every 25-30 minutes. Any inhibitors used were added prior to the

fourth contraction. The value of the contraction before the addition of BAY 58-2667 was set at 100%.

2.6.1.3 Organ bath studies: Endogenous NO limits the formation of vascular oxidized/heme-free sGC which is lost in the setting of hypertension. (Chapter 4)

Adult male WKY and SHR rats as well as adult male NZW rabbits were used in this study. Additionally, mice and SD rats were also employed and the functional protocol in relation to those experiments are described in section 2.6.2 which addresses the standard protocol for small vessel wire myography.

The effects of endothelial denudation, NOS (L-NAME, 100 $\mu\text{mol/L}$) and sGC (ODQ, 10 $\mu\text{mol/L}$) inhibition were assessed on the vasorelaxation responses to (i) the heme-independent sGC activator, BAY 58-2667, (ii) the NO donor, DEA/NO, (iii) the cGMP analogue 8-bromo-cGMP and (iv) the sGC-independent vasodilator, papaverine in the aortae of both WKY and SHR. Additionally, the effects of NOS inhibition (L-NAME) were assessed in the aortae and saphenous arteries of rabbits. Thus, following tissue isolation and the protocol outlined in sections 2.6.1.1 (rats) and 2.6.1.2 (rabbits), cumulative concentration-responses to the aforementioned vasodilators were constructed in arteries contracted to $\sim 50\% F_{\text{max}}$. L-NAME and ODQ were administered to the bath 30 minutes prior to pre-contraction with either U46619 and cirazoline (rats) or phenylephrine (rabbits). Refer to Table 3 and Figure 4 for an outline and the general schematic diagram respectively of the experimental protocol relating to the experiments performed in Chapter 4.

Table 3. Depicts the animals used (rats or rabbits), vessel type, endothelial status (+E or -E), inhibitors, vasodilators and the associated concentrations employed in this study. Refer to Figure 4 for the graphical representation of the general protocol involved. Note that endothelial integrity was only assessed in the aortic segments which had been manually denuded.

<i>Animal</i>	<i>Strain</i>	<i>Arteries</i> (+ E/ -E)	<i>Treatment</i>	<i>Vasodilators</i>
		+ E		
Rat	WKY	Aortae	Absence or presence	<i>BAY 58-2667</i>
Rat	SHR	Aortae	of L-NAME	
Rat	SD	Mesenteric arteries	(100 µmol/L)	
Rabbit	NZW	Aortae		
Rabbit	NZW	Saphenous arteries		
		- E		
Rat	WKY	Aortae	Absence or presence	<i>BAY 58-2667</i>
Rat	SHR	Aortae	of ODQ (10 µmol/L)	
		+ E		
Rat	WKY	Aortae	Absence or presence	<i>DEA/NO</i>
			of L-NAME	<i>8-bromo-cGMP</i>
			(100 µmol/L)	<i>Papaverine</i>
		-E		
Rat	WKY	Aortae	Absence or presence	<i>DEA/NO</i>
			of ODQ (10 µmol/L)	<i>8-bromo-cGMP</i>
				<i>Papaverine</i>
		+ E		<i>DEA/NO</i>
Rat	SHR	Aortae	—————	<i>8-bromo-cGMP</i>
				<i>Papaverine</i>

The following concentration range was used for the aforementioned vasodilators in Table 3: (i) BAY58-2667 (10 fmol/L-1 µmol/L), (ii) DEA/NO (0.1 nmol/L - 10 µmol/L), (iii) 8-bromo-cGMP (1 µmol/L-0.3 mmol/L) and (iv) papaverine (10 nmol/L - 10 µmol/L).

2.6.1.4 Organ bath studies: Role of the endothelium in regulating vasorelaxant responses to the sGC stimulator, BAY 41-2272 in hypertension (Chapter 5).

Adult male WKY and SHR were used in this study. The effects of endothelial denudation on cumulative concentration-responses to the (i) NO donor, DEA/NO, (ii) NO-independent and heme-dependent sGC stimulator, BAY 41-2272, (iii) cGMP analogue, 8-bromo-cGMP and (iv) sGC-independent vasodilator, papaverine were investigated in endothelium-intact and -denuded aortae from WKY and SHR rats. Additionally, the effects of sGC inhibition (ODQ, 10 $\mu\text{mol/L}$) in endothelium-denuded aortae, as well as NO (L-NAME, 100 $\mu\text{mol/L}$ and carboxy-PTIO, 200 $\mu\text{mol/L}$) or cyclo-oxygenase (3 mmol/L) inhibition in endothelium-intact aortae on the BAY 41-2272-induced relaxation were examined. Thus, following tissue isolation and the protocol outlined in section 2.6.1.1, cumulative concentration-responses to the aforementioned vasodilators were constructed in arteries contracted to $\sim 50\% F_{\text{max}}$. All inhibitors and scavengers were administered and allowed to incubate for 30 minutes prior to pre-contraction. Refer to Table 4 below for the protocol employed in this study and Figure 4 for the graphical representation of the general functional organ bath protocol.

Table 4. Depicts the animals, vessels, endothelial integrity (+E or -E) and the concentration - response curves performed using these vasodilators.

<i>Animal</i>	<i>Strain</i>	<i>Arteries</i> (+ E/ -E)	<i>Treatment</i>	<i>Vasodilators</i>
Rat	WKY	+ E & -E Aortae	—	<i>DEA/NO</i>
Rat	SHR	Aortae	—	(0.1 nmol/L – 10 µmol/L) <i>BAY 41-2272</i> (0.01 nmol/L – 10 µmol/L) <i>8-bromo-cGMP</i> (1 µmol/L – 0.3 µmol/L) <i>Papaverine</i> (10 nmol/L – 10 µmol/L)
Rat	WKY	-E Aortae	ODQ	<i>BAY 41-227</i>
Rat	SHR	Aortae	(10 µmol/L)	(0.01 nmol/L – 10 µmol/L)
Rat	WKY	+ E Aortae	Co-treatment	<i>BAY 41-2272</i>
Rat	SHR	Aortae	L-NAME (100 µmol/L) & Carboxy-PTIO (200 µmol/L)	(0.01 nmol/L – 10 µmol/L)
Rat	WKY	+ E Aortae	Indomethacin	<i>BAY 41-2272</i>
Rat	SHR	Aortae	(3 mmol/L)	(0.01 nmol/L – 10 µmol/L)

2.6.1.5 Organ bath studies: Cardio- and vaso-protective effects of the sGC stimulator, BAY 41-2272 and sGC activator, BAY 58-2667 in a model of hypertensive heart disease (Chapter 6)

This study investigated the potential cardio- and vaso-protective effects of chronic treatment with the NO-independent sGC stimulator (BAY 41-2272) or activator (BAY 58-2667) in an advanced model of hypertension. Aged WKY and SHR (20-22 months of age) were used in this study. Rats were administered either vehicle, BAY 41-2272 or BAY 58-2667 over a 4 week period (according to section 2.3), after which they were sacrificed (according to section 2.1.2) with all functional experiments being conducted in the thoracic aortae.

Aortic segments were isolated, mounted in organ baths and underwent the general experimental protocol outlined in section 2.6.1.1. All segments were left with the endothelium-intact and pre-contracted to ~50% F_{max} with U46619 and cirazoline. Subsequently, cumulative concentration-response curves to (i) DEA/NO (0.1 nmol/L – 10 μ mol/L), (ii) BAY 41-2272 (0.01 nmol/L – 10 μ mol/L), (iii) BAY 58-2667 (10 fmol/L – 1 μ mol/L), (iv) 8-bromo-cGMP (1 μ mol/L – 0.3 mmol/L), (v) papaverine (10 nmol/L – 10 μ mol/L), (vi) the phosphodiesterase V inhibitor, zaprinast (0.1 μ mol/L - 100 μ mol/L), (vii) ACh (1 nmol/L – 10 μ mol/L) and (viii) the vasoconstrictor, serotonin (5HT; 1 nmol/L – 10 μ mol/L) were constructed in the aortae of aged normotensive and hypertensive rats.

2.6.2 Small vessel wire myography

2.6.2.1 Small vessel wire myography (Chapters 3,4 & 7)

Mice and rats were killed as described in sections 2.1.1 and 2.1.2 respectively. Mouse abdominal aortae and carotid arteries and rat small mesenteric arteries, corresponding to the second order branch of the superior mesenteric artery, were excised, cleared of adherent fat and connective tissue and divided into ~2-3 mm long segments. Following isolation, where no prior treatment was required, mouse aortic and carotid segments and rat mesenteric segments were placed in ice-

cold Krebs's solution, bubbled continuously with carbogen (95% O₂, 5% CO₂) in individual 7ml myograph chambers and allowed to gradually warm to 37°C (~30 minutes) before mounting. Carotid arteries from C57BL/6J and Nox2^{-/-} mice, which underwent short-term (24 hour) *in vitro* treatment via organoid culture (section 2.2, Chapters 3 and 7), were transferred into a 7ml myograph chamber containing warm Krebs's solution at the end of the 24 hour treatment period and subsequently mounted.

Abdominal aortic segments (mouse) were mounted onto two stainless steel wire calipers (100 µm diameter, Figure 5, right panel), whilst each carotid (mouse) or mesenteric (rat) artery segment was setup between the mounting jaws by inserting two 40 µm stainless steel wires through the lumen of the vessel (Figure 5, left panel). One caliper/jaw (fixed end) was attached to an isometric force transducer (610M Multi-myograph, Denmark) and the other (adjustable end) to a micrometer. Mouse aortic and carotid segments were left to equilibrate for 30 minutes under zero tension, after which the vessels were stretched in a stepwise manner to achieve a final and optimal resting tension of 5mN. Changes in isometric tension were recorded using either a MacLab (MacLab ® data-acquisition system, AD Instruments, Pty.Ltd. interfaced with a Macintosh computer) or a four channel chart recorder (Model 3721, Yokogawa, Japan).

Following the 30-minute equilibration period at optimal resting tension, aortae and carotid vessels were maximally contracted (F_{max}) with U46619 (1 µmol/L). Once a plateau was reached, tissues were washed with warmed Krebs' solution and allowed to return to baseline tension (~30-40 minutes). Aortae (C57BL/6J, L-NAME, eNOS^{-/-}) and carotid arteries (C57BL/6J, Nox2^{-/-}, ApoE^{-/-}, Nox2^{-/-}/ApoE^{-/-}) were then pre-contracted to ~50% of the initial U46619 maximum (50% F_{max}) contraction using titrated concentrations of U46619 (0.1 nmol/L - 10 nmol/L). When a plateau was achieved, the integrity of the endothelium was tested in selected vessels via the addition of a bolus concentration of ACh (10 µmol/L). Following this response, vessels were

washed thoroughly with warm Krebs' solution and allowed to return to baseline over a subsequent 30-minute period. The general protocol is similar to that for the organ bath studies (Figure 4).

Rat mesenteric arteries were also allowed to equilibrate for a 30-minute period at zero force after which the internal diameter was normalized to an equivalent transmural pressure of 100 mmHg (D_{100} ; McPherson, 1992) or set to a resting tension of 3 mN. Changes in isometric tension were recorded using the CVMS data acquisition system (version 2.0, World Precision Instruments, USA) in addition to a hard copy obtained using a Panasonic KX-P3200 printer (USA).

Maximal contraction in the mesenteric arteries of rats was achieved using the high potassium depolarizing solution (KPSS) and once a plateau was established, vessels were washed and allowed to return to baseline tension. Following the 30 minute equilibration period, mesenteric arteries were contracted twice with U46619 (1 $\mu\text{mol/L}$), each contraction followed by a thorough washout period with warm Krebs' solution and a resting period of 30 minutes. This process of repeating the U46619 contractions was found to stabilise subsequent contractile responses and minimize the loss of tone whilst examining the slow vasorelaxant responses to BAY 58-2667. Similar to the rat aortae (refer to section 2.6.1.1), mesenteric arteries were pre-contracted with a combination of U46619 (2 nmol/L) and cirazoline (10 nmol/L - 1 $\mu\text{mol/L}$).

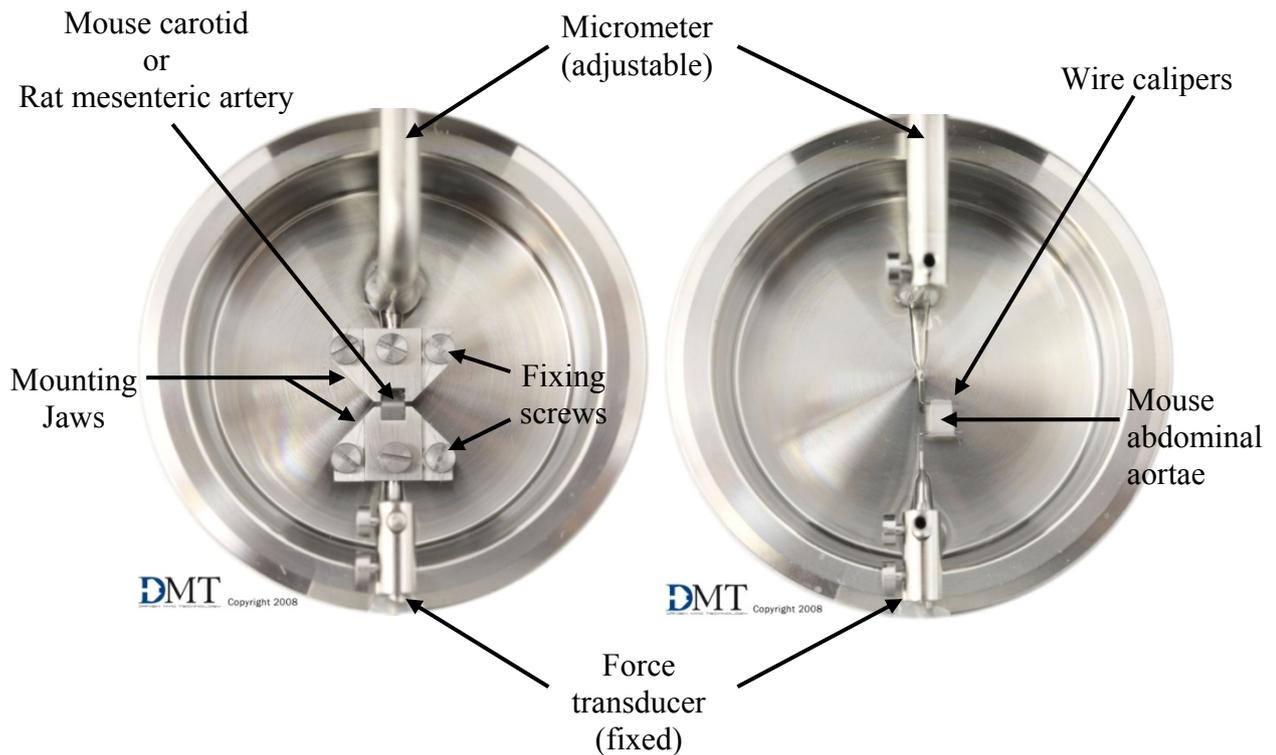


Figure 5. Graphical representation of the small vessel myograph setups, with the mounting jaws depicted on the left and stainless steel calipers shown on the right (Adapted from DMT, last updated 2011).

2.6.2.2 Examining endothelial integrity and NO bioavailability following 24-hour Ang II-treatment (Chapter 3)

NO bioavailability in isolated carotid arteries following 24-hour treatment with either the vehicle or Ang II in the absence or presence of Angeli's salt (refer to section 2.2 for the organoid culture protocol) was assessed by measuring and comparing the contractile responses to a single bolus concentration of the NO synthase inhibitor, L-NAME (100 $\mu\text{mol/L}$) or the vasorelaxant response to ACh.

Thus, following the general protocol outlined in section 2.6.2.1 and the 30-minute equilibration period, vessels were pre-contracted to ~40% of the initial U46619-induced maximal contraction (F_{max}) with titrated concentrations of U46619 (0.1 nmol/L - 10 nmol/L). Once a stable contraction was achieved, L-NAME (100 $\mu\text{mol/L}$) was administered and the subsequent

contraction measured over a 30 minute period. Arteries were then washed thoroughly with Krebs' solution and allowed to equilibrate for a further 30 minute period, after which, a second maximal contraction to 1 $\mu\text{mol/L}$ U46619 was obtained, as the contractile efficacy of U46619 was found to increase over the duration of the experiment. The U46619-mediated pre-contraction and L-NAME response were expressed as a percentage of the second maximum U46619 contraction. In a separate series of experiments, vasorelaxation responses to the endothelium-dependent vasodilator, ACh (0.1 nmol/L - 10 $\mu\text{mol/L}$) were assessed and compared in vehicle and Ang II-treated carotid arteries following pre-contraction to $\sim 50\%$ F_{max} with U46619 (refer to section 2.6.2.2 for the general protocol for small vessel wire myography).

2.6.2.3 Examining the vasorelaxant responses to BAY 58-2667 following acute and chronic NO synthase inhibition (Chapter 4)

Following the general protocol outlined in section 2.6.2.1 and the subsequent 30 minute equilibration period, C57BL/6J mouse aortae and SD rat mesenteric arteries were pre-contracted to $\sim 50\%$ of the initial maximum U46619/KPSS contraction respectively, after which cumulative concentration-response curves to BAY 58-2667 were constructed (C57BL/6J: 10 fmol/L - 1 nmol/L ; SD: 0.1 fmol/L - 10 nmol/L) in the absence and presence of L-NAME (100 $\mu\text{mol/L}$). L-NAME was administered to the myograph bath and allowed to incubate for 30 minutes prior to the U46619-induced pre-contraction. Furthermore, utilising the same protocol, cumulative concentration-response curves to BAY 58-2667 (10 fmol/L - 1 $\mu\text{mol/L}$) were also constructed in the abdominal aortae of C57BL/6J mice, C57BL/6J mice chronically treated with L-NAME (100 mg/kg/day for 28 days, refer to section 2.1.1.1) and $\text{eNOS}^{-/-}$ mice.

2.6.2.4 Examining the effect of NADPH oxidases on the vasorelaxant responses to BAY 58-2667 in both a short-term and chronic model of oxidative stress (Chapter 7)

The role of Nox2 containing NADPH oxidase in modulating the response to BAY 58-2667 was assessed in vehicle- and Ang II (24 hour)- treated carotid arteries of C57BL/6J and Nox2^{-/-} mice (treated according to section 2.2) and in freshly isolated carotid arteries from C57BL/6J on either normal (C57BL/6J ND) or high fat (C57BL/6J HFD) diet, ApoE^{-/-} and Nox2^{-/-}/ApoE^{-/-} mice, both on a high fat diet.

Following the general protocol described in section 2.6.2.1 and the 30-minute equilibration period, carotid segments were pre-contracted to ~50% F_{max} with titrated concentrations of U46619 (0.1 nmol/L - 10 nmol/L). Once a stable plateau was achieved, cumulative concentration-response curves to BAY 58-2667 (1 fmol/L - 1 μmol/L) were constructed in the carotid segments. In selected arteries, responses to BAY 58-2667 were also examined in presence of either the sGC inhibitor, ODQ (10 μmol/L) or the heme-free sGC antagonist, zinc protoporphyrin IX (Zn-PPIX, 10 μmol/L), which were added 30 minutes prior to pre-contraction with U46619. Cumulative concentration-responses curves to papaverine (10 nmol/L - 30 μmol/L) were also constructed in freshly isolated carotid arteries from ApoE^{-/-} HFD and C57BL/6J HFD mice as well as in vehicle and Ang II-treated (refer to section 2.2) carotid arteries from C57BL/6J ND mice.

Following the completion of the concentration-response curves to the selected vasodilators outlined in sections 2.6.2.2 - 2.6.2.4, maximal relaxation was achieved via the addition of SNP (10 μmol/L) and isoprenaline (1 μmol/L) in rat mesenteric artery or via the ATP-sensitive K⁺ channel opener, levcromakalim ((3S,4R)-3,4-dihydro-3-hydroxy-2,2-dimethyl-4-(2-oxo-1-pyrrolidinyl)-2H-1-benzopyran-6-carbonitrile, LKM, 10 μmol/L) in mouse arteries. For

functional experiments performed using small vessel wire myography, only one concentration-response curve was performed in each vessel.

2.6.3 General data analysis for functional blood vessel studies

Contractile responses were expressed as percentage of the maximum KPSS contraction (F_{\max}). Relaxation responses to the aforementioned vasodilators were expressed as a percentage reversal of the level of pre-contraction to U46619 (mouse aortae and carotid arteries), U46619 in combination with cirazoline (rat aortae and mesenteric arteries) or phenylephrine (rabbit aortae and saphenous arteries). Individual curves for each vasodilator were fitted to a non-linear sigmoidal logistic equation (Graphpad Prism, Version 4 and 5) and pEC_{50} values (concentration of agonist causing 50% relaxation) were calculated and expressed as $-\log M$. pEC_{50} values could only be obtained for curves which were sigmoidal and where a maximum response was obtained. Differences between mean pEC_{50} and maximum relaxation (R_{\max}) values were tested by using either a Student's unpaired t-test or a One-way ANOVA in combination with Bonferroni's multiple comparisons post-ANOVA test (Graphpad Prism, Version 4 and 5). A Two-way ANOVA (Sigma Stat, Version 1.0, Jandel Scientific) was used to compare concentration-response curves of agonists which were not sigmoidal in nature and hence where a pEC_{50} value could not be calculated. Where appropriate, the post-ANOVA Tukey's test was applied to identify the differences in response between treatment groups (Sigma Stat, Version 1.0, Jandel Scientific).

All results were expressed as mean \pm s.e.mean, with n representing the number of animals utilised per group. Statistical significance was accepted at the $P < 0.05$ level.

2.6.4 Drug dilutions for functional blood vessel studies

Stock solutions (10 mmol/L) and subsequent dilutions of DEA/NO were prepared fresh on the experimental day in 0.01 mol/L NaOH in order to avoid physiological decomposition. Stock solutions of ODQ (10 mmol/L) and U46619 (1 mmol/L) were made up in absolute ethanol (EtOH), levcromakalim (10 mmol/L) in absolute methanol (MeOH), indomethacin (100 mmol/L) in 1M Na₂CO₃, whilst zaprinast (10 mmol/L), Zn-PPIX (10 mmol/L), BAY 58-2667 (10 mmol/L) and BAY 41-2272 (10 mmol/L) were constituted in 100% dimethyl sulfoxide. To aid in dissolving these compounds on the experimental day, stock solutions of both BAY 58-2667 (10 mmol/L) and BAY 41-2272 (10 mmol/L) were initially diluted 1:10 in 50% DMSO to achieve a final concentration of 1 mmol/L. From here, all subsequent dilutions of BAY 58-2667 and BAY 41-2272 were made in distilled water (dH₂O). All other dilutions of stock solutions and all other drugs were constituted in dH₂O. With the exception of DEA/NO, papaverine, 8-bromo-cGMP and ACh, which were made up fresh, all other stock solutions could be prepared prior to the experimental day, divided into aliquots and stored at -20°C until ready for use.

2.7 RT-PCR array (Chapter 3)

2.7.1 RNA extraction from intact carotid arteries

In order to purify RNA from the carotid arteries, the RNeasy Micro Kit (Qiagen, Texas, USA) was utilized. Each kit consisted of the following contents:

Table 5. Contents of the RNeasy Micro Kit for RNA purification.

<i>RNeasy Micro Kit</i>	50
<i>Catalog no.</i>	74004
<i>Number of preps</i>	50
RNeasy MinElute Spin Columns (each in a 2 ml collection tube)	50
Collection tubes (1.5 ml)	50
Collection tubes (2 ml)	100
Buffer RLT	45 ml
Buffer RW1	45 ml
Buffer RPE (concentrate)	11 ml
RNase-free water	3 x 10 ml
Carrier RNA, poly-A	310 µg
RNase-free DNase Set	
• RNase-free DNase I (lyophilized)	1500 units
• Buffer RDD	2 x 2 ml
• RNase-free water	1.5 ml
Handbook	1

(Adapted from QIAGEN, 2007)

Tissue purification was conducted according to the fibrous tissue protocol outlined in the provided RNeasy Micro Kit handbook, pages 30-36 (Qiagen, USA). High quality RNA is

essential for obtaining accurate real-time PCR results, thus prior to starting, all equipment and bench tops were wiped down with RNaseZap (Ambion's RNaseZap®, Applied Biosystems) followed by dH₂O. C57BL/6J mice were sacrificed as described in section 2.1.1 with both the right and left carotids excised and subjected to organoid culture as described in section 2.2.3 and more specifically in section 2.2.4.3. As previously described, the entire lengths of carotid arteries (left and right) from two C57BL/6J mice were pooled and treated with either vehicle (50 nmol/L glacial acetic acid) or Ang II (10 nmol/L) in the absence or presence of Angeli's salt (0.3 μmol/L) and then snapped frozen 24 hours later as described in section 2.2.4.3.

Frozen tissue samples were homogenised into a powder by grinding the tissue with a glass pestal in an RNA-free eppendorf tube placed in a mortar containing liquid nitrogen. Once homogenized adequately, 150 μl of buffer RLT was added and the sample was further homogenised (0.5 cycles, 40 amps) using the hand held sonicator (Ultrasonic processor UP100H, Hielscher Ultrasonics, Germany). RNase-free water (295 μl) was then added to the homogenate along with 5 μl of QIAGEN Proteinase K to further aid in the digestion of the tissue.

Homogenate was then mixed thoroughly by gentle pipetting, after which it was incubated for 10 minutes at 55°C on a heat block. Following this incubation period, the homogenate was centrifuged for 3 minutes at 10,000 g at room temperature (15-25°C) allowing a small pellet of tissue debris to form. The supernatant was removed and placed into a new RNA free eppendorf tube, to which 225 μl of 96-100% ethanol was added to clear the lysate. The contents were mixed well by pipetting and then transferred to an RNeasy MinElute spin column placed in a 2 ml collection tube. The sample was then centrifuged for 15 seconds at 8000 g, removing the ethanol (flow through) whilst the RNA remained bound to the membrane of the RNeasy MinElute column. The flow through was then discarded and the collection tube reused.

As traces of DNA can contaminate the sample, a DNase digestion step was incorporated into the protocol in order to avoid genomic DNA contamination. Thus, for every sample, 10 μl of DNase I stock solution was added to 70 μl of Buffer RDD in an RNA free eppendorf tube. This DNase I incubation mix was then combined by gently inverting the tube, after which 80 μl was added to each sample and allowed to incubate for 15 minutes at room temperature (20-30°C). Following this period, 350 μl of Buffer RW1 was added to the RNeasy MinElute spin column and the sample centrifuged for 15 seconds at 8000 g in order to wash the membrane. Subsequently, the flow through and the collection tube were both discarded. A new 2 ml collection tube was attached to the RNeasy MinElute spin column containing the bound RNA, 500 μl of Buffer RPE added and centrifuged again for a further 15 seconds at 8000 g. The flow through was discarded, collection tube reattached and 500 μl of 80% ethanol added to the spin column. The sample was centrifuged again at 8000 g (2 minutes), after which the flow through and collection tube were discarded. During the final phases of this procedure, care was taken when removing the collection tube from the spin column as carry over of ethanol can occur affecting the purity of the sample. In order to remove residual ethanol from the sample, a new collection tube was attached to the spin column and the sample was centrifuged again with open lids at full speed for 7 minutes. The flow through and the collection tube were again discarded. Finally, a new 1.5 ml collection tube was attached to the spin column and 14 μl of RNase-free water was added directly to the membrane. The sample was then centrifuged for one minute at full speed to elute the RNA. It is expected that elution with 14 μl of RNase-free water will yield approximately 12 μl of eluate. The quantity of RNA (ng/ μl) in the 12 μl concentrated eluate was determined using the NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA).

2.7.2 Reverse transcription (RT) reaction

Following RNA extraction from tissue samples, a reverse transcription (RT) reaction was conducted to convert the purified RNA into single stranded cDNA. For the analysis of Nox2 and

p47^{phox} mRNA expression, RNA was reversed-transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, USA) whilst examination of the expression of genes involved in the nitric oxide signaling pathway was completed using the RT² First Strand Kit (SABiosciences, USA), both methods were conducted according to the manufacturer's specifications. As mentioned previously, accurate and reproducible PCR results are influenced by the purity of the RNA sample. Contamination of the assay with foreign DNA is one of the issues which could compromise the reliability of the assay as it is able to artificially elevate the SYBR Green signal resulting in skewed gene expression profiles in addition to generating false positive signals. Thus, in order to avoid DNA contamination, benches and all equipment required for this procedure were cleaned with 10% bleach which chemically inactivates and degrades any DNA prior to use.

The 12 µl eluate samples (concentrated RNA solutions) and the entire contents of the QuantiTect Reverse Transcription and RT² First Strand Kit were thawed on ice. Once thawed, the reagents were centrifuged briefly in order to collect residual liquid from the sides and then stored back on ice. Using the results obtained from the Nanodrop, the number of microliters required per sample to achieve an equivalent of 100 ng of RNA for the Nox2 and p47^{phox} analysis and/or 300 ng of RNA for the NO signaling pathway gene analysis was calculated. To eliminate genomic DNA contamination, the genomic DNA elimination mixture provided by each kit was combined with each RNA sample and RNase-free water. The entire contents were then mixed using gentle pipetting followed by a brief centrifugation. Samples were then allowed to incubate for 5 minutes at 42°C on the heat block. Immediately following this 5 minute period, samples were rested on ice and allowed to chill for at least one minute. To maintain consistency, all samples relating to the same gene analysis were converted to cDNA at the same time.

Once the samples had cooled sufficiently, the RT master mix (provided in the QuantiTect Reverse Transcription kit) or the RT cocktail mix (provided in the RT² First Strand kit) was added to the samples already containing the genomic DNA elimination mixture. Samples were mixed gently and incubated at 42°C for 15 minutes, immediately after which the reaction was stopped by increasing the temperature to 95°C for a further 5 minutes. This synthesis reaction resulted in 20 µl of cDNA to which 90 µl of RNase-free H₂O was added. These cDNA samples were stored at -20°C until ready for use for RT-PCR.

2.7.3 Quantitative real-time RT-PCR

Analysis of Nox2 and p47^{phox} gene expression were performed in triplicates using Taqman primers and probes and Taqman Universal Master Mix (Applied Biosystems) in accordance with the manufacturer's specifications. For the NO signaling pathway gene analysis, cDNA was analysed using the Mouse NO signaling pathway RT² Profiler PCR array and the RT² SYBR Green qPCR Master Mix (SABiosciences) where the reactions were prepared according to the manufacturer's instructions.

2.7.3.1 Taqman® Gene Expression Assay (Applied Biosystems)

The following was required to perform the real-time PCR:

Taqman gene expression assay: One 6-FAMTM dye-labeled, Taqman MGB probe

One 5-VIC[®] dye-labeled Taqman MGB probe for β-actin

Primer for Nox2 (reference sequence: NM_007807.4)

Primer for p47^{phox} (reference sequence: NM_010876.3)

Taqman Gene Expression Universal Master Mix

Taqman primers and probes, Taqman gene expression master mix (2x) and the appropriate cDNA samples (3x vehicle, 3x Ang II and 3x Ang II + Angeli's salt) were thawed on ice. Nox2 and p47^{phox} were prepared as PCR reaction mixtures (60 µl final volume) containing 1 x Taqman

Universal PCR master mix combined with primers for either Nox2 or p47^{phox} (1:10 dilution; 33 μ l) and the cDNA sample (27 μ l; 100 ng). No template controls (NTCs) were also used for each gene expression on the plate. NTCs reaction mixtures consisted of the Taqman Universal PCR master mix combined with either β -actin, Nox2 or p47^{phox} (33 μ l) and RNase free water (27 μ l) in a final volume of 60 μ l. Gene analysis were performed in 20 μ l triplicates (PCR reaction mixture) on a 96-well reaction plate according to the following PCR thermal cycle parameters: 2 minutes at 50°C, 10 minutes at 95°C followed by 40 cycles at 15 seconds each, also at 95°C and 60°C for 1 minute using the Bio-Rad CFX96 Real-Time PCR machine.

2.7.3.2 RT² Profiler PCR array: Mouse NO signaling pathway (SABiosciences)

The RT² Profiler PCR SuperArray is a 96-well plate containing primers for a thoroughly researched panel of relevant, pathway or disease-focused genes plus appropriate positive and negative controls associated with the NO signaling pathway. This is represented in Table 6A and B. One diluted cDNA sample (110 μ l: 20 μ l cDNA + 90 μ l dH₂O, generated in section 2.7.2) and one tube of SABiosciences RT² qPCR Master Mix (1 tube per sample) were thawed out on ice as only one PCR array could be performed at a time. Once thawed, the sample and reagent were briefly centrifuged in order to obtain all the residual liquid attached to the sides of the tubes. Following this, an experimental cocktail was prepared by combining the cDNA synthesis reaction and the master mix according to the following calculations:

SABiosciences RT ² qPCR Master Mix:	1375 μ l
Diluted first strand cDNA synthesis reaction:	110 μ l
H ₂ O:	1265 μ l

The experimental cocktail was mixed thoroughly using gentle pipetting. The PCR array was then removed from its sealed bag and placed on ice whilst 25 μ l of the experimental cocktail was pipetted carefully into each well. Once completed, the wells of the array were capped with the 8-cap strips provided in order to tightly seal the array which was then centrifuged to eliminate

any air bubbles and ensure that the cocktail mix settled towards the bottom of each well. The array was then wrapped in foil and placed on ice until the RT PCR detection was performed. PCR arrays containing the experimental cocktails were able to be stored at -20°C, wrapped in foil, for up to one week. RT PCR detection were performed using the Eppendorf Mastercycler Realplex® RT PCR machine via a two-step thermocycling program which involved:

Cycles	Duration	Temperature
1	10 minutes	95°C (denaturation)
40	15 seconds	95°C (denaturation)
	1 minute	60°C (annealing)

A melting curve was also conducted immediately after the above cycling program to ensure that no more than one peak was obtained from each of the 84 reactions at temperatures greater than 80°C. A single peak indicated a single melting event, thus verifying that a single gene-specific product was produced. This procedure was then repeated for all the other samples.

Data was analysed using the PCR Array Data Analysis Web Portal which was downloaded from the manufacturer's website and was tailored for that specific array: (<http://www.SABiosciences.com/pcrarraydataanalysis.php>). A positive reaction was expressed as a C_T (cycle threshold) value, which is defined as the number of cycles required for the fluorescent signal to exceed background levels. As each assay underwent 40 cycles, C_T values greater than 35 were considered as negative calls and may suggest some level of contamination in the sample. All C_T values were normalized to the housekeeping genes (Gusb, Hprt1, β -actin, Hsp90ab1 and GAPDH) and expressed as fold changes relative to control, using the formula $2^{-\Delta\Delta C_T}$. A fold change of ≥ 2 -fold was considered as statistically significant.

Table 6A. Functional gene groupings represented in the Mouse Nitric Oxide Signaling Pathway RT² Profiler PCR Array.

<i>Genes Involved in Nitric Oxide (NO) Biosynthesis</i>	
Genes with NO Synthase Activity:	Nos1, Nos2, Nos3
Genes with Oxidoreductase Activity:	Nos1, Nos2, Nos3.
Positive Regulators of NO Biosynthesis:	Ptx3
Negative Regulators of NO Biosynthesis:	Cav1, Dynll1 (Dncl1)
Other Genes Involved in NO Biosynthesis:	Ddah1, Ddah2, Hmgb1
<i>Genes Induced by NO</i>	
Apoptosis Genes:	Bax, Bcl2l1, Cdkn1a, Fas, Myc, Trp53
Cell Cycle Genes:	Ccnd1, Ccng1, Cdkn1a, Fos, Gadd45a, Myc, Rb1, Sfn, Trp53
Transcription Regulators:	Egr1, Fos, Myc, Rb1, Trp53
Other Genes Induced by NO:	Clcf1 (Bsf3), Irgm, Mdm2, Nedd1, Nnp1, Rprm
<i>Genes Suppressed by NO</i>	Capns1, Cd151, Hpn, Pea15, Rras, Ubc.
<i>Genes Involved in the NO Signaling Pathway</i>	Calm1, Dlg4, Grin1, Nos1, Ppp3ca, Prkaca.
<i>Genes Involved in Superoxide Metabolism</i>	
Genes Involved in Superoxide Release:	Nox1, Nox4, Sod
Genes with Oxidoreductase Activity:	Cyba, Nos1, Nos2, Nox4, Recql4, Scd1, Scd2, Scd3, Sod1, Sod2, Sod3
Genes with Superoxide Dismutase Activity:	Sod1, Sod2, Sod3
Genes with Antioxidant Activity:	Sod1, Sod3
Other Genes Involved in Superoxide Metabolism:	Ccs, Ncf2, Nox1, Nox4, Noxa1, Noxo1
<i>Genes Involved in Response to Oxidative Stress</i>	
Genes with Antioxidant Activity:	Prdx1, Prdx2, Prdx6, Sod1
Genes with Glutathione Peroxidase Activity:	Gpx1, Gpx2, Gpx3, Gpx4, Gpx5, Gpx6, Gpx7
Genes with Oxidoreductase Activity:	Aass, Cat, Epx, Gpx1, Gpx2, Gpx3, Gpx4, Gpx5, Gpx6, Gpx7, Idh1, Mpo, Prdx1, Prdx2, Prdx6, Sod1, Tpo, Txnrd2
Genes with Peroxidase Activity:	Aass, Cat, Ctsb, Epx, Gpx1, Gpx2, Gpx3, Gpx4, Gpx5, Gpx6, Gpx7, Mpo, Prdx1, Prdx2, Prdx6, Prpf4, Tpo.
Transcription Regulators:	Ercc2, Med4 (Vdrip)
Other Genes Involved in Oxidative Stress:	Als2, Apoe, Gab1, Nudt15, Ppp1r15b, Prnp, Psmb5, Txnip, Ucp3, Xpa
<i>Mouse Genomic DNA contamination</i>	MGDC
<i>Housekeeping Genes</i>	Gusb, Hpvt1, Hsp90ab1, Gapdh, Actb
<i>Reverse Transcription Control</i>	RTC
<i>Positive PCR Control</i>	PPC

Table 6B. Array/gene layout on the Mouse Nitric Oxide Signaling Pathway RT² Profiler PCR Array (PAMM-999, Format A). Note that the housekeeping genes, mouse genomic DNA contamination, reverse transcription control and positive PCR control are highlighted in row H.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Aass	Als2	Apoe	Bax	Bcl2l1	Clcf1	Calm1	Capns1	Cat	Cav1	Ccnd1	Ccng1
B	Ccs	Cd151	Cdkn1a	Ctsb	Cyba	Ddah1	Ddah2	Dlg4	Dynll1	Egr1	Epx	Ercc2
C	Fas	Fos	Gab1	Gadd45a	Gpx1	Gpx2	Gpx3	Gpx4	Gpx5	Gpx6	Gpx7	Grin1
D	Hmgb1	Hpn	Idh1	Irgm	Mdm2	Mpo	Myc	Ncf2	Nedd1	Nnp1	Nos1	Nos2
E	Nos3	Nox1	Nox4	Noxa1	Noxo1	Nudt15	Pea15	Ppp1r15b	Ppp3ca	Prdx1	Prdx2	Prdx6
F	Prkaca	Prnp	Prpf4	Psmb5	Ptx3	Rb1	Recql4	Rprm	Rras	Scd1	Scd2	Scd3
G	Sfn	Sod1	Sod2	Sod3	Tpo	Trp53	Txnip	Txnrd2	Ubc	Ucp3	Med4	Xpa
H	Gusb	Hprt1	Hsp90ab1	Gapdh	Actb	MGDC	RTC	RTC	RTC	PPC	PPC	PPC

2.8 UV/VIS Spectroscopy (Chapter 4)

UV/VIS spectra were recorded from 200 nm to 800 nm on an Agilent Technologies 8453 UV-Visible spectrophotometer. BAY 58-2667 was dissolved in DMSO at a concentration of 10 mmol/L. The compound was diluted to 10 $\mu\text{mol/L}$ in 50 mmol/L phosphate buffer solution (PBS) resulting in a final DMSO concentration of 0.1% (v/v). All controls were prepared with the same concentration of DMSO. NO was introduced via an aqueous solution of DEA/NO (1 $\mu\text{mol/L}$ and 10 $\mu\text{mol/L}$). All samples were incubated for 60 min at 37°C. Spectra were normalized to the absorbance at 800 nm, and the wavelength scans from the various DEA/NO concentrations were subtracted from the corresponding scans in the presence of BAY 58-2667 in order to determine the absorption of the compound.

2.9 Plasma lipid profile analysis (Chapter 7)

Plasma lipid profile of C57BL/6J (ND and HFD), ApoE^{-/-} (HFD) and Nox2^{-/-}/ApoE^{-/-} (HFD) mice were analysed. Once anaesthetized, blood was extracted from the vena cava of mice using a 26 gauge 1 ml syringe. The blood was centrifuged (4,000 x g, 4°C, 10 min) in order to isolate the plasma. Plasma samples were stored at -20°C before being sent to a commercial pathology laboratory (Gribbles Pathology Pty Ltd, Clayton). Plasma concentrations of total cholesterol, low density lipoprotein (LDL) cholesterol, high density lipoprotein (HDL) cholesterol, and triglycerides were determined using Roche CHOL, HDL-cholesterol plus, and TG assays, respectively (Roche, Australia). LDL cholesterol was calculated as the difference between total- and HDL-cholesterol concentrations.

2.10 Drug sources

Table 7. Represents the chemicals/drugs employed in this thesis and the companies from which they were obtained.

Companies	Chemicals
<i>Ajax Chemicals, Auburn, Australia</i>	Ethylenediaminetetra acetic acid disodium salt (EDTA).
<i>Applied Biosystems, Australia</i>	Taqman Gene Expression Assays (Nox2 and p47 ^{phox}).
<i>Australian Peptide Laboratories (AUSPEP), Australia</i>	Angiotensin II (Human).
<i>Bayer HealthCare, Germany</i>	BAY 58-2667 (4-[(4-carboxybutyl){2-[(4-phenethylbenzyl)oxy]phenethyl}mino)methyl[benzoic acid) and BAY 41-2272 (5-cyclopropyl-2-[1-(2-fluorobenzyl)-1H-pyrazolo[3,4-b]pyridine-3-yl]pyridine-4-ylamine).
<i>QIAGEN, USA SABiosciences, USA (A Qiagen Company)</i>	RNeasy Micro Kit and QuantiTect Reverse Transcription Kit Mouse nitric oxide signaling pathway PCR array, RT ² First strand kit and SYBR Green PCR master mixes.
<i>Tocris Bioscience, United Kingdom</i>	Levcromakalim ((3 <i>S</i> ,4 <i>R</i>)-3,4-dihydro-3-hydroxy-2,2-dimethyl-4-(2-oxo-1-pyrrolidinyl)-2 <i>H</i> -1-benzopyran-6-carbonitrile).

Table 7 continued

Companies	Chemicals
<i>Sapphire Bioscience, Australia</i>	U46619 (9-11-dideoxy-11 alpha, 9 alpha-epoxymethano-prostaglandin F2 alpha), ODQ ([1H-[1,2,4]Oxadiazolo[4,3-a]quinoxaline-1-one]), Angeli's salt (sodium trioxodinitrate, Na ₂ N ₂ O ₃), Diethylamine NONOate (DEA/NO) and Carboxy-PTIO potassium salt (1H-Imidazol-1-yloxy, 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-, 3-oxide, potassium salt).
<i>Sigma Chemical Co., USA</i>	sodium nitroprusside (SNP), isoprenaline ((-)-isoproterenol), diethylamine NONOate (DEA/NO), cirazoline, acetylcholine chloride (ACh), 1-[p-Chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid (indomethacin), N ^G -nitro-L-arginine methyl ester (L-NAME), serotonin (5-HT), papaverine, Dulbecco's Modified Eagle Medium (DMEM; 1000mg glucose/L, pyridoxine HCl, NaHCO ₃ , no L-glutamine or phenol red), penicillin/streptomycin solution, L-cysteine, lucigenin (bis-N-methylacridinium nitrate) and phosphate buffer solution (PBS), zaprinast, β-nicotinamide adenine dinucleotide 2'-phosphate (NADPH), zinc-protoporphyrin IX (Zn-PPIX), 8-bromoguanosine cyclic 3',5'-monophosphate (8-bromo-cGMP).
<i>Wako Pure Chemical Industries, Japan</i>	L-012 (8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4(2H,3H)dione)



CHAPTER 3

Nitroxyl (HNO), the novel redox sibling
of NO, suppresses vascular NADPH
oxidase-derived superoxide production



Nitroxyl (HNO), the reduced and protonated congener of nitric oxide (NO), displays distinct pharmacology and therapeutic advantages over its redox sibling, NO[•]. Unlike NO[•], HNO has positive cardiac inotropic effects, is resistant to scavenging by superoxide anion radical ([•]O₂⁻) and displays antioxidant properties in both yeast and cardiomyocytes. Here we explored the potential [•]O₂⁻ limiting effects of HNO in the vasculature. Vascular [•]O₂⁻ levels were measured in angiotensin II (Ang II; 10 nmol/L, 24 hrs)-treated common carotid arteries from C57BL/6J and Nox2^{-/-} mice using lucigenin (5 μmol/L)-enhanced chemiluminescence. Overnight treatment of arteries with Ang II caused a significant 4.3-fold increase in [•]O₂⁻ levels in C57BL/6J but not in the Nox2^{-/-} arteries suggesting a causal role of Nox2-containing NADPH oxidase in vascular [•]O₂⁻ generation. Such an increase in [•]O₂⁻ was not associated with a decrease in NO bioavailability (as assessed by L-NAME contraction) nor a change in Nox2 or p47^{phox} mRNA expression. Treatment with the HNO donor, Angeli's salt (1 nmol/L - 1 μmol/L, replenished 3x/day) during the 24-hour period decreased Ang II-stimulated [•]O₂⁻ production in carotid arteries from C57BL/6J mice in a concentration-dependent manner. Similarly, the HNO donor, isopropylamine NONOate (IPA/NO; 1 μmol/L, replenished 3x/day) also attenuated [•]O₂⁻ levels in these arteries. These effects were confirmed to be mediated by HNO as the HNO scavenger, L-cysteine (3 mmol/L) prevented the [•]O₂⁻ suppressing effects of both Angeli's salt and IPA/NO whilst the NO[•] scavenger, carboxy-PTIO (200 μmol/L) had no effect on either donors. Neither the sGC inhibitor, ODQ (10 μmol/L) nor the protein kinase G inhibitor, Rp-8-pCPT-cGMPS (10 μmol/L) altered the ability of Angeli's salt to suppress Ang II-stimulated [•]O₂⁻ generation, indicative of a cGMP-independent action of HNO. In conclusion, HNO donors attenuate [•]O₂⁻ production by Nox2-containing NADPH oxidase in the vasculature. Such an action may confer therapeutic potential for the use of HNO donors for the treatment of vascular pathologies associated with oxidative stress.

Nitric oxide (NO) plays an integral role in the control of cardiovascular homeostasis and most of its actions have been attributed to its uncharged state (NO[•]) (Andrews *et al.*, 2009; Irvine *et al.*, 2008). There exists, however, three different redox states of NO; nitric oxide (NO[•]), nitroxyl (NO⁻ or HNO) and the nitrosonium cation (NO⁺) (Irvine *et al.*, 2003). Recently, interest has grown in HNO, the one electron reduced and protonated congener of NO, given its putative endogenous formation, distinct pharmacology as compared with NO[•] and its therapeutic potential in the treatment of heart failure (Irvine *et al.*, 2008; Paolucci *et al.*, 2007).

Thus, unlike NO[•] donors, HNO donors are resistant to scavenging by superoxide anion radical (•O₂⁻) (Miranda *et al.*, 2003) and tolerance development (Irvine *et al.*, 2007; Irvine *et al.*, 2011) and interact directly with critical cysteine residues to modify thiol-containing proteins (Donzelli *et al.*, 2006; Paolucci *et al.*, 2007). Such thiol interactions of HNO are predominant in the heart where HNO directly targets the sarcoplasmic reticulum ryanodine receptors (RyRs) and sarcoplasmic reticulum calcium ATPase (SERCA) to facilitate calcium (Ca²⁺) release and it also sensitises the contractile myofilaments to Ca²⁺ leading to an increase in myocardial contractility (Dai *et al.*, 2007; Lancel *et al.*, 2009; Tochetti *et al.*, 2007). Such an action is not observed with NO[•] donors and consequently, the HNO donor, Angeli's salt has been shown to be protective in an experimental model of heart failure (Paolucci *et al.*, 2003).

In addition to increasing myocardial contractility, HNO also causes vasodilatation to unload the heart (Paolucci *et al.*, 2003). Thus, HNO is a potent vasodilator in large conduit (Ellis *et al.*, 2000) and small resistance-like arteries (Andrews *et al.*, 2009; Irvine *et al.*, 2003), mediating its vasorelaxant effects predominantly via activation of sGC and a subsequent increase in cGMP, yet it can also target other signalling pathways. These include the release of the vasodilator neuropeptide CGRP (Favaloro & Kemp-Harper, 2007; Paolucci *et al.*, 2001) and the activation

of K_v (Favaloro & Kemp-Harper, 2009; Irvine *et al.*, 2003) and K_{ATP} channels (Favaloro & Kemp-Harper, 2007).

Together with its cardio- and vasoprotective actions, evidence exists that HNO may also limit oxidative stress. Oxidative stress is a prominent feature of vascular disease states and is a term used to define the imbalance between oxidants and antioxidants, whereby an excessive production of reactive oxygen species (ROS), such as $\cdot O_2^-$ and hydrogen peroxide (H_2O_2), may overwhelm the antioxidant defence mechanisms of cells (Bengtsson *et al.*, 2003; Thomas *et al.*, 2008). Thus, HNO has been shown to decrease lipid peroxidation in yeast (Lopez *et al.*, 2007) and stimulate the expression and activity of the antioxidant protein, heme-oxygenase 1 in rat H9C2 cells (Naughton *et al.*, 2002). Moreover in rat neonatal cardiomyocytes, we have evidence that HNO reduces angiotensin II (Ang II)-induced $\cdot O_2^-$ production, possibly via decreasing the expression of the $\cdot O_2^-$ generating enzyme, Nox2-containing NADPH oxidase (Ritchie *et al.*, 2007).

NADPH oxidases are a family of multisubunit enzyme complexes to which the only ascribed function is the generation of ROS (Babior, 1999; Lassegue & Clempus, 2003). NADPH oxidases are comprised of a membrane-bound domain containing a Nox catalytic subunit and a $p22^{phox}$ subunit, up to three cytosolic subunits ($p47^{phox}$, $p67^{phox}$ and $p40^{phox}$) and a regulatory G-protein (Rac1 or Rac2) (Selemidis *et al.*, 2008). At least 3 isoforms of NADPH oxidase are expressed in the blood vessel wall, namely Nox1, Nox2 and Nox4-containing isoforms. While Nox4 is expressed in markedly high levels under physiological conditions, Nox1 and Nox2, are expressed at low levels in the vasculature under physiological conditions and are upregulated during cardiovascular disease (Selemidis *et al.*, 2008; Wind *et al.*, 2010).

Given the apparent ability of HNO to limit $\cdot\text{O}_2^-$ generation in cardiomyocytes (Ritchie *et al.*, 2007), we hypothesised that HNO may suppress vascular $\cdot\text{O}_2^-$ generation via a direct modulation of Nox2-containing NADPH oxidase. In order to test this hypothesis, a short-term model of oxidative stress was utilised in which carotid arteries from mice were treated for 24 hours with Ang II leading to a significant increase in $\cdot\text{O}_2^-$ production and a decrease in NO bioavailability (Chrissobolis *et al.*, 2008; Didion *et al.*, 2005), an effect that is dependent upon Nox2-containing NADPH oxidases (Scrader *et al.*, 2007). In the present study, we provide evidence that short-term (24hr) treatment with the HNO donor, Angeli's salt, suppresses vascular $\cdot\text{O}_2^-$ generation from Nox2-containing NADPH oxidase via a cGMP-independent mechanism.

Materials and Methods

This study was approved by the Pharmacology Animal Ethics Committee, Monash University, Australia and conforms to the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Tissue Preparation

Adult male C57BL/6J (10-14 weeks of age, n = 105) and Nox2^{-/-} mice (10-12 weeks of age, n = 6) were utilised for this study (see General Methods, section 2.1.1). On the experimental day, mice were killed humanely via isoflurane inhalation (2-4% in O₂) after which both the left and right carotid arteries were isolated.

Organoid Culture

Isolated carotid arteries from C57BL/6J and Nox2^{-/-} mice were maintained in Dulbecco's Modified Eagle's Medium (DMEM) and treated for 24 hours with either vehicle (50 nmol/L glacial acetic acid) or Ang II (10 nmol/L). In addition, carotid arteries from C57BL/6J mice were incubated for 24 hours with the following treatments:

- Ang II (10 nmol/L) + Angeli's salt (1 nmol/L - 1 µmol/L)
- Ang II (10 nmol/L) + Angeli's salt (0.3 µmol/L) in the absence or presence of either the:
 - (i) HNO scavenger, L-cysteine (3 mmol/L)
 - (ii) NO[•] scavenger, carboxy-PTIO (200 µmol/L)
 - (iii) sGC inhibitor, ODQ (10 µmol/L)
 - (iv) protein kinase G (PKG) inhibitor, Rp-8-pCPT-cGMPS (10 µmol/L)

- Ang II (10 nmol/L) + isopropylamine NONOate (IPA/NO, 1 μ mol/L) in the absence or presence of either:
 - (i) L-cysteine (3 mmol/L)
 - (ii) carboxy-PTIO (200 μ mol/L).
- Ang II (10 nmol/L) co-incubated with either:
 - (i) carboxy-PTIO (200 μ mol/L)
 - (ii) ODQ (10 μ mol/L)
 - (iii) Rp-8-pCPT-cGMPS (10 μ mol/L)

Treated carotid arteries were maintained in a CO₂ water-jacketed incubator (Forma Scientific) under sterile culture conditions (37°C, 5% CO₂ and 85% humidity; see General Methods, sections 2.2.3 - 2.2.5). All procedures from the dissection to the treatment of carotid arteries were conducted in the PS2 laminar flow hood (HWS120 Series, CLYBE-APAC Environmental Products Division) in order to maintain sterility as described in the General Methods, section 2.2. Due to their relatively short half-lives, both Angeli's salt and IPA/NO were administered 3 times over the 24 hour period at approximately 5 hour intervals. Furthermore, due to an expected and non-specific $\cdot\text{O}_2^-$ lowering action of sodium hydroxide (0.01 mol/L NaOH, Supplementary Figure 3), both Angeli's salt and IPA/NO were dissolved in sterilized saline immediately prior to each administration. All scavengers and inhibitors, however, were administered once only at the commencement of the 24 hour treatment period.

Following the 24-hour treatment period, carotid segments were used for either:

- (i) $\cdot\text{O}_2^-$ detection experiments via lucigenin (5 $\mu\text{mol/L}$)-enhanced (see General Methods, section 2.4.1) or L-012 (100 $\mu\text{mol/L}$)-enhanced (see General Methods, section 2.4.2, preliminary experiments only) chemiluminescence assay
- (ii) functional experiments using small vessel wire myography (see General Methods, sections 2.6.2.1 and 2.6.2.2)
- (iii) analysis of mRNA gene expression using RT-PCR (see General Methods, section 2.7).

Superoxide anion radical detection via lucigenin-enhanced chemiluminescence

Vascular $\cdot\text{O}_2^-$ levels were measured using NADPH-driven lucigenin (5 $\mu\text{mol/L}$)-enhanced chemiluminescence as described in the General Methods, sections 2.4.1 and 2.4.1.1. Vascular $\cdot\text{O}_2^-$ was detected using either the TopCount NXT single photon counter (Perkin Elmer) or the Hidex ChameleonTM Luminescence Plate Reader (Hidex Oy). Upon completion of the assay, rings were allowed to dry on pre-cut foil pieces overnight at 37°C. Dried carotid segments were then weighed the following day, background counts subtracted and $\cdot\text{O}_2^-$ normalised to dry tissue weight.

Vasorelaxation Experiments

Carotid artery segments (~2-3 mm long) were mounted in small vessel wire myographs, maintained in Krebs' solution at 37°C and bubbled continuously with carbogen (95% O₂, 5% CO₂) as described in the General Methods, section 2.6.2. NO bioavailability and endothelial integrity were assessed in isolated arteries via measurement of the contractile response to the NOS inhibitor, L-NAME (100 $\mu\text{mol/L}$) and concentration-dependent vasorelaxation response to acetylcholine (ACh) according to the protocol outlined in the General Methods, sections 2.6.2 and 2.6.2.1-2.6.2.2.

Quantitative RT-PCR

Levels of mRNA in the carotid arteries treated with either vehicle or Ang II (see General Methods, sections 2.2 and 2.2.4.3) were measured by quantitative real-time RT-PCR (refer to General Methods section 2.7). Total RNA was extracted and pooled from the carotids of two mice using the RNeasy Micro Kit (Qiagen). RNA (100ng or 300ng) was then reverse-transcribed using either QuantiTect Reverse Transcription (Qiagen) or the RT² First Strand Kit (SABiosciences), respectively, according to the manufacturer's specifications and the protocol outlined in the General Methods, section 2.7.2. Analysis of Nox2 and p47^{phox} mRNA expression were performed using Taqman primers and probes (Applied Biosystems) and 2 x Taqman Universal Master Mix (Applied Biosciences) as described in the General Methods section 2.7.3.1. Expression of genes involved in the NO signalling pathway (ie. Nox, NOS, SOD and Gpx) were analysed using the Mouse NO signalling pathway RT² Profiler PCR array and RT² SYBR Green/ROX qPCR Master Mix (SABiosciences; see General Methods, section 2.7.3.2).

Statistical Analysis

All of the data are expressed as mean \pm s.e. mean. Vasorelaxation responses to ACh were expressed as % reversal of the level of U46619-induced pre-contraction whilst contractions to L-NAME were expressed as a percentage of the maximum U46619 contraction (F_{max}). Comparisons between the treatment groups were made using either a Student's unpaired t-test or a One-way ANOVA followed by Bonferroni's multiple comparisons test (see General Methods, sections 2.4.3 and 2.6.3). Statistical significance was accepted at the $P < 0.05$ level. The analysis of genes involved in the NO signaling pathway was carried out using the PCR Array Data Analysis Web Portal. Data from the RT-PCR arrays was analysed as a ratio of the mRNA levels of the housekeeping genes supplied and expressed as a fold change relative to the vehicle (see General Methods, sections 2.7.3.1-2.7.3.2). A fold change of ≥ 2 -fold was considered as statistically significant.

Angiotensin II increases $\cdot\text{O}_2^-$ production by Nox2-containing NADPH oxidase in mouse carotid arteries

Short-term (24hr)-treatment with Ang II (10 nmol/L) significantly increased $\cdot\text{O}_2^-$ levels by ~4.3-fold ($P < 0.001$) in isolated carotid arteries from C57BL/6J mice (vehicle: $0.17 \pm 0.03 \times 10^3$ counts/mg, $n = 8$ vs Ang II: $0.59 \pm 0.05 \times 10^3$ counts/mg, $n = 25$) compared to the vehicle (Figure 1). This Ang II-mediated increase in $\cdot\text{O}_2^-$ was confirmed using L-012-enhanced chemiluminescence as an alternate probe (vehicle: $0.60 \pm 0.13 \times 10^3$ counts/mg vs Ang II: $1.12 \pm 0.17 \times 10^3$ counts/mg, $n = 9$, $P < 0.01$, Supplementary Figure 1) compared to the vehicle. Due to a non-specific interaction of L-012 with the HNO donor, Angeli's salt, such that background luminescence was elevated, lucigenin-enhanced chemiluminescence was used for all subsequent experiments (see Supplementary Figure 2).

The ability of Ang II to increase vascular $\cdot\text{O}_2^-$ was lost in the carotid arteries from Nox2^{-/-} mice (vehicle: $0.71 \pm 0.16 \times 10^3$ counts/mg vs Ang II: $0.49 \pm 0.15 \times 10^3$ counts/mg, $n = 4$, see Figure 4), yet Ang II did not alter the expression of either Nox2 or the cytosolic subunit, p47^{phox} at the level of the mRNA (Table 1). In addition, using Mouse NO signalling Pathway RT² Profiler PCR Arrays, the effects of Ang II-treatment on mRNA expression of 84 genes crucial to the NO signaling pathway were evaluated. Of particular interest were the NADPH oxidases (Noxs), superoxide dismutases (SODs), glutathione peroxidases (Gpxs) and NO synthases (NOS). The results revealed no significant changes in mRNA expression across the 84 different genes in response to short-term (24hrs) Ang II-treatment (Table 1).

Despite an ability of Ang II to increase $\cdot\text{O}_2^-$ production in carotid arteries, this was not accompanied by a change in NO bioavailability such that contraction to the NOS inhibitor, L-NAME (100 $\mu\text{mol/L}$) did not differ between vehicle ($38 \pm 2\%$ U46619 contraction, $n = 6$) or Ang II-treated ($42 \pm 5\%$, $n = 6$, figure not shown) vessels. Endothelium-dependent relaxation to

ACh ($pEC_{50} = 8.46 \pm 0.21$ $-\log M$, $R_{max} = 93 \pm 2\%$ reversal of the level of pre-contraction, $n = 7$) was also unchanged following Ang II-treatment ($pEC_{50} = 7.88 \pm 0.12$, $R_{max} = 95 \pm 1\%$, $n = 7$) of carotid arteries (figure not shown).

The HNO donor, Angeli's salt suppresses Ang II-stimulated $\cdot O_2^-$ production in isolated carotid arteries

The HNO donor, Angeli's salt (24 hrs) attenuated Ang II (10 nmol/L)-stimulated $\cdot O_2^-$ production in a concentration-dependent manner, such that 0.1, 0.3 and 1 $\mu\text{mol/L}$ Angeli's salt reduced $\cdot O_2^-$ levels by $\sim 40\%$ ($P < 0.05$), $\sim 65\%$ ($P < 0.001$) and $\sim 70\%$ ($P < 0.001$) respectively (Figure 1). A submaximal concentration of 0.3 $\mu\text{mol/L}$ Angeli's salt was chosen for subsequent experiments.

The $\cdot O_2^-$ suppressing actions of Angeli's salt and IPA/NO are mediated by HNO

The ability of Angeli's salt (0.3 $\mu\text{mol/L}$: $0.21 \pm 0.05 \times 10^3$ counts/mg, $n = 10$, $P < 0.001$) to suppress Ang II-induced $\cdot O_2^-$ production ($0.61 \pm 0.07 \times 10^3$ counts/mg, $n = 13$) was reversed in the presence of the HNO scavenger, L-cysteine (3 mmol/L: $0.57 \pm 0.09 \times 10^3$ counts/mg, $n = 5$, $P < 0.01$) but unchanged in the presence of the $NO\cdot$ scavenger, carboxy-PTIO (200 $\mu\text{mol/L}$: $0.12 \pm 0.03 \times 10^3$ counts/mg, $n = 5$, Figure 2A).

Similarly, the HNO donor IPA/NO (1 $\mu\text{mol/L}$, 24hrs) significantly suppressed Ang II-induced $\cdot O_2^-$ generation (Ang II: $0.84 \pm 0.10 \times 10^3$ counts/mg vs Ang II + IPA/NO: $0.26 \pm 0.03 \times 10^3$ counts/mg, $n = 16$, $P < 0.001$) in isolated carotid arteries. The effects of IPA/NO were reduced by L-cysteine (3 mmol/L: $0.65 \pm 0.15 \times 10^3$ counts/mg, $n = 9$, $P < 0.05$) but unchanged in the presence of carboxy-PTIO (200 $\mu\text{mol/L}$: $0.23 \pm 0.06 \times 10^3$ counts/mg, $n = 7$, $P < 0.001$, Figure 2B). Carboxy-PTIO alone did not alter Ang II-stimulated $\cdot O_2^-$ generation ($0.68 \pm 0.09 \times 10^3$ counts/mg, $n = 6$, data not shown).

The $\cdot\text{O}_2^-$ suppressing actions of Angeli's salt are cGMP-independent

Neither the sGC inhibitor, ODQ ($10 \mu\text{mol/L}$: $0.19 \pm 0.04 \times 10^3$ counts/mg, $n = 6$, $P < 0.001$), nor the PKG inhibitor, Rp-8-pCPT-cGMPS ($10 \mu\text{mol/L}$: $0.35 \pm 0.05 \times 10^3$ counts/mg, $n = 8$, $P < 0.01$) altered the ability of Angeli's salt ($0.3 \mu\text{mol/L}$: $0.20 \pm 0.05 \times 10^3$ counts/mg, $n = 6$, $P < 0.001$) to suppress Ang II-stimulated $\cdot\text{O}_2^-$ production ($0.92 \pm 0.12 \times 10^3$ counts/mg, $n = 17$, Figure 3) in carotid arteries from C57BL/6J mice. Furthermore, neither ODQ ($0.96 \pm 0.09 \times 10^3$ counts/mg, $n = 6$) nor Rp-8-pCPT-cGMPS ($0.72 \pm 0.11 \times 10^3$ counts/mg, $n = 8$) alone had any effect on Ang II-stimulated $\cdot\text{O}_2^-$ levels (data not shown).

Angeli's salt suppresses Nox2-containing NADPH oxidase $\cdot\text{O}_2^-$ generation

Treatment with Ang II (10 nmol/L , $0.49 \pm 0.15 \times 10^3$ counts/mg, $n = 4$) did not increase $\cdot\text{O}_2^-$ levels in isolated carotid arteries from Nox2^{-/-} mice compared to the vehicle ($0.71 \pm 0.16 \times 10^3$ counts/mg, $n = 4$). Similarly, the ability of Angeli's salt to limit the Ang II-stimulated $\cdot\text{O}_2^-$ production was absent in Nox2^{-/-} mice ($0.67 \pm 0.31 \times 10^3$ counts/mg, $n = 4$, Figure 4).

Table 1. The effects of angiotensin II (Ang II, 10 nmol/L, 24hrs)-treatment on mRNA gene expression of Nox2, p47^{phox} and genes involved in the NO signaling pathway.

Gene Expression	Symbol	Vehicle (50 nmol/L glacial acetic acid)	Ang II (10 nmo/L)
NADPH oxidase 2	Nox2	1	-0.59↔
p47 ^{phox}	p47 ^{phox}	1	-0.63↔
Gene Expression (selected from the NO signalling array)	Symbol	Vehicle (50 nmol/L glacial acetic acid)	Ang II (10 nmo/L)
NADPH oxidase 1	Nox 1	1	-1.18↔
NADPH oxidase 4	Nox 4	1	-1.06↔
NO synthase 1 (neuronal)	nNOS	1	N/A
NO synthase 2 (inducible)	iNOS	1	-1.17↔
NO synthase 3 (endothelial)	eNOS	1	-1.13↔
Superoxide dismutase 1 (soluble)	SOD 1	1	-1.11↔
Superoxide dismutase 2 (mitochondrial)	SOD 2	1	-1.05↔
Superoxide dismutase 3 (extracellular)	SOD 3	1	-1.24↔
Glutathione peroxidase 1	Gpx 1	1	-1.24↔
Glutathione peroxidase 2	Gpx 2	1	-1.04↔
Glutathione peroxidase 3	Gpx 3	1	-1.33↔
Glutathione peroxidase 4	Gpx 4	1	-1.04↔
Glutathione peroxidase 5	Gpx 5	1	N/A
Glutathione peroxidase 6	Gpx 6	1	N/A
Glutathione peroxidase 7	Gpx 7	1	N/A

Gene expression for Nox2 and p47^{phox} are normalised to β -actin while genes from the NO signaling pathway are normalised to a combine average of the housekeeping genes Gusb1, Hprt1, β -actin, Hsp90ab1 and GAPDH (n = 3 for all experiments).

Values are expressed as fold changes relative to the vehicle (represented as 1) using the formula $2^{-\Delta\Delta CT}$, indicating either a downregulation (\downarrow), upregulation (\uparrow) or no significant change (\leftrightarrow) in expression. C_T values reported as N/A represent values which were ≥ 35 cycles and considered as a negative call, indicating that the gene was not highly expressed.

Figure 1. Concentration-dependent effects of Angeli's salt (AS, 1 nmol/L - 1 μ mol/L, 24hrs, n = 7-9) upon angiotensin II (Ang II, 10 nmol/L, 24hrs, n = 25)-stimulated $\cdot\text{O}_2^-$ production in isolated carotid arteries from C57BL/6J mice. $\cdot\text{O}_2^-$ production is expressed as 10^3 counts per mg of dry tissue weight. Values are given as mean \pm s.e.mean, where n = number of mice. $^{###}P < 0.001$ versus vehicle; $^*P < 0.05$, $^{***}P < 0.001$ versus Ang II (One-way ANOVA, Bonferroni's multiple comparisons test). Vehicle = 50 nmol/L glacial acetic acid (n = 8).

Figure 1

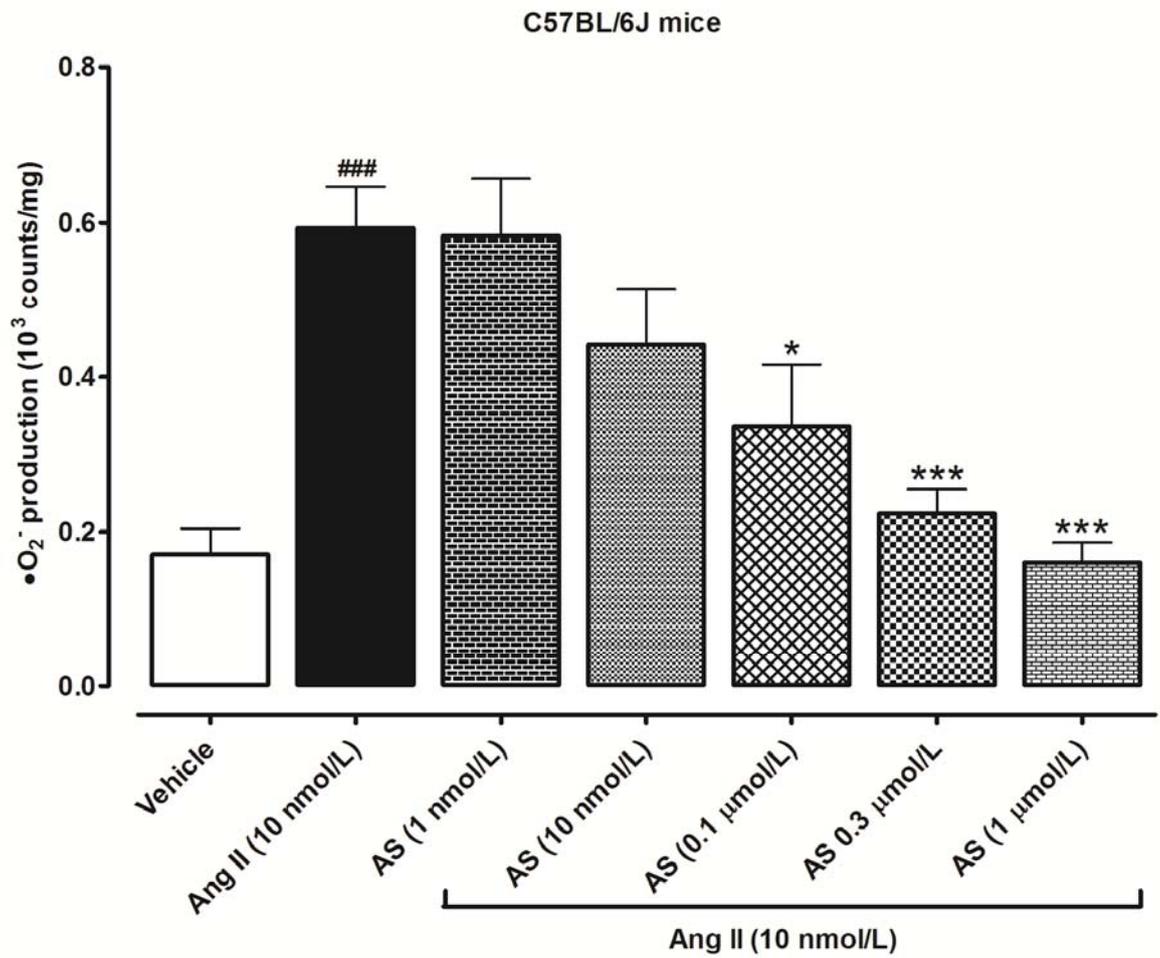


Figure 2. The effects of **(A)** Angeli's salt (AS, 0.3 $\mu\text{mol/L}$, 24hrs, n = 10) and **(B)** IPA/NO (1 $\mu\text{mol/L}$, 24hrs, n = 16) upon angiotensin II (Ang II, 10 nmol/L , 24hrs, n = 13-16)-stimulated $\cdot\text{O}_2^-$ production in isolated carotid arteries from C57BL/6J mice in the absence and presence of the HNO scavenger L-cysteine (3 mmol/L , 24hrs, n = 5-9) and the $\text{NO}\cdot$ scavenger, carboxy-PTIO (200 $\mu\text{mol/L}$, 24hrs, n = 5-7). $\cdot\text{O}_2^-$ production is expressed as 10^3 counts per mg of dry tissue weight. Values are given as mean \pm s.e.mean, where n = number of mice. *** $P < 0.001$ versus Ang II; # $P < 0.05$ versus **(A)** Ang II + AS and **(B)** Ang II + IPA/NO (One-way ANOVA, Bonferroni's multiple comparisons test).

Figure 2

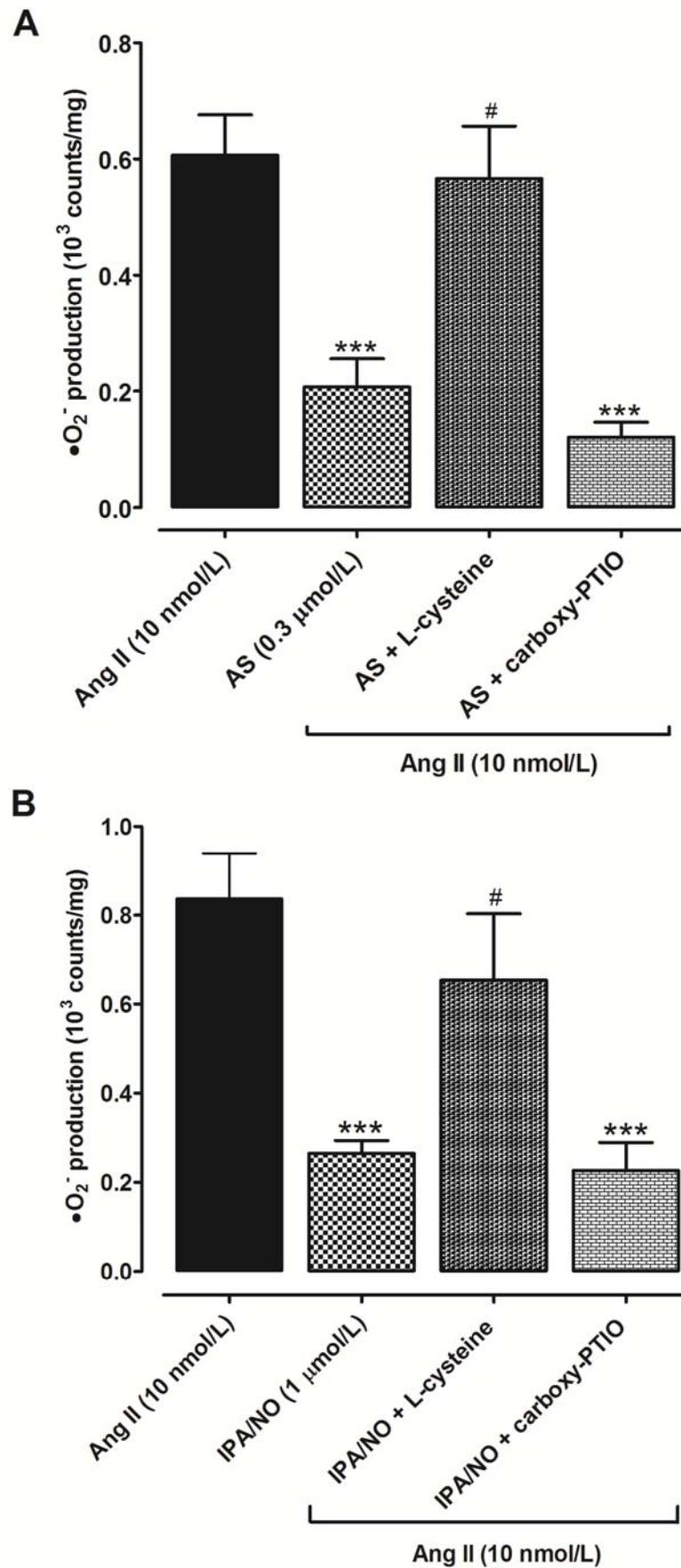


Figure 3. The effects of Angeli's salt (AS, 0.3 $\mu\text{mol/L}$, 24hrs, n = 6) upon angiotensin II (Ang II, 10 nmol/L, 24hrs, n = 17)-stimulated $\cdot\text{O}_2^-$ production in isolated carotid arteries from C57BL/6J mice in the absence and presence of the sGC inhibitor, ODQ (10 $\mu\text{mol/L}$, 24hrs, n = 6) or the protein kinase G inhibitor, Rp-8-pCPT-cGMPS (10 $\mu\text{mol/L}$, 24hrs, n = 8). $\cdot\text{O}_2^-$ production is expressed as 10^3 counts per mg of dry tissue weight. Values are given as mean \pm s.e.mean, where n = number of mice. ** $P < 0.01$, *** $P < 0.001$ versus Ang II (One-way ANOVA, Bonferroni's multiple comparisons test).

Figure 3

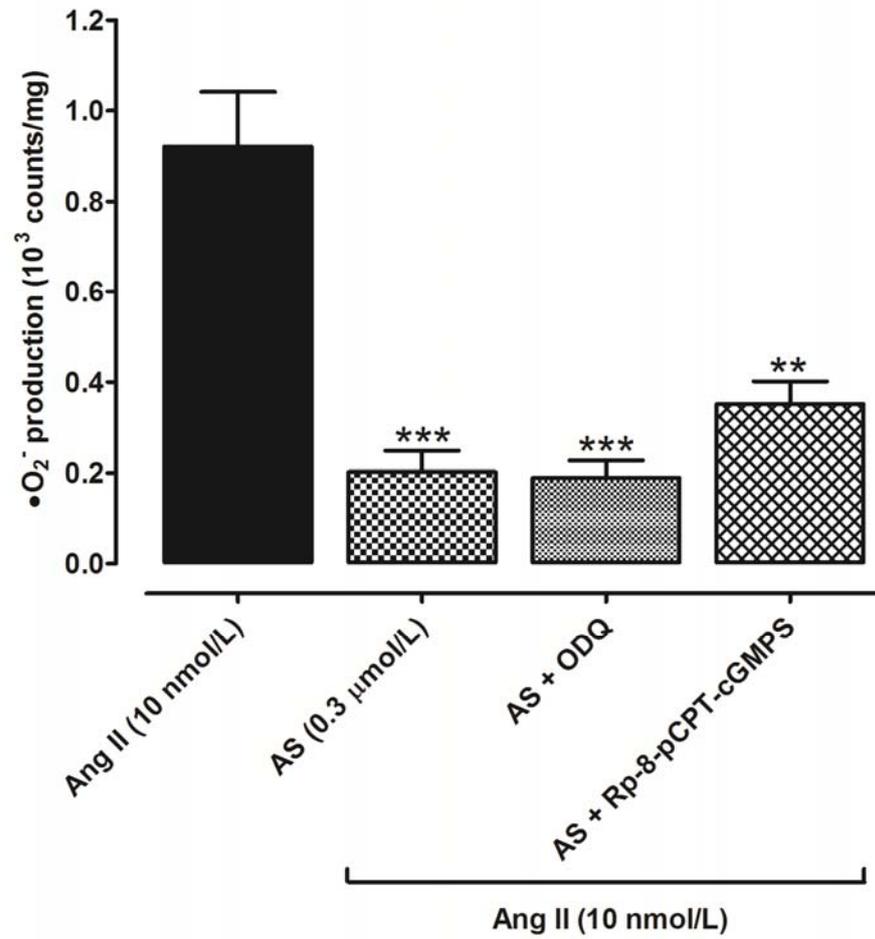
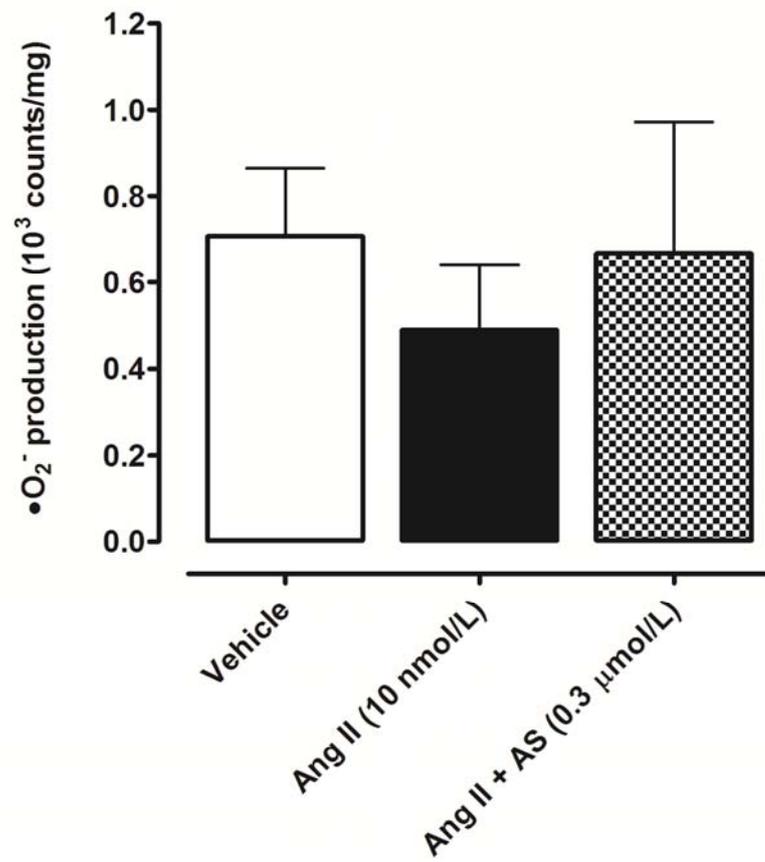


Figure 4. The effects of Angeli's salt (AS, 0.3 $\mu\text{mol/L}$, 24hrs, n = 4) upon angiotensin II (Ang II, 10 nmol/L, 24hrs, n = 4)-stimulated $\cdot\text{O}_2^-$ production in isolated carotid arteries from Nox2^{-/-} mice. $\cdot\text{O}_2^-$ production is expressed as 10^3 counts per mg of dry tissue weight. Values are given as mean \pm s.e.mean, where n = number of mice. Vehicle = 50 nmol/L glacial acetic acid (n = 4).

Figure 4



This study has provided the first evidence for an ability of HNO to limit vascular superoxide (O_2^-) generation via a cGMP-independent inhibition of Nox2-containing NADPH oxidase in response to Ang II. Such an effect appears to arise as a consequence of HNO decreasing the activity rather than the expression of Nox2 NADPH oxidase.

In the vasculature, NADPH oxidases represent the most quantitatively relevant sources of O_2^- (Drummond *et al.*, 2011; Selemidis *et al.*, 2007). NADPH oxidases play a central role in vascular oxidative stress and are upregulated in disease states associated with an increase in the renin-angiotensin system (RAS) (Cave *et al.*, 2006; Garrido & Griendling, 2009). Thus, the major effector of the RAS, angiotensin II (Ang II) targets the Ang II type 1 receptor (AT_1) to induce a protein kinase C (PKC)-dependent activation of NADPH oxidase and a subsequent increase O_2^- generation (Garrido & Griendling, 2009). Indeed, 24-hour treatment of isolated mouse common carotid arteries with Ang II (10 nmol/L) leads to a significant increase in O_2^- generation and is accompanied by a decrease in endogenous NO bioavailability (Chrissobolis *et al.*, 2008; Didion *et al.*, 2005). This model has been utilised in the current study to investigate an ability of HNO to limit O_2^- generation. In agreement with previous studies, Ang II (10 nmol/L, 24hrs) almost doubled O_2^- levels in both mouse common carotid arteries and aortae (data not shown), as detected using L-012- and lucigenin-enhanced chemiluminescence. Due to a non-specific interaction between the HNO donor, Angeli's salt and the luminescent probe L-012 (Supplementary Figure 2) in the absence of tissue, all subsequent experiments were conducted using lucigenin-enhanced chemiluminescence. Similarly, Tochetti and co-workers have previously reported a direct interaction between Angeli's salt and the calcium (Ca^{2+}) indicators fluo-4 and Indo-1, such that their fluorescent properties were altered (Tochetti *et al.*, 2007).

Despite the ability of Ang II to increase vascular O_2^- production, this was not associated with a decrease in endogenous NO bioavailability as assessed via contraction to the NOS inhibitor, L-

NAME. Furthermore, vasorelaxation to the endothelium-dependent vasodilator, ACh was unchanged in vehicle versus Ang II-treated carotid arteries. This is in contrast to previous studies which reported a correlation between an increase in Ang II-mediated $\cdot\text{O}_2^-$ generation and an approximate 50% impairment in endothelial function as assessed via ACh-mediated vasorelaxation (Didion *et al.*, 2005; Scrader *et al.*, 2007). Given that under the experimental conditions employed, we observed a similar increase in $\cdot\text{O}_2^-$ generation as in previous studies (Didion *et al.*, 2005), the reasons underlying the observed lack of endothelial dysfunction are unclear. One possible explanation may be related to the age of the mice used. While Didion and co-workers used slightly older ~7 month old mice (Didion *et al.*, 2005), the current study used young adult (10-14 weeks) C57BL/6J mice which may account for the preserved endothelial function following short-term Ang II-treatment. Slight variations in the setup and equilibration of the arteries used in the previous studies versus the conditions we employed may also explain the differences observed in NO bioavailability.

Importantly, the ability of Ang II to increase vascular $\cdot\text{O}_2^-$ was absent in carotid arteries from $\text{Nox2}^{-/-}$ mice, suggesting that the Ang II-mediated effect is Nox2-dependent. These findings concur with previous studies in mouse aortae (Scrader *et al.*, 2007) and heart homogenates (Bendall *et al.*, 2002) where the deletion of the Nox2 gene prevented Ang II-induced elevation in $\cdot\text{O}_2^-$ production. It should be noted that basal $\cdot\text{O}_2^-$ levels in carotid arteries from $\text{Nox2}^{-/-}$ mice were higher as compared to those recorded in C57BL/6J mice. Given previous studies in our laboratory have indicated that basal $\cdot\text{O}_2^-$ levels are similar in $\text{Nox2}^{-/-}$ compared with wildtype mice (Miller *et al.*, 2009), such differences may reflect differential sensitivities of the equipment employed. Thus the TopCount single photon Counter and the Hidex Chemiluminescence plate reader were used to detect $\cdot\text{O}_2^-$ levels in arteries from C57BL/6J and $\text{Nox2}^{-/-}$ mice, respectively. Indeed, subsequent studies utilising the Hidex Chemiluminescence plate reader indicated higher basal $\cdot\text{O}_2^-$ levels in carotid arteries from C57BL/6J mice (Figure 4A, Chapter 7) as compared

with measurements made with the TopCount single photon counter. Nevertheless, all experiments were internally controlled, such that findings were consistent and reliable.

Whilst the dependence of Ang II upon Nox2-containing NADPH oxidase for the increase in $\cdot\text{O}_2^-$ generation is evident, Ang II did not significantly alter the expression of Nox2 at the level of mRNA. Such findings suggest that short-term (24hr) treatment with Ang II increases the activity rather than the expression of Nox2-containing NADPH oxidase. Moreover, the translocation of the cytosolic subunit p47^{phox} to the membrane and its interaction with Nox2 is crucial for both the assembly and activation of NADPH oxidases (Selemidis *et al.*, 2008). Indeed, there is evidence that Ang II (100 nmol/L, 24hrs)-treatment of H9C2 cardiac muscle cells increases the mRNA expression of p47^{phox} (Qin *et al.*, 2006). Thus, we sought to determine if 24 hour Ang II-treatment would alter the mRNA expression of this regulatory subunit in mouse carotid arteries. Similar to Nox2, however, the expression of p47^{phox} was unaffected by short-term Ang II-treatment. A lack of effect of Ang II upon Nox2 and p47^{phox} mRNA is perhaps not so surprising given that changes in gene expression are generally observed following *in vitro* incubation for longer than 24 hours (Ritchie *et al.*, 2007) and in cultured cells (Qin *et al.*, 2006) as opposed to intact arteries *in vitro*. In fact it has been suggested that a study conducted *in vitro* does not necessarily reflect *in vivo* circumstances such that mRNA levels do not necessarily correspond to NADPH oxidase activity (Yokoyama & Inoue, 2004). In addition, the Mouse NO Signalling Pathway RT² Profiler PCR array kit was used to screen for changes in 84 different genes which play an important role in the NO signalling cascade with particular focus on the Noxs, NOS, superoxide dismutases (SODs) and glutathione peroxidases (Gpxs). The results again demonstrated that Ang II-treatment does not alter the expression of Noxs, NOS, SODs, Gpxs nor any of the genes represented on the superarray in mouse carotid arteries at the level of mRNA. Thus, our findings suggest that short-term Ang II-treatment of isolated carotid arteries is associated with a Nox2-dependent increase in $\cdot\text{O}_2^-$ production, yet no change in NO

bioavailability nor gene expression, at the level of mRNA, of the NADPH oxidase subunits Nox2 and p47^{phox}.

To examine the ability of HNO to limit vascular $\cdot\text{O}_2^-$ generation, the well characterised HNO donor, Angeli's salt was used. Interestingly, 24-hour treatment with Angeli's salt (1 nmol/L - 1 $\mu\text{mol/L}$) caused a concentration-dependent decrease in Ang II-stimulated $\cdot\text{O}_2^-$ levels with concentrations of $\geq 0.1 \mu\text{mol/L}$ having the most significant effect. Subsequent studies utilised 0.3 $\mu\text{mol/L}$ Angeli's salt, which caused an ~65% decrease in vascular $\cdot\text{O}_2^-$ generation. Importantly, such an effect of Angeli's salt did not appear to occur as a consequence of HNO directly scavenging $\cdot\text{O}_2^-$, as previous studies in our laboratory have shown that unlike superoxide dismutases (SODs), Angeli's salt was unable to directly scavenge $\cdot\text{O}_2^-$ generated via xanthine (100 $\mu\text{mol/L}$)/xanthine oxidase (50 mU/ml or 10 mU/ml) in cell-free assays (Bullen *et al.*, unpublished observations). Rather, the findings suggest a direct interaction with NADPH oxidase itself.

Given that Angeli's salt can decompose to generate both HNO and nitrite (NO_2^-) (Miranda *et al.*, 2005), and NO_2^- can modulate vascular function (Demoncheaux *et al.*, 2002), it was important to confirm that this $\cdot\text{O}_2^-$ -suppressing action of Angeli's salt was not attributed to NO_2^- . Thus, a second donor, isopropylamine NONOate (IPA/NO) was employed which spontaneously decomposes to at physiological pH to generate HNO but not NO_2^- , with a comparable half-life (2.3 mins), vasodilator potency and efficacy to Angeli's salt (data not shown). Importantly, IPA/NO decreased Ang II-mediated $\cdot\text{O}_2^-$ production to levels comparable to those observed with Angeli's salt.

These findings with Angeli's salt and IPA/NO suggest that the $\cdot\text{O}_2^-$ suppressing action is a class effect of HNO donors. However, given that HNO may undergo oxidation to NO^\bullet both

extracellularly (Ellis *et al.*, 2001; Nelli *et al.*, 2001) and intracellularly (Dierks & Burstyn, 1996; Zeller *et al.*, 2009), and we have shown that the NO[•] donor DETA/NONOate can suppress endothelial NADPH-derived [•]O₂⁻ (Selemidis *et al.*, 2007), it was important to confirm that the observed effects were due to HNO rather than NO[•]. Extracellular oxidation of HNO was limited via the inclusion of the Cu²⁺ chelator, EDTA (0.026 mmol/L) in the experimental media (Irvine *et al.*, 2003) and our finding that the [•]O₂⁻ suppressing actions of Angeli's salt were maintained in the presence of NO[•] scavenger, carboxy-PTIO (200 μmol/L) yet reversed by the HNO scavenger, L-cysteine (3 mmol/L) (Irvine *et al.*, 2007; Irvine *et al.*, 2003; Wanstall *et al.*, 2001) confirmed that HNO mediated such an action. It is still possible, however, that HNO may be converted to NO[•] intracellularly, however, this cannot be ascertained currently. A recent study by Rosenthal and co-workers have identified a fluorescent molecular probe, Tripodal copper (II) BODIPY complex (Cu^{II}[BOT1]) with visible excitation and emission profiles for detecting HNO in living biological systems (Rosenthal & Lippard, 2010). Such a technique may be of use in future studies, perhaps in determining if HNO is being oxidised within the cell or if HNO targets specific subcellular localisations. Nevertheless, the finding that L-cysteine reversed the ability of both Angeli's salt and IPA/NO to limit [•]O₂⁻ production in the current study suggests that effects of these donors were due to HNO.

These findings concur with our previous observation in cultured neonatal cardiomyocytes in which Angeli's salt (1 μmol/L, 48hrs) suppressed the Ang II (1 μmol/L)-mediated increase in cellular [•]O₂⁻. Moreover Angeli's salt was found to also decrease the expression of Nox2 mRNA, indicative of an ability of HNO to modulate Nox2-containing NADPH oxidase (Ritchie *et al.*, 2007). Similarly in isolated carotid arteries, the ability of Angeli's salt to limit [•]O₂⁻ production was absent in Nox2^{-/-} mice. Moreover, the effects of HNO appear to be specific for Nox2-containing NADPH oxidase as the residual [•]O₂⁻ signal in the carotid arteries from Nox2^{-/-} mice, which is possibly due to either Nox1- or Nox4- NADPH oxidase, was unchanged by Angeli's

salt. Alternatively, given the reciprocal relationship between Nox enzymes and other ROS producing systems (i.e. mitochondrial, uncoupled eNOS) (Griendling & Brown, 2009), it would be of interest for future studies to investigate, following 24-hour Ang II-treatment, if HNO acts by attenuating Nox activity and/or if multiple sources of ROS are involved. Indeed, Ang II stimulation has been reported to result in mitochondrial ROS production and eNOS uncoupling which occurs downstream of the initial Nox activation, indicating the important roles of Nox enzymes as both initiators and integrators of redox signaling (Griendling & Brown, 2009). However, pilot studies conducted in our laboratory have shown that both Angeli's salt and IPA/NO rapidly suppressed Ang II-mediated $\cdot\text{O}_2^-$ production in the cerebral and peripheral vasculature (Miller *et al.*, unpublished observations), presumably suggesting that HNO may be able to directly inhibit Nox2 activity.

It is well documented that HNO can modulate cellular function via cGMP-dependent and -independent pathways (Irvine *et al.*, 2008). Thus, vasorelaxation in response to HNO donors is mediated predominantly via the activation of sGC and a subsequent increase in cGMP (Favaloro & Kemp-Harper, 2007; Fukuto *et al.*, 1992; Irvine *et al.*, 2007; Irvine *et al.*, 2003), yet HNO can also target K_v and K_{ATP} channels (Irvine *et al.*, 2003) and stimulate CGRP release (Paolocci *et al.*, 2003; Paolocci *et al.*, 2001) to modulate vascular function. The current study revealed that neither the sGC inhibitor, ODQ nor the protein kinase G (PKG) inhibitor, Rp-8-pCPT-cGMPS altered the ability of Angeli's salt to suppress the Ang II-mediated increase in vascular $\cdot\text{O}_2^-$. Such findings suggest that HNO modulates NADPH oxidase activity via a cGMP-independent mechanism.

Given that HNO interacts directly with thiol groups to modify thiol-containing proteins and receptors such as the cardiac (RyR2) ryanodine receptor (Tochetti *et al.*, 2007), we hypothesise that HNO modulates NADPH oxidase activity via a direct thiol interaction. Indeed, evidence

exists to indicate that post-translational modification of reactive cysteine thiols within NADPH oxidase can modify its activity. Indeed, NO^\bullet has been reported to reduce NADPH oxidase-dependent O_2^\bullet production in human endothelial cells via S-nitrosylation of the NADPH cytosolic subunit, p47^{phox} (Selemidis *et al.*, 2007). In addition, p47^{phox} contains up to 4 cysteine residues (ie. Cys98, Cys111, Cys196, Cys 378), and in leukocytes, mutation of both Cys111 and Cys378 leads to suppressed NADPH oxidase activity (Babior, 2002; Inanami *et al.*, 1998). Furthermore, given that Cys378 is in close proximity to essential serine residues (Ser303-379) which are required for the phosphorylation, translocation and binding of p47^{phox} to the Nox2/p22^{phox} complex (El-Benna *et al.*, 2009), its modification may lead to attenuated NADPH oxidase function. Unlike NO^\bullet , HNO interacts directly with thiols to generate a disulfide (a reversible process) or sulfinamide (an irreversible process) (Fukuto *et al.*, 2009), and we anticipate that it may interact with cysteine residues on p47^{phox} resulting in altered phosphorylation, translocation and or binding of p47^{phox} to the Nox2/p22^{phox} complex.

In order to test this hypothesis, future studies will need to determine if the ability of HNO to suppress O_2^\bullet is lost in p47^{phox-/-} mice. It would also be of interest to investigate if HNO alters the phosphorylation and/or translocation of p47^{phox} and its association with Nox2. Generation of the sulfinamide moiety is specific for HNO and novel mass-spectrometry based methods (Hoffmann *et al.*, 2009) could be applied to detect HNO-mediated modification of cysteine residues on NADPH oxidase subunits.

A further possible mechanism by which HNO could suppress the activity of NADPH oxidase is via the induction of heme-oxygenase 1 (HO-1) (Naughton *et al.*, 2002). HO-1 catalyses the degradation of heme to produce iron, carbon monoxide and biliverdin, which in turn is rapidly converted to bilirubin (Otterbein *et al.*, 2003). HO-1 demonstrates powerful antioxidant properties via the generation of the ROS scavenger, bilirubin and a direct modulation of NADPH

oxidase activity (Minetti *et al.*, 1998; Taille *et al.*, 2004). Thus, heme incorporation is essential for the activity and stability of Nox2 as well as its association with p22^{phox} (Taille *et al.*, 2004). Indeed, induction of HO-1 in macrophages leads to a decrease in cellular heme content and reduced Nox2-containing NADPH oxidase activity and protein expression (Taille *et al.*, 2004). In addition, the HO-1 products bilirubin (Minetti *et al.*, 1998) and carbon monoxide (Otterbein, 2002) have also been shown to inhibit NADPH oxidase activity. Thus, given that HNO has been shown to increase the activity, protein and mRNA expression of HO-1 in rat H9C2 cells (Naughton *et al.*, 2002), it may suppress vascular NADPH oxidase activity, in part, via the induction of HO-1. This hypothesis, however, remains to be tested.

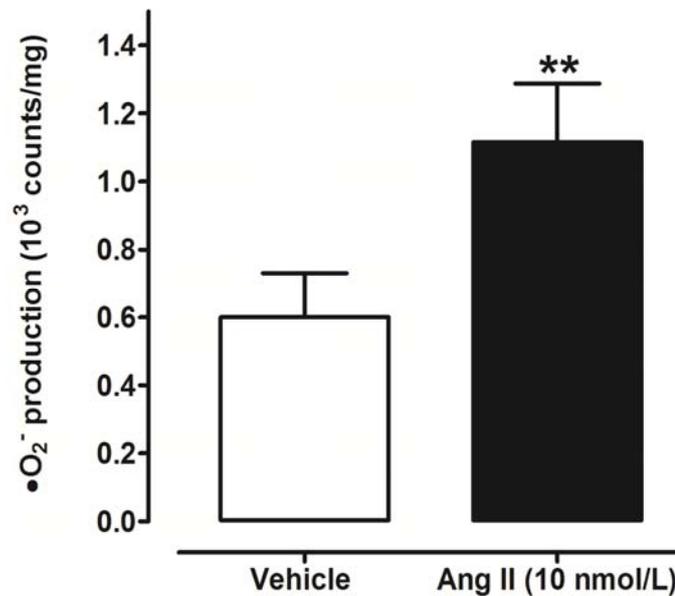
In our discussion to date, we have assumed that the inhibitory action of HNO upon vascular $\cdot\text{O}_2^-$ generation is indicative of a direct modulation of Nox2-containing NADPH oxidase. However, it is also possible that HNO may inhibit signal transduction elements upstream of NADPH oxidase such as the AT₁ receptor. Future studies utilising an alternate stimuli such as endothelin-1 (ET-1) or a PKC activator which directly phosphorylates p47^{phox} (ie. phorbol dibutyrate; PDB) are required to confirm that HNO has a direct effect on one or more of the NADPH oxidase subunits.

Moreover, despite the clear ability of NO \cdot to also suppress NADPH oxidase-mediated vascular $\cdot\text{O}_2^-$ generation, we propose that HNO may be more advantageous due to its resistance to $\cdot\text{O}_2^-$ scavenging and the lack of tolerance development both *in vitro* (Irvine *et al.*, 2007) and *in vivo* (Irvine *et al.*, 2011), two factors which limit the current clinical use of nitrovasodilators. In addition, recent studies in our laboratory have revealed that HNO reduces NADPH oxidase activity almost immediately, and more potently than does NO \cdot (Miller *et al.*, unpublished observations). Taken together, the study reveals that HNO suppresses Ang II-stimulated $\cdot\text{O}_2^-$ production in mice. This effect is independent of an ability of HNO to directly scavenge $\cdot\text{O}_2^-$, and

rather is indicative of a direct modulation of Nox2-containing NADPH oxidase activity by HNO. Such an action of HNO is cGMP-independent and may arise as a consequence of direct modification of thiols on regulatory subunits of NADPH oxidase. Thus, the concomitant ability of HNO to cause vasorelaxation (Ellis *et al.*, 2000; Favalaro & Kemp-Harper, 2007; Irvine *et al.*, 2007; Irvine *et al.*, 2003), inhibit platelet aggregation (Bermejo *et al.*, 2005) and decrease $\cdot\text{O}_2^-$ generation (Lopez *et al.*, 2007; Ritchie *et al.*, 2007) may confer therapeutic potential in the treatment of vascular dysfunction associated with cardiovascular diseases such as hypertension and atherosclerosis.

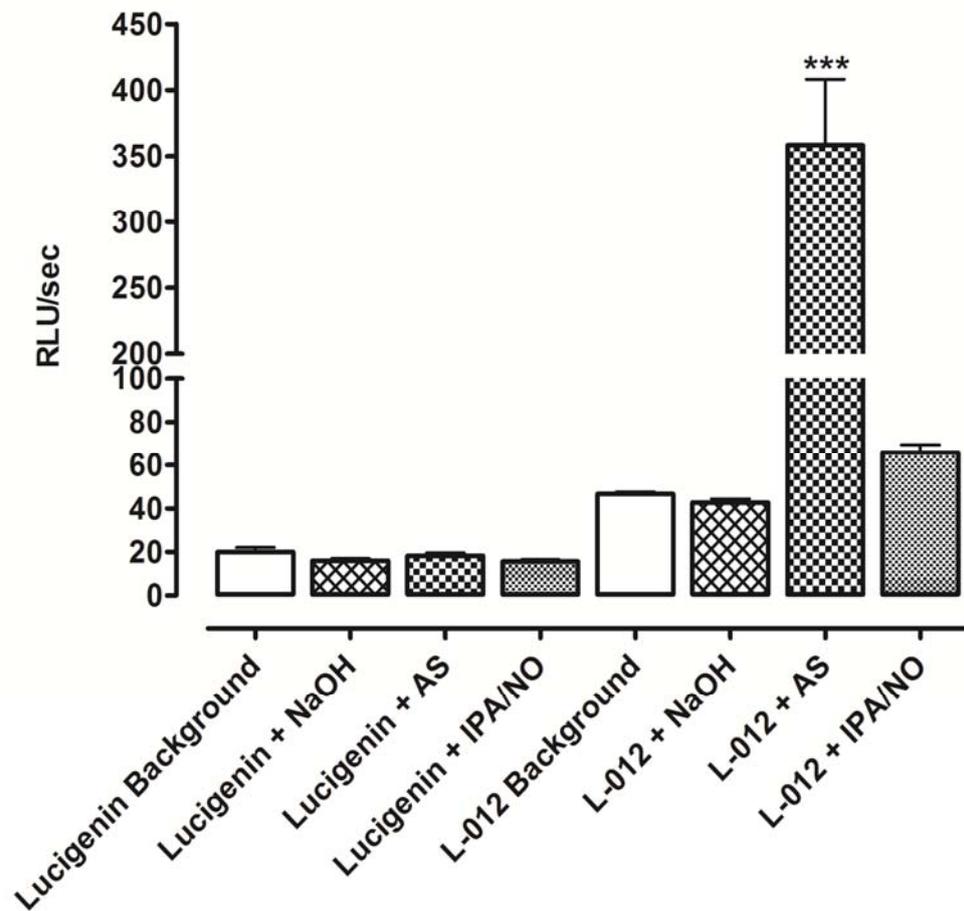
Additional Figures and Supporting Information

Figure 1



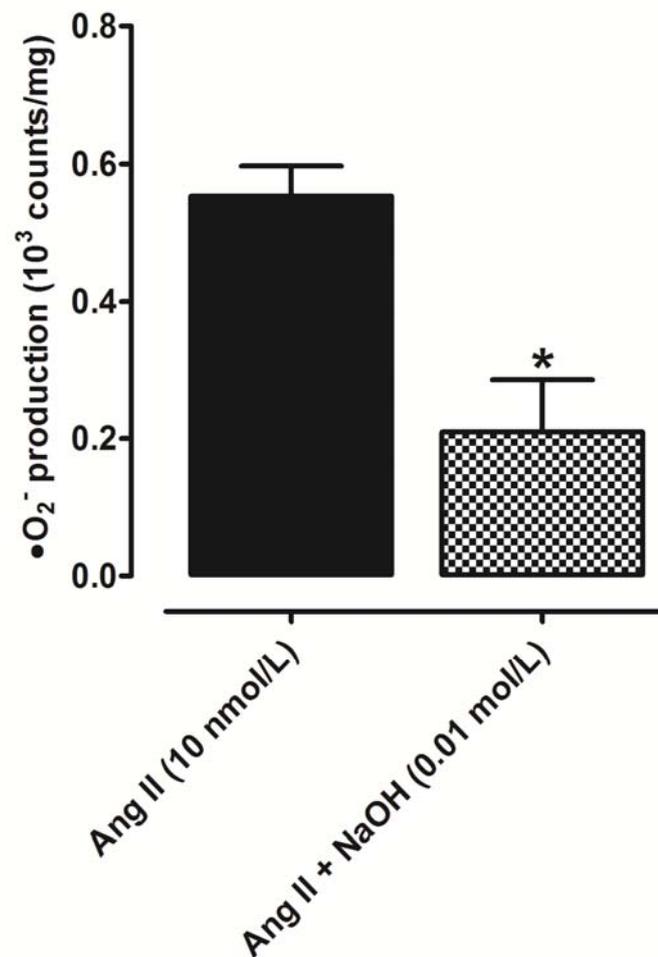
Supplementary Figure 1. The effects of angiotensin II (Ang II, 10 nmol/L, 24hrs, n = 9) on $\bullet\text{O}_2^-$ production in isolated carotid arteries from C57BL/6J mice as measured by L-012 (100 $\mu\text{mol/L}$)-enhanced chemiluminescence and expressed as 10^3 counts per mg of dry tissue weight. Values are given as mean \pm s.e.mean, where n = number of mice. ** $P < 0.01$ versus vehicle (Student's unpaired t-test). Vehicle = 50 nmol/L glacial acetic acid (n = 9).

Figure 2



Supplementary Figure 2. The effects of sodium hydroxide (0.01 mol/L NaOH, $n = 3$), Angeli's salt (AS, 0.3 $\mu\text{mol/L}$, $n = 3$) or IPA/NO (1 $\mu\text{mol/L}$, $n = 3$) on background chemiluminescence in response to lucigenin (5 $\mu\text{mol/L}$) and L-012 (100 $\mu\text{mol/L}$) in cell-free assays. Background values for both lucigenin- and L-012-enhanced chemiluminescence are shown for comparison ($n = 3$). Luminescence are expressed as relative light units per second (RLU/s). Values are given as mean \pm s.e.mean, where $n =$ number of assays performed. *** $P < 0.01$ versus L-012 background (One-way ANOVA, Bonferroni's multiple comparisons test).

Figure 3



Supplementary Figure 3. The effects of sodium hydroxide (0.01 mol/L NaOH, 24hrs, n = 4) on angiotension II (Ang II, 10 nmol/L, 24hrs, n = 3)-mediated $\cdot\text{O}_2^-$ production in isolated carotid arteries from C57BL/6J mice. $\cdot\text{O}_2^-$ production was detected via lucigenin-enhanced chemiluminescence and expressed as 10^3 counts per mg of dry tissue weight. Values are given as mean \pm s.e.mean, where n = number of mice. * $P < 0.05$ versus Ang II (One-way ANOVA, Bonferroni's multiple comparisons test).

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CHAPTER 4

Endogenous NO may limit the formation of vascular oxidized/heme-free sGC, an effect which is lost in the setting of hypertension



Endothelial-derived nitric oxide (NO) mediates vascular smooth muscle relaxation via activation of the reduced-heme (Fe^{2+}) containing form of soluble guanylyl cyclase (sGC) and a subsequent increase in cGMP. Disease states associated with an increase in oxidative stress interfere with this NO/sGC/cGMP signaling pathway, either by scavenging NO or by converting sGC into the NO-insensitive, oxidized (Fe^{3+})/heme-free form. The recently developed NO-independent heme mimetic, BAY 58-2667 has been shown to target these altered states of sGC, resulting in the preferential vasorelaxation of diseased blood vessels. Here we examined the potential protective role of endothelial-derived NO in limiting the accumulation of oxidized/heme-free sGC using BAY 58-2667-mediated vasorelaxation as a marker of oxidized/heme-free sGC. In endothelium-intact aortae from adult normotensive WKY rats, BAY 58-2667 was a potent relaxant ($\text{pEC}_{50} = 9.83 \pm 0.36$ -log M) eliciting near maximal relaxation ($89 \pm 5\%$, $n = 9$). Removal of the endothelium or NOS inhibition (L-NAME 10 μM /L) enhanced the potency of BAY 58-2667 by approximately 29 and 417-fold ($P < 0.05$), respectively. Conversely, responses to the exogenous NO donor, DEA/NO, the cGMP analogue, 8-bromo-cGMP or the sGC-independent vasodilator, papaverine were unchanged following endothelial removal or NOS inhibition. Similarly, the potency of BAY 58-2667 was augmented in L-NAME-treated rat isolated small mesenteric arteries ($P < 0.05$) and in rabbit isolated aortae and saphenous arteries ($P < 0.05$) compared to their age-matched controls. This effect, however was not observed in mice, where the response to BAY 58-2667 was unchanged following either acute (L-NAME, 100 μM /L) or chronic (L-NAME-treated, 100 mg/kg/day for 28 days) NOS inhibition or upon the deletion of eNOS (eNOS^{-/-}). Thus, our results suggest that the regulatory role of endogenous NO in the vasculature was somewhat species specific. A potential direct chemical interaction between NO with BAY58-2667 was excluded via UV/VIS spectroscopy. Furthermore, in isolated aortae from spontaneously hypertensive rats (SHR), in which endothelial dysfunction (ACh vasorelaxation decreased by 36%, $P < 0.05$, $n = 6 - 9$) and increased basal $\cdot\text{O}_2^-$ production (67% increase compared with WKY, $P < 0.05$, $n = 5$ for each group) was apparent, the vasorelaxant response to

BAY 58-2667 was markedly enhanced ($pEC_{50} = 11.34 \pm 0.33 -\log M$, $P < 0.05$ $n = 5$; $R_{max} = 97 \pm 0.5\%$ reversal of pre-contraction) compared to the response in WKY. Conversely, the vasorelaxant responses to DEA/NO, 8-bromo-cGMP and papaverine were unchanged in isolated endothelium-intact aortae between WKY and SHR. Moreover, in contrast to the findings in WKY aortae, neither removal of the endothelium, NOS (L-NAME) nor sGC (ODQ) inhibition improved the response to BAY 58-2667 compared to that observed in isolated endothelium-intact aortae from SHR. Collectively our data suggests that endothelial-derived NO may modulate the vasorelaxant response to BAY 58-2667, possibly via limiting the accumulation of oxidized/heme-free sGC in the vasculature. Therefore, we propose that under disease conditions, a loss of endogenous NO may contribute to an increase in the pools of oxidized/heme-free sGC and under such circumstances the response to BAY 58-2667 will be enhanced.

In the cardiovascular system, soluble guanylyl cyclase (sGC) serves as the primary receptor for endothelial-derived nitric oxide (NO) (Ignarro, 1999; Moncada *et al.*, 1991). sGC is a heterodimeric heme protein consisting of a large α and a smaller heme-containing β subunit (Schmidt *et al.*, 2004). NO binds to the prosthetic heme group on the β subunit (Feil & Kemp-Harper, 2006) when in its ferrous state, inducing a conformational change in the enzyme and activation of sGC (Friebe & Koesling, 2003). Upon activation, sGC catalyses the enzymatic conversion of guanosine-5'-triphosphate (GTP) to form the second messenger cyclic guanosine-3',5'-monophosphate (cGMP), which plays an important role in the regulation of a number of vascular functions such as platelet aggregation, cell growth and differentiation and smooth muscle relaxation (Kemp-Harper & Schmidt, 2008). Thus, the NO/sGC/cGMP signaling pathway is crucial for the maintenance of cardiovascular homeostasis (Evgenov *et al.*, 2006). However, under pathophysiological vascular conditions such as hypertension and atherosclerosis, the NO/sGC/cGMP signaling pathway is impaired as evidenced by attenuated endothelium-dependent relaxation and decreased responsiveness to exogenous NO (Cai & Harrison, 2000; Munzel *et al.*, 2003). Furthermore, the excessive generation of reactive oxygen species (ROS) has also been reported to interfere with this signaling cascade (Armitage *et al.*, 2009).

Indeed, three underlying mechanisms involved in the dysfunction of the NO/sGC/cGMP pathway have been proposed. First, ROS can indirectly affect and reduce NO synthesis via the uncoupling of endothelial NOS (eNOS). Second, an increase in vascular superoxide anion radical (O_2^-) generation can result in enhanced scavenging of NO, forming the powerful oxidant peroxynitrite (ONOO^-) and consequently decreasing NO bioavailability (Armitage *et al.*, 2009; Evgenov *et al.*, 2006). Lastly, excessive ROS can also alter both the expression and activity of sGC via a mechanism which involves the oxidation of the heme of sGC to an NO-insensitive oxidised (Fe^{3+})/heme-free state (Armitage *et al.*, 2009; Evgenov *et al.*, 2006; Stasch *et al.*, 2006).

Indeed, ONOO⁻ can oxidise the ferrous (Fe²⁺) heme group of sGC into the ferric (Fe³⁺) form and ultimately the heme group is lost, rendering the enzyme unresponsive to NO (Weber *et al.*, 2001). Thus, a loss of vasodilator efficacy to conventional NO donors in disease (Preik *et al.*, 1996) may be due, in part, to an increase in the pools of NO-insensitive, oxidised/heme-free sGC.

Excitingly, a novel class of NO-independent compounds which preferentially target the NO-unresponsive, heme-oxidised (Fe³⁺) or heme-free forms of sGC have recently been identified (Evgenov *et al.*, 2006). Two structurally distinct members of this class, BAY 58-2667 (*Cinaciguat*) and HMR 1766 (*Ataciguat*) have been shown to activate purified sGC more potently once the heme-group is oxidised or the enzyme rendered heme-deficient (Schindler *et al.*, 2005; Schmidt *et al.*, 2009; Stasch *et al.*, 2002). Although the precise mechanisms via which HMR 1766 activates sGC remains to be elucidated, BAY 58-2667 mimics the spatial structure of the prosthetic heme group, competing with heme anchoring residues tyrosine 135 and arginine 139 on the β_1 subunit of sGC (Martin *et al.*, 2010; Schmidt *et al.*, 2004). As such, BAY 58-2667 activates heme-free sGC by a direct interaction with the unoccupied heme-binding pocket or by displacing the weakly bound oxidised heme (Stasch *et al.*, 2006). This unique property of BAY 58-2667 confers advantages over traditional nitrovasodilators and allows it to serve as a novel tool to probe for oxidised/heme-free forms of sGC. As such, BAY 58-2667 provides non-invasive quantification of the intracellular redox states of sGC (Stasch *et al.*, 2006).

Indeed in the vasculature, the ability of BAY 58-2667 to stimulate endothelial cell cGMP accumulation and elicit relaxation is enhanced following oxidation of sGC by the sGC inhibitor, ODQ or ONOO⁻ (Stasch *et al.*, 2006). Moreover, under pathological conditions associated with oxidative stress, the vasodilator potency of BAY 58-2667 is dramatically increased (Stasch *et al.*, 2006). Specifically BAY 58-2667-mediated relaxation is augmented in saphenous arteries from

Watanabe heritable hyperlipidemic (WHHL) rabbits, aortae from ApoE^{-/-} mice and spontaneously hypertensive (SHR) rats as well as mesocolon arteries from Type 2 diabetic patients as compared to their respective healthy controls (Stasch *et al.*, 2006). Whilst it is clear that vascular disease is associated with an increase in the pool of oxidised/heme-free sGC, little is known with respect to the factors contributing to, or protecting against, such changes in intracellular sGC redox equilibrium. Cardiovascular disease is associated with increased levels of ROS such as $\cdot\text{O}_2^-$ and ONOO⁻ of which the later may directly oxidise sGC (Stasch *et al.*, 2006). In addition, $\cdot\text{O}_2^-$ scavenges endogenous NO to limit its bioavailability (Armitage *et al.*, 2009). Whether a loss in NO *per se* influences the ratio of reduced to oxidised sGC in the vasculature is unknown.

Thus, using the vasodilator capacity of BAY 58-2667 as a tool to identify oxidised/heme-free sGC in the vasculature, this study aimed to determine if endothelial-derived NO limits the accumulation of oxidised/heme-free sGC and if its loss, in the setting of hypertension, contributes to augmented vasorelaxant responses to BAY 58-2667.

Materials and Methods

This study was approved by the Pharmacology Animal Ethics Committee, Monash University, Australia and conforms to the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Tissue Preparation

This study utilised isolated thoracic aortae from adult male WKY (15-17 weeks of age, n = 29) and SHR (15-17 weeks of age, n = 27) rats, small mesenteric arteries from SD rats (12-16 weeks of age, n = 7) and the thoracic aortae and saphenous arteries of adult New Zealand (NZW) rabbits (1.5 years of age, n = 18). Additionally, isolated abdominal aortae from adult male C57BL/6J (10-14 weeks of age, n = 14), N^G-nitro-L-arginine methyl ester (L-NAME) treated C57BL/6J (10-14 weeks of age, 100 mg/kg/day for 28 days refer to General Methods section 2.1.1.1, n = 4) and eNOS^{-/-} knockout (10-13 weeks of age, n = 6) mice were employed in this study. All animals were killed humanely as described in the General Methods sections 2.1.1 (mice), 2.1.2 (rats) and 2.1.3 (rabbits).

Functional Blood Vessel Studies

Isolated arteries were mounted either in organ baths (rat and rabbit thoracic aortae and saphenous arteries) or in small vessel wire myographs (mice abdominal aortae and rat small mesenteric arteries) as described in the General Methods, sections 2.6.1 and 2.6.2 respectively. Vessels were maintained in physiological Krebs' solution (rat and mouse arteries) or in Krebs-Henseleit solution containing 0.01% BSA (rabbit arteries) and bubbled continuously with carbogen (95% O₂ and 5% CO₂) at 37°C (see General Methods, sections 2.6.1.1 and 2.6.2.1).

The effects of endothelial denudation, NOS (L-NAME, 100 µmol/L or 100 mg/kg/day for 28 days) or sGC (ODQ, 10 µmol/L) inhibition on cumulative concentration-dependent

vasorelaxation responses to selected vasodilators were examined as described in the General Methods, sections 2.6.1.3 (WKY and SHR rats, NZW rabbits) and 2.6.2.3 (SD rats, C57BL/6J and eNOS^{-/-} mice).

Tail -Cuff Systolic Blood Pressure Measurements (SBP)

Systolic blood pressure (SBP) was monitored in a subgroup of rats (WKY and SHR) via the tail-cuff method (see General Methods, section 2.5). The average of at least five SBP recordings was taken for each rat.

Superoxide anion radical detection via Lucigenin-enhanced Chemiluminescence

Aortic segments from WKY and SHR rats were used to assess vascular $\cdot\text{O}_2^-$ levels using the lucigenin (5 $\mu\text{mol/L}$)-enhanced chemiluminescence assay (see General Methods, sections 2.4.1 and 2.4.1.2). Vascular $\cdot\text{O}_2^-$ was detected using the Hidex ChameleonTM Luminescence Plate Reader (Hidex Oy). Upon completion of the assay, aortic segments were allowed to dry on pre-cut foil pieces overnight at 37°C. Dried aortic segments were weighed the following day, background counts subtracted and $\cdot\text{O}_2^-$ production normalized for tissue dry weight.

UV/VIS Spectroscopy

The possible interaction between BAY 58-2667 and NO was examined via UV/VIS spectroscopy according to the protocol outlined in the General Methods section 2.8.

Statistical analysis

A Student's unpaired t-test was used to compare SBP, aortic basal $\cdot\text{O}_2^-$ production and vasorelaxation to ACh (10 $\mu\text{mol/L}$) in WKY versus SHR. Responses in SHR were expressed as a % of control (WKY). Vasorelaxation responses were expressed as a percentage reversal of the level of pre-contraction (ie. U46619 + cirazoline in rats; phenylephrine in rabbits and U46619 in

mice). Individual relaxation curves for each vasodilator were fitted to a non-linear sigmoidal logistic equation (Graphpad Prism, Version 4) and pEC₅₀ values (concentration of agonist causing 50% relaxation) were calculated and expressed as -log M. pEC₅₀ values could only be obtained for concentration-response curves which were sigmoidal in nature and where a maximum response was obtained. Differences between mean pEC₅₀ and maximum relaxation (R_{max}) values between two groups were tested using a Student's unpaired t-test, whilst a one-way ANOVA (Graphpad Prism version 4.0) with Bonferroni's multiple comparisons post-ANOVA test was used to compare differences in pEC₅₀ values and R_{max} values between three or more experimental groups. A two-way ANOVA was used to compare concentration-response curves in which pEC₅₀ values could not be determined (Sigma Stat, Version 1.0, Jandel Scientific). Where appropriate, the post-ANOVA Tukey's test was applied to identify differences between treatment groups (Sigma Stat, Version 1.0, Jandel Scientific). All results were expressed as mean ± s.e. mean, with n representing the number of animals per group. Statistical significance was accepted at the $P < 0.05$ level.

Endothelial-derived NO modulates relaxant responses to BAY 58-2667 in isolated aortae from WKY rats

The NO-independent sGC activator, BAY 58-2667 caused concentration-dependent relaxation of endothelium-intact aortae from WKY rats ($pEC_{50} = 9.83 \pm 0.36$, $-\log M$; $R_{max} = 87 \pm 5\%$ reversal of the level of pre-contraction, $n = 9$). BAY 58-2667 was up to 350 – 600, 000-fold more potent at eliciting vasorelaxation as compared to other NO-dependent and –independent vasodilators with the rank order of potency of BAY 58-2667 \gg DEA/NO ($pEC_{50} = 7.28 \pm 0.47$, $n = 6$) $>$ papaverine $>$ 8-bromo-cGMP (Figure 1).

In isolated aortae from WKY, removal of the endothelium largely abolished the response to the endothelium-dependent vasodilator, ACh (10 μ mol/L) from $64 \pm 4\%$ ($n = 9$) to $10 \pm 3\%$ ($n = 12$). In addition, endothelial denudation increased the sensitivity to BAY 58-2667 by 30-fold ($P < 0.05$, Figure 1A) with no change in maximum response. This effect was specific for the sGC activator, as relaxant responses to DEA/NO, 8-bromo-cGMP and papaverine were unchanged in endothelium-denuded versus endothelium-intact arteries (Figures 1B-D). Like endothelial denudation, NOS inhibition (L-NAME, 100 μ mol/L) in endothelium-intact aortae, caused a significant leftward shift to the concentration-response curve to BAY 58-2667 ($P < 0.001$, $n = 9$, Figure 1A), yet augmented BAY 58-2667-mediated vasorelaxation to a greater extent ($pEC_{50} = 12.44 \pm 0.33$) than endothelial denudation alone ($pEC_{50} = 11.29 \pm 0.35$, $n = 12$).

Treatment of endothelial denuded aortae with the sGC oxidant, ODQ increased the potency of BAY 58-2667 further ($pEC_{50} = 12.71 \pm 0.35$, $n = 6$, Figure 1A) such that vasorelaxant responses were similar to those obtained in the presence of L-NAME. Conversely ODQ decreased both the sensitivity and maximum response to DEA/NO (Figure 1B) yet had no effect on the vasorelaxant responses to either of the sGC-independent vasodilators, 8-bromo-cGMP or papaverine (Figures 1C-D).

Inhibition of endogenous NO augments relaxant responses to BAY 58-2667 in isolated arteries from rats and rabbits but not mice

Similar to the rat aortae, L-NAME enhanced vasorelaxation to BAY 58-2667 in isolated endothelium-intact rat small mesenteric arteries, increasing the potency (pEC_{50}) from 12.94 ± 0.17 ($n = 7$) to 14.37 ± 0.11 ($n = 5$, $P < 0.05$, Figure 2A). In addition, in both rabbit isolated aortae and saphenous artery, the sensitivity of BAY 58-2667 was enhanced by up to 30-fold ($P < 0.05$, Figures 2B-C) by L-NAME. In contrast, relaxation to BAY 58-2667 in mouse isolated abdominal aortae ($pEC_{50} = 12.89 \pm 0.11$, $n = 5$) was unchanged in the presence of L-NAME (Figure 3A). Moreover, chronic loss of endogenous NO induced by either long-term L-NAME treatment (100 mg/kg/day for 28 days, Figure 3B) or deletion of endothelial NOS (eNOS^{-/-}, Figure 3C) did not modulate BAY 58-2667-mediated relaxation in mouse isolated aortae. Loss of endothelium-derived NO in the aortae from L-NAME-treated and eNOS^{-/-} mice was confirmed with responses to ACh (10 μ mol/L) abolished as compared with aortae from C57BL/6J control mice (ACh: $70 \pm 3\%$, $n = 7$).

NO does not interact directly with BAY 58-2667

A potential direct interaction between BAY 58-2667 and NO was assessed using UV/VIS spectroscopy. In a cell free assay, incubation of BAY 58-2667 with either 1 μ mol/L or 10 μ mol/L DEA/NO for a 60 minute period did not alter the absorption spectrum of BAY 58-2667 (Figure 4).

Endothelial-derived NO does not modulate relaxant responses to BAY 58-2667 in isolated aortae from hypertensive (SHR) rats

Systolic blood pressure (SBP: 217 ± 7 mmHg; $n = 5$, $P < 0.05$) and aortic $\cdot O_2^-$ levels ($62 \pm 6 \times 10^3$ counts/mg, $n = 5$, $P < 0.05$) in SHR were significantly higher than in WKY rats (SBP: 114 ± 5 mmHg; $\cdot O_2^-$: $37 \pm 10 \times 10^3$ counts/mg, $n = 5$). In addition, relaxation to the endothelium-

dependent vasodilator, ACh (10 $\mu\text{mol/L}$) was attenuated by 36% in isolated aortae from SHR versus WKY. ($P < 0.05$, Figure 5).

BAY 58-2667-mediated relaxation was markedly augmented in endothelium-intact aortae from SHR ($\text{pEC}_{50} = 11.34 \pm 0.33$, $n = 5$, Figure 6B) as compared with WKY ($\text{pEC}_{50} = 9.83 \pm 0.36$, $n = 9$; Figure 6A: data reproduced from Figure 1A for comparison). Conversely, relaxation responses to DEA/NO ($\text{pEC}_{50} = 7.44 \pm 0.11$, $R_{\text{max}} = 98 \pm 1\%$, $n = 4$, Figure 7A), 8-bromo-cGMP and papaverine (Figures 7B-C) were unchanged in SHR versus WKY aortae. Note that in Figures 7A-C, the WKY data was reproduced from Figures 1B-D for comparison and are represented by broken lines.

In contrast to findings in aortae from WKY, neither endothelial denudation ($\text{pEC}_{50} = 10.93 \pm 0.34$, $n = 5$) nor L-NAME treatment ($\text{pEC}_{50} = 11.51 \pm 0.31$, $n = 6$) potentiated the response to BAY 58-2667 in SHR (Figure 6B). Similarly, in endothelium-denuded aortae from SHR, ODQ ($\text{pEC}_{50} = 11.43 \pm 0.44$, $n = 6$, Figure 6B) did not augment BAY 58-2667-mediated relaxation.

Figure 1. Concentration-dependent vasorelaxation responses to the (A) NO-independent sGC activator, BAY 58-2667 (10 fmol/L - 1 μ mol/L, n = 7-12), (B) NO donor, DEA/NO (0.1 nmol/L - 10 μ mol/L, n = 6-8), (C) cGMP analogue, 8-bromo-cGMP (1 μ mol/L - 0.3 mmol/L, n = 4-6) and (D) sGC-independent vasodilator, papaverine (10 nmol/L - 10 μ mol/L, n = 5-6) in endothelium-intact (+E, ○) and endothelium-denuded (-E, ●) aortae from WKY rats in the absence and presence of L-NAME (100 μ mol/L, □) or ODQ (10 μ mol/L, ■). Responses are expressed as percentage reversal of pre-contraction and given as mean \pm s.e.mean, where n = number of rats. ⁺ P <0.05, ⁺⁺⁺ P <0.001 for pEC₅₀ value versus +E control; ^ψ P <0.05 for pEC₅₀ value versus -E (One-way ANOVA, Bonferroni's multiple comparisons test); ^{*} P <0.05 for concentration-response curve versus +E control (Two-way ANOVA, Tukey Test); [#] P <0.05 for response at 10 μ mol/L DEA/NO versus +E control (Student's unpaired t-test).

Figure 1

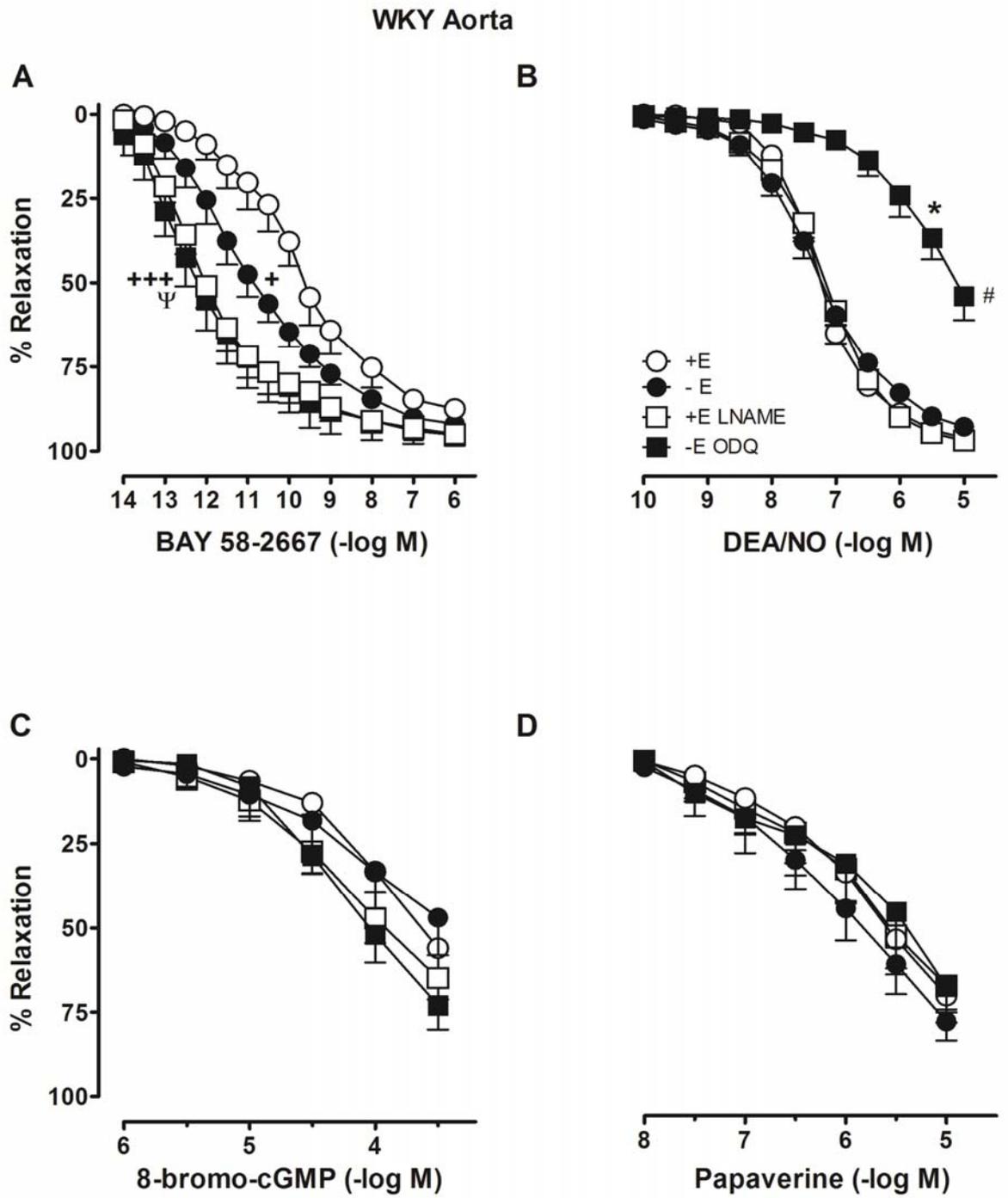


Figure 2. Concentration-response curves to BAY 58-2667 in endothelium-intact **(A)** rat small mesenteric arteries (n = 5-7), **(B)** rabbit aortae (n = 7-8) and rabbit saphenous arteries (n = 10) in the absence (○) and presence of L-NAME (100 μmol/L, □). Responses are expressed as percentage reversal of pre-contraction and given as mean ± s.e.mean, where n = number of animals. **P*<0.05 for concentration-response curves versus untreated control (Two-way ANOVA, Tukey test); +*P*<0.001 for pEC₅₀ value versus untreated control (Student's unpaired t-test).

Figure 2

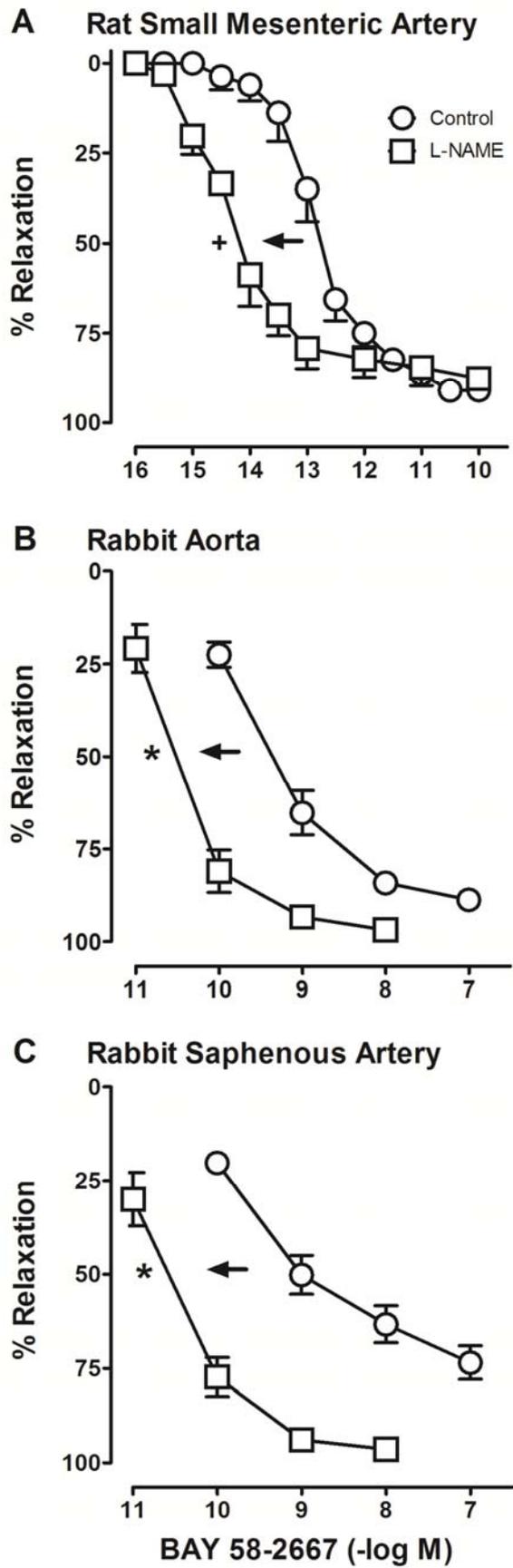


Figure 3. The effects of (A) acute (L-NAME, 100 μ mol/L, \square , n = 5-6) and (B) chronic (L-NAME treatment, 100 mg/kg/day for 28 days; \square , n = 4) NOS inhibition and (C) eNOS deletion (eNOS^{-/-}, \square , n = 5-6) upon vasorelaxation responses to BAY 58-2667 in mouse aortae. Responses are expressed as percentage reversal of pre-contraction, given as mean \pm s.e.mean, where n = number of mice.

Figure 3

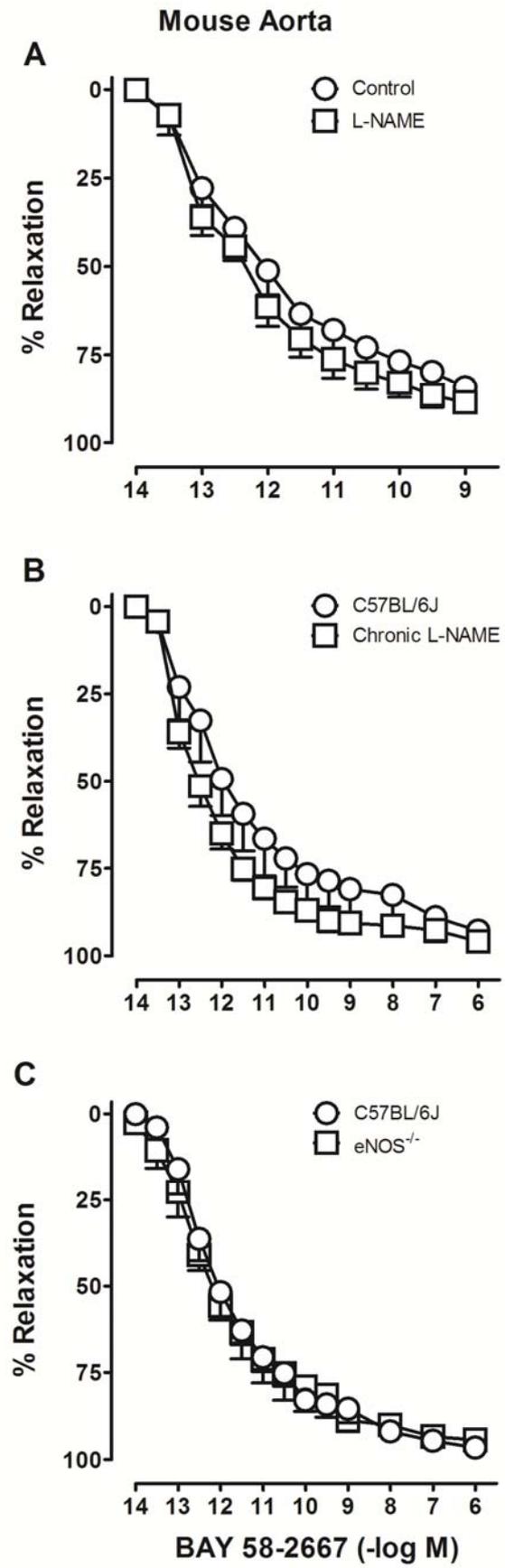


Figure 4

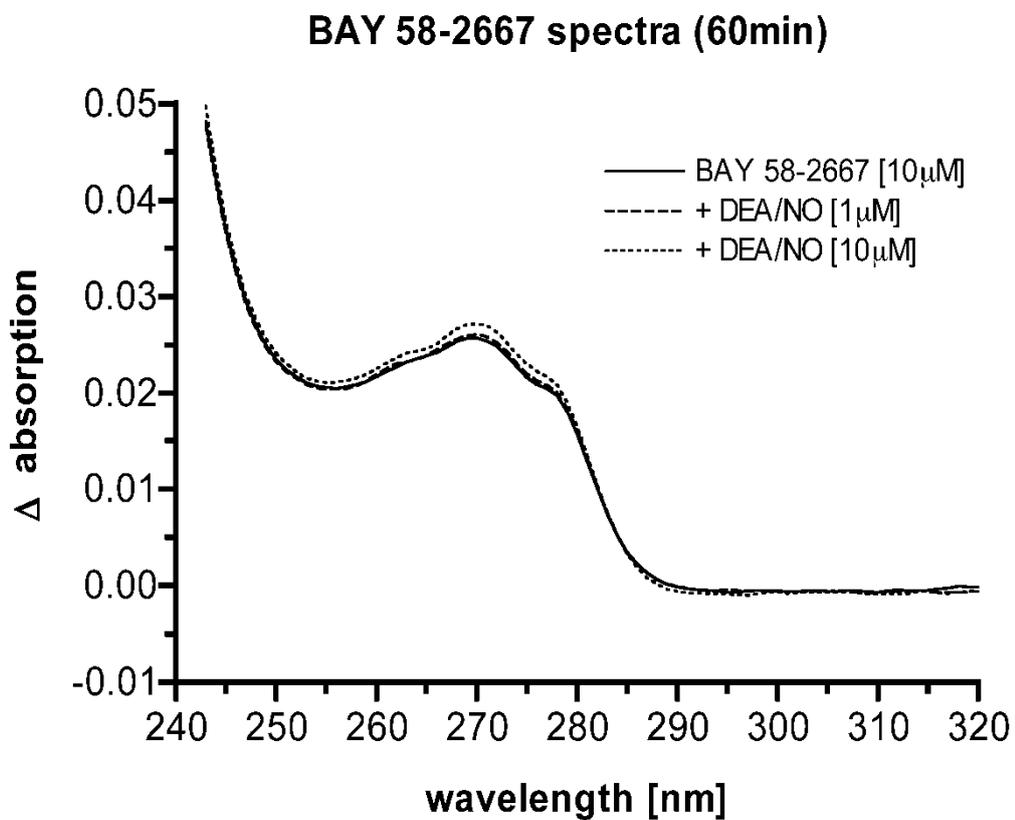


Figure 4. Absorption spectra of BAY 58-2667 (10 μ mol/L) in the absence (—) and presence of 1 μ mol/L (---) and 10 μ mol/L (.....) DEA/NO.

Figure 5

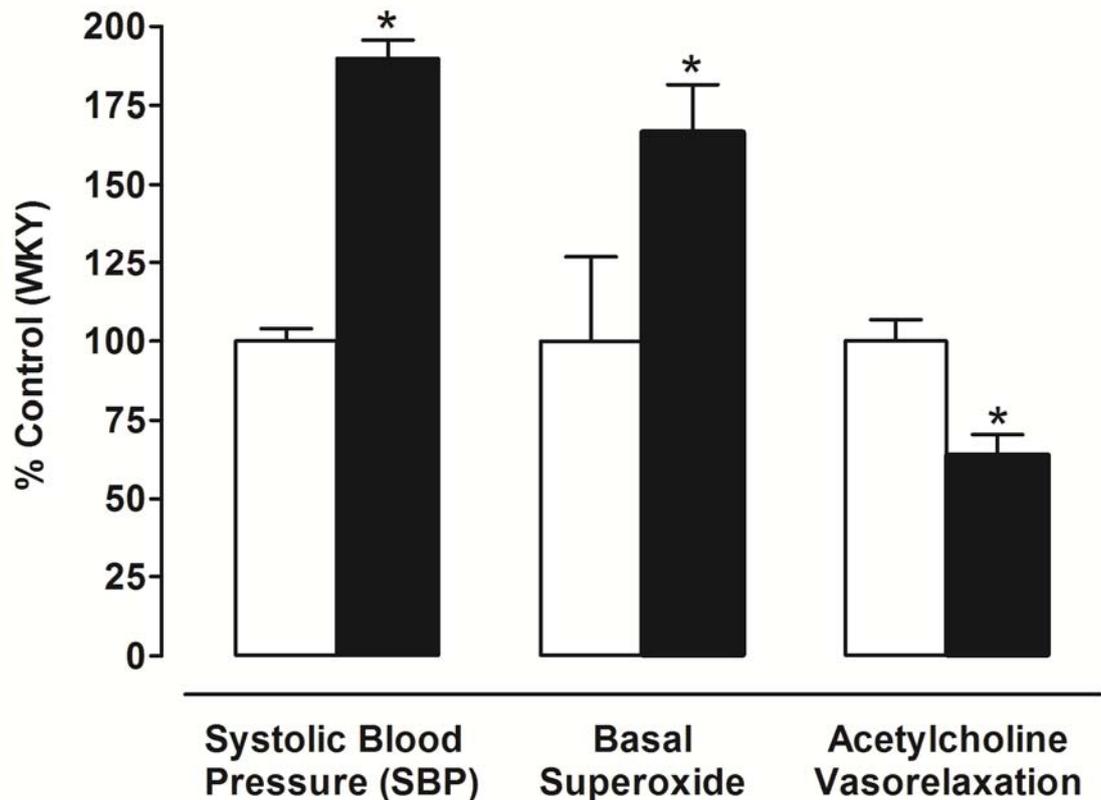


Figure 5. Measurements of systolic blood pressure (SBP, $n = 5$), aortic superoxide anion radical levels ($n = 5$) and endothelium-dependent, ACh-mediated aortic relaxation ($n = 6-9$) in normotensive (WKY, open bars) and hypertensive (SHR, closed bars) rats. Responses in WKY rats are designated as 100% and those in SHR as a percentage of that in WKY. All values are given as mean \pm s.e.mean, where $n =$ number of rats. * $P < 0.05$ versus WKY (Student's unpaired t-test).

Figure 6. The effects of endothelial denudation, NOS (L-NAME, 100 $\mu\text{mol/L}$, \square , $n = 6$) and sGC (ODQ, 10 $\mu\text{mol/L}$, \blacksquare , $n = 6$) inhibition on the vasorelaxation responses to BAY 58-2667 (10 fmol/L - 1 $\mu\text{mol/L}$) in isolated endothelium-intact (+E, \circ , $n = 5$) and -denuded aortae (-E, \bullet , $n = 5$) from WKY (**A**) and SHR (**B**) rats. Note that Figure 6A has been reproduced from Figure 1A for comparison. Responses are expressed as percentage reversal of pre-contraction and given as mean \pm s.e.mean, where $n =$ number of rats. Broken lines provide an indication of the pEC_{50} values for BAY 58-2667 in endothelium-intact aortae from WKY (**A**) and SHR (**B**) in the absence (+E) and presence of L-NAME (+E L-NAME). $^{+++}P < 0.001$, $^{+}P < 0.05$ for pEC_{50} value versus +E control and $^{\Psi}P < 0.05$ for pEC_{50} value versus -E (One-way ANOVA, Bonferroni's multiple comparisons test).

Figure 6

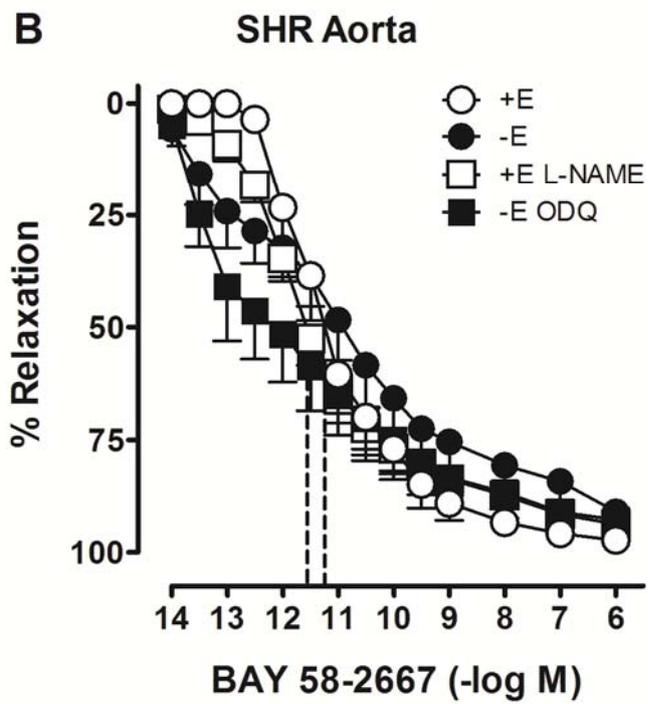
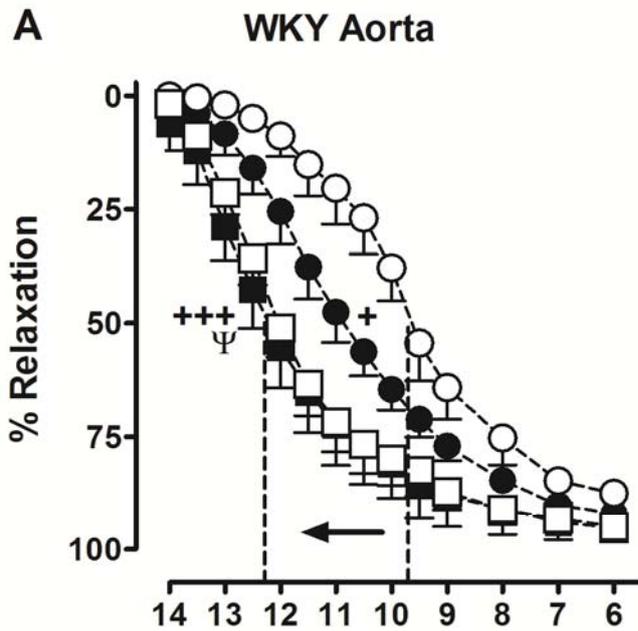
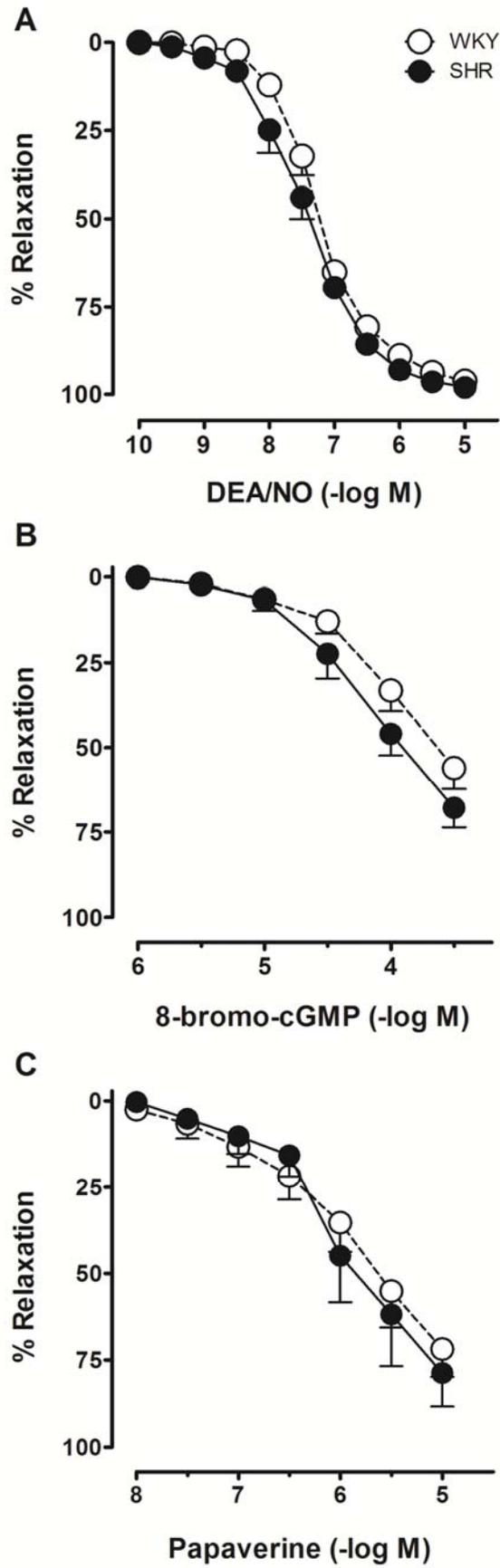


Figure 7. Concentration-dependent vasorelaxation responses to (A) DEA/NO (0.1 nmol/L - 10 μ mol/L, n = 4-6), (B) 8-bromo-cGMP (1 μ mol/L - 0.3 mmol/L, n = 4-5) and (C) papaverine (10 nmol/L - 10 μ mol/L, n = 4-5) in endothelium-intact aortae from WKY (○) and SHR (●) rats. Note that the responses to DEA/NO, 8-bromo-cGMP and papaverine in endothelium-intact aortae from WKY rats were reproduced from Figures 1B-D respectively for comparison. Responses are expressed as percentage reversal of pre-contraction and given as mean \pm s.e.mean, where n = number of rats.

Figure 7



The key findings of this study revealed that firstly, endothelial-derived NO modulates the vasorelaxant response to the novel NO-independent sGC activator, BAY 58-2667 in rat and rabbit arteries, such that its potency is enhanced following a loss of NO. These findings suggest that endogenous NO may limit the formation of oxidized/heme-free sGC. Furthermore, under conditions of chronic oxidative stress such as hypertension, where there is a decrease in endogenous NO bioavailability, the vasorelaxant potency of BAY 58-2667 is markedly augmented. Thus, endogenous NO may serve to maintain sGC in its reduced (Fe^{2+}) state such that a disease-associated loss of NO may facilitate the formation of oxidized (Fe^{3+})/heme-free sGC.

Spectroscopic studies using purified sGC have shown that sGC can exist in three different redox states: the reduced (Fe^{2+}), oxidized (Fe^{3+}) or heme-free form, the latter two states being insensitive to NO (Schmidt *et al.*, 2004; Stasch *et al.*, 2006). Indeed, physiological oxidants such as ONOO^- can oxidize the ferrous (Fe^{2+}) heme group of sGC (Stasch *et al.*, 2006; Weber *et al.*, 2001) and may contribute to the decreased efficacy of nitrovasodilators in cardiovascular disease (Preik *et al.*, 1996; Williams *et al.*, 1996). The identification of the novel NO-independent sGC activator, BAY 58-2667 which preferentially targets the oxidized/heme-free forms of sGC, now provides a valuable tool to probe for pools of oxidized/heme-free sGC in living cells and explore their modulation under disease conditions. Indeed, using the vasorelaxant activity of BAY 58-2667 as a marker for oxidized/heme-free sGC, previous studies have shown that pools of oxidized/heme-free sGC exist under physiological conditions and are further enhanced under disease conditions (Stasch *et al.*, 2006).

In accordance with previous reports (Stasch *et al.*, 2006), BAY 58-2667 was found to be a potent vasodilator under physiological conditions with EC_{50} values in the picomolar (rat small mesenteric arteries and mouse aortae) to nanomolar (rat aortae, rabbit aortae and saphenous

arteries) range. Such differences in vasorelaxant potency to BAY 58-2667 may be indicative of heterogeneous populations of oxidized/heme-free sGC between vascular beds and species. Indeed, one may speculate that these differences in BAY 58-2667 responses may also reflect variations in the overall concentration of sGC present, however, if this were true, one may also expect to observe variations in the responses to vasodilators such as DEA/NO across the different vascular beds and species, which is not the case. Nevertheless, this theory remains to be tested.

It is well recognized that endothelial-derived NO can modulate the response to NO donors/nitrovasodilators such that removal of the endothelium, or NOS inhibition, can potentiate the sensitivity and response to exogenous NO (Brandes *et al.*, 2000; Faraci *et al.*, 1998; Moncada *et al.*, 1991). The phenomenon where exogenous NO is hypersensitive under NO-deficient conditions may involve a number of different mechanisms such as an increased release of NO from the nitrovasodilator, a decreased activity of phosphodiesterase 5 (PDE5), sensitization of sGC or increased sensitivity of PKG and cGMP (Gupta *et al.*, 2008). However, it is currently unknown if sGC activators are modulated in a similar manner. Interestingly, endothelial denudation and NOS inhibition markedly augmented the vasorelaxant response to BAY 58-2667 in isolated aortae from normotensive rats (up to 407-fold increase in potency). Whilst the NOS inhibitor, L-NAME increased the potency of BAY 58-2667 to a slightly greater extent as compared to endothelial denudation alone, this may reflect incomplete endothelial removal and production. Similarly in endothelium-denuded aortae, the sGC inhibitor and oxidant, ODQ, further augmented BAY 58-2667-mediated vasorelaxation. Such an effect may arise as a consequence of ODQ negating the effects of residual NO or oxidizing sGC (Stasch *et al.*, 2006). Taken together, these results suggest that a loss of endogenous NO may lead to an enhanced pool of oxidized/heme-free sGC in the vasculature.

In the current study, the ability of endothelial-derived NO to modulate vasodilator responses appears to be specific for BAY 58-2667 as neither endothelial removal nor NOS inhibition affected vasorelaxant responses to the NO donor, DEA/NO, cGMP-analogue, 8-bromo-cGMP or the sGC-independent vasodilator, papaverine. Whilst endothelial removal/NOS inhibition has previously been shown to augment vasorelaxant responses to NO donors such as SNP (Faraci *et al.*, 1998; Moncada *et al.*, 1991), NONOates such as DEA/NO appear to be somewhat resistant to such modulation. Thus, in agreement with the current findings, previous studies in rabbit aortae have shown that neither endothelial denudation nor NOS inhibition (L-NMMA) had any effect on the vasorelaxant potency to DEA/NO (Morley *et al.*, 1993). Similarly, in endothelium-denuded rat pulmonary artery (Homer & Wanstall, 2000) and L-NAME-treated rabbit cavernosal strips (Kalsi *et al.*, 2004), the potency of spermine-NONOate-mediated relaxation was also shown to be unchanged. Although vasorelaxant responses to DEA/NO in this study were not modulated by endogenous NO, its sensitivity to inhibition by ODQ confirmed its ability to target reduced sGC.

We next sought to determine if the ability of endogenous NO to modulate the vasorelaxant responses to BAY 58-2667 was species- or vascular bed-specific. Analogous to the results observed in WKY aortae, L-NAME augmented the vasorelaxant response to BAY 58-2667 in rat small mesenteric arteries as well as in rabbit aortae and saphenous arteries. Surprisingly, L-NAME did not potentiate the vasorelaxant response to BAY 58-2667 in mouse aortae. Moreover, chronic loss of NO, induced either via long term *in vivo* L-NAME treatment or endothelial NOS (eNOS) deletion, did not modulate the response to BAY 58-2667 in mice. The reasons underlying a lack of NO modulation of responses to BAY 58-2667 in mouse aortae are unclear. Whilst NO may be derived from other NOS isoforms (i.e. nNOS) (Meng *et al.*, 1998; Yatera *et al.*, 2007), the non-specific NOS inhibitor, L-NAME should inhibit the generation of NO from these sources. Alternatively, NO may be derived from NOS-independent sources such as S-

nitrosothiols (Andrews *et al.*, 2002). However, such speculations require further investigation and future studies employing NO scavenging compounds such as haemoglobin (Hb) and/or carboxy-PTIO in L-NAME-treated mice may provide better insight.

To exclude a potential direct chemical interaction between BAY 58-2667 and NO, UV/Vis spectroscopic analysis was employed. Given that the absorption spectrum of BAY 58-2667 was unchanged in the presence of increasing concentrations of NO (DEA/NO; 1 μ M and 10 μ M), endogenous NO is unlikely to directly interact with BAY 58-2667. Rather, NO is likely to target sGC itself to potentially protect the protein from oxidation.

The mechanisms via which NO serves to limit vasorelaxant responses to BAY 58-2667 are currently unknown. However, there may be a possible mechanism by which endogenous NO is modulating vascular pools of oxidized/heme-free sGC. Previous studies in isolated endothelium-denuded bovine pulmonary arteries have identified a cytosolic NADPH-dependent flavoprotein-containing methemoprotein reductase which plays an important role in maintaining sGC in its ferrous NO-sensitive state (Gupte *et al.*, 1999; Wolin, 2009). Thus, oxidation of sGC may be a reversible process with a putative reductase converting oxidized (Fe³⁺) sGC back to its reduced ferrous (Fe²⁺) state. Whether endogenous NO stimulates the activity of an endogenous reductase remains to be determined. Additionally, it has also been suggested that the oxidation of sGC with ODQ is competitive with NO. Therefore, perhaps the same concept can be applied to endogenous oxidants such as ONOO⁻, where NO competes with oxidants for binding to ferrous sGC, thereby limiting oxidation of the heme group (Schrammel *et al.*, 1996).

It is clear from our findings that a loss in endogenous NO augments vasorelaxant responses to BAY 58-2667 under physiological conditions. Moreover, it is well established that the vasodilator response to BAY 58-2667 is enhanced in cardiovascular diseases associated with

oxidative stress and a loss of endogenous NO bioavailability (Stasch *et al.*, 2006). This raises the interesting idea that a disease-associated loss in NO, *per se*, may contribute to augmented pools of oxidized/heme-free sGC. Thus, this concept was investigated in the setting of hypertension, where mean systolic blood pressure (SBP) was markedly elevated, vascular $\cdot\text{O}_2^-$ levels were 67% higher and endogenous NO was reduced by 36% as evidenced by attenuated ACh-mediated relaxation in SHR as compared with WKY. Although not pursued in the current study, the detection of 3-nitrotyrosine (3-NT), the biomarker for the nitrative pathology caused by ONOO^- , could provide further verification of the elevated oxidative stress exhibited by the SHR and may be implemented in future studies. Moreover, in accordance with previous studies (Stasch *et al.*, 2006), a clear augmentation in the vasorelaxant response to BAY 58-2667 was observed in isolated endothelium-intact aortae from SHR compared to WKY, such that 3 nmol/L BAY 58-2667 achieved $85 \pm 6\%$ versus $54 \pm 8\%$ relaxation, respectively. Indeed, Stasch and colleagues have reported markedly higher levels of cGMP in SHR aortae upon BAY 58-2667-mediated activation of sGC compared to WKY (Stasch *et al.*, 2006). Conversely, vasorelaxant responses to DEA/NO, papaverine and 8-bromo-cGMP were unchanged in isolated endothelium-intact aortae from SHR compared to WKY rats. Whilst it may have been anticipated that the increased $\cdot\text{O}_2^-$ generation in SHR would lead to an attenuated response to the NO donor, DEA/NO, the preserved vasorelaxation may be indicative of an ability of this nitrovasodilator to activate cGMP-independent mechanisms (Hempelmann *et al.*, 2000; Sampson *et al.*, 2001). Indeed, previous studies in other disease models associated with oxidative stress have reported that endothelium-independent relaxation to DEA/NO was preserved, with similar responses observed in the aortic rings of sham-operated and myocardial infarct rats (Sartorio *et al.*, 2007). Similarly, the ability of DEA/NO to increase cGMP levels is unchanged in the internal mammary artery of non-diabetic and Type 2 diabetic patients (Witte *et al.*, 2004). Indeed, it has been suggested that NO donors which ‘spontaneously’ generate NO (i.e. DEA/NO) appear to exhibit a larger component of cGMP-independent vasorelaxation when compared to nitrovasodilators that

require bioactivation in the tissue (ie GTN), and that this component is more prevalent in animal models of disease (Wanstall *et al.*, 2005).

Thus, a loss of endogenous NO *per se* in vascular disease may contribute to an increase in the pools of oxidized/heme-free sGC. Indeed, our results demonstrated close similarities in the potency of BAY 58-2667 between endothelium-intact aortae from SHR and endothelium-denuded aortae from WKY rats, again highlighting that endogenous NO bioavailability is compromised during hypertension. However in contrast to the WKY, a further loss of endogenous NO in SHR aortae, either via endothelium removal, NOS inhibition (L-NAME) and/or the oxidation of sGC (ODQ) did not potentiate the response to BAY 58-2667 further. These findings suggest that in the setting of hypertension, endogenous NO does not modulate BAY 58-2667 responsiveness, possibly as NO production is already compromised due to eNOS uncoupling and scavenging by $\cdot\text{O}_2^-$. Thus, it is likely that pools of oxidized/heme-free sGC are already maximally augmented in SHR.

Using BAY 58-2667 as a tool to probe for oxidized/heme-free sGC, the results from the current body of work suggest that endogenous NO may play a protective role in the vasculature, possibly by limiting the oxidation of sGC and the subsequent accumulation of pools of oxidized/heme-free sGC. This effect appears to be lost with the onset of disease. The exact mechanisms by which endogenous NO may be eliciting its protective role remains unclear and further experiments will be required to delineate its actions. Our findings indicate that the augmented responsiveness to BAY 58-2667 and, via inference, pools of oxidized/heme-free sGC observed in disease conditions associated with oxidative stress may arise, at least in part, as a consequence of a loss in endogenous NO. These findings further support the concept that BAY 58-2667, and compounds with a similar mechanism of action, offer conceptionally novel therapeutic approaches in the treatment of vascular diseases.

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CHAPTER 5

Role of the endothelium in
regulating vasorelaxant responses
to BAY 41-2272 in hypertension



The NO-independent, heme-dependent sGC stimulator, BAY 41-2272 represents a potential therapeutic alternative to traditional nitrovasodilator therapy in the treatment of cardiovascular diseases due to its resistance to scavenging by superoxide anion radicals ($\cdot\text{O}_2^-$), lack of tolerance development and ability to also target sGC/cGMP-independent mechanisms. However, several studies have also reported that BAY 41-2272 mediates vasorelaxation in a synergistic manner with NO, such that removal of the endothelium results in an attenuation of its response. This synergy with NO, in addition to the associated loss in endothelial function and subsequently NO bioavailability in disease states, may lead to a reduced efficacy of BAY 41-2272 in hypertension. Thus, this study examined the modulatory role of the endothelium on the vasodilator capacity of BAY 41-2272 in aortae from normotensive (WKY) and hypertensive (SHR) rats. Firstly, removal of the endothelium in both WKY and SHR aortae did not alter vasorelaxant responses to either the NO donor, DEA/NO or sGC-independent vasodilator, papaverine, while a small, yet significant ($P < 0.05$), impairment in the response to the cGMP analogue, 8-bromo-cGMP was observed in WKY, but not SHR aortae. BAY 41-2272-mediated relaxation was equipotent in isolated endothelium-intact aortae from WKY and SHR rats, eliciting near maximal relaxation of $91 \pm 3\%$ ($n = 9$) and $87 \pm 4\%$ ($n = 5$), respectively. Interestingly, removal of the endothelium in the SHR, but not in the WKY, saw a significant reduction in both the potency ($P < 0.01$) and maximum response ($55 \pm 6\%$, $n = 6$, $P < 0.01$) to BAY 41-2272. Interestingly, this effect was not due to a loss of endogenous NO as treatment with the NOS inhibitor, L-NAME (100 $\mu\text{mol/L}$) alone, or in combination with the NO \cdot scavenger, carboxy-PTIO (200 $\mu\text{mol/L}$), did not alter relaxant responses to BAY 41-2272 in endothelium-intact aortae from either strain. Furthermore, in the presence of the sGC inhibitor ODQ (10 $\mu\text{mol/L}$), both the potency ($P < 0.01$) and response ($P < 0.01$) to 10 $\mu\text{mol/L}$ BAY 41-2272 were markedly attenuated in endothelium-denuded WKY aortae. This attenuation, however, was not observed in the SHR, where ODQ treatment did not impair relaxant responses to BAY 41-2272 further than endothelium denudation alone. Such findings in SHR indicate that a cGMP-independent mechanism may be up-regulated to maintain

BAY 41-2272-mediated vasorelaxation. In addition, the presence of the cyclo-oxygenase inhibitor, indomethacin (3 $\mu\text{mol/L}$) augmented vasorelaxant responses to BAY 41-2272 by ~13-fold ($P<0.05$) and ~63-fold ($P<0.01$) in endothelium-intact aortae from WKY and SHR rats respectively. These findings are indicative of the increased release of vasoconstrictor prostanoids in hypertension, which may counteract the relaxant effects of endogenous vasodilators as well as BAY 41-2272. Thus, the main findings of this study suggest that BAY 41-2272-mediated vasorelaxation is preserved in hypertension due, in part, to its synergistic action with a NO-independent endothelial-derived factor in addition to an up-regulation of cGMP-independent signaling mechanisms.

Endothelium-derived nitric oxide (NO) is a major regulator of smooth muscle tone, mediating its effects via activation of soluble guanylyl cyclase (sGC) and resulting in the subsequent accumulation of cyclic guanosine-3',5'-monophosphate (cGMP) (Tang & Vanhoutte, 2010; Thuillez & Richard, 2005). In vascular disease states such as hypertension, the NO/cGMP signaling pathway appears to be impaired such that endothelium-dependent relaxation is compromised (Vanhoutte, 2006). Indeed, endothelial dysfunction is a prominent feature of cardiovascular diseases. It is often characterised by impaired NO bioavailability, most notably due to an increase in superoxide anion radical ($\cdot\text{O}_2^-$) production and/or release of vasoconstrictor prostanoids, which counteract the vasoprotective and vasorelaxant effects of the endothelium (Feletou *et al.*, 2009; Priviero *et al.*, 2009; Versari *et al.*, 2009). Furthermore, an increase in vascular $\cdot\text{O}_2^-$ levels leads to the enhanced scavenging of NO, forming the oxidant peroxynitrite (ONOO^-), which itself, is capable of oxidising the heme moiety of sGC (Weber *et al.*, 2001). Such oxidation renders sGC insensitive to both endogenous and exogenous NO-induced activation via the conversion of its reduced (Fe^{2+}) heme group to its oxidised (Fe^{3+})/heme-free state (Evgenov *et al.*, 2006). Therefore, a loss in the protective role of the endothelium and the associated impairment of the NO/sGC/cGMP pathway is evident in hypertension.

As such the use of traditional NO donors such as organic nitrates (ie. GTN) in the treatment of hypertension is limited due to potential decrease in efficacy caused by the scavenging of NO, tolerance with continued use and the presence of dysfunctional sGC (Evgenov *et al.*, 2006). The development of NO-independent sGC stimulators such as BAY 41-2272 may provide a therapeutically superior alternative due to their resistance to scavenging by $\cdot\text{O}_2^-$ and lack of tolerance development. BAY 41-2272 stimulates the cysteine 238 and cysteine 243 spanning region of the α -subunit of sGC in an NO-independent, but heme-dependent, manner (Stasch *et al.*, 2001). In addition, BAY 41-2272 has also been shown to cause vasorelaxation via cGMP-

independent mechanisms of action such as blocking calcium influx into the vascular smooth muscle (Teixeira *et al.*, 2006).

Importantly, BAY 41-2272 induces vasorelaxation in a synergistic fashion with NO such that endothelial removal diminishes its response (Priviero *et al.*, 2005; Priviero *et al.*, 2009; Stasch *et al.*, 2001). As such we postulate that in hypertension, a decrease in endogenous NO bioavailability may result in attenuated responses to BAY 41-2272. Indeed, subsequent to the completion of the current study, a report by Priviero and co-workers described the impairment of cGMP-dependent and -independent relaxation of BAY 41-2272 in the superior mesenteric arteries of SHR rats, presumably due to the reduction in NO bioavailability and increase in vasoconstrictor prostanoids associated with hypertension (Priviero *et al.*, 2009). This study has examined the vasorelaxant responses to BAY 41-2272 in the aortae from normotensive WKY and SHR rats to elucidate the modulatory role of the endothelium in both settings.

Materials and Methods

This study was approved by the Pharmacology Animal Ethics Committee, Monash University, Australia and conforms to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Tissue Preparation

This study utilised isolated thoracic aortae from adult male WKY (15-16 weeks of age, n = 58) and SHR (15-16 weeks of age, n = 30). All rats were sacrificed humanely as described in the General Methods section 2.1.2.

Functional blood vessel studies

Isolated aortic segments were mounted in 10ml water-jacketed organ baths for the measurement of isometric tension (see General Methods, section 2.6.1.1). Vessels were maintained in Krebs' solution at 37°C and bubbled continuously with carbogen (95% O₂ and 5% CO₂) as described in the General Methods, section 2.6.1.1. The endothelium was deliberately removed from selected aortic segments by gentle rolling around the wire hooks. The absence of endothelium was confirmed by the lack of response to the endothelium-dependent vasodilator, acetylcholine (ACh, 10 µmol/L) according to the General Methods section 2.6.1.1.

The effects of endothelial denudation on cumulative concentration-dependent vasorelaxation responses to the **(i)** NO donor, DEA/NO, **(ii)** NO-independent sGC stimulator, BAY 41-2272, **(iii)** cGMP analogue, 8-bromo-cGMP and **(iv)** sGC-independent vasodilator, papaverine were examined in isolated aortae from WKY and SHR rats (see General Methods, section 2.6.1.4). Additionally, the effects of sGC (ODQ, 10 µmol/L), NO (L-NAME, 100 µmol/L and carboxy-PTIO, 200 µmol/L) and cyclo-oxygenase (indomethacin, 3 µmol/L) inhibition on cumulative

concentration-dependent vasorelaxation responses to BAY 41-2272 were also examined in isolated aortae from WKY and SHR rats as described in the General Methods, section 2.6.1.4.

Statistical analysis

Vasorelaxation responses were expressed as a percentage reversal of the level of pre-contraction. Individual relaxation curves for each vasodilator were fitted to a non-linear sigmoidal logistic equation (Graphpad Prism, Version 4) and pEC₅₀ values (concentration of agonist causing 50% relaxation) were calculated and expressed as $-\log M$. pEC₅₀ values could only be obtained for concentration-response curves which were sigmoidal in nature and where a maximum response was obtained. Differences between mean pEC₅₀ and maximum relaxation (R_{max}) values between two groups were tested using a Student's unpaired t-test (GraphPad Prism version 4.0). A two-way ANOVA was used to compare concentration-response curves in which pEC₅₀ values could not be determined (Sigma Stat, Version 1.0, Jandel Scientific). Where appropriate, the post-ANOVA Tukey's test was applied to identify differences between treatment groups (Sigma Stat, Version 1.0, Jandel Scientific). A one-way ANOVA (Graphpad Prism version 4.0) with Bonferroni's multiple comparisons post-ANOVA test was used to compare differences in R_{max} values between more than two experimental groups. All results were expressed as mean \pm s.e. mean, with n representing the number of animals per group. Statistical significance was accepted at the $P < 0.05$ level.

Endothelial denudation alters relaxant responses to DEA/NO or papaverine in isolated aortae from WKY and SHR rats

Endothelial denudation almost abolished responses to the endothelium-dependent vasodilator, ACh (10 $\mu\text{mol/L}$) in isolated aortae from WKY and SHR rats, thus verifying the absence of the endothelium (Table 1). Furthermore, endothelium-denudation did not alter the sensitivity nor maximum responses to the NO donor, DEA/NO (n = 4-6) or the sGC-independent vasodilator, papaverine (n = 4-6) in WKY and SHR aortae (Figure 1A-B, E-F, Table 1). Whilst a small, but significant, decrease in vasorelaxant potency to 8-bromo-cGMP was observed in endothelial-denuded aortae from WKY, endothelial denudation did not alter responses to 8-bromo-cGMP in SHR aortae (Figure 1C-D, Table 1).

Endothelial denudation attenuates the vasorelaxant response to BAY 41-2272 in isolated aortae from SHR

In isolated endothelium-intact aortae, the NO-independent sGC stimulator, BAY 41-2272 produced equipotent concentration-dependent relaxation in both WKY and SHR (Figure 2A-B, Table 1). Removal of the endothelium had no effect on relaxant responses to BAY 41-2272 in aortae from WKY, however reduced both the sensitivity ($P < 0.01$) and response to 10 $\mu\text{mol/L}$ BAY 41-2272 (+E: $R_{\text{max}} = 87 \pm 4\%$ reversal of pre-contraction vs -E: $59 \pm 6\%$ reversal of pre-contraction, n = 5, $P < 0.01$) in SHR aortae (Figure 2A-B).

Whilst the sGC inhibitor, ODQ caused a significant rightward shift of the concentration-response curve to BAY 41-2272 in endothelium-denuded WKY aortae ($P < 0.01$), responses to BAY 41-2272 in endothelium-denuded SHR aortae were unchanged by ODQ.

A loss of endogenous NO does not account for the attenuated response to BAY 41-2272 in SHR aortae

To determine if the modulatory effect of endothelial removal upon BAY 41-2272-mediated vasorelaxation in SHR aortae was due to a loss in endogenous NO, the NOS inhibitor, L-NAME and NO scavenger, carboxy-PTIO were employed. In contrast to endothelial removal (Figure 2B), neither L-NAME alone (BAY 41-2272: $R_{\max} = 87 \pm 4\%$, $n = 5$ vs BAY 41-2272 + L-NAME: $R_{\max} = 81 \pm 4\%$, $n = 14$) nor in combination with carboxy-PTIO (Figure 2D), altered vasorelaxation to BAY 41-2272 in endothelium-intact aortae from SHR.

Similarly, endothelial denudation (Figure 2A) and treatment of endothelial intact arteries with L-NAME did not change responses to BAY 41-2272 in WKY aortae. While combined treatment with L-NAME and carboxy-PTIO tended to reduce the response to 10 $\mu\text{mol/L}$ BAY 41-2272 in endothelium-intact arteries from WKY, this did not reach statistical significance (Figure 2C).

Vasoconstrictor prostanoids may modulate the BAY 41-2272-mediated response in endothelium-intact aortae from WKY and SHR rats

To determine the modulatory effect of vasoconstrictor prostanoids upon BAY 41-2272-mediated vasorelaxation in SHR aortae, the cyclo-oxygenase (COX) inhibitor, indomethacin was employed. Indomethacin (3 $\mu\text{mol/L}$) significantly enhanced the potency of BAY 41-2272 by ~13-fold ($P < 0.05$) and ~63-fold ($P < 0.01$) in endothelium-intact aortae from WKY and SHR rats, respectively (Figure 2E-F).

Table 1. Effects of endothelial removal on vasorelaxation responses to selected vasodilators in isolated aortae from WKY and SHR rats.

VASODILATORS	WKY			SHR	
	<i>Endothelium-intact/denuded</i>	<i>pEC₅₀</i> (-log M)	<i>R_{max}</i> (% reversal of pre-contraction)	<i>pEC₅₀</i> (-log M)	<i>R_{max}</i> (% reversal of pre-contraction)
ACh	+E	7.29 ± 0.10	64 ± 4% (n = 9)	6.80 ± 0.28	41 ± 4% (n = 6) ^{ΨΨ}
ACh (bolus 10 μmol/L)	-E	—	9 ± 2% (n = 24)	—	3 ± 1% (n = 29) ^{ΨΨ}
DEA/NO	+E	7.21 ± 0.21	98 ± 1% (n = 4)	6.78 ± 0.21	92 ± 1% (n = 6)
DEA/NO	-E	7.02 ± 0.16	94 ± 3% (n = 4)	6.48 ± 0.32	87 ± 3% (n = 4)
BAY 41-2272	+E	ND ~6.98	91 ± 3% (n = 9)	ND~6.51	87 ± 4% (n = 5)
BAY 41-2272	-E	ND ~7.46	90 ± 6% (n = 8)	ND~7.03**	55 ± 6% (n = 6) ^{##}
8-bromo-cGMP	+E	ND~3.98	59 ± 6% (n = 4)	ND~4.15	61 ± 5% (n = 4)
8-bromo-cGMP	-E	ND~3.68*	47 ± 9% (n = 4)	ND~4.23	54 ± 6% (n = 6)
Papaverine	+E	ND~6.21	66 ± 12% (n = 4)	ND~5.98	70 ± 4% (n = 6)
Papaverine	-E	ND~5.92	74 ± 5% (n = 4)	ND~6.00	69 ± 7% (n = 6)

Values are given as mean ± s.e.mean, where n = number of rats. +E = endothelium-intact, -E = endothelium-denuded

ND = not determined, pEC₅₀ values could not be calculated so estimated values are given

^{ΨΨ}P<0.01 for responses at 10 μmol/L ACh versus WKY (Student's unpaired t-test)

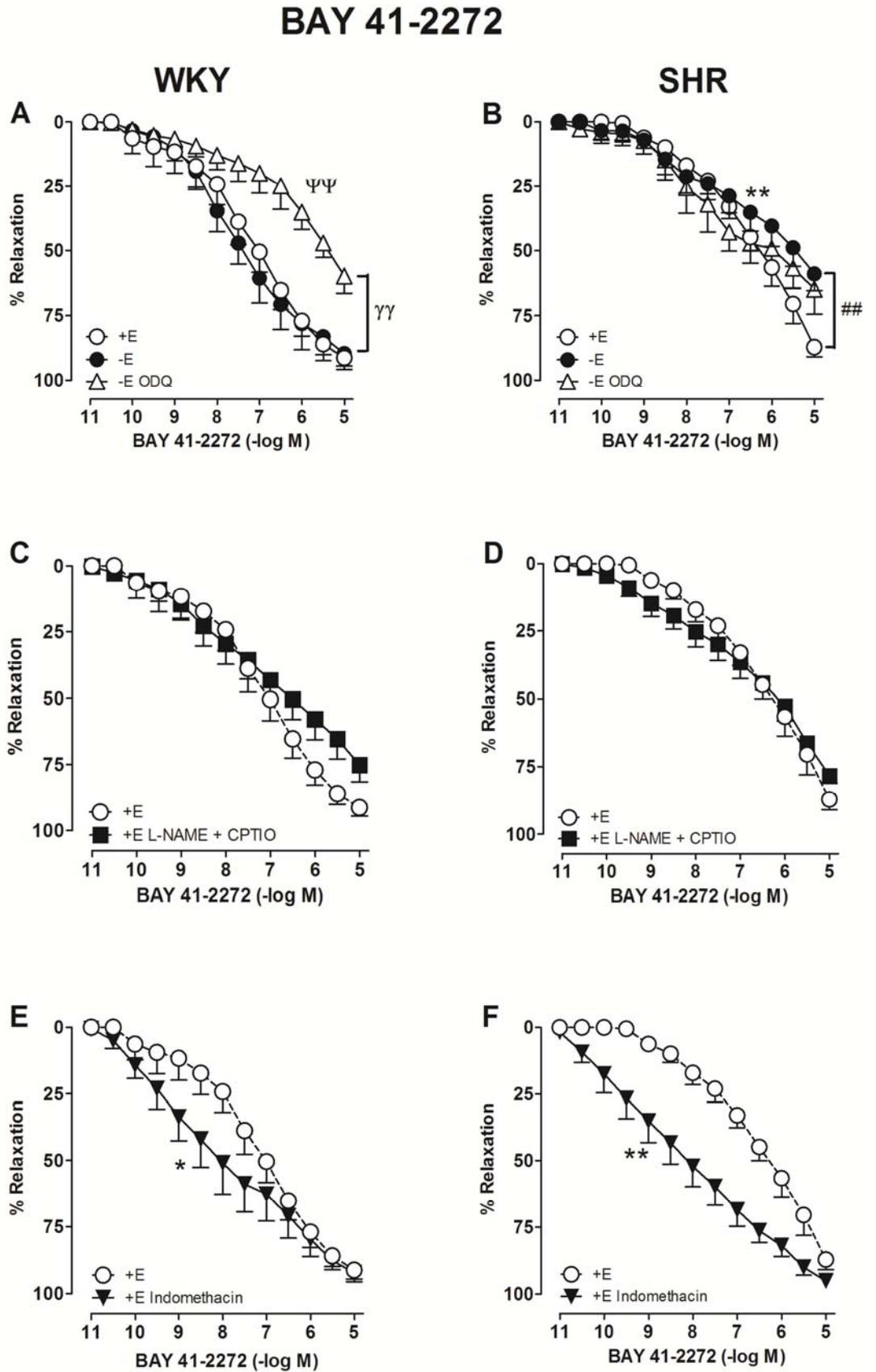
*P<0.05, **P<0.01 for concentration-response curve versus +E (Two-way ANOVA, Tukey Test)

^{##}P<0.01 for responses at 10 μmol/L BAY 41-2272 versus + E (One-way ANOVA, Bonferroni's multiple comparisons test)

Figure 1. Concentration-dependent vasorelaxation responses to the NO donor, DEA/NO (0.1 nmol/L - 10 μ mol/L, n = 4-6, **A-B**), cGMP analogue, 8-bromo-cGMP (1 μ mol/L - 3 mmol/L, n = 4-6, **C-D**) and sGC-independent vasodilator, papaverine (10 nmol/L - 10 μ mol/L, n = 4-6, **E-F**) in endothelium-intact (\circ) and -denuded (\bullet) aortae from WKY (left panel) and SHR (right panel) rats. Responses are expressed as percentage reversal of pre-contraction and given as mean \pm s.e.mean, where n = number of rats. * P <0.05 for concentration-response curve versus +E (Two-way ANOVA, Tukey Test).

Figure 2. Concentration-response curves to BAY 41-2272 (0.01 nmol/L – 10 μ mol/L) in endothelium-intact (+E, \circ , n = 5-9) and endothelium-denuded (-E, \bullet , n = 6-8) aortae from WKY (left panel) and SHR (right panel) rats in the absence and presence of ODQ (10 μ mol/L, Δ , n = 4, **A-B**), L-NAME (100 μ mol/L) and carboxy-PTIO (CPTIO, 200 μ mol/L) in combination (\blacksquare , n = 8-9, **C-D**) or indomethacin (3 μ mol/L, \blacktriangledown , n = 4-5, **E-F**). Note that the responses to BAY 41-2272 in endothelium-intact aortae from WKY (2C + E) and SHR (2D + F) rats were reproduced from Figures 2A + B respectively for comparison and are represented by dashed lines. Responses are expressed as percentage reversal of pre-contraction and given as mean \pm s.e.mean, where n = number of rats. * P <0.05, ** P <0.01 for concentration-response curve versus +E (Two-way ANOVA, Tukey Test). $^{###}P$ <0.01 for responses at 10 μ mol/L BAY 41-2272 versus +E (One-way ANOVA, Bonferroni's multiple comparisons test). $^{***}P$ <0.01, $^{**}P$ <0.01 for concentration-response curve (Two-way ANOVA, Tukey Test) and responses at 10 μ mol/L BAY 41-2272 (One-way ANOVA, Bonferroni's multiple comparisons test) versus -E, respectively.

Figure 2



The key findings of this study revealed that vasorelaxant responses to BAY 41-2272 are preserved in SHR aortae despite an attenuation in endothelial function, and apparent increase in the release of vasoconstrictor prostanoids. Furthermore, in the setting of hypertension, sGC/cGMP-independent relaxation to BAY 41-2272 appears to be augmented. Interestingly, an NO-independent endothelial-derived factor serves to maintain vasorelaxant responses to BAY 41-2272 in SHR.

Disease states such as hypertension are associated with attenuated endothelium-dependent relaxation and decreased responsiveness to NO (Cai & Harrison, 2000). In addition, sGC function is compromised due to a reduction in its expression and susceptibility to oxidation, changes which may contribute to the decreased efficacy of traditional NO vasodilators as well as tolerance development with continued use (Evgenov *et al.*, 2006). Whilst the NO-independent sGC stimulator, BAY 41-2272 may circumvent many of these issues, its synergism with endogenous NO and targeting of reduced (Fe^{2+}) sGC may limit its efficacy under pathophysiological conditions. Thus, the objective of this study was to investigate the modulatory role of the endothelium on responses to BAY 41-2272 in WKY and SHR aortae.

We (Chapter 4) and others (Cuzzocrea *et al.*, 2004) have previously shown the occurrence of endothelial dysfunction and an increase in vascular $\cdot\text{O}_2^-$ generation in SHR aortae, while a decrease in sGC expression has also been reported (Kloß *et al.*, 2000; Ruetten *et al.*, 1999). In the present study, we observed that vasorelaxant responses to the endothelium-dependent vasodilator, ACh were markedly attenuated in SHR versus WKY aortae. This change was specific for the endothelium-dependent vasodilator with responses to the endothelium-independent vasodilators, DEA/NO (NO donor), 8-bromo-cGMP (cGMP analogue) and papaverine (sGC-independent vasodilator) unchanged in WKY versus SHR aortae. Together such findings indicate that in the SHR, sGC/cGMP signaling is preserved. Although impaired

relaxation to NO donors and/or a decrease in sGC expression has been previously reported in SHR, this appears to occur in more advanced stages of the disease accompanied with aging (Kloß *et al.*, 2000) or in resistance arteries (Priviero *et al.*, 2009).

Despite the preserved sGC/cGMP signaling at the level of the vascular smooth muscle in SHR, impaired vasorelaxation to the NO-independent sGC stimulator, BAY 41-2272 may be anticipated given its synergistic action with NO. Thus, BAY 41-2272 targets the reduced (Fe^{2+}) state of sGC to elevate cGMP and synergises with both endogenously (Priviero *et al.*, 2009; Teixeira *et al.*, 2006) and exogenously (Stasch *et al.*, 2001; Teixeira *et al.*, 2006) generated NO to mediate vasorelaxation and cGMP elevation. Specifically, endogenous NO released from the vascular endothelium has been reported to contribute, to some degree, to vasorelaxant responses to BAY 41-2272 in rabbit aortae (Priviero *et al.*, 2005), rat basilar artery (Teixeira *et al.*, 2006) and rat mesenteric arteries (Priviero *et al.*, 2009). In the setting of hypertension, we have previously demonstrated an increase in O_2^- generation and decreased vasorelaxation to ACh, presumably due to a loss in NO bioavailability in SHR aortae (Chapter 4). In addition, we also have evidence that a loss in endogenous NO may facilitate sGC oxidation (Chapter 4). Interestingly, however, in the current study, we show that BAY 41-2272-induced relaxation is equipotent in isolated endothelium-intact aortae from WKY and SHR rats. These findings contrast those of Priviero and co-workers in which BAY 41-2272-induced vasorelaxation in endothelium-intact superior mesenteric arteries was attenuated in SHR ($R_{\text{max}} = 70 \pm 5\%$) versus WKY ($R_{\text{max}} = 90 \pm 5\%$) (Priviero *et al.*, 2009). The reasons underlying such differences remain to be elucidated, however, they may be indicative of distinct mechanisms of vasorelaxation to BAY 41-2272 in aortae versus mesenteric arteries from SHR. Thus, BAY 41-2272 has also been reported to activate sGC/cGMP-independent pathways as demonstrated by its resistance to ODQ in rat basilar arteries (Teixeira *et al.*, 2006) and rabbit cavernosal strips (Baracat *et al.*, 2003). While Priviero and co-workers reported that the cGMP-independent component to the BAY 41-

2272-mediated relaxation was reduced in SHR mesenteric arteries (Priviero *et al.*, 2009), the current study showed that ODQ reduced vasorelaxant responses to BAY 41-2272 in WKY aortae, yet did not alter responses in SHR aortae. Our findings are indicative of a potential increase in the ability of BAY 41-2272 to target sGC-independent pathways in the aortae of hypertensive rats, an action which may contribute to its preserved efficacy in SHR.

Upon initial consideration, our studies indicate that in rat aortae, endothelial-derived NO does not synergise with BAY 41-2272 as neither the removal of the endothelium nor treatment with the NOS inhibitor, L-NAME potentiated vasorelaxation to the sGC stimulator in WKY. Furthermore, while the combined treatment of L-NAME with the NO scavenger, carboxy-PTIO tended to attenuate the maximum response to BAY 41-2272 in WKY aortae, this did not reach statistical significance. This slight attenuation may reflect a small role of NOS-resistant preformed stores of NO (i.e. S-nitrosothiols) in regulating BAY 41-2272-mediated vasorelaxation. However, given that endothelial-denudation did not mimic the actions of L-NAME and carboxy-PTIO in combination, a regulatory role of preformed stores of NO is probably not apparent following endothelial denudation as their loss may be compensated for by a concomitant reduction in endothelial-derived vasoconstrictor prostanoids, as discussed later.

Indeed, an interesting observation to arise from the current study pertains to the regulatory role of the endothelium in the setting of hypertension. Whilst endothelial denudation did not alter BAY 41-2272-mediated relaxation in WKY, it did lead to a significant attenuation in response in SHR. This effect was specific for BAY 41-2272 as responses to DEA/NO, 8-bromo-cGMP and papaverine were unchanged. Moreover, such an observation did not reflect a loss of synergy with endogenous NO as combined treatment of endothelium-intact aortae from SHR with L-NAME and carboxy-PTIO did not mimic the effects of endothelial removal. Rather, it appears that in the setting of hypertension, the endothelium itself and/or an NO-independent endothelial-

derived factor facilitates BAY 41-2272-mediated relaxation, an effect not observed in normotension.

Indeed, it is well documented that when NO bioavailability is compromised, the endothelium is capable of activating various compensatory factors such as endothelium-derived hyperpolarising factor (EDHF), which aid in partially maintaining endothelium-dependent vasodilation (Versari *et al.*, 2009). Purely speculatively, EDHF-mediated hyperpolarisation of vascular smooth muscle may augment the closure of voltage-operated Ca^{2+} channels, thereby, potentially synergising with the cGMP-independent mechanisms of BAY 41-2272. To date, however, conflicting evidence exists with regards to the contribution of EDHF across different vascular beds and in hypertension. Indeed, an impairment in EDHF-mediated response has been reported in the mesenteric arteries of SHR (Fujii *et al.*, 1992) and stroke-prone SHR (Sunano *et al.*, 1999). On the other hand, others have reported a positive correlation between the contribution of EDHF and the presence of myoendothelial gap junctions in vascular preparations, which facilitate the electrical and/or chemical coupling between the endothelium and the vascular smooth muscle, such that its increased incidence maintains the functional activity for EDHF in the caudal artery of SHR (Sandow *et al.*, 2003). While the importance of EDHF in maintaining vascular tone is well recognised, its contribution is thought to predominate in the microvasculature, such that its role increases as vessel size decreases (Shimokawa *et al.*, 1996; Takaki *et al.*, 2008). Therefore, given the relatively small contribution of EDHF reported in conduit arteries such as the aorta, it seems unlikely that the identity of the endothelial-derived factor modulating the response to BAY 41-2272 in SHR is EDHF. Nevertheless, future studies using combined treatment with small- (SK_{Ca}) and intermediate (IK_{Ca})-conductance calcium-activated potassium channel inhibitors, apamin and TRAM-34 (1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole) respectively, should be implemented to examine the effect of EDHF on BAY 41-2272-mediated relaxation in SHR.

In addition to NO and EDHF, vasodilatory prostaglandins constitute one of the three endothelium-derived vasorelaxant factors. It is however, unlikely that vasodilatory prostaglandins are facilitating the response to BAY 41-2272, given that cyclo-oxygenase (COX) inhibition with indomethacin potentiated, rather than diminished, BAY 41-2272-mediated relaxation in SHR. In agreement with Priviero and co-workers, the use of indomethacin in the current study confirms the enhanced release of vasoconstrictor prostanoids in SHR which may contribute to the impairment of vasorelaxant mechanisms. Indeed, an augmented release of vasoconstrictor prostanoids in SHR may be related to increased $\cdot\text{O}_2^-$ generation, resulting in enhanced activation of COX (Priviero *et al.*, 2009; Vanhoutte *et al.*, 2005).

Furthermore, antioxidants such as superoxide dismutase (SOD) have been shown to improve BAY 41-2272-mediated relaxation in SHR (Priviero *et al.*, 2009). The observation that antioxidants modulate the responsiveness to BAY 41-2272 raises an interesting concept. Given the recognised antioxidant capacity of the endothelium (i.e. SOD), it may be postulated that in the setting of hypertension, endothelial denudation augments oxidative stress in the vascular wall, thereby leading to sGC oxidation and attenuated BAY 41-2272-mediated vasorelaxation. Moreover, Xu and co-workers have shown that an unidentified endothelial-derived factor, distinct from NO, hydroxyeicosatetraenoic acid (HETE) or prostacyclins (PGI), up-regulates the activity and expression of the antioxidant enzyme, thioredoxin in the VSM (Xu *et al.*, 2009). Thus, the endothelium may facilitate antioxidant mechanisms in the VSM (i.e. thioredoxin), which aid to maintain BAY 41-2272-mediated relaxation either at the level of sGC and/or via a cGMP-independent pathway. Certainly future studies will be required to identify the antioxidant(s) present in rat aortae, and determine how their expression and activity are regulated by the endothelium in both normotension (WKY) and hypertension (SHR). Alternatively, it may also be possible that hydrogen peroxide (H_2O_2), the dismutation product of $\cdot\text{O}_2^-$, is responsible for the sustained response to BAY 41-2272 in hypertension. Indeed, H_2O_2

has not always been documented to play a protective role in hypertension, and its ability to modify vascular tone may be dependent on the vascular bed and experimental conditions involved (Rodriguez-Martinez *et al.*, 1998). However, previous study by Thomas and co-workers have reported that H₂O₂-treatment in endothelial cells enhances BAY 41-2272-mediated activation of sGC and increased cGMP accumulation, whilst having no effect on endothelium-independent relaxation by DEA/NO (Thomas *et al.*, 2006). While the exact mechanisms remain unclear and may relate to BAY 41-2272's synergism with NO, it nevertheless warrants further investigation into the possibility that endothelial-derived H₂O₂ maintains BAY 41-2272-mediated relaxation in SHR.

Thus, in SHR aortae, BAY 41-2272 sustains vasodilatory capacity despite endothelial dysfunction and an increase in vasoconstrictor prostanoids, which may be due in part, to an increased utilisation of cGMP-independent pathways. Moreover in hypertension, the endothelium facilitates the relaxant response to BAY 41-2272, possibly by augmenting the antioxidant capacity of the vessel. Indeed, future studies are crucial in order to determine if the vasodilatory capacity of BAY 41-2272 can be sustained in more advanced stages of hypertension, in which pronounced oxidative stress and increased release of vasoconstrictor prostanoids as well as sGC dysfunction per se are evident. Thus, future treatment strategies utilising BAY 41-2272 may need to consider the severity of hypertension.

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CHAPTER 6

BAY 41-2272 and BAY 58-2667

attenuate cardiac hypertrophy

independently of blood pressure in aged

hypertensive rats



In the setting of chronic hypertension, targeting the NO/sGC/cGMP pathway confers protection with cGMP having anti-hypertrophic, vasodilatory and anti-aggregatory properties. The NO-independent sGC stimulator, BAY 41-2272 and the sGC activator, BAY 58-2667 may have therapeutic advantages over traditional nitrovasodilators in chronic hypertension as they are resistant to scavenging by $\cdot\text{O}_2^-$ and tolerance development. Moreover, BAY 58-2667 can target the oxidized/heme-free form of sGC and its efficacy is increased in disease. To assess the potential cardio- and vaso-protective actions of BAY 41-2272 and BAY 58-2667 in chronic hypertensive heart disease, aged spontaneously hypertensive rats (SHR; 20-22 months) were instrumented with telemetric probes and administered either vehicle (i.p), BAY 41-2272 (1 mg/kg/day, i.p) or BAY 58-2667 (0.3 or 1 mg/kg/day, i.p) for 4 weeks. Vehicle-treated, aged-matched normotensive Wistar-Kyoto rats (WKY) were also employed in the study. Mean arterial blood pressure (MABP) was measured via radiotelemetry in all treatment groups over the 4 week period. At the conclusion of the treatment period, the hearts were removed and heart weight:body weight measured, whilst the aortae were subsequently removed for basal $\cdot\text{O}_2^-$ measurements (L-012-enhanced chemiluminescence) and functional blood vessel studies. Aged SHR displayed elevated MABP, cardiac hypertrophy, increased vascular $\cdot\text{O}_2^-$ and reduced cGMP-mediated vasorelaxation compared with the age-matched WKY. Bolus intraperitoneal injections of BAY 41-2272 (1 mg/kg/day) and BAY 58-2667 (0.3 and 1 mg/kg/day, $P < 0.001$) transiently reduced MABP in aged SHR during the initial administration, however this effect was not retained throughout the entire 4 week treatment period, indicative of *in vivo* tolerance development. Despite the absence of a maintained anti-hypertensive effect, both BAY 41-2272 (1 mg/kg/day) and BAY 58-2667 (0.3 and 1 mg/kg/day) significantly reduced cardiac hypertrophy. Additionally, chronic treatment with 1 mg/kg/day BAY 58-2667 significantly decreased basal $\cdot\text{O}_2^-$ levels in isolated aortae of aged SHR by approximately 60%, however, this effect was not observed with 0.3 mg/kg/day BAY 58-2667 or 1 mg/kg/day BAY 41-2272. Vasorelaxation responses to the endothelium-dependent vasodilator, ACh, the NO donor,

DEA/NO, the sGC stimulator, BAY 41-2272, the sGC activator, BAY 58-2667 and the sGC-independent vasodilators, papaverine and zaprinast were unchanged in aged SHR following chronic treatment with either BAY 41-2272 (1 mg/kg/day) or BAY 58-2667 (0.3 or 1 mg/kg/day). Furthermore, chronic treatment with BAY 41-2272 (1 mg/kg/day) or BAY 58-2667 (0.3 or 1 mg/kg/day) did not reverse the augmented vasoconstrictor responses to serotonin in the aged SHR aortae, yet restored vasodilator responses to the cGMP analogue, 8-bromo-cGMP, such that the vasodilator profile was similar to that observed in the WKY. These findings in chronic hypertensive heart disease suggest that chronic stimulation and/or activation of sGC is cardioprotective, markedly reversing cardiac hypertrophy in the absence of a maintained anti-hypertensive effect. Although *in vivo* tolerance to vasodepressor responses to BAY 41-2272 and BAY 58-2667 were observed, this was not evident in the isolated vasculature where neither tolerance nor cross tolerance was observed in response to chronic treatment with these novel agents. Moreover, BAY 58-2667 is able to attenuate vascular $\cdot\text{O}_2^-$ generation and both chronic stimulation of reduced and oxidized/heme-free sGC restored vasorelaxation to a cGMP analogue in SHR. Taken together, chronic stimulation and/or activation of sGC with these novel agents confers cardioprotection, is not associated with vascular tolerance development and may offer novel therapeutic avenues in the treatment of hypertensive heart disease.

Hypertension is a disorder of the heart and blood vessels (Mayet & Hughes, 2003) and is characterised by cardiac hypertrophy and vascular dysfunction (Lassegue & Griendling, 2004). Cardiac hypertrophy, in the form of wall thickening and reduced cavity size, is a mechanism by which the heart adapts its structure in response to pressure overload (Doggrell & Brown, 1998), and involves cardiomyocyte hypertrophy, cardiac fibroblast proliferation, fibrosis and cell death (apoptotic and necrotic) (McMullen *et al.*, 2005). With respect to the hypertensive vasculature, endothelial dysfunction is apparent as a consequence of a decrease in nitric oxide (NO) synthesis (Lassegue & Griendling, 2004) and an increased scavenging of NO by reactive oxygen species (ROS) such as vascular superoxide anion radical ($\cdot\text{O}_2^-$) (Cuzzocrea *et al.*, 2004).

Indeed, NO is a vital signaling molecule which can activate soluble guanylyl cyclase (sGC) at low nanomolar concentrations, subsequently inducing the generation of the second messenger, cyclic guanosine-3',5'-monophosphate (cGMP) which is crucial for endothelial, vascular smooth muscle and cardiomyocyte function (Friebe & Koesling, 2003; Tsai & Kass, 2009). At the level of the cardiomyocyte, cGMP confers protection with cGMP elevating agents such as NO \cdot (sGC) and natriuretic peptides (particulate GC) exhibiting anti-hypertrophic actions (Ritchie *et al.*, 2009). Moreover, the vasoprotective actions of cGMP include vasodilation and inhibition of platelet aggregation and vascular smooth muscle growth (Ritchie *et al.*, 2009; Tsai & Kass, 2009). However, accumulating evidence suggests that the disruption of NO/sGC/cGMP signaling and a loss in NO bioavailability, due to an increase in oxidative stress (i.e. $\cdot\text{O}_2^-$), are key contributors to the pathogenesis of hypertension (Pacher *et al.*, 2007). Moreover, dysfunction may also occur at the level of sGC itself, whereby its oxidation and subsequent heme loss renders the enzyme insensitive to NO (Evgenov *et al.*, 2006; Schmidt *et al.*, 2004). Indeed, the susceptibility of the heme group of sGC to undergo oxidation is thought to principally occur under pathophysiological conditions, where there is an up-regulation of ROS (Stasch *et al.*, 2006). These effects of hypertension are further augmented with aging, with aged

spontaneously hypertensive rats (SHR) exhibiting a decrease in vascular sGC expression at both the mRNA and protein level (Kloß *et al.*, 2000).

To overcome dysfunctional NO/sGC/cGMP signaling in the setting of hypertension, nitrovasodilators have been employed (Moncada, 1999). However, despite the clear therapeutic value of nitrovasodilators, their clinical use is limited due to the development of tolerance as well as a decrease in efficacy with the onset of oxidative stress (Parker & Gori, 2001). Such a decrease in efficacy of these compounds is believed to arise as a consequence of an increase in NO scavenging by $\cdot\text{O}_2^-$ and/or oxidation of sGC, rendering it insensitive to NO (Evgenov *et al.*, 2006). Thus, given the limitations associated with the utility of traditional nitrovasodilators, NO-independent stimulators (BAY 41-2272) and activators (BAY 58-2667) of sGC may represent novel agents to overcome these problems. Like NO, BAY 41-2272 stimulates sGC in its reduced (Fe^{2+}) state yet is not susceptible to scavenging by $\cdot\text{O}_2^-$ or tolerance development (Stasch *et al.*, 2001). Furthermore, BAY 58-2667 is unique in that it preferentially targets the oxidised (Fe^{3+})/heme-free form of sGC and its efficacy is enhanced under conditions of oxidative stress and disease (Stasch *et al.*, 2006). Both BAY 41-2272 and BAY 58-2667 have been reported to exhibit favourable hemodynamic properties for the treatment of chronic hypertension, with an ability to vasodilate the isolated vasculature (Stasch *et al.*, 2006; Straub *et al.*, 2001), and acutely decrease MABP in both normotensive and hypertensive rats (Stasch *et al.*, 2006; Stasch *et al.*, 2002; Straub *et al.*, 2001) as well as in patients with acute decompensated heart failure (Lapp *et al.*, 2009). In addition both BAY 41-2272 and BAY 58-2667 have been shown to exhibit anti-remodeling effects (Dumitrascu *et al.*, 2006). Thus, chronic (14 day) treatment with BAY 41-2272 decreases cardiac fibrosis in angiotensin II-induced hypertensive rats (Masuyama *et al.*, 2006) and pressure-overloaded rats (Masuyama *et al.*, 2009). Furthermore, chronic treatment (18 weeks) with BAY 58-2667 has been reported to lower blood pressure and reduce left ventricular hypertrophy in a rat model of renal disease (Kalk *et al.*, 2006), improve cardiac performance and

reduce necrosis in a canine model of myocardial ischemia (Korkmaz *et al.*, 2009), as well as reverse the hemodynamic and structural changes associated with rodent models of pulmonary hypertension (Dumitrascu *et al.*, 2006).

Taken together, the data suggests that chronic stimulation of reduced (BAY 41-2272) and oxidised/heme-free sGC (BAY 58-2667) by NO-independent agents may confer cardio- and vasoprotection in the setting of advanced hypertensive heart disease. To test this hypothesis, BAY 41-2272 and BAY 58-2667 were chronically administered (4 weeks) to aged SHR rats (20-22 months old), a well established model of advanced hypertension with associated heart failure (Doggrell & Brown, 1998).

Materials and Methods

This study was approved by the Pharmacology Animal Ethics Committee, Monash University, Australia and conforms to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Chronic treatment with BAY 41-2272 and BAY 58-2667

Aged, male normotensive Wistar-Kyoto (WKY, 20-22 months, n = 10) and age-matched spontaneously hypertensive rats (SHR, n = 40) were used in this study (refer to General Methods section 2.1.2). Under isoflurane anaesthesia (2-4% in O₂), a midline incision was made and radiotelemetry probes (TA11-PAC40, USA) placed around the distal end of the abdominal aortae of WKY and SHR (see General Methods, section 2.3). Following a one week recovery period, WKY rats were treated with vehicle (0.1 ml/100g/day of PEG400/glycerol/H₂O in 1% DMSO, i.p., n = 10) while SHR rats were treated with either:

(i) vehicle (0.1 ml/100g/day of PEG400/glycerol/H₂O in 1% DMSO, i.p., n = 10)

(ii) 1 mg/kg/day BAY 41-2272 (i.p., n = 10)

(iii) 0.3 mg/kg/day BAY 58-2667 (i.p., n = 10)

(iv) 1 mg/kg/day BAY 58-2667 (i.p., n = 10)

for a 4 week period according to the General Methods, section 2.3.

The doses of BAY 41-2272 and BAY 58-2667 were chosen based on previous studies where similar doses were reported to exhibit cardio- and vaso-protective properties.

Telemetric measurement of mean arterial blood pressure (MABP)

Radiotelemetry (TA11-PAC40, USA) was used to continuously record heart rate and both systolic (SBP) and diastolic (DBP) blood pressures in treated rats throughout both the baseline (1 week recovery period) and the following 4 week treatment period. Sampling was carried out for

10 seconds every 10 minutes, 24 hours a day. MABP was calculated daily from SBP and DBP recordings (see General Methods, section 2.3).

Tissue Preparation

Following the four week treatment period, aged WKY and SHR were anaesthetised with isoflurane (2-4% in O₂) and sacrificed via decapitation (see General Methods, section 2.3). Thoracic aortae were isolated, sectioned into ~5mm ring segments (endothelium intact) and used in either $\cdot\text{O}_2^-$ detection experiments using L-012-enhanced chemiluminescence assays (see General Methods, section 2.4.2) or in functional experiments using organ baths (see General Methods, section 2.6.1.1 and 2.6.1.5). Additionally, hearts from treated animals were isolated and weighed and the heart to body weight ratio (HW:BW) expressed as mg/g.

Superoxide anion radical detection via L-012-enhanced chemiluminescence

Vascular $\cdot\text{O}_2^-$ levels were measured using L-012 (100 $\mu\text{mol/L}$)-enhanced chemiluminescence in aortae from vehicle-treated WKY or SHR treated with either the vehicle, BAY 41-2272 (1 mg/kg/day) or BAY 58-2667 (0.3 or 1 mg/kg/day). Vascular $\cdot\text{O}_2^-$ was detected using the Hidex ChameleonTM Luminescence Plate Reader (Hidex Oy). Upon completion of the assay, rings were allowed to dry on pre-cut foil pieces overnight at 37°C. Dried aortic segments were then weighed the following day, background counts subtracted and $\cdot\text{O}_2^-$ normalised to dry tissue weight (see General Methods, section 2.4.2)

Functional Blood Vessel Studies

Aortic segments were mounted in 10ml water-jacketed organ baths for the measurement of isometric tension (see General Methods, section 2.6.1.1). Vessels were maintained in Krebs' solution at 37°C and bubbled continuously with carbogen (95% O₂, 5% CO₂) as described in the General Methods, section 2.6.1.1. Vasorelaxant responses to the (i) endothelium-dependent

vasodilator, ACh, **(ii)** NO donor, DEA/NO, **(iii)** sGC stimulator, BAY 41-2272, **(iv)** sGC activator, BAY 58-2667 and **(v)** sGC-independent vasodilators, papaverine, zaprinast and the cGMP analogue, 8-bromo-cGMP were examined in isolated aortae from vehicle-treated WKY and SHR treated with either vehicle, BAY 41-2272 (1 mg/kg/day) or BAY 58-2667 (0.3 or 1 mg/kg/day). Additionally, contractile responses to the vasoconstrictor serotonin were also assessed in isolated aortae from rats in the aforementioned treatment groups (see General Methods, section 2.6.1.5).

Statistical analysis

The effect of drug treatments on MABP (mmHg) over time was assessed by Two-way ANOVA (GraphPad Prism, Version 4). The effects of drug treatments on peak MABP at the end of each treatment week and differences in morphometric data between treatment groups were determined using a One-way ANOVA followed by Bonferroni's multiple comparisons test (GraphPad Prism, Version 4). $\cdot\text{O}_2^-$ generation was expressed as 10^3 counts per mg of dry tissue weight and statistical differences were measured using a One-way ANOVA followed by Bonferroni's multiple comparisons test. Contractions to serotonin were expressed as a percentage of the maximum KPSS contraction (F_{max}). Relaxation responses were expressed as a percentage reversal of the level of pre-contraction. Individual curves for each vasodilator were fitted to a non-linear sigmoidal logistical equation (Graphpad Prism, Version 4) and pEC_{50} values (concentration of agonist causing 50% relaxation) were calculated and expressed as $-\log M$. pEC_{50} values could only be obtained for curves which were sigmoidal and where a maximum response was obtained. Where appropriate, a One-way ANOVA followed by Bonferroni's multiple comparisons test was used to compare differences between three or more treatment groups. A Two-way ANOVA followed by a Tukey test was used to compare vasodilator concentration-response curves which were not sigmoidal in nature and where a pEC_{50} could not be calculated (Sigma Stat, Version 1.0, Jandel Scientific). All results were expressed as mean \pm

s.e. mean, with n representing the number of animals per group. Statistical significance was accepted at the $P < 0.05$ level.

Chronic treatment with BAY 41-2272 and BAY 58-2667 does not reduce mean arterial blood pressure in aged SHR

Mean arterial blood pressure (MABP) was significantly higher in aged SHR (149 ± 9 mmHg, $n = 7$, $P < 0.01$) compared to the age-matched WKY (122 ± 3 mmHg, $n = 6$) and this trend was sustained throughout the 4 week study period (Table 1). Bolus intraperitoneal injection of BAY 58-2667 (0.3 or 1 mg/kg/day) transiently reduced MABP (~ 27 - 37 mmHg respectively) in aged SHR, however this effect was not sustained such that MABP returned to baseline ~ 4 - 5 hours following the administration (Figure 1A). BAY 41-2272 (1 mg/kg/day) also caused a transient decrease in MABP (~ 11 mmHg), however this effect did not reach statistical significance during the initial administration. Results are represented from Day 3, allowing for the animals to acclimatise to handling and treatment (Figure 1A).

Moreover, the ability of BAY 58-2667 to acutely decrease MABP upon administration was lost over the 4 week period (Figure 1B), such that 1 mg/kg/day BAY 58-2667 acutely decreased MABP by approximately 34 mmHg ($P < 0.001$), 37 mmHg ($P < 0.001$), 14 mmHg and 4 mmHg on day 7, 14, 21 and 28 of the treatment period, respectively.

When considering the effects of BAY 41-2272 and BAY 58-2667 on MABP *per se* during chronic administration, both agents did not lead to a significant decrease in baseline MABP in the aged SHR following the 4 weeks of treatment (Table 1).

Chronic treatment with BAY 41-2272 and BAY 58-2667 significantly reverses cardiac hypertrophy in SHR

Chronic stimulation/activation of sGC significantly attenuated cardiac hypertrophy in aged SHR such that the heart weight:body weight ratio (SHR vehicle: 5.9 ± 0.3 mg/g, $n = 8$) was reduced following 4 weeks treatment with either 1mg/kg/day BAY 41-2272 (4.6 ± 0.2 mg/g, $n = 7$, $P < 0.05$) or BAY 58-2667 at doses of 0.3 mg/kg/day (5.1 ± 0.2 mg/g, $n = 8$, $P < 0.05$) and 1 mg/kg/day (5.0 ± 0.2 mg/g, $n = 8$, $P < 0.05$). Neither BAY 41-2272 nor BAY 58-2667 returned the heart weight:body weight ratio to levels observed in the vehicle-treated WKY (3.5 ± 0.3 mg/g, $n = 7$, Figure 2).

Chronic treatment with BAY 58-2667 but not BAY 41-2272 suppressed vascular $\cdot\text{O}_2^-$ levels in isolated aortae from SHR

$\cdot\text{O}_2^-$ production in isolated rat aortae was significantly higher in aged SHR (4743 ± 651 10^3 counts/mg, $n = 7$) compared to WKY (2251 ± 593 10^3 counts/mg, $n = 7$, $P < 0.05$). Chronic activation of sGC by 1 mg/kg/day BAY 58-2667 (1767 ± 376 10^3 counts/mg, $P < 0.01$) significantly suppressed $\cdot\text{O}_2^-$ production. Although chronic treatment with both 1 mg/kg/day BAY 41-2272 (3272 ± 896 10^3 counts/mg, $n = 6$) and 0.3 mg/kg/day BAY 58-2667 (2767 ± 305 10^3 counts/mg, $n = 5$) tended to lower $\cdot\text{O}_2^-$ generation, this change failed to reach statistical significance (Figure 3).

Chronic treatment with BAY 41-2272 and BAY 58-2667 does not normalise contractile responses to serotonin in isolated aortae from SHR

Despite a similar contraction to 124 mM K^+ (KPSS, Figure 4A) in isolated aortae from vehicle-treated WKY versus SHR, the maximum response to the vasoconstrictor, serotonin was significantly increased in the SHR ($77 \pm 8\%$ KPSS, $n = 5$, $P < 0.05$) compared with WKY ($46 \pm 7\%$ KPSS, $n = 6$, Figure 4B). Chronic treatment of aged SHR with either BAY 41-2272 (1

mg/kg/day) or BAY 58-2667 (0.3 and 1 mg/kg/day) did not alter vasoconstrictor responses to serotonin.

Chronic treatment with BAY 41-2272 and BAY 58-2667 does not alter endothelial function or vasorelaxant responses to DEA/NO in SHR

Endothelial function was similar between endothelium-intact aortae from vehicle-treated aged WKY and SHR such that the endothelium-dependent vasodilator, ACh (10 μ mol/L) caused vasorelaxation of $58 \pm 4\%$ (n = 5) and $53 \pm 4\%$, n = 6), respectively. Furthermore, chronic treatment with either BAY 41-2272 (1 mg/kg/day) or BAY 58-2667 (0.3 and 1 mg/kg/day) did not alter ACh-mediated relaxation in isolated aortae from SHR (Figure 5A).

Vasorelaxation to the NO donor, DEA/NO was similar in aortae from vehicle-treated aged WKY ($pEC_{50} = 7.31 \pm 0.11$ $-\log$ M; $R_{max} = 91.7 \pm 4\%$, n = 6) and SHR ($pEC_{50} = 6.94 \pm 0.21$; $R_{max} = 95.3 \pm 7\%$, n = 5, Figure 5B). Furthermore, chronic stimulation and activation of sGC with BAY 41-2272 and BAY 58-2667, respectively did not alter vasorelaxation responses to DEA/NO in SHR.

Chronic treatment with BAY 41-2272 and BAY 58-2667 does not induce vascular tolerance in SHR

Concentration-dependent vasorelaxation responses to BAY 41-2272 and BAY 58-2667 *per se* were unchanged in vehicle-treated aged SHR as compared with WKY. Moreover, chronic treatment of aged SHR with either BAY 41-2272 (1 mg/kg/day) or BAY 58-2667 (0.3 or 1 mg/kg/day) did not alter subsequent vasorelaxant responses to these sGC stimulators and activators in isolated aortae, indicative of a lack of tolerance and cross-tolerance development (Figures 6A-B).

Chronic treatment with BAY 41-2272 and BAY 58-2667 augments 8-bromo-cGMP-mediated vasorelaxation in SHR

Concentration-dependent relaxation responses to the sGC-independent vasodilators, papaverine and zaprinast were preserved, yet relaxation to the cGMP analogue, 8-bromo-cGMP was attenuated in vehicle-treated aged SHR ($R_{\max} = 30 \pm 7\%$, $n = 6$, $P < 0.05$) compared to WKY ($R_{\max} = 56 \pm 5\%$, $n = 5$). Whilst chronic treatment with either BAY 41-2272 (1 mg/mg/day) or BAY 58-2667 (0.3 or 1 mg/kg/day) in SHR did not alter vasorelaxant responses to papaverine and zaprinast, they significantly improved vasorelaxation to 8-bromo-cGMP (BAY 41-2272 1 mg/kg/day treatment: $R_{\max} = 50 \pm 4\%$, $n = 6$; BAY 58-2667 0.3 and 1 mg/kg/day treatment: $R_{\max} = 51 \pm 4\%$ and $53 \pm 5\%$ respectively, $n = 6$) such that responses were similar to those in WKY (Figures 7A-C).

Table 1. Telemetric measurements of mean arterial blood pressures (MABP) in conscious vehicle-treated aged WKY and aged SHR treated with either vehicle, 1 mg/kg/day BAY 41-2272, 0.3 mg/kg/day or 1 mg/kg/day BAY 58-2667. Shown are baseline MABP recordings established prior to (baseline) treatment and at day 7, 14, 21 and 28 following treatment.

<i>Animals</i>	<i>Baseline</i> (mmHg)	<i>Week 1(Day 7)</i> (mmHg)	<i>Week 2 (Day 14)</i> (mmHg)	<i>Week 3 (Day 21)</i> (mmHg)	<i>Week 4 (Day 28)</i> (mmHg)
<i>WKY vehicle</i>	122 ± 3 (n = 6)	119 ± 3 (n = 6)	121 ± 1 (n = 6)	118 ± 3 (n = 6)	117 ± 1 (n = 6)
<i>SHR vehicle</i>	149 ± 9* (n = 7)	152 ± 12 (n = 5)	165 ± 4*** (n = 5)	150 ± 11** (n = 6)	144 ± 11 (n = 7)
<i>SHR BAY 41-2272</i> (1 mg/kg/day)	157 ± 7** (n = 5)	153 ± 7* (n = 5)	152 ± 7*** (n = 5)	149 ± 2** (n = 5)	153 ± 6* (n = 5)
<i>SHR BAY 58-2667</i> (0.3 mg/kg/day)	159 ± 4** (n = 6)	154 ± 5* (n = 6)	164 ± 5*** (n = 6)	157 ± 4*** (n = 6)	160 ± 6** (n = 6)
<i>SHR BAY 58-2667</i> (1 mg/kg/day)	144 ± 6 (n = 7)	143 ± 3 (n = 7)	148 ± 2*** (n = 7)	144 ± 3* (n = 7)	155 ± 3** (n = 7)

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus baseline WKY vehicle

All analyses were conducted using a One-way ANOVA, Bonferroni's multiple comparisons test

Figure 1. Telemetric measurement of mean arterial blood pressure (MABP, mmHg) in conscious vehicle-treated aged WKY (●, n = 5-6) and aged SHR treated with either vehicle (●, n = 6-7), 1 mg/kg/day BAY 41-2272 (■, n = 5), 0.3 mg/kg/day (▼, n = 6) or 1 mg/kg/day (▲, n = 7) BAY 58-2667. Shown are MABP recordings taken at 1h prior to and 6hrs post bolus intraperitoneal injection of either vehicle, BAY 41-2272 or BAY 58-2667 on (A) Day 3 and (B) Day 28 of the treatment period. Bolus administration of vehicle, BAY 41-2272 or BAY 58-2667 was at time = 0 hrs, as indicated by arrows. Values are presented as mean ± s.e.mean and where n = number of rats. *** $P < 0.001$ versus SHR vehicle, (Two-way ANOVA).

Figure 1

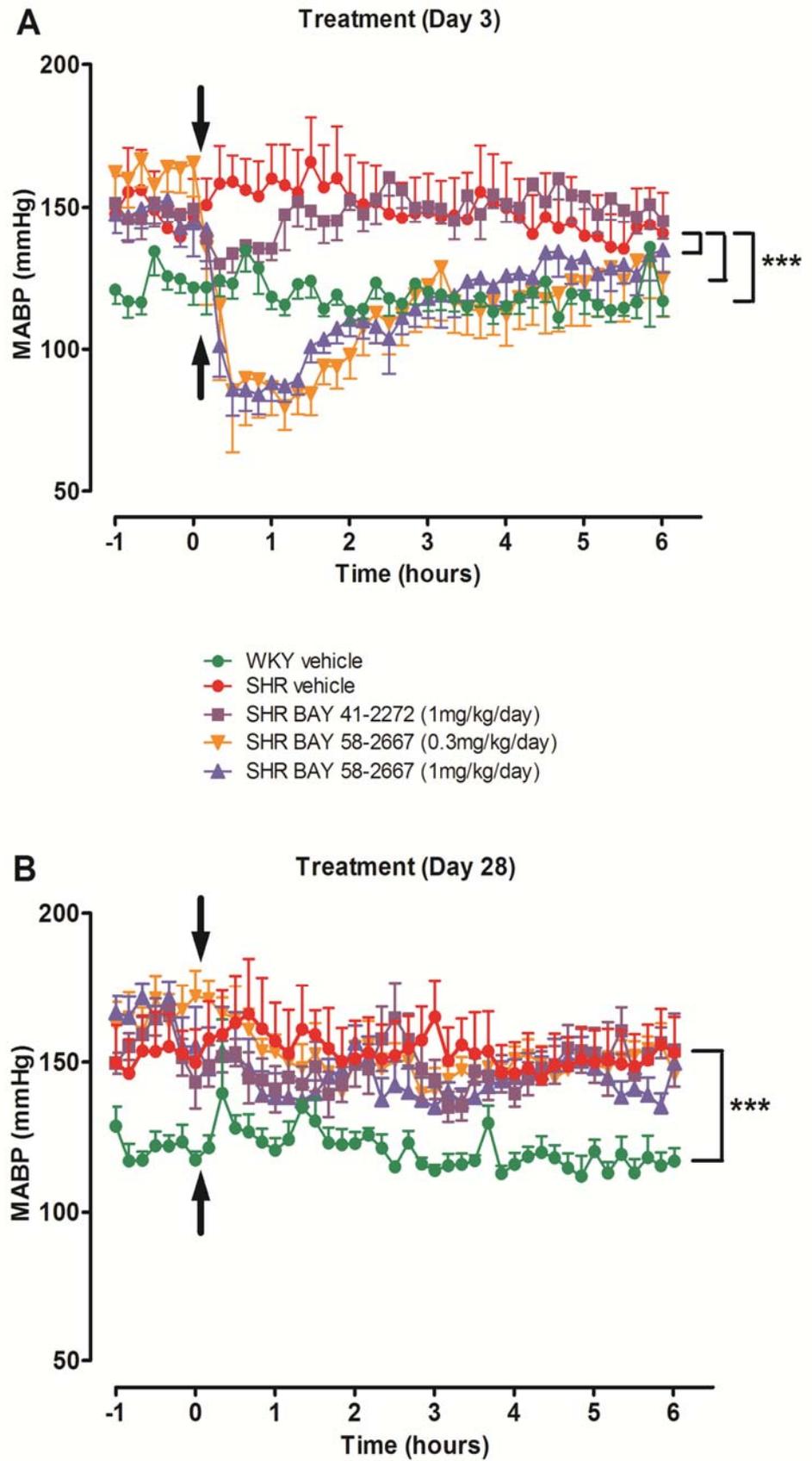


Figure 2. The effects of chronic treatment (4 weeks) with either 1 mg/kg/day BAY 41-2272 (■, n = 7), 0.3 mg/kg/day (▣, n = 8) or 1 mg/kg/day (▤, n = 7) BAY 58-2667 on cardiac hypertrophy, as measured via heart weight:body weight (HW:BW) ratio, in aged SHR (vehicle, ■, n = 8). HW:BW ratios for age-matched WKY (vehicle, □, n = 7) are shown for comparison. Results are expressed as heart weight:body weight ratio (mg/g) and presented as mean ± s.e.mean, where n = number of rats. #*P*<0.05 versus WKY vehicle, **P*<0.05 versus SHR vehicle, (One-way ANOVA, Bonferroni's multiple comparisons test).

Figure 2

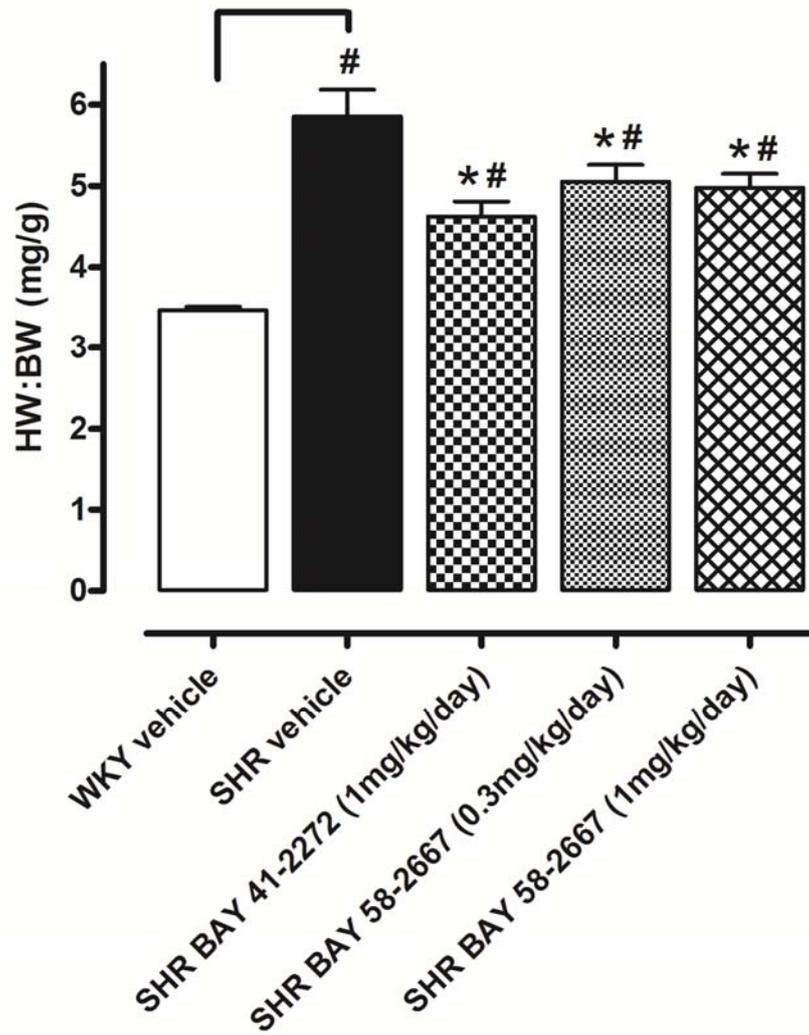


Figure 3. The effects of chronic treatment (4 weeks) with either 1 mg/kg/day BAY 41-2272 ( , n = 6), 0.3 mg/kg/day ( , n = 5) or 1 mg/kg/day ( , n = 6) BAY 58-2667 on vascular $\cdot\text{O}_2^-$ production, in aged SHR (vehicle,  , n = 7). $\cdot\text{O}_2^-$ production for WKY (vehicle,  , n = 7) are shown for comparison. Results are expressed as 10^3 counts per mg of dry tissue weight and presented as mean \pm s.e.mean, where n = number of rats. [#] $P < 0.05$ versus WKY vehicle, ^{**} $P < 0.01$ versus SHR vehicle, (One-way ANOVA, Bonferroni's multiple comparisons test).

Figure 3

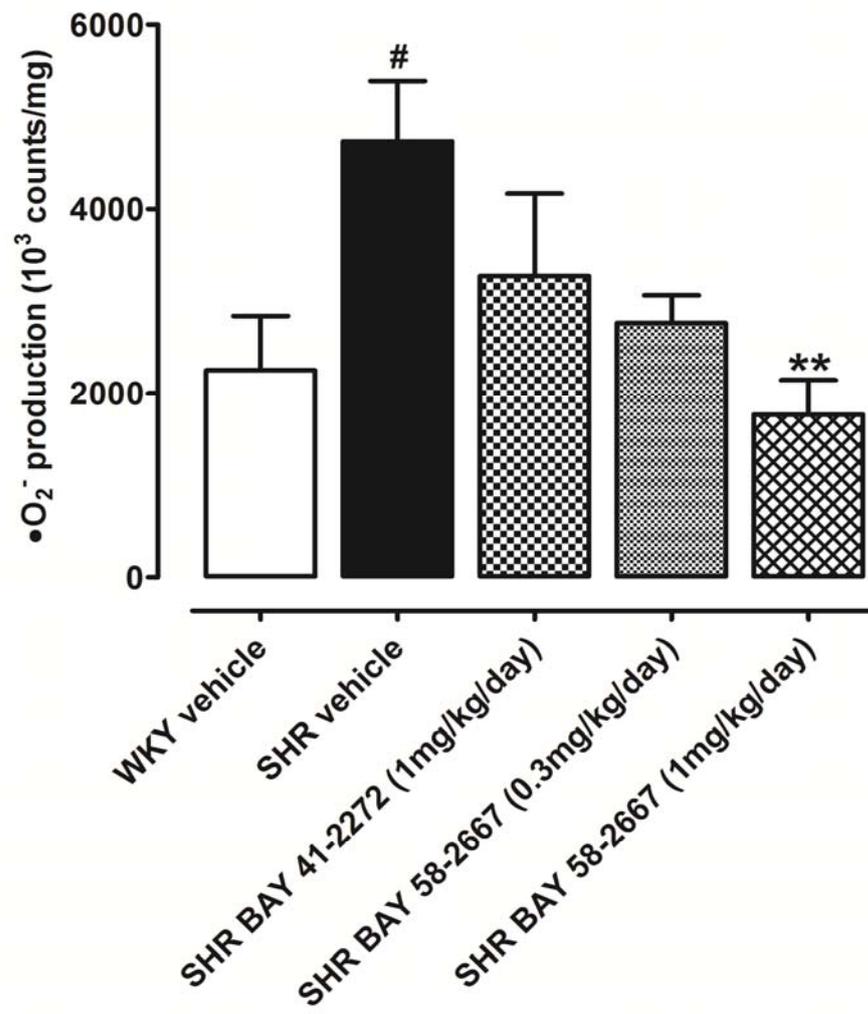


Figure 4. Vasoconstrictor responses to (A) 124 mM K⁺ solution (KPSS) and (B) serotonin (1 nmol/L - 10 μmol/L) in endothelium-intact aortae isolated from vehicle-treated aged WKY (□, ○, n = 6) and aged SHR treated chronically (4 weeks) with either vehicle (■, ●, n = 5), 1 mg/kg/day BAY 41-2272 (▣, □, n = 5), 0.3 mg/kg/day (▤, ▽, n = 4) or 1 mg/kg/day (▥, ▲, n = 5) BAY 58-2667. Responses are expressed as (A) g force and (B) percentage of the maximum contraction to KPSS. Values are given as mean ± s.e.mean and where n = number of rats. #*P*<0.05 for responses at 10 μmol/L serotonin versus WKY vehicle (One-way ANOVA, Bonferroni's multiple comparisons test).

Figure 4

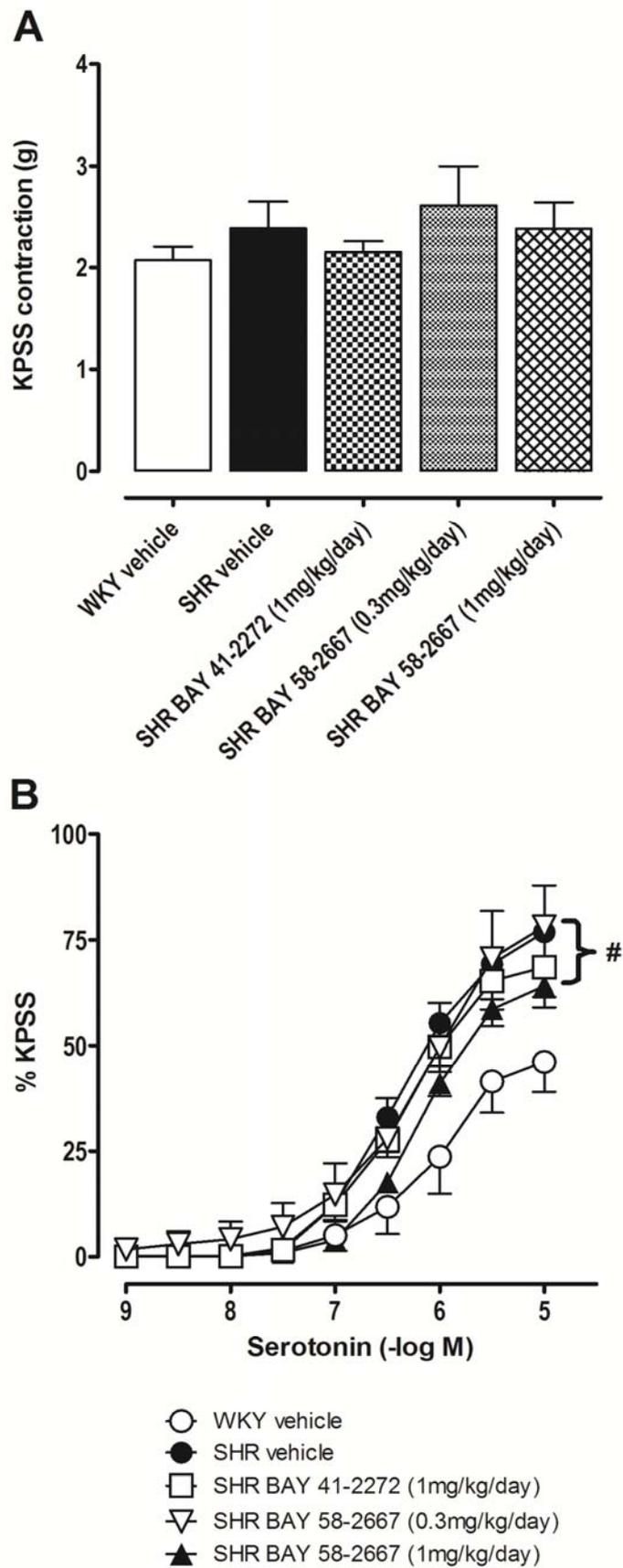


Figure 5. Vasorelaxant responses to **(A)** ACh (1 nmol/L - 10 μ mol/L) and **(B)** DEA/NO (0.1 nmol/L - 10 μ mol/L) in endothelium-intact aortae isolated from vehicle-treated aged WKY (\circ , n = 5) and aged SHR treated chronically (4 weeks) with either vehicle (\bullet , n = 6), 1 mg/kg/day BAY 41-2272 (\square , n = 5), 0.3 mg/kg/day (∇ , n = 6) or 1 mg/kg/day (\blacktriangle , n = 6) BAY 58-2667. Responses are expressed as a percentage reversal of pre-contraction and presented as mean \pm s.e.mean, where n = number of rats.

Figure 5

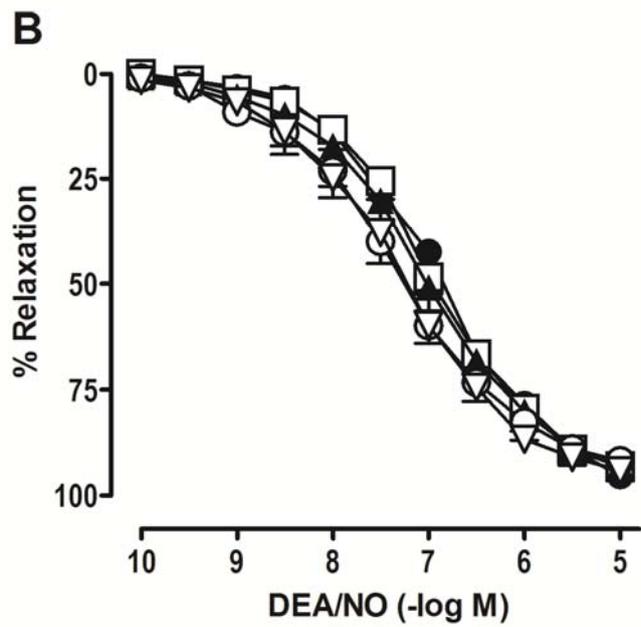
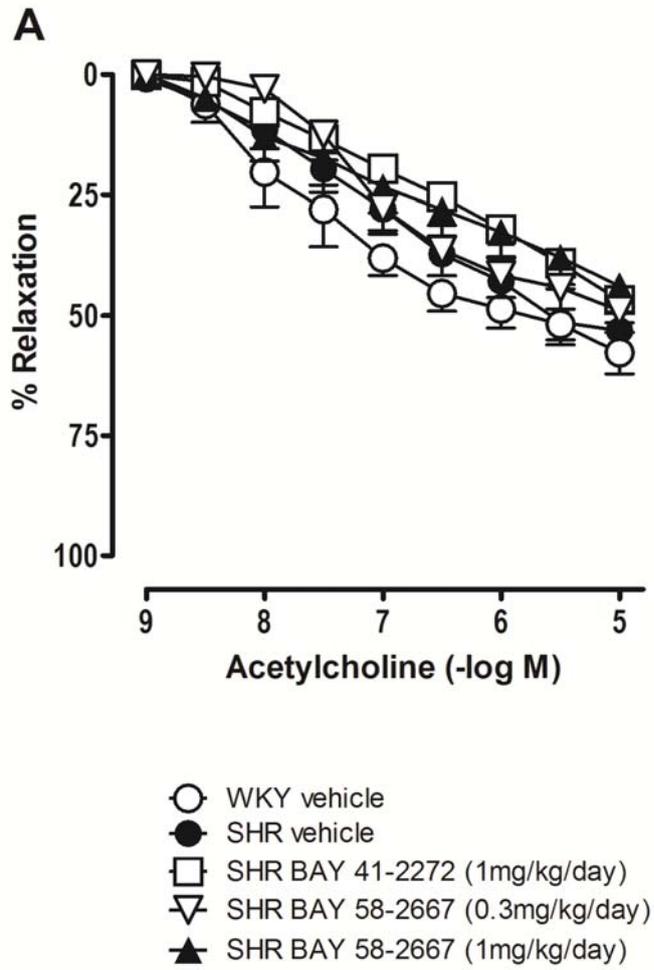


Figure 6. Vasorelaxant responses to **(A)** BAY 41-2272 (0.01 nmol/L - 10 μ mol/L) and **(B)** BAY 58-2667 (0.01 pmol/L - 1 μ mol/L) in endothelium-intact aortae isolated from vehicle-treated aged WKY (\circ , n = 5) and aged SHR treated chronically (4 weeks) with either vehicle (\bullet , n = 5), 1 mg/kg/day BAY 41-2272 (\square , n = 6), 0.3 mg/kg/day (∇ , n = 4) or 1 mg/kg/day (\blacktriangle , n = 5-6) BAY 58-2667. Responses are expressed as a percentage reversal of pre-contraction and presented as mean \pm s.e.mean, where n = number of rats.

Figure 6

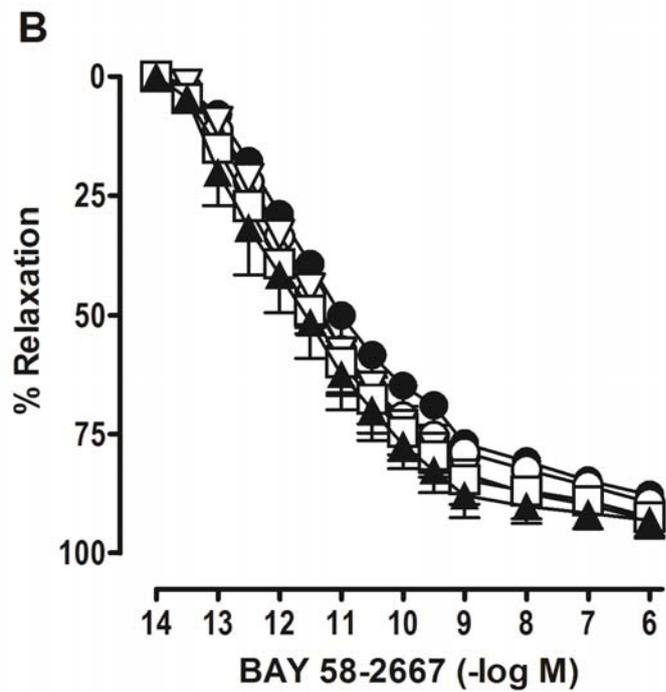
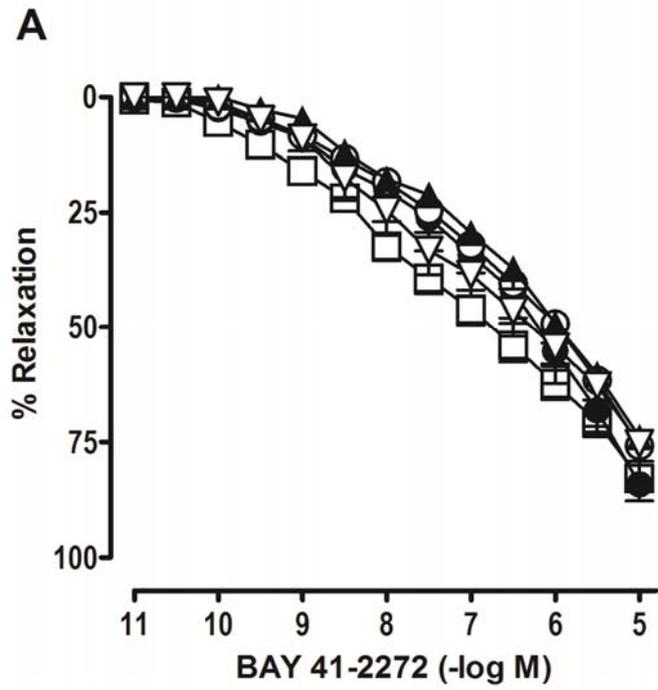
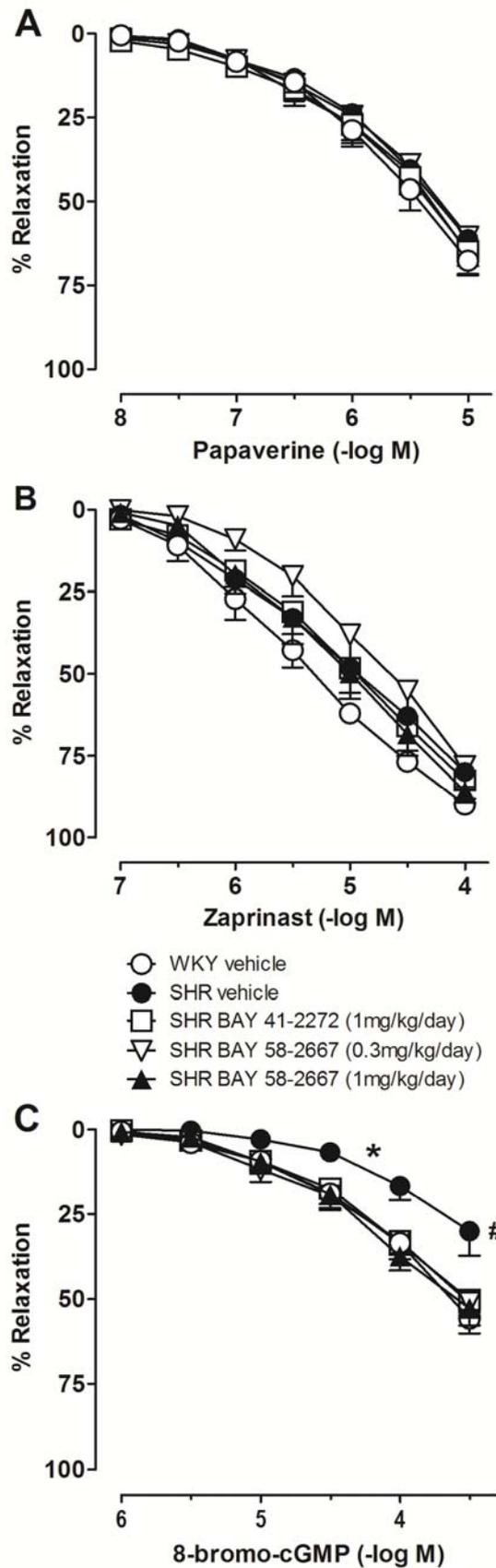


Figure 7. Vasorelaxant responses to (A) papaverine (10 nmol/L – 10 μ mol/L), (B) zaprinast (0.1 μ mol/L - 100 μ mol/L) and (C) 8-bromo-cGMP (1 μ mol/L – 300 μ mol/L) in endothelium-intact aortae isolated from vehicle-treated aged WKY (\circ , n = 5) and aged SHR treated chronically (4 weeks) with either vehicle (\bullet , n = 2-5), 1 mg/kg/day BAY 41-2272 (\square , n = 6), 0.3 mg/kg/day (∇ , n = 4-6) or 1 mg/kg/day (\blacktriangle , n = 6) BAY 58-2667. Responses are expressed as a percentage reversal of pre-contraction and presented as mean \pm s.e.mean, where n = number of rats. * P <0.05 for concentration-response curve versus WKY vehicle (Two-way ANOVA, Tukey Test), # P <0.05 for response at 300 μ mol/L versus WKY vehicle (One-way ANOVA, Bonferroni's multiple comparisons test).

Figure 7



The key findings of this study revealed that chronic treatment with either the NO-independent sGC stimulator, BAY 41-2272 or the sGC activator, BAY 58-2667 attenuates cardiac hypertrophy in aged SHR rats. Such an effect was independent of an ability of either BAY 41-2272 or BAY 58-2667 to chronically lower blood pressure. Whilst both BAY 41-2272 and BAY 58-2667 caused a transient decrease in mean arterial blood pressure (MABP) in aged SHR upon administration, this effect was lost over the 4 week treatment period, indicative of *in vivo* tolerance development. However, vascular tolerance and cross-tolerance development to these sGC stimulators and activators was not evident *in vitro* with vasorelaxant responses to BAY 41-2272 and BAY 58-2667 maintained in isolated aortae from SHR following chronic *in vivo* treatment. We also showed that chronic BAY 58-2667 treatment has vasoprotective actions in aged SHR, suppressing vascular O_2^- production and restoring vasorelaxation to the cGMP analogue, 8-bromo-cGMP. Like BAY 58-2667, BAY 41-2272 treatment also improved the vasodilator response to 8-bromo-cGMP yet neither the sGC stimulator nor activator reversed the augmented serotonin-mediated vasoconstriction in aged SHR. The findings from the current study suggest that in this model of chronic hypertensive heart disease, targeting reduced/oxidised heme-free sGC using NO-independent agents may confer cardioprotection.

In order to evaluate and compare the potential cardio- and vaso-protective actions of BAY 41-2272 and BAY 58-2667, a model of hypertensive heart disease was employed. Aged (20-22 months) SHR exhibited elevated MABP and vascular O_2^- production, cardiac hypertrophy and vascular dysfunction with increased vasoconstrictor responses to serotonin as well as reduced vasorelaxation to 8-bromo-cGMP, as compared to age-matched normotensive WKY. Similarly, previous studies have also reported a significant increase in MABP and heart weight/body weight ratio in aged (16 month) SHR compared to age-matched WKY (Bauersachs *et al.*, 1998). Whilst Kloß and colleagues also reported a decrease in aortic sGC expression and attenuated vasorelaxation to endothelium-dependent (ACh) and -independent (SNP) vasodilators in aged

SHR, such observations were not made in the current study (Kloß *et al.*, 2000). As will be discussed later, aging *per se* may induce such dysfunction in NO/sGC/cGMP signaling, thus accounting for the lack of change to both NO-dependent and -independent vasodilators in the current study. Our finding that vasoconstrictor responses were augmented in aortae from aged SHR concurs with previous studies in adult SHR versus WKY. Such an effect has been attributed to an increase in $\cdot\text{O}_2^-$ production and/or the release of vasoconstrictor prostanoids, both of which counteract the modulatory effects of NO (Budzyn *et al.*, 2008; Ibarra *et al.*, 2006).

In this model of hypertensive heart disease, BAY 58-2667 caused a transient decrease in MABP following intraperitoneal administration, an effect which lasted ~4-5 hours. While BAY 41-2272 also showed a similar trend to decrease MABP following the initial administration, this did not achieve statistical significance. These findings concur with those in conscious mice (Dumitrascu *et al.*, 2006) and SHR rats (Stasch *et al.*, 2006) in which both BAY 41-2272 and BAY 58-2667 have been shown to acutely decrease blood pressure using similar doses. Moreover, vasodepressor actions of BAY 58-2667 were evident in humans, with a 6-hour infusion of the sGC activator potently unloading the heart, increasing cardiac output and decreasing MABP in patients with acute decompensated heart failure (ADHF) (Lapp *et al.*, 2009). In the current study, BAY 58-2667 was found to be more efficacious at lowering blood pressure compared to BAY 41-2272, suggesting the possible existence of a substantial pool of oxidised/heme-free sGC in this model of hypertensive heart disease. Although both BAY 41-2272 and BAY 58-2667 caused an acute depressor response, this was not sustained for the entire 28 day period such that MABP returned to baseline following 2 and 3 weeks of administration, respectively. This loss in the acute blood pressure lowering effects of both BAY 41-2272 and BAY 58-2667 was indicative of tolerance development to these drug-induced hemodynamic changes. Similarly, in an angiotensin II (Ang II) infusion model of hypertension in rats, the ability of BAY 41-2272 to attenuate

systolic blood pressure is lost following 14 days oral administration of this sGC stimulator (Masuyama *et al.*, 2006).

Importantly, such tolerance was not observed at the level of the isolated artery, where we found vasorelaxant responses to both BAY 41-2272 and BAY 58-2667 were preserved in isolated aortae from SHR chronically treated with either the sGC stimulator or activator, suggesting that chronic treatment with these compounds do not induce vascular tolerance or cross-tolerance. Indeed, pre-clinical observations have shown that vasorelaxant response to BAY 58-2667 were preserved under nitrate tolerant conditions (Stasch *et al.*, 2002), whilst radioligand binding studies have confirmed that BAY 58-2667's interaction with sGC is highly specific in contrast to the lack of specificity observed with nitrovasodilators (Lapp *et al.*, 2009). BAY 58-2667 treatment, therefore, appears to be devoid of potential off-target clinical effects (Lapp *et al.*, 2009). Thus, the hemodynamic tolerance observed in these SHR rats may reflect homeostatic reflex mechanisms such as activation of the renin-angiotensin system (RAS) or baroreflex-induced changes in heart rate, which may counteract the fall in MABP during chronic therapy without evoking the additional vascular tolerance (Serone *et al.*, 1996). Indeed, 'vascular tolerance' is generally based on isolated large artery experiments whilst 'hemodynamic adjustments' are assessed in the intact animal or human subjects (Serone *et al.*, 1996). Given cGMP elevating agents (i.e. endogenous and exogenous NO) have been shown to have direct effects on the sympathetic nerves of the rat heart (Schwarz *et al.*, 1995), perhaps sGC stimulators/activators may also do the same, thereby evoking hemodynamic, but not vascular tolerance, following prolonged use. This study, therefore, highlights the importance of characterising the vasodilator capacity of these compounds both *in vivo* and *in vitro*, and certainly, future studies investigating the exact mechanisms underlying the hemodynamic tolerance development to BAY 41-2272 and BAY 58-2667 are warranted. With regards to therapeutic use, we need to be mindful that if the main therapeutic aim is an anti-hypertensive

effect, then there may be some caveats associated with the chronic use of sGC stimulators/activators.

Importantly, this study showed that neither chronic administration of neither BAY 41-2272 (1 mg/kg/day) nor BAY 58-2667 (0.3 and 1 mg/kg/day) in conscious aged SHR lead to a change in baseline MABP following the 4 week treatment period. Although it has previously been reported that administration of BAY 41-2272 (10 mg/kg/day) for 10 days in Ang II-infused rats leads to a suppression of systolic blood pressure, this study used a higher dose of the sGC stimulator as compared to the current study. In addition, while the current study commenced treatment after the full establishment of hypertension, Masuyama and co-workers administered BAY 41-2272 concomitantly with Ang II infusion (Masuyama *et al.*, 2006). Despite neither BAY 41-2272 nor BAY 58-2667 causing a sustained decrease in MABP, both compounds significantly reduced the heart weight:body weight ratio in SHR, indicative of an anti-hypertrophic action. Indeed, Masuyama and co-workers demonstrated that BAY 41-2272 (10 mg/kg/day) significantly reduced both the Ang II-induced increase in left ventricular hypertrophy and cardiomyocyte size (Masuyama *et al.*, 2006). Furthermore, BAY 58-2667 has been shown to reduce left ventricular hypertrophy in rats with 5/6 nephrectomy (Kalk *et al.*, 2006), while both BAY 41-2272 and BAY 58-2667 decreased right ventricular hypertrophy in rodent models of pulmonary hypertension (Dumitrascu *et al.*, 2006). The anti-hypertrophic actions of BAY 41-2272 and BAY 58-2667 observed in the current study may have arisen as a consequence of a direct action of these cGMP-elevating agents at the level of the cardiomyocyte rather than as a result of a hemodynamic change. Although myocardial cGMP levels were not measured, previous studies have shown that BAY 41-2272 (Masuyama *et al.*, 2006) and BAY 58-2667 (Krieg *et al.*, 2009) increase cGMP levels in myocardial tissue. Indeed, the anti-hypertrophic actions of cGMP elevating drugs such as the pGC stimulator, ANP and the PDE inhibitor, sildenafil are well documented (Ritchie *et al.*, 2009) and cardiac hypertrophy is augmented in various knockout

mice models lacking elements of the cGMP signaling pathway (Tsai & Kass, 2009). Thus, our findings provide further support for the concept that activation of sGC and a subsequent increase in cGMP can attenuate cardiac remodelling associated with hypertension.

Having clearly demonstrated the cardioprotective actions of both BAY 41-2272 and BAY 58-2667 in this model of hypertensive heart disease, we sought to explore the potential vasoprotective actions of these compounds. Indeed, a hallmark of hypertension is an elevation in the generation of ROS, such as $\cdot\text{O}_2^-$ leading to a decrease in endogenous NO bioavailability (Cuzzocrea *et al.*, 2004). In accordance with previous studies (Bauersachs *et al.*, 1998; Cuzzocrea *et al.*, 2004), we found basal $\cdot\text{O}_2^-$ production by aortae from SHR to be ~52% greater than in WKY. This increase in ROS production in the SHR has been reported to be associated with an enhanced aortic expression of the NADPH oxidase subunits Nox1, Nox2, Nox4 and p22^{phox} as well as the additional contribution from uncoupled eNOS (Li *et al.*, 2006). Certainly, evidence to date has supported the role of xanthine oxidase, uncoupled NOS and in particular NADPH oxidase in the production of ROS in the SHR (Paravicini & Touyz, 2006). Interestingly, chronic treatment with only the high dose of BAY 58-2667 (1 mg/kg/day) suppressed basal $\cdot\text{O}_2^-$ levels in SHR aortae, returning $\cdot\text{O}_2^-$ levels to baseline. While both 0.3 mg/kg/day BAY 58-2667 and 1 mg/kg/day BAY 41-2272 tended to lower basal $\cdot\text{O}_2^-$ generation, these results did not achieve statistical significance. In contrast, $\cdot\text{O}_2^-$ lowering effects of BAY 41-2272 have been previously reported in mouse isolated cavernosal smooth muscle, where BAY 41-2272 inhibited U46619-induced $\cdot\text{O}_2^-$ formation and NADPH oxidase expression in a cGMP-dependent and ODQ-sensitive manner (Teixera *et al.*, 2007). It should be noted, however, that these studies were conducted acutely *in vitro*.

Although not tested directly in the current study, it is likely that BAY 58-2667 suppresses basal $\cdot\text{O}_2^-$ levels via a cGMP-dependent mechanism. Indeed, the ROS-limiting effects of cGMP-

elevating agents are well documented with the nitrovasodilator, morpholinosydnonimine (SIN-1) (Muzaffar *et al.*, 2004), PDE V inhibitor, sildenafil (Koupparis *et al.*, 2005) and pGC stimulator, ANP (Laskowski *et al.*, 2006), all shown to reduce NADPH oxidase activity via a cGMP-dependent manner in porcine pulmonary artery VSMC, rabbit cavernosal smooth muscle cells and neonatal rat cardiomyocytes, respectively. The mechanisms by which this occurs are yet to be elucidated, however, it has been suggested that the downstream activation of cGMP-dependent protein kinases (cGK-1) suppresses both the induction and activity of NADPH oxidase (Ritchie *et al.*, 2009). Moreover, the substantial $\cdot\text{O}_2^-$ limiting actions of BAY 58-2667, as compared to BAY 41-2272, may be indicative of its ability to activate sGC to elevate cGMP to a greater extent (Stasch *et al.*, 2006) and the need for a threshold level of cGMP to be reached to instigate NADPH oxidase suppression. Alternatively, BAY 58-2667 may suppress $\cdot\text{O}_2^-$ production via a cGMP-independent mechanism. Certainly, future studies will need to determine if this $\cdot\text{O}_2^-$ suppressing effect of BAY 58-2667 is mediated via a cGMP-dependent or -independent mechanism of action.

The results thus far reveal that chronic sGC stimulation and/or activation exhibits anti-hypertrophic effects in the absence of a maintained anti-hypertensive effect, whilst BAY 58-2667 alone was capable of limiting basal vascular $\cdot\text{O}_2^-$ generation. With an ability to suppress basal vascular $\cdot\text{O}_2^-$, coupled with the recent finding that BAY 58-2667 may be cytoprotective, as evidenced by its ability to preserve endothelial function in rat coronary arteries following acute exposure to peroxynitrite (ONOO \cdot) (Korkmaz *et al.*, 2009), we sought to determine if chronic activation of sGC by BAY 58-2667 would normalise vascular function in aged SHR. As mentioned previously, the model of hypertensive heart disease employed in the current study is associated with augmented vasoconstrictor responses to serotonin, which may occur as a consequence of a loss of endogenous endothelial-derived NO together with an augmented release of vasoconstrictor prostanoids from the endothelium and/or vascular smooth muscle (Budzyn *et*

al., 2008; Ibarra *et al.*, 2006). Additionally, we have also demonstrated, both in this study and in a previous study, utilising adult SHR rats (Budzyn *et al.*, 2008), that this enhanced contractility in isolated SHR aortae is selective for serotonin with contractile responses to a high potassium (K^+) solution (KPSS) unaltered in SHR as compared to WKY aortae. Neither chronic treatment with BAY 41-2272 (1 mg/kg/day) nor BAY 58-2667 (0.3 or 1 mg/kg/day) normalised the contractile response to serotonin in isolated aortae from aged SHR. Thus, it appears that neither the ability of BAY 58-2667 to decrease vascular $\cdot O_2^-$ production nor activate sGC is sufficient to counteract the potential loss of endogenous NO and the augmented release of vasoconstrictor prostanoids in aged SHR. A possible extension of the current study would be to confirm a decrease in endogenous NO bioavailability via examination of contractile responses to the NOS inhibitor, L-NAME.

It is well documented that hypertension is associated with impaired endothelial function as a consequence of a decreased release of endothelial-derived NO and an increased release of vasoconstrictor prostanoids (Tang & Vanhoutte, 2010; Versari *et al.*, 2009). Indeed, we (Chapter 4) and others (Chen *et al.*, 1997; Lui *et al.*, 2001) have reported impaired endothelium-dependent vasorelaxation to ACh in isolated aortae from adult SHR compared with normotensive WKY. Moreover, independent of hypertension, aging *per se* is associated with endothelial dysfunction (Taddei *et al.*, 2006; Vanhoutte, 2002) which may arise as a consequence of decreased eNOS activity, elevated $\cdot O_2^-$ generation and increased susceptibility of endothelial cells to apoptosis (Asai *et al.*, 2000; Hoffman *et al.*, 2001). This is predominantly due to an decrease in NO production, which would normally interfere with apoptosis via the blockade of caspase activity by S-nitrosylation of the essential cysteine residues (Hoffman *et al.*, 2001). Indeed, aged endothelial cells appear to lack the ability to recruit sufficient amounts of NO which, may be related to the impaired phosphorylation and expression of eNOS, deficiency in essential co-factors (BH_4) and/or enhanced ROS formation induced by age-related mitochondrial damage

(Hoffman *et al.*, 2001). Furthermore, the hypertensive phenotype, together with aging, did not attenuate ACh-mediated vasorelaxation further than aging alone with vasorelaxant responses to ACh in aortae being similar between aged WKY and SHR rats. Chronic treatment with BAY 41-2272 and BAY 58-2667 did not improve endothelial function in aged hypertensive rats such that ACh-mediated vasorelaxation was similar in aortae from vehicle- and sGC stimulator/activator-treated SHR. While BAY 58-2667 at least may preserve endogenous NO bioavailability via a suppression of O_2^- production, this may not be sufficient to restore endothelial dysfunction in the setting of aging and hypertension, particularly if endothelial dysfunction arises as a consequence of apoptosis (Asai *et al.*, 2000; Hoffman *et al.*, 2001).

To explore the potential changes in vascular NO/sGC/cGMP signaling beyond the endothelium in the setting of hypertensive heart disease, the NO donor, DEA/NO, NO-independent sGC stimulator, BAY 41-2272, NO-independent sGC activator, BAY 58-2667, phosphodiesterase V (PDE5) inhibitor, zaprinast, sGC-independent vasodilator, papaverine and the cGMP analogue, 8-bromo-cGMP were employed. The current study revealed no differences in vasorelaxation responses to DEA/NO, BAY 41-2272, BAY 58-2667, zaprinast or papaverine in vehicle-treated aged SHR as compared to vehicle-treated aged WKY.

Although studies have reported decreased sGC expression, attenuated vasorelaxant responses to NO donors (Kloß *et al.*, 2000) and BAY 41-2272 (Priviero *et al.*, 2009) and an increased vasodilator capacity of BAY 58-2667 in either adult or aged SHR (Stasch *et al.*, 2006), these findings were compared to adult WKY. Thus, it is likely that alterations in NO/sGC/cGMP signaling occur with aging alone and thus, were not apparent in the current study when comparisons were made between aged WKY and aged SHR. Indeed, Ruetten and co-workers observed an aging-induced downregulation of sGC at the mRNA and protein level in aortic rings from aged WKY rats (Ruetten *et al.*, 1999), suggesting that aging itself can lead to an

impairment in the NO/sGC/cGMP pathway. Furthermore, we have evidence that aging *per se* can alter NO/sGC/cGMP signaling. Thus, the vasodilator potency of BAY 41-2272 in aged WKY appears to be ~8-fold less than that in adult WKY (Chapter 5), indicative of dysfunction at the level of sGC itself with age. Although vasorelaxation to DEA/NO did not appear to be changed between adult (Chapter 4) and aged WKY, this may be indicative of an ability of DEA/NO to target cGMP-independent mechanisms (Hempelmann *et al.*, 2000; Sampson *et al.*, 2001). By contrast, vasorelaxation responses to BAY 58-2667 were ~72-fold more potent in aged WKY (pEC₅₀ value of 11.69 ± 0.30 $-\log$ M, n = 5) compared to adult WKY (9.83 ± 0.36 , n = 9, Chapter 4). Together these findings suggest that aging itself can alter the NO/sGC/cGMP signalling pathway, possibly resulting in considerable pools of oxidised sGC, such that the vasorelaxant response to BAY 58-2667 is markedly enhanced.

The predominant dysfunction observed with regards to vascular NO/sGC/cGMP signaling, in the setting of aging and hypertension, was impaired vasorelaxation to 8-bromo-cGMP in aged SHR versus aged WKY. Such an alteration was unlikely to be attributed to an increase in cGMP degradation by PDE V in aged SHR as vasorelaxant responses to zaprinast were unaltered. Interestingly, impaired relaxation to 8-bromo-cGMP has also been previously reported in isolated aorta from salt-loaded stroke-prone spontaneously hypertensive rats (SHRSP), an effect attributed to a decrease in PKG activity (Kagota *et al.*, 2007). Chronic treatment with both BAY 41-2272 and BAY 58-2667 improved vasorelaxation to 8-bromo-cGMP in aged SHR, such that responses were similar to that in vehicle-treated aged WKY. The mechanisms underlying such protective actions of BAY 41-2272 and BAY 58-2667 in the present study are currently unknown, yet may be indicative of a synergistic action of these sGC stimulators and activators with 8-bromo-cGMP, leading to further augmentation of cellular cGMP levels. Presumably, 8-bromo-cGMP is less efficacious at stimulating downstream targets compared to DEA/NO, BAY

41-2272 and BAY 58-2667 which may also explain why its effects are more readily compromised in the aged SHR.

Taken together, the results from the current study revealed that chronic targeting of reduced (BAY 41-2272) and oxidised/heme-free (BAY 58-2667) sGC in hypertensive heart disease exerts an equivalent anti-hypertrophic effect that is not dependent on a lowering in MABP. Furthermore, targeting oxidised/heme-free sGC can limit vascular $\cdot\text{O}_2^-$ generation whilst both BAY 41-2272 and BAY 58-2667 augmented cGMP-dependent signaling. From a therapeutic perspective, tolerance may develop to the depressor responses, however regardless, sGC stimulators and activators may represent novel therapeutic strategies for hypertensive heart disease.

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CHAPTER 7

NADPH oxidase & the Western-style
high fat diet independently modulate
responses to the sGC activator,

BAY 58-2667



The NO- and heme-independent sGC activator, BAY 58-2667, preferentially targets NO-insensitive oxidised/heme-free sGC, such that its vasorelaxant potency is enhanced in both human and animal models of disease. Moreover, previous studies have shown that the vasorelaxant response to BAY 58-2667 is augmented in disease states that are associated with an elevation in vascular superoxide anion radical ($\bullet\text{O}_2^-$) generation. Indeed, NADPH oxidases have been noted as the principal source of reactive oxygen species (ROS) such as vascular $\bullet\text{O}_2^-$, and have been identified as key players in the development of oxidative stress. However, it is currently unknown if NADPH oxidase influences the pools of oxidised/heme-free sGC. Thus, the aim of this study was to determine if an increase in NADPH oxidase-derived $\bullet\text{O}_2^-$ production results in an enhancement in the pools of oxidised/heme-free sGC, as assayed via the responsiveness to BAY 58-2667. In endothelium-intact carotid arteries from C57BL/6J mice, BAY 58-2667 proved to be a potent vasodilator achieving approximately 50% relaxation in the nanomolar range. This potency was further augmented in the presence of the sGC inhibitor, ODQ (10 $\mu\text{mol/L}$) and decreased in the presence of the heme-free sGC antagonist, Zn-PPIX (10 $\mu\text{mol/L}$), indicative of the existence of pools of oxidised/heme-free sGC under physiological conditions. Furthermore, in a model of short-term oxidative stress induced by 24-hour Ang II (10 nmol/L)-treatment and associated with an approximate 2-fold increase in NADPH oxidase-driven $\bullet\text{O}_2^-$ production, the vasodilator response to BAY 58-2667 was enhanced by 16-fold compared to the vehicle (50 nmol/L glacial acetic acid)-treated carotid arteries. Such results are indicative of a correlation between an increase in NADPH oxidase activity and an enhanced response to BAY 58-2667. More specifically, the role of Nox2-containing NADPH oxidases were implicated in both the short-term model of oxidative stress (Nox2^{-/-} mice, Ang II treatment) and the chronic model of disease (Nox2^{-/-}/ApoE^{-/-} mice, HFD), whereby the deletion of the Nox2 gene reduced $\bullet\text{O}_2^-$ production and impairment of endothelial function relative to their respective controls. However, while the potentiated response to BAY 58-2667 was lost in Ang II-treated arteries from Nox2^{-/-} mice, vasorelaxant responses to BAY 58-2667 appeared to be equipotent in

all mice groups fed the high fat diet (C57BL/6J, ApoE^{-/-} and Nox2^{-/-}/ApoE^{-/-}) for 7 weeks, regardless of the varying levels of $\cdot\text{O}_2^-$ detected between the strains. Indeed, BAY 58-2667-induced relaxation was more potent in C57BL/6J mice receiving a high fat diet compared to those fed the normal chow diet, despite no differences in $\cdot\text{O}_2^-$ production recorded. Thus, perhaps the most intriguing finding of this study was that the Western-style high fat diet itself was also able to augment the vasorelaxant responses to BAY 58-2667 irrespective of $\cdot\text{O}_2^-$ generation in mouse carotid arteries. Taken together, our results suggest that Nox2-containing NADPH oxidase and the Western-style high fat diet may independently play an important role in modulating the relaxation responses to BAY 58-2667, possibly by regulating the vascular pools of oxidised/heme-free sGC.

Nitric oxide (NO) is an important signaling molecule in the vasculature (Moncada & Higgs, 2006), mediating most of its effects via the activation of its intracellular receptor, soluble guanylyl cyclase (sGC). sGC is a heterodimeric protein comprising of an α and heme-containing β -subunit (Denninger & Marletta, 1999; Hobbs, 2002). NO binds to the ferrous heme (Fe^{2+}) group leading to sGC activation and the subsequent generation of the second messenger, cyclic guanosine-3',5'-monophosphate (cGMP) (Ballou *et al.*, 2002). This interaction in turn is responsible for the regulation of platelet aggregation, vascular tone and neurotransmission (Denninger & Marletta, 1999; Gao, 2009). Thus, the importance of this NO signalling pathway in the maintenance of vascular homeostasis has been well established.

Furthermore, the impairment of the NO/sGC/cGMP signalling cascade under conditions of increased oxidative stress, as associated with several disease states, has also been well documented (Melichar *et al.*, 2004; Napoli & Ignarro, 2001; Ruetten *et al.*, 1999). Indeed, oxidative stress results from an imbalance between reactive oxygen species (ROS) generation and inactivation, whereby NO bioavailability is thus compromised due to scavenging by ROS, namely superoxide anion radical (O_2^-), resulting in the formation of the powerful oxidant peroxynitrite (ONOO^-) (Armitage *et al.*, 2009; Stasch *et al.*, 2006). Furthermore, ONOO^- has been shown to oxidise purified sGC, rendering it insensitive to NO (Stasch *et al.*, 2006). As such, disease conditions may be associated with impaired NO bioavailability and/or dysfunction at the level of its receptor (Evgenov *et al.*, 2006). Excitingly, the recent development of novel NO-independent sGC activators such as BAY 58-2667 (Stasch *et al.*, 2006; Stasch *et al.*, 2002) and HMR 1766 (Schindler *et al.*, 2006) may overcome such limitations. BAY 58-2667 preferentially targets the oxidised/heme-free form of sGC, mimicking the spatial structure of the heme and interacting with the unoccupied heme motif, Y-x-S-x-R (tyrosine₁₃₉, serine₁₃₇ and arginine₁₃₉) to activate the enzyme (Martin *et al.*, 2010; Schmidt *et al.*, 2004). Thus, its ability to activate sGC has been shown to be enhanced following oxidation with sGC inhibitors (ODQ) and ONOO^-

(Stasch *et al.*, 2006; Stasch *et al.*, 2002). Moreover, in disease states associated with an increase in oxidative stress such as hypertension and diabetes, the vasorelaxant potency to BAY 58-2667 is augmented (Stasch *et al.*, 2006). BAY 58-2667, therefore represents a valuable pharmacological tool for probing the intracellular pools of oxidised/heme-free sGC under both physiological and pathological conditions (Schmidt *et al.*, 2004; Stasch *et al.*, 2006; Stasch *et al.*, 2002). It has generally been assumed that the disease associated increase in responsiveness to BAY 58-2667 arises as a consequence of ROS-mediated oxidation of sGC, leading to enhanced pools of this redox state of the protein (Stasch *et al.*, 2006). To date, however, this concept has not been tested directly.

ROS generation in the vasculature is derived predominantly via NADPH oxidases, whose primary action is the production of $\cdot\text{O}_2^-$ (Armitage *et al.*, 2009; Lassegue & Clempus, 2003). Briefly, NADPH oxidases are comprised of a membrane-bound domain containing Nox (Nox1, Nox2 or Nox4) and p22phox subunits, a cytosolic domain containing p67^{phox}, p47^{phox} subunits, the G regulatory protein Rac as well as a catalytic domain. NADPH oxidases generate $\cdot\text{O}_2^-$ by transferring electrons from its substrate NADPH to molecular oxygen via the Nox subunit (Lassegue and Clempus, 2003). The expression of Nox2-containing NADPH oxidase, in particular, is up-regulated in the presence of cardiovascular risk factors (Selemidis *et al.*, 2008). The relationship between Nox2-containing NADPH oxidase activity and the redox regulation of sGC (responsiveness to BAY 58-2667) is currently unknown. In order to explore this concept, this study has utilised both a short term and chronic model of oxidative stress in which Nox2-containing NADPH plays a predominant role. Specifically, organoid culture was used to establish a model of short term oxidative stress as previously described by Chrissobolis and co-workers, in which 24-hour treatment of mouse carotid arteries with Ang II (10 nmol/L) substantially increases Nox2-containing NADPH-driven $\cdot\text{O}_2^-$ production with a concomitant decrease in NO bioavailability (Chrissobolis *et al.*, 2008; Scradler *et al.*, 2007). Furthermore, a

strong correlation between the progression of atherosclerosis, endothelial dysfunction and Nox2-containing NADPH oxidase activity has been proposed (Guzik *et al.*, 2000; Judkins *et al.*, 2009), and as such ApoE^{-/-} mice on a high fat diet were utilised as a model of chronic oxidative stress.

Thus, using BAY 58-2667 as a tool to identify the oxidised/heme-free sGC, the aim of the current study was to determine if Nox2-containing NADPH oxidases are able to modulate the vasorelaxant responses to BAY 58-2667 in a model of short-term oxidative stress and chronic disease, by potentially regulating the pools of oxidised/heme-free sGC.

Materials and Methods

This study was approved by the Pharmacology Animal Ethics Committee, Monash University, Australia and conforms to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

This study utilised common carotid arteries isolated from adult (10-14 weeks of age) male C57BL/6J (n = 31) and Nox-2^{-/-} (n = 6) mice maintained on a normal chow diet (ND), and C57BL/6J (n = 9), ApoE^{-/-} (n = 11) and Nox-2^{-/-}/ApoE^{-/-} (n = 10) mice that were fed a high fat diet for 7 weeks (HFD; see General Methods, section 2.1.1). Additionally, plasma lipid profile analysis was conducted in C57BL/6J (ND and HFD), ApoE^{-/-} and Nox2^{-/-}/ApoE^{-/-} mice prior to sacrifice and vessels isolated according to the General Methods, section 2.9.

Organoid Culture

All mice were killed via anaesthetic overdose with inhaled isoflurane (2-4% in O₂, Isorane, Baxter HealthCare PTY Ltd) after which both common carotid arteries were isolated. Isolated carotid arteries from C57BL/6J and Nox2^{-/-} (both fed a normal diet, ND) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) and incubated with either Ang II (10 nmol/L) or vehicle (50 nmol/L glacial acetic acid) for 24 hours in a CO₂ water-jacketed incubator (Forma Scientific) under sterile culture conditions (37°C, 5% CO₂ and 85% humidity; see General Methods, section 2.2.3 - 2.2.5). All procedures, from the dissection to the treatment of carotid arteries, were conducted in the PS2 laminar flow hood (HWS120 Series, CLYBE-APAC Environmental Products Division) in order to maintain sterility as described in the General Methods, section 2.2.

Treated (24 hrs, Ang II or vehicle) and freshly isolated carotid arteries were used in either •O₂⁻ detection experiments via lucigenin (5 µmol/L)-enhanced chemiluminescence assay (see General

Methods, section 2.4.1) or functional experiments using small vessel wire myography (see General Methods, sections 2.6.2 and 2.6.2.4).

Superoxide anion radical detection via lucigenin-enhanced chemiluminescence

Vascular $\cdot\text{O}_2^-$ levels were measured using lucigenin (5 $\mu\text{mol/L}$)-enhanced chemiluminescence in carotid arteries following either 24-hour treatment (either Ang II or vehicle) from C57BL/6J (ND) and Nox-2^{-/-} (ND) mice or in freshly isolated carotid arteries from C57BL/6J (ND and HFD), ApoE^{-/-} (HFD) and Nox-2^{-/-}/ApoE^{-/-} (HFD) mice (see General Methods, section 2.4.1 and 2.4.1.1). Vascular $\cdot\text{O}_2^-$ was detected using either the TopCount NXT single photon counter (Perkin Elmer) or the Hidex ChameleonTM Luminescence Plate Reader (Hidex Oy). Upon completion of the assay, rings were allowed to dry on pre-cut foil pieces overnight at 37°C. Dried carotid segments were then weighed the following day, background counts subtracted and $\cdot\text{O}_2^-$ normalised to dry tissue weight.

Vasorelaxation Experiments

Twenty-four hour vehicle or Ang II-treated (C57BL/6J ND and Nox-2^{-/-} ND) and freshly isolated C57BL/6J (ND and HFD), ApoE^{-/-} (HFD) and Nox-2^{-/-}/ApoE^{-/-} (HFD) carotid artery segments were mounted in small vessel wire myographs containing physiological Krebs' solution, continuously bubbling with carbogen (95% O₂ and 5% CO₂) and maintained at 37°C (see General Methods, section 2.6.2).

Cumulative concentration-dependent vasorelaxation responses to the sGC activator, BAY 58-2667 were examined in:

(i) 24-hour vehicle and Ang II-treated carotid arteries from C57BL/6J (ND) and Nox2^{-/-} (ND) mice. Additionally, in Ang II-treated carotid arteries from C57BL/6J mice, the effects of sGC

inhibition (ODQ, 10 $\mu\text{mol/L}$) and heme-free sGC antagonism (Zn-PPIX, 10 $\mu\text{mol/L}$) on BAY 58-2667-mediated relaxation were examined.

(ii) freshly isolated carotid arteries from C57BL/6J (ND and HFD), ApoE^{-/-} (HFD) and Nox2^{-/-}/ApoE^{-/-} (HFD) mice. In ApoE^{-/-} (HFD) mice, the effects of ODQ (10 $\mu\text{mol/L}$) on responses to BAY 58-2667 were also examined (see General Methods, section 2.6.2.4)

Furthermore, endothelium-dependent relaxation to 10 $\mu\text{mol/L}$ ACh was tested in endothelium-intact carotid arteries from C57BL/6J (ND and HFD), ApoE^{-/-} (HFD) and Nox2^{-/-}/ApoE^{-/-} (HFD) mice. Additionally, concentration-dependent vasorelaxation responses to the sGC-independent vasodilator, papaverine were also examined in:

(i) 24-hour vehicle and Ang II-treated carotid arteries from C57BL/6J mice

(ii) freshly isolated carotid arteries from C57BL/6J (HFD) and ApoE^{-/-} (HFD) mice (see General Methods, section 2.6.2.4).

Statistical analysis

All values were expressed as mean \pm s.e. mean, where n = number of mice. $\cdot\text{O}_2^-$ generation was expressed as 10^3 counts per mg of dry tissue weight. Vasorelaxation responses to BAY 58-2667, ACh and papaverine were expressed as percentage reversal of the level of the U46619-induced pre-contraction. A student's unpaired t-test was used when comparing 2 treatment groups, whilst a one-way ANOVA followed by Bonferroni's multiple comparisons posthoc test was used when comparing 3 or more groups, only where an EC₅₀ for the concentration-response curves could be established. Finally, where a sigmoidal curve or an EC₅₀ could not be determined, a two-way ANOVA followed by a Tukey test was employed. Statistical significance was accepted at the level of $P < 0.05$.

BAY 58-2667 is a potent vasodilator in isolated carotid arteries of C57BL/6J mice

BAY 58-2667 was found to be a potent vasodilator of mouse carotid arteries, with an EC_{50} value of 9.78 ± 0.50 -log M and R_{max} value of $83 \pm 6\%$ reversal of the pre-contraction (Figure 1, $n = 5$). The potency of BAY 58-2667 was further enhanced by ~1800-fold in the presence of the sGC inhibitor, ODQ ($10 \mu\text{mol/L}$; $pEC_{50} = 13.04 \pm 0.56$ -log M, $R_{max} = 97 \pm 2\%$ reversal of pre-contraction, $n = 5$, $P < 0.01$), with no change to the maximum response, whilst a rightward shift was observed in the presence of the heme-free sGC antagonist, Zn-PPIX (data not shown).

Ang II (10 nmol/L, 24hr)-treatment augments NADPH-driven $\cdot\text{O}_2^-$ production and relaxation responses to BAY 58-2667 in isolated carotid arteries of C57BL/6J mice

Short-term Ang II (10 nmol/L , 24)-treatment of isolated carotid arteries from C57BL/6J mice caused over a 2-fold increase ($P < 0.05$) in $\cdot\text{O}_2^-$ production ($0.55 \pm 0.10 \times 10^3$ counts/mg, $n = 6$) compared to the vehicle (50 nmol/L glacial acetic acid, $0.26 \pm 0.05 \times 10^3$ counts/mg, $n = 6$, Figure 2A). Similarly, BAY 58-2667-mediated relaxant responses were significantly potentiated by ~16-fold ($P < 0.05$) in Ang II-treated ($pEC_{50} = 11.21 \pm 0.53$, $n = 9$) compared to the vehicle-treated ($pEC_{50} = 10.01 \pm 0.71$, $n = 9$) carotid arteries (Figure 2B). In Ang II-treated carotid arteries, ODQ ($10 \mu\text{mol/L}$) augmented the response to BAY 58-2667 by ~2000-fold ($pEC_{50} = 14.51 \pm 0.37$, $n = 5$, $P < 0.001$), such that 1 pmol/L BAY 58-2667 achieved a near maximum relaxation response of $90 \pm 4\%$ whilst only $15 \pm 3\%$ relaxation was achieved at the same concentration in Ang II-treated carotids alone. Conversely, the vasorelaxant response to BAY 58-2667 in Ang II-treated carotids was significantly right-shifted ($P < 0.001$) in the presence of Zn-PPIX ($10 \mu\text{mol/L}$, Figure 2B). Relaxant responses to papaverine in carotid arteries ($pEC_{50} = 6.42 \pm 0.19$, $R_{max} = 95 \pm 1\%$, $n = 5$) were unchanged following 24-hour treatment with Ang II ($pEC_{50} = 6.44 \pm 0.20$, $R_{max} = 95 \pm 1\%$, $n = 5$, Figure 2C).

Nox2 gene deletion reduces Ang II-mediated $\cdot\text{O}_2^-$ production and relaxant response to BAY 58-2667 in isolated carotid arteries from Nox2^{-/-} mice

Unlike the findings in C57BL/6J mice, Ang II did not increase $\cdot\text{O}_2^-$ production in Nox2^{-/-} mice. Rather, it unexpectedly led to a significant decrease in $\cdot\text{O}_2^-$ levels ($0.35 \pm 0.09 \times 10^3$ counts/mg, $n = 6$, $P < 0.01$) compared to the vehicle ($0.67 \pm 0.10 \times 10^3$ counts/mg, $n = 6$, Figure 3A). Similarly, in Nox2^{-/-} mice, Ang II-treatment had no effect on the BAY 58-2667-mediated vasorelaxant response (Figure 3B).

The Western-style high fat diet elevates plasma levels of total cholesterol, triglycerides and low-density lipoprotein (LDL)

Plasma levels of total cholesterol (~3-fold), triglycerides (~1.3-fold) and LDL cholesterol (~8-fold) tended to be higher in C57BL/6J mice on HFD compared to those fed a normal chow diet, however, these changes failed to reach statistical significance (Table 1). In ApoE^{-/-} mice on a HFD, total cholesterol ($P < 0.001$), triglycerides ($P < 0.05$) and LDL cholesterol ($P < 0.001$) levels were significantly elevated, while HDL cholesterol ($P < 0.001$) levels were lower compared to C57BL/6J mice on a HFD (Table 1). Surprisingly, both total cholesterol ($P < 0.01$) and LDL cholesterol ($P < 0.001$) levels were further augmented by approximately 1.4-fold in Nox2^{-/-}/ApoE^{-/-} compared to ApoE^{-/-} mice on HFD, whilst HDL and triglyceride levels remained unchanged.

The Western-style high fat diet potentiates the vasorelaxant response to BAY 58-2667 independently of NADPH oxidase-derived $\cdot\text{O}_2^-$ production

Vascular $\cdot\text{O}_2^-$ levels were no different in isolated carotid arteries from C57BL/6J mice fed a normal diet (C57BL/6J ND: $1.18 \pm 0.27 \times 10^3$ counts/mg, $n = 5$) compared to those fed a high fat diet (C57BL/6J HFD: $0.97 \pm 0.25 \times 10^3$ counts/mg, $n = 4$, Figure 4A). Although the high fat diet did not lead to an increase in $\cdot\text{O}_2^-$ generation, the vasorelaxant potency of BAY 58-2667 was

significantly enhanced in carotid arteries from these animals (C57BL/6J ND: $pEC_{50} = 10.26 \pm 0.35$ vs C57BL/6J HFD: $pEC_{50} = 12.09 \pm 0.37$, $n = 5-6$, $P < 0.01$, Figure 4B).

$\cdot O_2^-$ production was significantly ($P < 0.01$) elevated in carotid arteries from ApoE^{-/-} mice on a HFD ($2.76 \pm 0.24 \times 10^3$ counts/mg, $n = 5$) as compared to C57BL/6J (HFD) mice, yet relaxant responses to BAY 58-2667 were similar between the strains. In ApoE^{-/-} mice, ODQ (10 μ mol/L) caused ~54-fold leftward shift in the potency to BAY 58-2667 (ApoE^{-/-} HFD: $pEC_{50} = 12.40 \pm 0.24$, vs ApoE^{-/-} HFD + ODQ: $pEC_{50} = 14.13 \pm 0.65$, $n = 5-6$, $P < 0.05$, Figure 4B).

The increase in $\cdot O_2^-$ generation observed in the ApoE^{-/-} (HFD) was absent in the Nox2^{-/-}/ApoE^{-/-} on a HFD ($1.38 \pm 0.35 \times 10^3$ counts/mg, $n = 5$, $P < 0.05$, Figure 4A) such that $\cdot O_2^-$ levels in isolated arteries from these mice were comparable with that observed in C57BL/6J on both the normal or HFD. The vasodilator ability of BAY 58-2667 in the Nox-2^{-/-}/ApoE^{-/-} tended to be right shifted ($pEC_{50} = 11.68 \pm 0.30$, $n = 5$) as compared to ApoE^{-/-} (HFD), however, this did not reach statistical significance (Figure 4B).

Endothelial dysfunction in the ApoE^{-/-} ($n = 6$, $P < 0.01$) was confirmed by the impaired relaxation to ACh (10 μ mol/L) achieving only $55 \pm 10\%$ relaxation compared to over 90% observed in the C57BL/6J on both the normal and HFD (Figure 4C). Again, this impairment in ACh response in the ApoE^{-/-} was absent in Nox2^{-/-}/ApoE^{-/-} such that $87 \pm 1\%$ relaxation was achieved ($n = 6$, $P < 0.01$, Figure 4C).

Vasorelaxation to papaverine in isolated carotid arteries did not differ C57BL/6J ($pEC_{50} = 6.13 \pm 0.16$, $R_{max} = 88 \pm 3\%$, $n = 5$) or ApoE^{-/-} ($pEC_{50} = 6.30 \pm 0.17$, $R_{max} = 92 \pm 2\%$, $n = 4$, Figure 4D) mice on a HFD.

Table 1. Plasma lipid profiles of C57BL/6J mice on a normal (ND) and high fat diet (HFD), as well as ApoE^{-/-} and Nox2^{-/-}/ApoE^{-/-} mice on a HFD (7 weeks).

	<i>C57BL/6J ND</i> (mmol/L) n = 4	<i>C57BL/6J HFD</i> (mmol/L) n = 7	<i>ApoE^{-/-} HFD</i> (mmol/L) n = 6	<i>Nox2^{-/-}/ApoE^{-/-} HFD</i> (mmol/L) n = 5
<i>Triglycerides</i>	1.20 ± 0.04	1.57 ± 0.33	2.73 ± 0.40*	2.84 ± 0.28
<i>Total cholesterol</i>	1.88 ± 0.23	5.69 ± 0.36	30.67 ± 2.73***	42.04 ± 2.45###
<i>HDL</i>	1.05 ± 0.14	2.77 ± 0.14 ^{γγγ}	0.33 ± 0.06***	0.37 ± 0.04
<i>LDL</i>	0.28 ± 0.11	2.20 ± 0.21	29.08 ± 2.59***	40.37 ± 2.37###
<i>LDL/HDL ratio</i>	0.26 ± 0.09	0.79 ± 0.06	119.90 ± 41.95	115.6 ± 13.13

^{γγγ}*P*<0.001 for levels versus C57BL/6J mice on ND

P*<0.05, **P*<0.001 for levels versus C57BL/6J mice on HFD

###*P*<0.01, ###*P*<0.001 for levels versus ApoE^{-/-} mice on HFD

n = number of mice

Figure 1

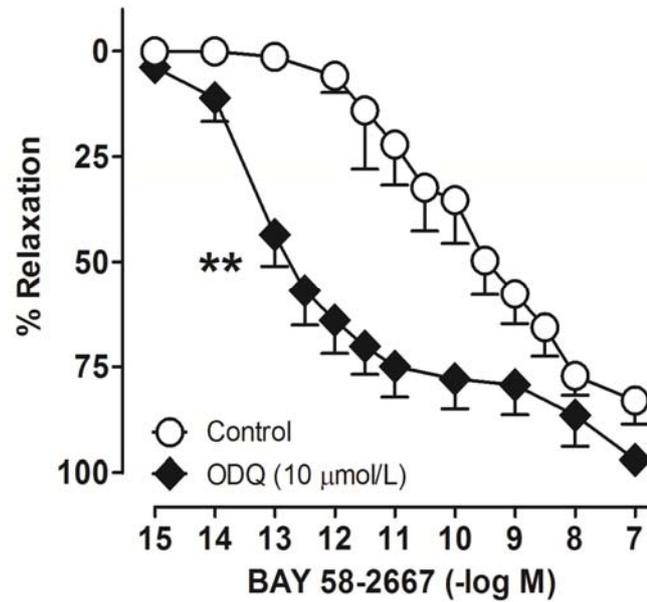


Figure 1. Concentration-dependent vasorelaxation responses to the sGC activator, BAY 58-2667 (1 fmol/L – 1 $\mu\text{mol/L}$, \circ , $n = 5$) in endothelium-intact carotid arteries from C57BL/6J mice in the absence and presence of the sGC inhibitor, ODQ (10 $\mu\text{mol/L}$, \blacklozenge , $n = 5$). Responses are expressed as percentage reversal of pre-contraction and given as mean \pm s.e.mean, where $n =$ number of mice. $^{**}P < 0.01$ for $p\text{EC}_{50}$ values versus control (Student's unpaired t-test).

Figure 2. The effects of 24hr treatment with either vehicle (50 nmol/L glacial acetic acid, open bars, ○) or Ang II (10 nmol/L, closed bars, ●) on (A) NADPH (100 μmol/L)-driven $\cdot\text{O}_2^-$ production (n = 6) and concentration-dependent vasorelaxation responses to (B) BAY 58-2667 (1 fmol/L - 1 μmol/L, n = 9) and (C) papaverine (10 nmol/L - 30 μmol/L, n = 5) in endothelium-intact carotid arteries from C57BL/6J mice. Vasorelaxant responses to BAY 58-2667 in Ang II-treated carotid arteries from C57BL/6J mice were also performed in the presence of ODQ (10 μmol/L, ◆, n = 5) or Zn-PPIX (10 μmol/L, ▲, n = 9). $\cdot\text{O}_2^-$ production is expressed as 10^3 counts per mg of dry tissue weight while responses to BAY 58-2667 and papaverine are expressed as percentage reversal of pre-contraction. All values are given as mean \pm s.e.mean, where n = number of mice. * $P < 0.05$ versus vehicle (Student's unpaired t-test); # $P < 0.05$ for concentration-response curve versus vehicle; $\Psi\Psi\Psi P < 0.001$ for concentration-response curve versus Ang II (Two-way ANOVA, Tukey test).

Figure 2

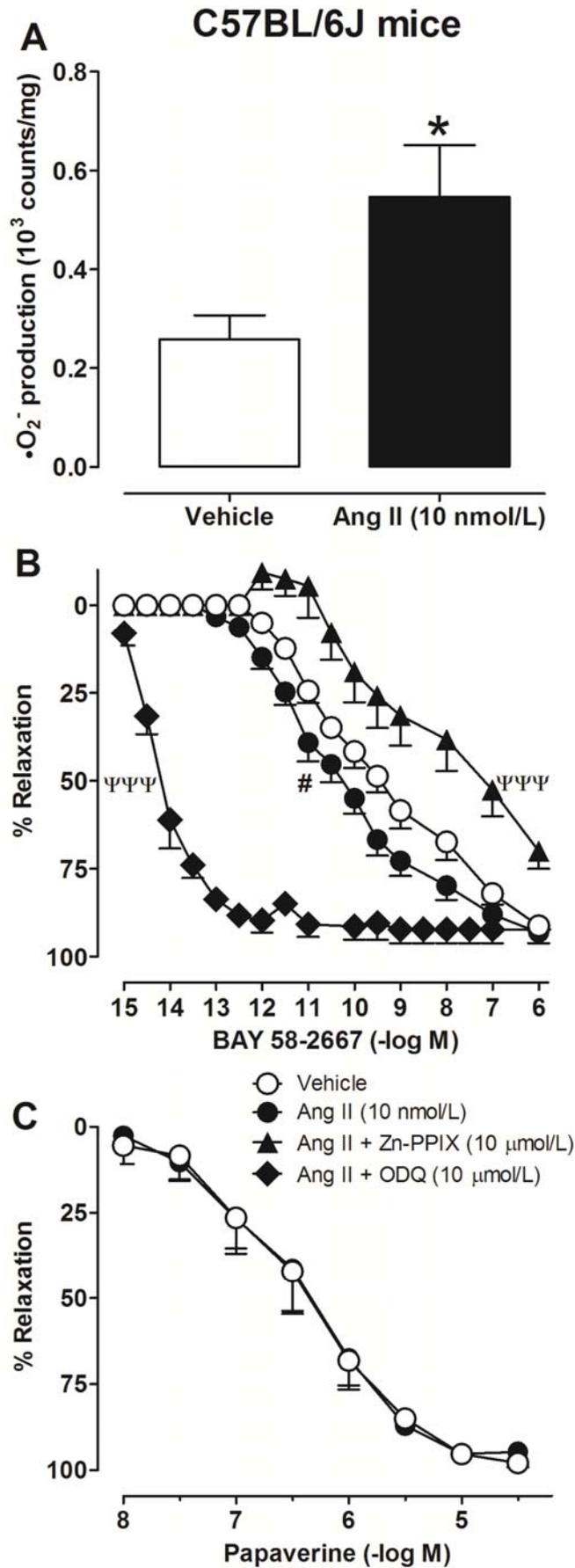


Figure 3. The effects of 24hr treatment with either vehicle (50 nmol/L glacial acetic acid, open bars, ○) or Ang II (10 nmol/L, closed bars, ●) on **(A)** NADPH (100 μmol/L)-driven $\cdot\text{O}_2^-$ production (n = 5) and **(B)** concentration-dependent vasorelaxation responses to BAY 58-2667 (0.1 pmol/L – 1 μmol/L, n = 6) in endothelium-intact carotid arteries from Nox-2^{-/-} mice. $\cdot\text{O}_2^-$ production is expressed as 10^3 counts per mg of dry tissue weight while responses to BAY 58-2667 are expressed as percentage reversal of pre-contraction. All values are given as mean ± s.e.mean, where n = number of mice. ** $P < 0.01$ versus vehicle (Student's unpaired t-test).

Figure 3

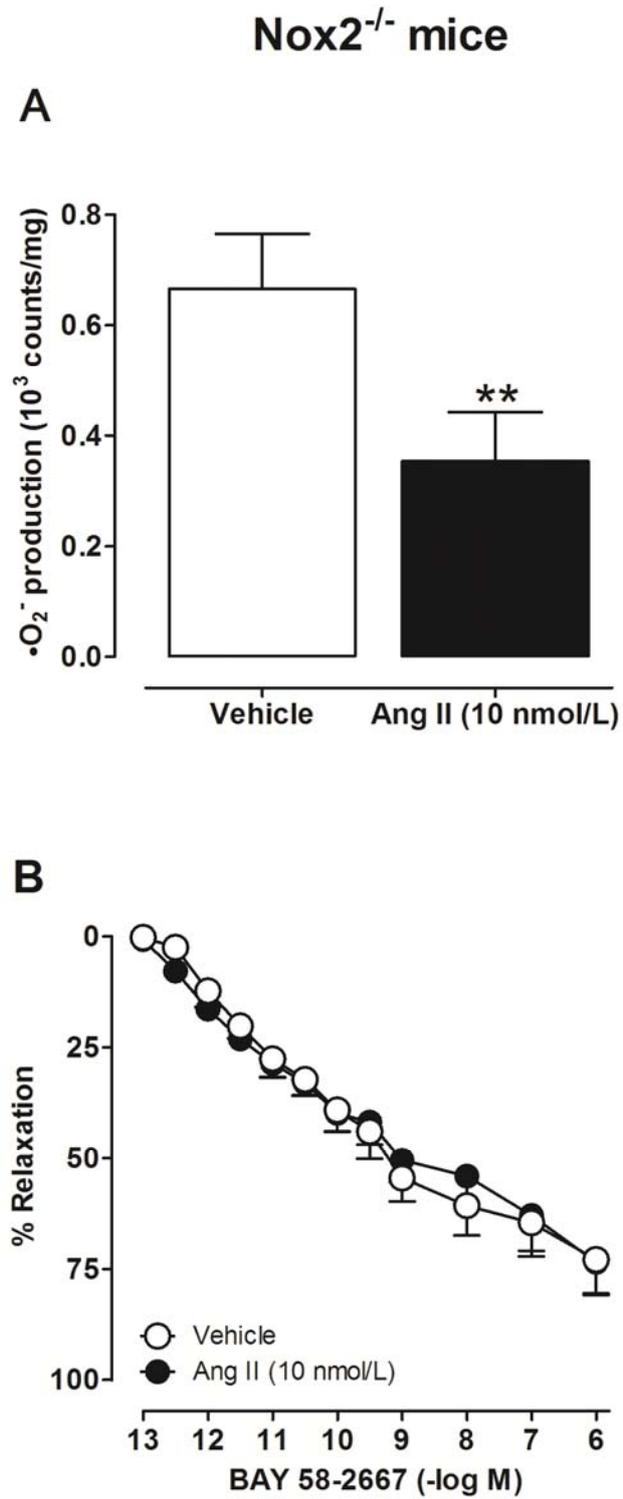
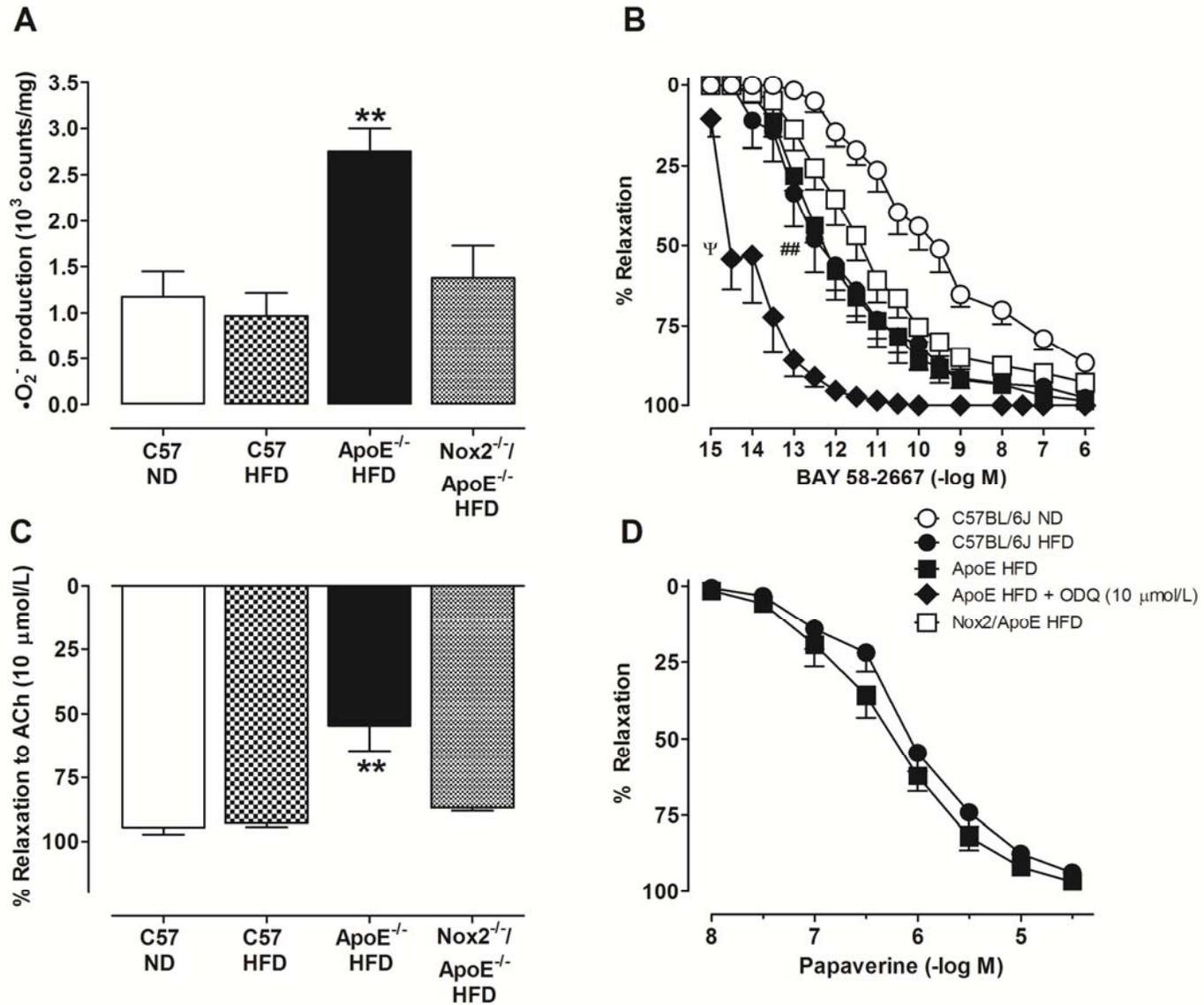


Figure 4. (A) NADPH (100 $\mu\text{mol/L}$)-driven $\cdot\text{O}_2^-$ production and relaxation responses to (B) BAY 58-2667 (1 fmol/L – 1 $\mu\text{mol/L}$), (C) ACh (10 $\mu\text{mol/L}$) and (D) papaverine (10 nmol/L – 30 $\mu\text{mol/L}$) in endothelium-intact carotid arteries from C57BL/6J mice on a normal diet (C57 ND, open bars, \circ , n = 5-6) and high fat diet (C57 HFD, chequered bars, \bullet , n = 4-5), and ApoE^{-/-} (HFD, closed bars, \blacksquare , n = 5-6) and Nox2^{-/-}/ApoE^{-/-} (HFD, hashed bars, \square , n = 5) mice on a high fat diet (7 weeks). Vasorelaxant responses to BAY 58-2667 in ApoE^{-/-} HFD was also performed in the presence of ODQ (\blacklozenge , n = 5). $\cdot\text{O}_2^-$ production is expressed as 10^3 counts per mg of dry tissue weight while relaxation responses are expressed as percentage reversal of pre-contraction. All values are given as mean \pm s.e.mean, where n = number of mice. ** $P < 0.01$ for $\cdot\text{O}_2^-$ production and relaxation to 10 $\mu\text{mol/L}$ ACh versus C57BL/6J ND and HFD and Nox2^{-/-}/ApoE^{-/-} HFD; ## $P < 0.01$ for pEC₅₀ values versus C57BL/6J ND; $^\Psi P < 0.05$ for pEC₅₀ values versus ApoE^{-/-} HFD (One-way ANOVA, Bonferroni's multiple comparisons post test).

Figure 4



The key findings of the current study revealed that NADPH oxidase modulates the vasorelaxant response to the novel sGC activator, BAY 58-2667 in mouse carotid arteries such that an increase in NADPH-driven $\cdot\text{O}_2^-$ production is concomitant with an enhanced potency to BAY 58-2667. This NADPH-driven effect was shown to be Nox2-dependent as deletion of the gene suppressed $\cdot\text{O}_2^-$ production and restored endothelial function in models of short-term oxidative stress (Ang II-treatment in Nox2^{-/-} mice) and chronic disease (Nox2^{-/-}/ApoE^{-/-}). Indeed, a reduction in Ang II (10 nmol/L)-mediated $\cdot\text{O}_2^-$ generation in the Nox2^{-/-} mice saw a loss in the augmented vasorelaxant potency to BAY 58-2667. These findings suggest that Nox2-containing NADPH oxidase may promote the formation of oxidised (Fe³⁺)/heme-free sGC in the vasculature. Furthermore, this study also revealed that the Western-style high fat diet was able to modulate vasorelaxant responses to BAY 58-2667 such that mice fed this diet (C57BL/6J, ApoE^{-/-} and Nox2^{-/-}/ApoE^{-/-}) exhibited a similar enhanced potency to BAY 58-2667, irrespective of strain or $\cdot\text{O}_2^-$ levels detected, compared to those fed a normal diet (C57BL/6J). Thus, using BAY 58-2667 as a marker for oxidised/heme-free sGC, this study shows that NADPH oxidase and the Western-style high fat diet may independently facilitate the formation of oxidised/heme-free sGC.

In accordance with previous studies (Stasch *et al.*, 2006), BAY 58-2667 was found to be a potent vasodilator, relaxing mouse isolated carotid arteries, under physiological conditions, with an EC₅₀ value in the nanomolar range. In the presence of zinc-protoporphyrin IX (PPIX), a full antagonist at the heme-binding pocket of sGC, both the potency and maximum response to BAY 58-2667 were significantly impaired. Similarly, Stasch and co-workers have previously shown that Zn-PPIX abolished both BAY 58-2667-induced activation of heme-free purified rat sGC and the increase in cellular cGMP in endothelial cells (Stasch *et al.*, 2006). Conversely, vasorelaxant responses to BAY 58-2667 were significantly augmented in the presence of the sGC inhibitor and heme-oxidant, ODQ, which facilitates the oxidation of the sGC heme from its reduced (Fe²⁺)

to its NO-insensitive ferric (Fe^{3+}) state, subsequently promoting the eventual loss of the prosthetic heme group from sGC (Evgenov *et al.*, 2006; Garthwaite *et al.*, 1995; Schrammel *et al.*, 1996). These findings concur with those in rat isolated aortae where ODQ enhances both the vasorelaxant response to BAY 58-2667 and its ability to increase cGMP levels (Stasch *et al.*, 2006). Thus, the results from the current study supports the existence of oxidised/heme-free sGC in the vasculature of mice under physiological conditions.

Given that vasorelaxant responses to BAY 58-2667 are further augmented in disease states associated with oxidative stress (Stasch *et al.*, 2006), we next sought to determine if an elevation in reactive oxygen species (ROS) is directly responsible for an increased pool of oxidised/heme-free sGC. A short-term model of oxidative stress was initially employed, whereby, carotid arteries were treated for 24 hours with Ang II (10 nmol/L), a potent stimulator of Nox2-containing NADPH oxidase. This model has previously been shown to be associated with an elevation in $\cdot\text{O}_2^-$ production coinciding with endothelial dysfunction, as evidenced by the impaired relaxation to the endothelium-dependent vasodilator, ACh (Chrissobolis *et al.*, 2008; Didion *et al.*, 2005). Such an effect may arise due to the direct scavenging of NO by $\cdot\text{O}_2^-$ forming the strong oxidant peroxynitrite (ONOO^-) (Hoffmann *et al.*, 2009). Indeed, ONOO^- can facilitate the oxidation of reduced (Fe^{2+}) heme of sGC into an NO-insensitive, oxidised/heme-free state, with the ONOO^- donor, SIN-1 enhancing the sensitivity to BAY 58-2667, whilst leading to attenuated vasorelaxant responses to ACh and the NO donor DEA/NO, both of which activate reduced sGC (Stasch *et al.*, 2006). In agreement with previous studies (Chrissobolis *et al.*, 2008; Didion *et al.*, 2005), 24-hour Ang II (10 nmol/L)-treatment elevated $\cdot\text{O}_2^-$ production by approximately 2-fold in mouse carotid arteries. This increase in NADPH-driven $\cdot\text{O}_2^-$ production was associated with a 16-fold increase in the vasorelaxant potency to BAY 58-2667. Furthermore, the ability of NADPH oxidase to modulate vasodilator responses appears to be specific for BAY 58-2667 as an increase in Ang II-mediated $\cdot\text{O}_2^-$ production did not affect

responses to the phosphodiesterase (PDE) inhibitor, papaverine. Together, these findings suggest that NADPH oxidases may play a role in enhancing pools of oxidised/heme-free sGC in the vasculature. Indeed, quantitative measurement of 3-nitrotyrosine (3-NT) levels, the biomarker for nitrating oxidants, in both vehicle and Ang II-treated vessels should be implemented in future studies in order to gain some information on the level of ONOO⁻ produced. Remarkably, ODO augmented the response to BAY 58-2667 in Ang II-treated vessels, increasing the potency to the sGC activator an additional 200-fold, indicating that the pools of sGC could be further manipulated to enhance the response to BAY 58-2667. Similar to that observed in untreated carotid arteries of C57BL/6J mice, the presence of Zn-PPIX markedly reduced the sensitivity of BAY 58-2667 in Ang II-treated rings. Thus, the results indicate that an increase in NADPH oxidase activity can augment the vasodilator response to BAY 58-2667 and that this modulation is likely to occur at the level of sGC, possibly via the regulation of pools of oxidised/heme-free sGC.

Schrader and co-workers have previously demonstrated that the increase in NADPH-driven $\cdot\text{O}_2^-$ generation in Ang II-treated vessels is Nox2 dependent as this elevation in $\cdot\text{O}_2^-$ levels was absent in the carotid arteries of Nox2^{-/-} mice (Schrader *et al.*, 2007). Similarly, in Nox2^{-/-} carotid arteries, Ang II did not elevate $\cdot\text{O}_2^-$ levels, rather surprisingly, it appeared to lower $\cdot\text{O}_2^-$ production in the Nox2^{-/-} mice. Despite the reasons behind such a drop in Ang II-mediated $\cdot\text{O}_2^-$ production compared to vehicle-treated vessels from Nox2^{-/-} mice remaining unclear, we have nevertheless demonstrated that Ang II-mediated NADPH oxidase-driven $\cdot\text{O}_2^-$ production is Nox2 dependent as a loss of the gene does not increase $\cdot\text{O}_2^-$ generation to levels above the vehicle-treated response. Furthermore, the 16-fold augmented relaxant response to BAY 58-2667 achieved following Ang II-treatment (10 nmol/L, 24hr) in C57BL/6J mice was lost upon deletion of the Nox2 gene, such that responses to BAY 58-2667 in the Nox2^{-/-} were similar to that exhibited in the vehicle-treated arteries from C57BL/6J mice. These findings imply a direct role of Nox2-

containing NADPH oxidase in modulating vasorelaxant responses to BAY 58-2667 and thereby pools of oxidised/heme-free sGC, at least under conditions of short-term ROS elevation.

The present study also highlighted the important contribution of Nox2-containing NADPH oxidase in elevating ROS levels and vascular dysfunction in cardiovascular disease. Specifically, in ApoE^{-/-} mice on a high fat diet, NADPH-stimulated $\cdot\text{O}_2^-$ production was increased while endothelium-dependent relaxation to ACh was attenuated. Such changes, however, were absent in Nox2^{-/-}/ApoE^{-/-} mice, implicating a causal role of Nox2-containing NADPH oxidase in vascular dysfunction associated with atherosclerosis. Our results are in agreement with Judkins and co-workers who demonstrated lower $\cdot\text{O}_2^-$ production and enhanced NO bioavailability in Nox2^{-/-}/ApoE^{-/-} as compared with ApoE^{-/-} mice (Judkins *et al.*, 2009).

Given our finding that short-term stimulation of Nox2-containing NADPH oxidase with Ang II lead to the augmented response to BAY 58-2667, it was somewhat surprising that the vasorelaxant response to BAY 58-2667 did not differ between Nox2^{-/-}/ApoE^{-/-} and ApoE^{-/-} mice, both of which received the same high fat diet for 7 weeks, despite the lowering of $\cdot\text{O}_2^-$ generation following Nox2 deletion. Such observations indicate that under chronic disease conditions, an elevation in $\cdot\text{O}_2^-$ alone does not account for an augmentation in pools of oxidised/heme-free sGC. In further support of this concept was the observation that the response to BAY 58-2667 was unchanged between C57BL/6J and ApoE^{-/-} mice, both on a high fat diet, regardless of the higher levels of $\cdot\text{O}_2^-$ and the impairment in endothelial function exhibited by the ApoE^{-/-} mice. Interestingly, no differences were observed in $\cdot\text{O}_2^-$ production between C57BL/6J mice on normal chow diet compared to those on a high fat diet, however the vasodilator response to BAY 58-2667 was ~67-fold more potent in the arteries from mice fed the high fat diet. Similarly, Stasch and co-workers have previously reported that the vasorelaxant effects of BAY 58-2667 were markedly enhanced in the aortae from ApoE^{-/-} mice fed on a high fat diet compared to

ApoE^{-/-} on the standard chow, highlighting the ability of the diet itself to potentially regulate pools of oxidised/heme-free sGC and subsequently the response to BAY 58-2667. Again, the relaxant response to papaverine was unaffected by diet indicating that the diet-induced influence was likely to be exclusive to the level of sGC. Similar to that observed in the Ang II-treated arteries, the presence of ODQ in the arteries of ApoE^{-/-} mice enhanced the vasorelaxation response to BAY 58-2667 by ~54-fold, indicating the extent by which these pools of sGC could be further manipulated. Collectively, the results indicated that the Western-style high fat diet itself may induce sGC oxidation, independent of NADPH oxidase activity and the subsequent $\cdot\text{O}_2^-$ production.

It has previously been shown that total cholesterol, low-density lipoprotein (LDL) plasma levels and body weight are higher in C57BL/6J mice fed a high fat diet compared to those fed a normal chow diet, yet the aortae of these mice do not display any histological evidence of atherosclerosis (Zhao *et al.*, 2008). Indeed, high serum levels of LDL is one of the leading risk factors in the development of atherosclerosis, with the oxidative modification of LDL (oxLDL) being implicated as a major pathological factor in the early development of atherosclerotic lesions (Meyer & Schmitt, 2000). Oxidised LDLs are rapidly taken up by monocytes/macrophages by scavenger receptors via a process which converts them into foam cells, which are essential components of fatty streaks and fibrofatty plaques (Holvoet *et al.*, 1998). Indeed, although not statistically significant, plasma levels of LDL cholesterol were higher in C57BL/6J mice fed a high fat diet compared to those on standard chow diet, correlating with an increase in BAY 58-2667-induced relaxation. Furthermore, while we observed that vasorelaxant response to BAY 58-2667 were equipotent in arteries from C57BL/6J, ApoE^{-/-} and Nox2^{-/-}/ApoE^{-/-} mice, all fed the high fat diet, interestingly, LDL cholesterol levels were shown to be significantly elevated in ApoE^{-/-} and even further in Nox2^{-/-}/ApoE^{-/-} mice. These results suggest that perhaps a threshold level of LDL cholesterol is required to trigger a change in the

pools of oxidised/heme-free sGC, such that once this level is achieved, a further augmentation in LDL levels has no additional influences on these pools of altered sGC. This concept, however, remains to be tested. Currently, the reason for the substantial increase in LDL cholesterol levels in Nox2^{-/-}/ApoE^{-/-} mice cannot be explained, however, factors such as animal weight may contribute to this observation. Indeed, not documenting the weights of the mice used was a limitation of the current study.

While, the mechanisms by which the Western-style high fat diet may be regulating pools of oxidised/heme-free sGC and thereby modulating vasorelaxant responses to BAY 58-2667 are currently unknown, there may be a few possible explanations. Firstly, in addition to its role in the initiation and development of atherosclerosis, hyperlipidemia has also been reported to induce the expression of the antioxidant stress protein, heme oxygenase-1 (HO-1) in aortic smooth muscle cells (Anwar *et al.*, 2005). HO-1, induced in vascular cells, counteracts the noxious effects of oxidants in the vessel wall (Anwar *et al.*, 2005; Siow *et al.*, 1999) as well as functions as a key regulator of cellular heme levels (Mingone *et al.*, 2008). Thus, while HO-1 constitutes a protective response in various experimental models (Anwar *et al.*, 2005), an increase in HO-1 activity is also associated with a depletion of heme (Abraham *et al.*, 2002; Mingone *et al.*, 2008). Indeed, prolonged exposure of cultured rat pulmonary endothelial cells to increased levels of HO-1 has been shown to reduce sGC activity by limiting the availability of cellular heme (Abraham *et al.*, 2002). Furthermore, a study in organ cultured bovine pulmonary arteries have reported that the induction of HO-1 activity attenuated vasorelaxant responses to the NO donor, spermine NONOate, independent of an increase in $\cdot\text{O}_2^-$ production (Mingone *et al.*, 2008). Thus, perhaps in the current study, a hyperlipidemia-induced increase in HO-1 activity promotes heme degradation and/or impaired incorporation of heme into sGC, thereby influencing the pools of oxidised/heme-free sGC and subsequently modulating vasorelaxant responses to BAY 58-2667 in mouse isolated carotid arteries. However, a recent study by Jones and co-workers contradicts

this concept, revealing that the potency of the heme-free sGC activator, BAY 60-2270 was greater in the superior mesenteric arteries of HO-1 deficient (HO-1^{-/-}) mice (Jones *et al.*, 2010). The authors proceeded to suggest that the antioxidant properties of HO-1 may be important in preserving the heme of sGC in its reduced state (Jones *et al.*, 2010). Additionally, other studies have also suggested that HO-1 and heme degradation products may improve vascular function, at least in part, by preserving vascular NO (Pae *et al.*, 2010). In any case, investigating the activity and expression of HO-1 in the arteries of these mice and its correlation with hyperlipidemia in the current study may be of interest for future studies.

Furthermore, given we have shown in Chapter 4 that a loss of endogenous NO results in the augmented response to BAY 58-2667, another possible explanation may be that hyperlipidemia limits endogenous NO synthesis and/or production, thereby resulting in the accumulation of oxidised/heme-free sGC. In fact, hyperlipidemia has been demonstrated to impair NO-dependent relaxation through a variety of mechanisms including a decrease in eNOS expression, the uncoupling of G₁ protein-dependent signal transduction or via the reduction in eNOS substrate (tetrahydrobiopterin, BH₄) availability (Feron *et al.*, 1999; Harrison, 1997). Additionally, Michel and co-workers have shown that the caveola protein, caveolin directly binds to eNOS and regulates the generation of NO (Michel *et al.*, 1997). Caveolae are known signal transducing molecules enriched in cholesterol and the microdomains to which eNOS binds, and are found in most cells. Indeed, hyperlipidemia has been shown to inhibit eNOS activity by depleting caveolae of cholesterol, causing eNOS to translocate, thereby disrupting the association between eNOS and caveolae (Blair *et al.*, 1999). However, it should be noted that vasorelaxant responses to ACh in the current study were preserved in C57BL/6J and Nox2^{-/-}/ApoE^{-/-} mice on a high fat diet, despite cholesterol levels being elevated in these mice, suggesting that perhaps under our experimental settings, only basal NO release may be reduced. Alternatively, in Chapter 4, we discussed the possibility of the existence of an endogenous reductase (NADPH-dependent

methemoprotein reductase), which may aid in preserving the heme of sGC in its reduced NO-sensitive state (Gupte *et al.*, 1999; Wolin, 2009). While we have proposed that endogenous NO may stimulate the activity of this endogenous reductase, thereby limiting the accumulation of oxidised/heme-free sGC, it may be possible that hyperlipidemia attenuates the activity of this reductase, whether directly or indirectly, via the inhibition of endogenous NO. However, the exact mechanisms and extent via which diet is influencing these redox pools of sGC, remains unclear and further studies are warranted.

This study, however, supports the existence of oxidised/heme-free sGC in blood vessels under both physiological and pathophysiological conditions. As NADPH oxidases represent the major source of vascular ROS, it seems likely that they may play a role in regulating the pools of oxidised/heme-sGC. Indeed we have provided evidence for this concept using a short-term model of vascular oxidative stress where an increase in Nox2-containing NADPH oxidase activity is associated with an augmented vasodilator response to BAY 58-2667. Interestingly, however, our results have also revealed that the Western-style high fat diet, crucial for the development of atherosclerosis, was also able to influence the vasorelaxant response to BAY 58-2667, independent of NADPH oxidase activity and the subsequent generation of $\cdot\text{O}_2^-$. The exact mechanisms by which the diet was able to induce such changes, however are yet to be elucidated. Taken together, our results suggest that Nox2-containing NADPH oxidases and the Western-style high fat diet may independently play an important role in modulating the relaxation responses to BAY 58-2667, possibly by regulating the vascular pools of oxidised/heme-free sGC.

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CHAPTER 8

General Discussion



8. GENERAL DISCUSSION

It is well recognised that cardiovascular disorders such as angina, heart failure and arterial and pulmonary hypertension stem from a dysfunction in the NO/sGC/cGMP signaling pathway. To date, the limitations associated with the current clinical use of nitrovasodilators, most notably the multifactorial phenomenon of tolerance development (Evgenov *et al.*, 2006; Parker & Gori, 2001), has resulted in the increased interest in the use of NO-independent sGC ligands as alternate and potentially more beneficial therapeutic avenues in the treatment of cardiovascular diseases. Such non-NO based ligands include the one electron reduced protonated congener of NO, nitroxyl (HNO) as well as the direct stimulators and activators of sGC, BAY 41-2272 and BAY 58-2667, respectively, which display unique biochemical and pharmacological properties and may therefore overcome the obstacles posed by traditional nitrovasodilator therapy. Thus, the aim of this thesis was to characterise the vasoprotective actions of these novel NO mimetics.

8.1 HNO donors suppresses vascular $\cdot\text{O}_2^-$ generation

The redox siblings HNO and $\text{NO}\cdot$, exhibit quite distinct biological and pharmacological properties (Irvine *et al.*, 2008; Kemp-Harper, 2011), and as such HNO donors have emerged as promising therapeutic agents in the treatment of cardiovascular diseases. Indeed, in contrast to $\text{NO}\cdot$, HNO interacts readily with thiols (Donzelli *et al.*, 2006), exhibits positive cardiac inotropic activity (Paolucci *et al.*, 2003; Paolucci *et al.*, 2001), increases plasma levels of calcitonin gene-related peptide (CGRP) (Paolucci *et al.*, 2003), is resistant to $\cdot\text{O}_2^-$ scavenging (Miranda *et al.*, 2002), targets vascular K_v channels (Irvine *et al.*, 2003), and importantly does not induce tolerance, even remaining equipotent in animals made tolerant to GTN (Irvine *et al.*, 2007; Irvine *et al.*, 2011). Furthermore, evidence to date has highlighted the antioxidant properties of HNO donors, including the ability to decrease lipid peroxidation in the yeast *Saccharomyces cerevisiae* model system (Lopez *et al.*, 2007), increasing heme oxygenase (HO-1) activity in rat H9c2 cells (Naughton *et al.*, 2002) and more recently reducing angiotensin II (Ang II) (Ritchie *et al.*, 2007)

and endothelin-1 (ET-1) (Ritchie *et al.*, 2009) induced NADPH-driven $\cdot\text{O}_2^-$ production in neonatal rat cardiomyocytes. Thus, we hypothesised that HNO may also limit oxidative stress in the vasculature. Whilst $\text{NO}\cdot$ has also been reported to reduce NADPH oxidase-dependent $\cdot\text{O}_2^-$ production in human cultured endothelial cells (Selemidis *et al.*, 2007), we propose that HNO may be more advantageous due to its resistance to $\cdot\text{O}_2^-$ scavenging (Miranda *et al.*, 2002) and lack of tolerance development (Irvine *et al.*, 2007; Irvine *et al.*, 2011).

Subsequently, this thesis (Chapter 3) demonstrated that HNO donors (Angeli's salt and IPA/NO) suppressed Ang II-mediated $\cdot\text{O}_2^-$ generation in the vasculature of mice. Given that HNO can exert sGC-dependent and -independent actions (Irvine *et al.*, 2008), it was important to assess the contribution of sGC/cGMP signaling to the actions of HNO under our experimental setting. Indeed, to the best of our knowledge, Chapter 3 demonstrates for the first time that HNO suppresses $\cdot\text{O}_2^-$ production in the vasculature via a cGMP-independent mechanism of action. The exact mechanism(s) via which HNO elicits these effects are still a matter of contention, however, there are a few plausible explanations.

As mentioned previously, $\text{NO}\cdot$ has also been shown to limit $\cdot\text{O}_2^-$ levels in human endothelial cells via *S*-nitrosylation of the cytosolic organiser subunit of NADPH oxidase, p47^{phox} (Selemidis *et al.*, 2007). However, in addition to its resistance to scavenging by $\cdot\text{O}_2^-$ and tolerance development (Irvine *et al.*, 2008), studies in our laboratory (Miller *et al.*, unpublished observations) have also shown that HNO causes an immediate and rapid (within minutes) decrease in $\cdot\text{O}_2^-$ production, whilst $\text{NO}\cdot$ requires a longer duration (up to 6 hours, Selemidis *et al.*, 2007), further adding to the advantage of using HNO versus $\text{NO}\cdot$. Furthermore, evidence in leukocytes has demonstrated that mutation of cysteine residues (Cys111 and Cys378) on p47^{phox} was able to suppress NADPH oxidase activity (Babior, 2002; Inanami *et al.*, 1998). Given that HNO is highly thiophilic, we proposed that it may be suppressing the activity of NADPH

oxidase in the vasculature by interacting directly with cysteine residues on p47^{phox}, thereby interfering with either the phosphorylation, translocation and/or binding of p47^{phox} to the Nox2/p22^{phox} complex of NADPH oxidase. Indeed, immunoprecipitation experiments may be employed to determine if HNO interferes with the association of p47^{phox} with either p22^{phox} and/or Nox2. Furthermore, future studies using p47^{phox}^{-/-} mice to examine $\cdot\text{O}_2^-$ generation and the ability of HNO to suppress it in these mice may also be required to test this hypothesis. Additionally, HNO interacts directly with thiols generating a disulfide or sulfinamide via a reversible and irreversible process, respectively (Fukuto *et al.*, 2009). Given the generation of sulfinamide is specific for HNO, mass-spectrometry techniques (Hoffman *et al.*, 2009) could also be employed to detect HNO-mediated modification of cysteine residues on NADPH oxidase.

Given that HNO readily reacts with metalloproteins, a further possibility is that HNO may inhibit Nox2 activity via a direct reaction with the heme of this enzyme, thereby forming an inhibitory nitrosyl-heme adduct. Indeed, the heme molecules on Nox2 are thought to be responsible for the transfer of electrons from NADPH to oxygen to generate $\cdot\text{O}_2^-$, as well as maintaining protein stability and formation with p22^{phox} (Selemidis *et al.*, 2008). Thus, it is predicted that a disruption in heme homeostasis would affect the entire assembly and activation of the Nox2-containing NADPH oxidase complex (Selemidis *et al.*, 2008). Alternatively, HNO may also interfere with NADPH oxidase activity via the induction of the antioxidant, heme-oxygenase 1 (HO-1), which is involved in heme degradation and the maintenance of cellular heme content. Moreover, Taille and co-workers have previously demonstrated, both *in vitro* and *in vivo*, that the induction of HO-1 leads to a decrease in the expression of the heme-containing subunit of NADPH oxidase, Nox2 (Taille *et al.*, 2004). Indeed, HNO has been previously reported to increase the activity, protein and mRNA expression of HO-1 in rat H9c2 cells (Naughton *et al.*, 2002), and therefore, future studies investigating the effect of HNO on HO-1 expression and activity in the vasculature under our experimental settings would be of value. It should, however,

be noted that we have so far made the assumption that HNO limits $\cdot\text{O}_2^-$ production by modulating Nox2-containing NADPH oxidase. However, it is also possible that the $\cdot\text{O}_2^-$ suppressing effects of HNO are occurring further upstream of NADPH oxidase, perhaps at the level of the AT_1 receptor and therefore future studies employing the use of an alternate stimuli such as endothelin-1 (ET-1) and the protein kinase C (PKC) activator, phorbol-12, 13-dibutyrate (PDB) are necessary to firstly confirm the interaction between HNO and NADPH oxidase. Whilst the precise mechanisms via which HNO elicits its vasoprotective effects remain to be determined, this chapter has nevertheless demonstrated that HNO is able to suppress $\cdot\text{O}_2^-$ generation in a cGMP-independent manner. Such an effect, in addition to its distinct pharmacological properties and other vasoprotective actions, may prove to be therapeutically beneficial in the treatment of cardiovascular diseases associated with an increase in ROS generation such as hypertension.

8.2 BAY 58-2667 as a pharmacological tool to probe for oxidised/heme-free sGC

Importantly, one major prerequisite for the NO-induced activation of sGC is the presence of the reduced (Fe^{2+}) heme moiety, such that its oxidative stress/disease-associated oxidation (Fe^{3+}) or removal abolishes NO-induced enzyme activation (Evgenov *et al.*, 2006). The recent development of NO-independent sGC activators, that preferentially target the oxidised/heme-free state of sGC, has provided valuable tools to probe the redox states of sGC in the vasculature. Previous findings that such compounds serve as potent vasodilators in the non-diseased vasculature are indicative of the existence of pools of oxidised/heme-free sGC under physiological conditions. Future development of fluorescently tagged BAY 58-2667, or similarly functioning compounds, may aid in the identification and localisation of these redox altered pools of sGC in intact cells. Indeed, we have shown that BAY 58-2667 vasodilates vessels from normotensive WKY rats (Chapter 4) and C57BL/6J mice (Chapter 7) with a potency in the nanomolar range. Furthermore, while the presence of the sGC inhibitor, ODQ potentiated BAY

58-2667-induced relaxation, the heme-free antagonist, zinc protoporphyrin IX (Zn-PPIX) significantly blocked the response, thus confirming the existence of oxidised/heme-free sGC under physiological conditions.

Disease states, particularly those coupled with an increase in oxidative stress, are also associated with augmented responsiveness to BAY 58-2667, and thus by implication, elevated pools of oxidised/heme-free sGC (Stasch *et al.*, 2006). Similarly, we observed that BAY 58-2667-induced relaxation was significantly more potent in the vasculature of spontaneously hypertensive rats (SHR, Chapter 4) and ApoE^{-/-} mice (Chapter 7), both well-established models of disease (Nakashima *et al.*, 1994; Udenfriend & Spector, 1972), compared to their respective controls. To date, it has been assumed that an elevation in $\cdot\text{O}_2^-$ and the generation of oxidants such as ONOO⁻ results in oxidation of the heme moiety of sGC, thereby accounting for augmented BAY 58-2667-induced relaxation. Studies in Chapter 7 directly assessed the causal relationship between NADPH-driven $\cdot\text{O}_2^-$ generation and BAY 58-2667 responsiveness.

Currently, little is known with regards to the endogenous factors involved in the regulation of the relative ratio of reduced to oxidised/heme-free sGC. The possibility that the oxidation of the heme moiety of sGC may be a reversible process, such that an endogenous reductase, as discussed in Chapter 4, may function to preserve the heme of sGC in its reduced state also requires further investigation. Future studies are also required to determine if the scavenging of NO by $\cdot\text{O}_2^-$ and the subsequent formation of ONOO⁻ is predominantly responsible for the elevated pools of oxidised/heme-free sGC. Indeed, such information may be gained via the measurement of 3-nitrotyrosine (3-NT) levels in the studied vessels. However, when applying this technique to future studies, it is important to note that while measurement of 3-NT levels are generally taken as evidence for ONOO⁻ generation in tissues, 3-NT is simply an indicator for the

formation of reactive nitrogen species and not unique for ONOO⁻. Thus, 3-NT can also be formed by heme peroxidase, independent of the ONOO⁻ pathway (van der Vliet *et al.*, 1997).

8.3 Modulation of oxidised/heme-free sGC by endogenous NO

The results from Chapter 4 revealed an ability of endogenous, endothelial-derived NO to modulate the vasodilator actions of BAY 58-2667. Using UV/VIS spectroscopy, we show that this modulation of BAY 58-2667 was not due to a direct chemical interaction with NO, rather we speculated that NO may protect sGC from oxidation, thus limiting the formation and accumulation of oxidised/heme-free sGC and thereby the response to BAY 58-2667. Interestingly, we found the ability of endogenous NO to attenuate BAY 58-2667-induced relaxation was species specific. Thus, whilst a modulatory role of NO was observed in rat and rabbit arteries, neither acute *in vitro* or long-term *in vivo* treatment with the NOS inhibitor, L-NAME nor endothelial NOS (eNOS^{-/-}) deletion had any effect on the response to BAY 58-2667 in isolated mouse aortae. While the reasons underlying this apparent lack of modulation by NO in mouse arteries was not investigated in the current study, we propose that endogenous NO derived from preformed L-NAME-resistant NO stores (Andrews *et al.*, 2002) may persist in the face of NOS inhibition and thereby modulate the response to BAY 58-2667. Indeed, future studies incorporating co-treatment with NO scavenging compounds, such as haemoglobin and/or carboxy-PTIO, with L-NAME in mice may provide a clearer insight.

The mechanisms via which endogenous NO may be influencing pools of oxidised/heme-free sGC remains to be elucidated. However, recent evidence has identified the existence of cytosolic NADPH-dependent flavoprotein-containing methemoprotein reductase, which was shown to maintain sGC in its reduced state (Gupte *et al.*, 1999; Wolin, 2009). Thus, we proposed that the oxidation of sGC may be reversible process, whereby endogenous NO is able to stimulate the activity of this endogenous reductase to either preserve reduced sGC or convert sGC back to its

reduced state. While this NADPH-dependent reductase has yet to be fully characterised, Gupte and colleagues suggest that it is present in sufficient amounts to control the redox status of the heme of sGC (Gupte *et al.*, 1999). Thus, in order to test this hypothesis under our experimental settings, future experiments will need to determine if NADPH facilitates NO stimulation of sGC, as assayed via enzyme immunoassay measurement of cGMP formation, in L-NAME- and ferricyanide-treated arteries.

These findings raise the interesting concept that a disease-associated loss in endogenous NO may lead to increased pools of oxidised/heme-free sGC, thereby contributing to the enhanced vasorelaxant potency of BAY 58-2667 observed. Indeed, in SHR, NOS inhibition failed to augment the response to BAY 58-2667 further, and this is likely due to the fact that NO is already compromised in hypertension (Chatterjee & Catravas, 2008; Dominiczak & Bohr, 1995).

8.4 Modulation of BAY 41-2272-induced relaxation by the endothelium in hypertension

Whilst a disease associated loss of NO may augment the response to BAY 58-2667, it may also lead to decreased responsiveness to the new generation of NO-independent sGC stimulators, such as BAY 41-2272, which synergise with endogenous NO to elicit vasorelaxation. Interestingly, Chapter 5 revealed that endogenous NO does not modulate the response to BAY 41-2272 in isolated aortae from either WKY or SHR rat. More importantly, although endogenous NO did not appear to play a role in maintaining BAY 41-2272-mediated relaxation, this study identified an unknown, NO-independent endothelial-derived factor in SHR, which serves to maintain, in part, responses to BAY 41-2272 in hypertension.

The identity of such a factor is yet to be elucidated. One possibility is hydrogen peroxide (H₂O₂) which, has been shown to augment sGC activation by BAY 41-2272 in endothelial cells (Thomas *et al.*, 2006). Whether this is the case in hypertension remains to be examined and is a

point for future studies. However, given that cardiovascular diseases such as hypertension are associated with an enhanced production of ROS in conjunction with a reduction in antioxidant systems (Lee & Griendling, 2008), we propose that a loss of antioxidant systems upon removal of the endothelium in the SHR, which would normally counteract the increased generation of $\cdot\text{O}_2^-$, may be responsible for the impaired vasorelaxant response to BAY 41-2272 in this study. Such antioxidant systems include the family of superoxide dismutases (SOD; Cu/Zn-SOD, Mn-SOD, EC-SOD), catalase, glutathione (GSH) system, thioredoxin, peroxiredoxin as well as ROS scavengers like vitamins A, C and E (Lee & Griendling, 2008; Szasz *et al.*, 2007). Indeed, studies in a human co-cultured system demonstrated that endothelial cells protected the vascular smooth muscle against oxidative stress via the up-regulation of antioxidant systems (Cu/Zn-SOD and thioredoxin), an effect attributed to an unknown endothelial-derived factor unrelated to NO, prostacyclins or hydroxyeicosatetraenoic acid (HETE) (Xu *et al.*, 2009).

In order to confirm this hypothesis, future studies will need to firstly elucidate if the endothelium protects against oxidative stress in the vascular smooth muscle of SHR via the up-regulation of antioxidant systems as well as delineate the specific or primary antioxidant system(s) involved. Additionally, examining mRNA/protein expression of specific antioxidants such as SODs, catalase and/or thioredoxin in endothelium-denuded versus -intact aortae from both WKY and SHR may provide a clearer insight. Certainly, the identity of this protective endothelial-derived factor and the mechanisms by which it mediates its response need to be determined. Nevertheless, while BAY 41-2272 does confer advantages over traditional nitrovasodilator therapy, its beneficial effects may be strongly enhanced if administered in conjunction with antioxidant compounds.

8.5 Modulation of oxidised/heme-free sGC by Nox2-containing NADPH oxidase and the Western-style high fat diet

It has generally been assumed that augmented responsiveness to BAY 58-2667 in disease is due to an increased generation of ROS, such as $\cdot\text{O}_2^-$, the subsequent formation of peroxynitrite (ONOO) and the oxidation of sGC. This concept, however, has not been tested directly and this thesis utilised Nox2^{-/-} mice to address this issue. As the primary function of NADPH oxidase is the generation of ROS (Cave *et al.*, 2006), we sought to determine if NADPH oxidase was capable of modulating vasorelaxant responses to BAY 58-2667, possibly by influencing the pools of oxidised/heme-free sGC. Thus, this chapter (Chapter 7) demonstrated that a short-term Ang II-mediated increase in vascular $\cdot\text{O}_2^-$ production was mirrored by an enhanced vasorelaxant potency to BAY 58-2667, an effect dependent on Nox2-containing NADPH oxidase. Indeed, this chapter has provided evidence for a causal role of Nox2-derived ROS in augmenting the responsiveness to BAY 58-2667, and therefore via implication, pools of oxidised sGC. However, in the setting of chronic disease, ROS alone do not appear to account for the augmented vasorelaxant potency to BAY 58-2667. Specifically, the findings in C57BL/6J mice highlighted that in a model of atherosclerosis (ApoE^{-/-}), BAY 58-2667-induced relaxation was not influenced by ROS with similar responses to the sGC activator observed in arteries from C57BL/6J, ApoE^{-/-} and Nox2^{-/-}/ApoE^{-/-}, all on a high fat diet (HFD), despite distinct vascular $\cdot\text{O}_2^-$ levels. Interestingly, HFD alone appeared to influence pools of oxidised/heme-free sGC and subsequently BAY 58-2667 responsiveness.

While the mechanisms via which diet is modulating BAY 58-2667-induced relaxation is currently unknown, there may be a few potential explanations. As one of the leading factors in the progression of atherosclerosis (Meyer & Schmitt, 2000), low density lipoproteins (LDL) and its oxidative modification, oxLDL may be responsible for the diet-induced modulation of responses to BAY 58-2667. First, oxLDL has been shown to increase HO-1 activity in aortic

smooth muscle cells (Anwar *et al.*, 2005), a key regulator of cellular heme levels (Mingone *et al.*, 2008). Indeed, in cultured rat pulmonary endothelial cells, an increase in HO-1 activity has been shown to reduce sGC activity by limiting the availability of cellular heme (Abraham *et al.*, 2002), allowing us to speculate that in doing so, HO-1 may also result in the increased pools of oxidised/heme-free sGC. To date, however, there exists conflicting evidence with regards to the inhibitory versus protective effects of HO-1 in the vasculature. In fact, we have proposed (Chapter 3) that one of the possible mechanisms by which HNO may be limiting NADPH oxidase activity is via HO-1-mediated reduction of Nox2 heme content. Thus, we need to be mindful when interpreting such results as HO-1 products have also been shown to inhibit NADPH oxidase activity (Minetti *et al.*, 1998; Otterbein, 2002), while we have observed that the diet-induced effects were neither protective nor dependent on NADPH oxidase activity. However, it would still be of benefit to examine HO-1 expression and/or activity in the arteries of mice receiving the high-fat diet.

Alternatively, while we have discussed the possible ability of endogenous NO to preserve the heme of sGC in its reduced state via the induction of an endogenous NADPH-dependent reductase (Chapter 4), we speculate that it may also be possible that hyperlipidemia promotes the accumulation of oxidised/heme-free sGC perhaps by attenuating the activity of this endogenous reductase. Certainly, further experiments are required. Nevertheless, this chapter has revealed that two factors, namely O_2^- and HFD, are capable of independently modulating BAY 58-2667-induced responses. Moreover, given the augmented responsiveness to BAY 58-2667 observed in pre-atherosclerotic mice (ie. C57BL/6J mice on HFD), these findings highlight the potential use of sGC activators as diagnostic tools to identify blood vessels at risk of disease-associated dysfunction.

8.6 Cardio- and vasoprotective actions of chronic treatment with BAY 41-2272 and BAY 58-2667

Having ascertained that the sGC stimulator, BAY 41-2272 and sGC activator, BAY 58-2667 demonstrated sustained and enhanced efficacy, respectively, under acute oxidative stress conditions (Chapter 4 and 7) as well as identifying an ability of BAY 41-2272 to signal via cGMP-dependent and -independent mechanisms (Chapter 5), we sought to assess their protective actions following chronic administration. Indeed, BAY 41-2272 has been shown to reduce Ang II-induced increase in left ventricular hypertrophy and cardiomyocyte size (Masuyama *et al.*, 2006), while BAY 58-2667 was shown to reduce left ventricular hypertrophy in rats with 5/6 nephrectomy (Kalk *et al.*, 2006). Additionally, in rodent models of pulmonary hypertension, both BAY 41-2272 and BAY 58-2667 reduced right ventricular hypertrophy (Dumitrascu *et al.*, 2006), further highlighting the cardioprotective effects of both compounds in various models of hypertension.

Thus, in Chapter 6, we demonstrated that chronic stimulation of sGC (reduced and oxidised/heme-free) and the subsequent elevation of cGMP, with BAY 41-2272 (1 mg/kg/day) and BAY 58-2667 (0.3 and 1 mg/kg/day) throughout a 4-week period conferred cardio- and vaso-protection in an advanced model of hypertensive heart disease (20-22 months old aged SHR). BAY 41-2272 and BAY 58-2667-treatment transiently reduced mean arterial blood pressure (MABP) in aged SHR rats following intraperitoneal administration, however this effect only lasted for a few hours and was lost by the end of the 4 week treatment period, indicative of hemodynamic tolerance development. Such tolerance development appeared to be exclusive to *in vivo* depressor responses as vasorelaxant responses to both BAY 41-2272 and BAY 58-2667 in isolated aortae from SHR were resistant to tolerance and cross-tolerance development. Presently, the mechanisms underlying hemodynamic, but not vascular tolerance, are unknown and require further investigation. Nevertheless, this study highlights the importance of

characterising the vasodilator capacity of sGC stimulators and activators *in vivo* and *in vitro*, and indicates that the efficacy of these compounds as long-term anti-hypertensive agents may be limited.

Importantly, both BAY 41-2272 and BAY 58-2667 exhibited anti-hypertrophic actions, which, were independent of a blood pressure lowering action, and thus indicative of a direct interaction at the level of the cardiomyocyte, possibly via an increase in myocardial cGMP levels (Krieg *et al.*, 2009; Ritchie *et al.*, 2009). Moreover, both BAY 41-2272 and BAY 58-2667 restored vasorelaxant responses to the cGMP-analogue, 8-bromo-cGMP, presumably in a synergistic manner resulting in enhanced cGMP levels. Certainly, future studies quantifying cGMP levels in isolated conduit arteries as well as myocardial cGMP levels in aged SHR following chronic stimulation/activation of sGC would provide a better insight. Surprisingly, BAY 58-2667 limited cardiac hypertrophy to a similar extent as that observed with BAY 41-2272. This result was somewhat unexpected given we anticipate that pools of oxidised/heme-free sGC are augmented in disease. Perhaps, a critical level of cGMP elevation is required to confer cardioprotection and treatment with both BAY 41-2272 and BAY 58-2667 were able to achieve this level. Additionally, only BAY 58-2667 (1 mg/kg/day) was able to significantly suppress $\cdot\text{O}_2^-$ production in SHR aortae, and given its ability to elevate cGMP, we propose that these effects may be cGMP-dependent. However, there is also the possibility that, like HNO (Chapter 3), the $\cdot\text{O}_2^-$ limiting effects of BAY 58-2667 may be mediated via cGMP-independent mechanisms. While we believe that HNO may directly oxidise thiols on p47^{phox} to limit NADPH oxidase activity, cGMP-independent actions of BAY 58-2667 are yet to be reported. Indeed, future studies employing the use of a protein kinase (PKG) inhibitor, will be required to investigate this concept.

Thus, Chapter 6 demonstrates that while tolerance to the acute blood pressure lowering actions of sGC stimulators and activators may occur, these compounds with an ability to limit cardiac hypertrophy and exhibit resistance to scavenging by $\cdot\text{O}_2^-$, may still confer therapeutic advantages over traditional nitrovasodilator therapy in the treatment of hypertensive heart disease. Indeed, BAY 58-2667 (Cinaciguat) is currently in Phase IIb clinical trials for the treatment of acute decompensated heart failure (Hingorany & Frishman, 2011).

8.7 Current limitations and future directions

8.7.1 HNO Study

While HNO offers new strategies in the treatment of cardiovascular diseases, there exists a number of limitations associated with investigating the therapeutic potential of HNO. First, the inability to store HNO due to its rapid dimerisation and dehydration necessitates the use of donor compounds to introduce HNO into biological systems (Miranda *et al.*, 2005c).

Of the HNO donors available, Angeli's salt represents the most convenient, well-studied and utilised choice, yet it is not a pure HNO donor, decomposing to generate both HNO and nitrite (NO_2^-) (Irvine *et al.*, 2008; Paolucci *et al.*, 2007). Although IPA/NO does not decompose to generate NO_2^- , it does produce a nitrosamine by-product and generates NO^\bullet at $\text{pH} < 7$ (Dutton *et al.*, 2006; Miranda *et al.*, 2005b; Paolucci *et al.*, 2007). However, given that both donors were susceptible to scavenging by L-cysteine in our study verifies that their effects were mediated predominantly by HNO. It should be noted that both Angeli's salt and IPA/NO have short half-lives ($< 3\text{min}$) and in order for the therapeutic utility of HNO to be investigated further, there is a need for the development of pure, long-lasting HNO donors (DuMond & King, 2011; Miranda *et al.*, 2005a). Acyloxy-nitroso compounds may form the basis for the development of such alternate donors for HNO (DuMond & King, 2011; Irvine *et al.*, 2008).

A further hurdle when proposing the therapeutic benefits of HNO is its capacity to also produce non-specific and toxic effects. Indeed, early investigations involving Chinese hamster V79 lung fibroblasts revealed that high concentrations of Angeli's salt (2-4 mM) decreased cellular viability, depleted cellular glutathione (GSH) levels as well as elicited breaks in double-stranded DNA (Wink *et al.*, 1998), which is presumably related to its propensity to react with thiols and thiol proteins. Furthermore, Angeli's salt has been reported to induce neurotoxicity in dopaminergic neurons (Vaananen *et al.*, 2003) whilst also capable of aggravating ischaemic-reperfusion injury in both the brain (Choe *et al.*, 2009) and heart (Ma *et al.*, 1999). Indeed, some of the toxic effects elicited by HNO under the aforementioned conditions may be attributed to the exceedingly high concentrations used, which are not relevant for biological systems. Thus, the potential HNO-induced cytotoxicity may be limited via controlling the dose utilised. Certainly, more work is required in assessing the therapeutic benefits of HNO.

Moreover, whether HNO is produced endogenously remains a contentious issue. Although numerous biochemical pathways have been proposed for its generation under physiological conditions, the lack of direct detection methods for HNO in the intact cell has prevented definitive evidence for its existence (Irvine *et al.*, 2008). Nevertheless, biochemical studies indicate that HNO can be generated from both NOS-dependent and -independent sources (Fukuto *et al.*, 2005; Irvine *et al.*, 2008). Furthermore, the possibility of HNO serving as an EDRF has been entertained by several studies with a recent study by Andrews and co-workers suggesting that HNO appears to be derived entirely from NOS in mouse mesenteric arteries, while non-NOS stores may also contribute to endogenous HNO production in rat mesenteric arteries (Andrews *et al.*, 2009). Thus, while indirect methods of HNO detection do exist and there is pharmacological evidence to suggest that HNO is produced endogenously, such techniques cannot yet be applied to intact cells. However, a recent study has identified a membrane permeable metal-based fluorescent probe, Tripodal copper (II) BODIPY complex

(Cu^{II}[BOT]) capable of specifically detecting HNO in living cells (Rosenthal & Lippard, 2010). Such probes, if of sufficient sensitivity, may facilitate the detection of HNO *in vitro* and *in vivo*, as well as provide long awaited confirmation of its role as an endogenous signaling molecule.

8.7.2 Redox regulation of sGC

Similar to the lack of detection methods for HNO, to date, it is currently not possible to quantify the pools of oxidised/heme-free sGC within living cells with sufficient sensitivity given the vast amounts of cellularly expressed heme proteins and the susceptibility of sGC to oxidation during the extraction process (Stasch *et al.*, 2006; Wolin *et al.*, 1982). Indeed, the precise mechanisms pertaining to the higher abundance of oxidised/heme-free sGC under disease conditions remains to be fully elucidated, with roles for oxidative stress and insufficient heme biosynthesis or heme incorporation proposed. Additionally, given the wide distribution of NO/sGC/cGMP signaling, future studies determining the localisation and expression levels of oxidised/heme-free sGC is also of therapeutic importance (Kemp-Harper & Schmidt, 2009). Presently, the NO- and heme-independent sGC activator, BAY 58-2667 represents the first and only reliable non-invasive pharmacological tool to probe for the intracellular pools of oxidised/heme-free sGC (Stasch *et al.*, 2006). Moreover, the interaction between BAY 58-2667 and sGC has been reported to be highly specific, while BAY 58-2667 itself is devoid of clinically off-target effects (Lapp *et al.*, 2009) and does not appear to interact with cytochrome P450, making the risk of drug to drug interaction less problematic (Frey *et al.*, 2008; Hingorany & Frishman, 2011). Thus, the preferential activation of oxidised/heme-free sGC makes BAY 58-2667 a beneficial pharmacological therapy for a range of diseases associated with oxidative stress (Stasch *et al.*, 2006).

Despite the potential therapeutic utility of this compound, the precise target of BAY 58-2667 (oxidised or heme-free species of sGC or both) remains a contentious issue. Originally, it was

suggested that following oxidation (Fe^{3+}), the bond between sGC and its heme group is weakened, allowing for the easy displacement of the heme and consequently activation of sGC by BAY 58-2667 (Schmidt *et al.*, 2004). Recent studies have, however, challenged this proposal suggesting that BAY 58-2667 is only able to target heme-free sGC and is otherwise unable to discriminate between oxidised (Fe^{3+}) versus reduced (Fe^{2+}) sGC (Roy *et al.*, 2008). While the authors concede that oxidation indeed weakens the bond between sGC and the heme, they argue that the resultant spontaneous loss of the heme rather than the active drug-induced displacement of the heme, allows BAY 58-2667 to bind to the unoccupied heme pocket of sGC (Roy *et al.*, 2008). If this theory holds true, it would indicate that the activity of BAY 58-2667 observed under physiological, pathological and drug-induced oxidative stress (i.e. ODQ) conditions reflects the presence of heme-free sGC in all these circumstances. Furthermore, heme biosynthesis and its incorporation is essential for the activation of sGC by NO. The precursor to heme, protoporphyrin IX (PPIX) is generated from δ -aminolevulinic acid (ALA), where heme is synthesised via the insertion of ferrous iron into PPIX by ferrochelatase, a process which is reliant upon iron availability (Mingone *et al.*, 2006). Indeed, a decline in heme biosynthesis is reported to be associated with diseases of aging (Atamna *et al.*, 2002). Moreover, given that heme biosynthesis occurs in the mitochondria and mitochondrial dysfunction has been reported in several vascular diseases, it may also pose as another possible factor in impairing the synthesis of heme (Mingone *et al.*, 2008). Thus, it is also possible that the process and rate of heme biosynthesis and/or incorporation into sGC may be impacted during vascular disease. While these concepts remain to be conclusively proven, either way BAY 58-2667 proves to be effective in conditions associated with enhanced heme loss or deficient heme incorporation.

Indeed, the future of BAY 58-2667-mediated therapy looks promising with early Phase I and II trials using intravenous BAY 58-2667 (Cinaciguat) already completed, with an additional Phase II clinical program underway for the treatment of acute decompensated heart failure (ADHF)

(Hingorany & Frishman, 2011). Using a single-blind, randomised, placebo-controlled, dose-escalating protocol, an early Phase I study demonstrated that intravenous infusions of BAY 58-2667 exhibited potent preload- and afterload-reducing effects, favourable safety profile and was well-tolerated in healthy male subjects (Frey *et al.*, 2008). Subsequently, a non-randomised, uncontrolled, unblinded, dose-escalating multicentre Phase II study was carried out to assess the therapeutic potential of intravenous BAY 58-2667 infusion (6 hour) in the treatment of ADHF (Lapp *et al.*, 2009). Of the 60 patients participating in the trial, and who received BAY 58-2667 treatment, 13 of them reported 14 drug-related treatment-emergent adverse events which ranged from mild to moderate intensity, the most common of which was hypotension. Indeed, in our study using a model of advanced hypertensive heart disease, we demonstrated that BAY 58-2667 induced a significant 37 mmHg drop in MABP following the initial intraperitoneal administration, lowering MABP below that recorded in normotensive, age-matched WKY (Chapter 6), and thus suggestive of excessive vasodilation by this compound. While these effects only persisted for approximately 4-5 hours and were eventually lost with continued, chronic administration, they highlight the potential for BAY 58-2667 to induce hypotension and the possibility of rebound hypertension occurring in response to BAY 58-2667 administration. Furthermore, in both Phase I and II studies, the positive hemodynamic effects of BAY 58-2667 infusion were quickly reversed following the cessation of its infusion (Frey *et al.*, 2008; Lapp *et al.*, 2009). This fast on and off effect of BAY 58-2667 therapy has been identified by Frey and co-workers as the key for the prevention of hypotension and the safe management of this vasodilating therapy (Frey *et al.*, 2008). Nevertheless, intravenous BAY 58-2667 infusion has been shown to facilitate potent cardiac unloading and importantly improve cardiac output while preserving renal function in patients with ADHF as well as in an experimental canine model of severe congestive heart failure (CHF) (Boerrigter *et al.*, 2007; Lapp *et al.*, 2009). Thus, from a clinical perspective, BAY 58-2667 showed favourable hemodynamic efficacy, overall was well-tolerated in patients with ADHF and appears to confer protection in cardiorenal disease states

(Boerrigter *et al.*, 2007; Lapp *et al.*, 2009). In fact, Phase IIb clinical trials are currently in progress incorporating longer infusion periods (24-48 hours) and, considering the adverse hypotensive episodes reported, such studies will also be employing lower infusion doses (Hingorany & Frishman, 2011).

8.8 Conclusions

In conclusion, this thesis has highlighted the vasoprotective properties of NO-independent ligands and their potential in the treatment of cardiovascular diseases. Firstly, the redox sibling of NO, HNO was shown to suppress vascular $\cdot\text{O}_2^-$ production in an NO- and cGMP-independent manner, possibly via a direct interaction with NADPH oxidase itself. Such findings further add to the vaso-protective armoury of HNO (ie. vasodilatory effects, anti-aggregatory and anti-proliferative properties) and lend support for its use in the treatment of vascular dysfunction. Moreover, utilising the novel sGC activator, BAY 58-2667 as a pharmacological tool to probe for oxidised/heme-free sGC, this thesis provides further evidence for the existence of these redox altered states of sGC in the vasculature under both physiological and pathological conditions. Indeed, we have shown that endothelial-derived NO may play a crucial and protective role in limiting the accumulation of oxidised/heme-free sGC, such that an oxidative stress- or disease-initiated loss of NO enhances BAY 58-2667-induced relaxation. Importantly, we have demonstrated that oxidative stress *per se* is not the sole factor capable of enhancing the pool of oxidised/heme-free sGC, with a Western-style high fat diet also capable of initiating such changes independently of an elevation in $\cdot\text{O}_2^-$ generation.

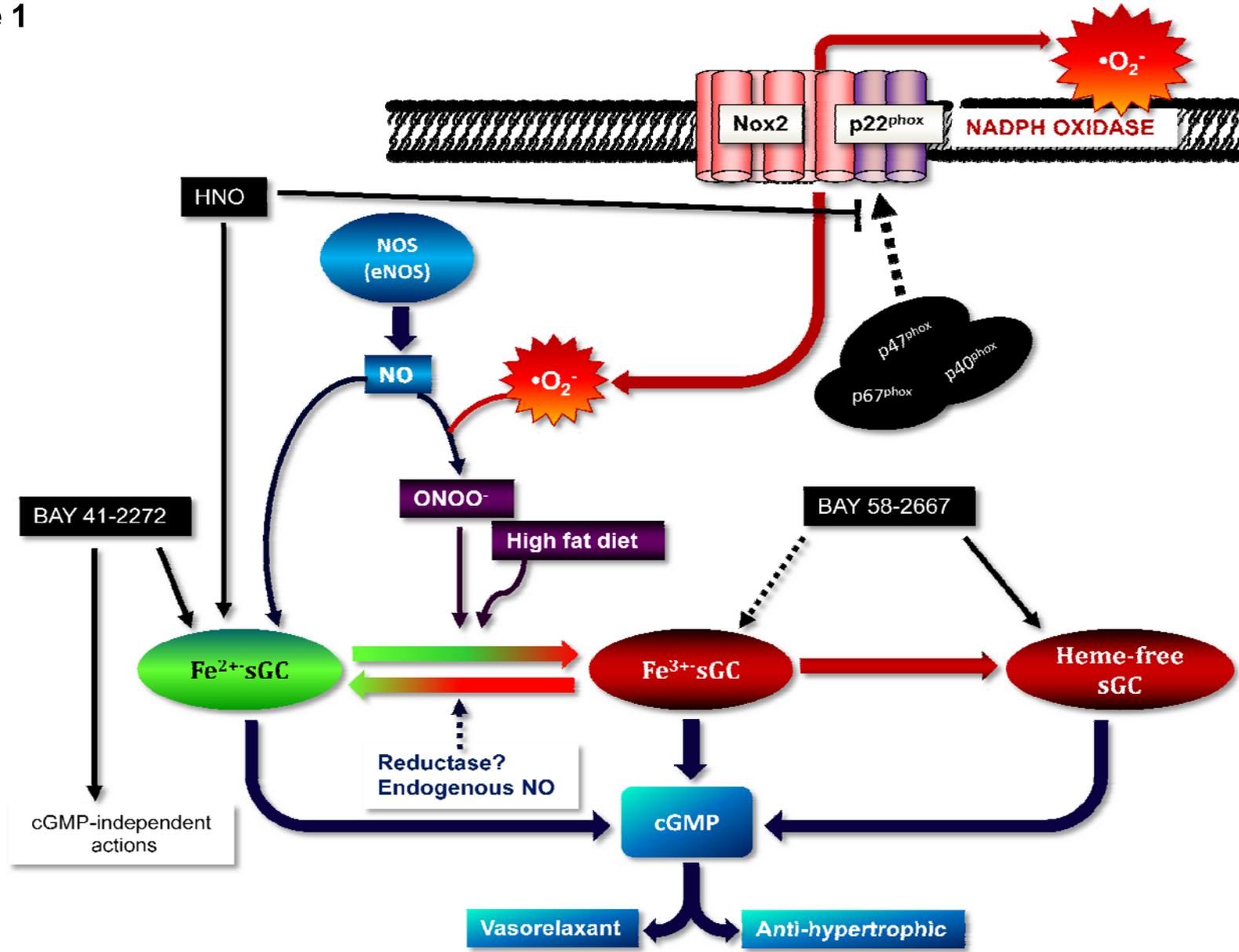
From a clinical perspective, our finding that the vasorelaxant response is augmented in the arteries from C57BL/6J mice on a HFD, suggests that BAY 58-2667 may not only serve as a novel therapeutic avenue in the treatment of cardiovascular diseases such as hypertension and atherosclerosis but may also prove to be a valuable diagnostic marker for the early detection of

some conditions. Furthermore, while the use of either BAY 41-2272 or BAY 58-2667 as anti-hypertensive agents has been cautioned due to the potential development of hemodynamic tolerance, we have nevertheless provided evidence for the cardio- and vaso-protective effects of both compounds in an advanced model of hypertensive heart disease.

Given that the NO/sGC/cGMP signaling pathway is impaired in disease and traditional nitrovasodilator therapy is compromised, therapies which function independently of NO such as HNO donors, and NO-independent sGC stimulators and activators, may offer potential benefits and alternatives over traditional nitrovasodilators in the management of cardiovascular disorders.

Figure 1. Schematic diagram representing the main findings of this thesis. It is well-documented that in disease, the NO/sGC/cGMP pathway is dysfunctional and traditional nitrovasodilator therapy is limited due to the development of tolerance. Thus, this thesis firstly demonstrates that HNO is able to suppress NADPH-driven $\cdot\text{O}_2^-$ production in the vasculature in a manner which is independent of NO and cGMP. Due to its highly thiophilic nature, it may be possible that HNO interacts directly with thiol groups on one or more of the regulatory subunits of NADPH oxidase, thereby limiting its effects. Furthermore, using the NO-independent sGC activator, BAY 58-2667 as a pharmacological probe, this thesis provides further evidence for the existence of oxidised/heme-free sGC under both physiological and pathophysiological conditions. While it has been assumed that an increase in vascular $\cdot\text{O}_2^-$ levels results in enhanced pools of oxidised/heme-free sGC in disease, interestingly this thesis demonstrates that NADPH oxidase (predominant generator of ROS) and the Western-style high fat diet independently modulate the redox pools of sGC, and by inference the responsiveness to BAY 58-2667. In addition, we also provide evidence which suggests that endothelial-derived NO may limit the accumulation of oxidised/heme-free sGC, possibly via the activation of an endogenous reductase. Such findings indicate that the oxidation of the heme group of sGC may be a reversible process and, that endogenous NO may aid in preserving the heme of sGC in its reduced state. Collectively, this thesis highlights the protective role of the endothelium and endothelial-derived factors in limiting the accumulation of oxidised/heme-free sGC and facilitating sGC/cGMP signaling. While there currently exists no direct detection method for quantifying the relative pools of oxidised/heme-free sGC, this thesis has nevertheless identified a number of factors (endogenous NO, NADPH oxidase and Western-style high fat diet) capable of putatively regulating the redox state of sGC and subsequently influencing BAY 58-2667-induced relaxation. Thus, given their resistance to $\cdot\text{O}_2^-$ scavenging, vascular tolerance development and the potential to elicit their effects via cGMP-independent mechanisms, as well as the ability to preferentially target oxidised/heme-free sGC (BAY 58-2667), such NO-independent compounds may offer alternate and possibly more advantageous therapeutic avenues in the treatment of cardiovascular diseases.

Figure 1





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