VASOPROTECTIVE ACTIONS OF NITROXYL (HNO) IN VASCULAR DISEASE

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Amendments:

ERRATA

P24, 2nd line from bottom: “vasodilatory capacity of HNO” for “vasodilatory capacity o HNO”

P32, line 6: “An increase in VSMC” for “An increased in VSMC”

P76, line 13: “and it has” for “and it jas”

P122, line 22: “While” for “Whilst”

P136, Fig 4 Headings: “Lipid content” and “Macrophage content” for “Collagen content” and “α-SMC actin content”

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General Declaration

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's regulations the following declarations are made:

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.

This thesis includes 2 original papers published in peer reviewed journals and 3 unpublished publications. The core theme of the thesis is "Vasoprotective actions of nitroxyl (HNO) in vascular disease". The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Pharmacology Department under the supervision of Dr Barbara Kemp-Harper and Dr Alyson Miller.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of Chapters 1-5 my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status*	Nature and extent of candidate's contribution
1	Nitroxyl (HNO) as a vasoprotective signaling molecule	Published	Prepared and wrote the manuscript for the review article, with intellectual advice and editorial assistance from co-authors
2	Vasorelaxant and anti-aggregatory actions of the nitroxyl donor isopropylamine NONOate are maintained in	Published	Conducted all experiments, analysed results and wrote manuscript, with with intellectual advice and editorial assistance from co-authors from co-

	hypercholesterolemia		authors
3	The vasoprotective actions of endogenous and exogenously generated nitroxyl (HNO) are preserved in advanced atherosclerosis	Ready for submission	Conducted all experiments except those for total plasma cholesterol and some tissue harvesting, analysed results and wrote manuscript, with intellectual advice and editorial assistance from co-authors
4	Platelet nitric oxide resistance in hypercholesterolemia is circumvented with the HNO donor isopropylamine NONOate and the sGC stimulator BAY 41-2272	Ready for submission	Conducted all experiments, analysed results and wrote manuscript, with intellectual advice and editorial assistance from co-authors
5	Nox2 deficiency improves plaque stability in advanced atherosclerosis	Ready for submission	Conducted all experiments except those for plasma cholesterol and some tissue harvesting, analysed results and wrote manuscript, with intellectual advice and editorial assistance from co-authors

I have / have not (circle that which applies) renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: **……**

MICHELLE LEANNE BULLEN

Date: ……

Summary

Nitroxyl (HNO), the one electron reduced and protonated congener of $NO^{\bullet}$ has recently gained therapeutic interest as HNO exhibits similar vasoprotective actions to that of $NO^{\bullet}$, but does not share its limitations. Additionally, HNO displays unique pharmacological actions that may enable its effects to be preserved and/or enhanced in disease states where $NO^{\bullet}$ function is compromised. This thesis examined whether the vasoprotective actions of endogenous and exogenous HNO were maintain in the setting of hypercholesterolemia and advanced atherosclerosis (apolipoprotein E-deficient mice maintained on a high fat diet for 7 and 21 weeks, respectively; $ApoE^{-/-}$-HFD) and these effects were directly compared to $NO^{\bullet}$. Given the role that oxidative stress plays in the pathogenesis of these diseases, this thesis also determined whether the absence of the superoxide ($^{\cdot}O_2^-$) generating enzyme Nox2-NADPH oxidase, conferred vasoprotection in advanced atherosclerosis.

In **Chapter 2**, we confirmed that the vasorelaxant and anti-aggregatory effects of the HNO donors, Angeli's salt and isopropylamine NONOate (IPA/NO) were mediate via HNO and were dependent upon sGC/cGMP signalling. Furthermore, in hypercholesterolemic mice ($ApoE^{-/-}$-HFD mice) we observed that vasorelaxation responses to IPA/NO and the clinically used $NO^{\bullet}$ donor, glyceryl trinitrate (GTN) were conserved. However, GTN, but not IPA/NO, was susceptible to vascular tolerance development and its anti-aggregatory response was abolished in hypercholesterolemia.

Given the vasoprotective actions of HNO were preserved in hypercholesterolemia, **Chapter 3** sought to compare the vasoprotective actions of HNO and $NO^{\bullet}$ in advanced atherosclerosis ($ApoE^{-/-}$-HFD mice). In these mice, IPA/NO and the $NO^{\bullet}$ donor, diethylamine NONOate (DEA/NO) maintained their ability to induce relaxation in carotid arteries, inhibit platelet aggregation, supress $^{\cdot}O_2^-$ generation and did not develop vascular tolerance. Whilst relaxation responses to GTN were sustained, GTN developed vascular tolerance and it's anti-aggregatory and $^{\cdot}O_2^-$ limiting actions were compromised in atherosclerotic mice. Additionally, the contribution of HNO to endothelium-dependent relaxation was enhanced in advanced atherosclerosis and may compensate for a reduction in endogenous $NO^{\bullet}$.

In **Chapter 4** we found that the ability of the $NO^{\bullet}$ donor sodium nitroprusside (SNP) to inhibit platelet aggregation and elevate cGMP levels was impaired in hypercholesterolemic ($ApoE^{-/-}$-HFD) mice,

indicating a model of platelet $NO^{\bullet}$ resistance. In this model, dysfunction at the level of sGC and/or downstream mediators of cGMP appeared to contribute to resistance. Our main finding was that the anti-platelet effects of $NO^{\bullet}$-independent sGC stimulators IPA/NO and BAY 41-2272 were maintained in hypercholesterolemic mice, indicating that IPA/NO and BAY 41-2272 can circumvent platelet $NO^{\bullet}$ resistance.

In **Chapter 5** we examined the effect of genetic deletion of the $^{\cdot}O_2^-$ generating enzyme Nox2-NADPH oxidase upon vascular function and plaque morphology in advanced atherosclerosis ($ApoE^{-/-}$ mice on a HFD for 21 weeks). We observed that Nox2 oxidase deletion had no effect upon endogenous $NO^{\bullet}$ generation or endothelium-dependent and –independent relaxation responses but reduced vascular $^{\cdot}O_2^-$ generation in advanced atherosclerosis. Moreover, in aortae and innominate arteries Nox2 deletion did not reduce lesion size but did lead to an increase in plaque stability and appeared to promote an anti-inflammatory (M2) macrophage phenotype. These findings suggest that, Nox2 oxidase plays an important role in plaque instability in atherosclerosis.

In conclusion, the findings of this thesis indicate that the vasoprotective actions of endogenous and exogenously generated HNO are sustained or augmented, in the setting of hypercholesterolemia and advanced atherosclerosis. These properties, coupled with the lack of tolerance development, resistance to scavenging by $^{\cdot}O_2^-$ and an ability of HNO to circumvent platelet $NO^{\bullet}$ resistance and target sGC/cGMP-independent signalling pathways confers advantages over traditional nitrovasodilators. Thus, HNO donors represent an innovative pharmacotherapy for the treatment of cardiovascular diseases.

Publications arising from this thesis

Manuscripts

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Abbreviations

1-NCA	1-nitrosocyclohexyl acetate
ACE	angiotensin-converting enzyme
ACh	acetylcholine
Ang II	angiotensin II
$ApoE^{-/-}$	apolipoprotein E-deficient
Arg1	arginase 1
AS	angeli's salt
α-SMC actin	alpha smooth muscle cell actin
AUC	area under the curve
Ca^{2+}	calcium
carboxy-PTIO	2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxy-3-oxide
cAK	cAMP-dependent protein kinases
cDNA	complementary DNA
cGK	cGMP-dependent protein kinases
CGRP	calcitonin gene related peptide
Cu	copper
C-X-C motif ligand 2	CxCl2
DEA/NO	diethylamine NONOate
dH_2O	distilled water
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDRF	endothelium-dependent relaxation factor
EDTA	ethylenediaminetetraacetic acid
EIA	enzyme immunoassay
eNOS	endothelial nitric oxide synthase
EtOH	ethanol
Fe	iron
Fe^{2+}	ferrous haem

Fe^{3+}	ferric haem
Fizz1	resistin-like molecule alpha 1
F_{max}	maximum contraction
GSH	glutathione
GTN	glyceryl trinitrate
HDL	high density lipoprotein
HFD	high fat diet
$H_2N_2O_2$	hyponitrous acid
HNO	nitroxyl
H_2O_2	hydrogen peroxide
HXC	hydroxocobalamin
IBMX	3-isobutyl-1-methyl-xanthine
ICAM-1	intracellular adhesion molecule 1
IFN-γ	interferon gamma
IL	interleukin
IPA/NO	isopropylamine NONOate
K^+	potassium
K_{ATP}	ATP-sensitive potassium channels
K_V	voltage-sensitive potassium channels
L-cys	L-cysteine
LDL	low density lipoprotein
L-NAME	N^{ω}-nitro-L-arginine methyl ester
MMPs	matrix metalloproteinases
N_2O	nitrous oxide
NAC	*N*-acetyl-L-cysteine
NaOH	sodium hydroxide
ND	normal diet of standard chow
NF-κB	nuclear factor-kappa B
NO	nitrogen oxide
$NO^{\bullet}$	nitric oxide

NO^+	nitrosonium cation
NO^-	nitroxyl anion
NO_2^-	nitrite
$Nox2^{-/-}$	Nox2 oxidase deficient
$^{\cdot}O_2^-$	superoxide
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
ox-LDL	oxidised low density lipoproteins
PBS	phosphate buffered saline solution
PDB	phorbol 12,13-dibutyrate
PDE	phosphodiesterase
pEC_{50}	concentration of agonist causing 50% relaxation
PEG-CAT	polyethylene glycol-catalase
PEG-SOD	polyethylene glycol-superoxide dismutase
POLDIP2	polymerase δ-interacting protein 2
PRP	platelet rich plasma
qPCR	quantitative real-time polymerase chain reaction
RLU	relative light units
R_{max}	maximum relaxation
ROS	reactive oxygen species
RyR	ryanodine receptors
SEM	standard error of mean
SERCA2a	ATP-dependent SR Ca^{2+}-ATPase
sGC	soluble guanylyl cyclase
SNP	sodium nitroprusside
SR	sarcoplasmic reticulum
TGF-β	transforming growth factor beta
TNFα	tumour necrosis factor alpha
VASP	vasodilator-stimulated phospho protein
VSMC	vascular smooth muscle cell

Monash University

Declaration for Thesis Chapter 1

Declaration by candidate

In the case of Chapter 1, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
Prepared and wrote the manuscript for the review article	80%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	**Nature of contribution**	**Extent of contribution (%) for student co-authors only**
Alyson Miller	Provided intellectual advice during manuscript preparation stages and assisted with the editorial process.	
Karen Andrews		
Jennifer Irvine		
Rebecca Ritchie		
Christopher Sobey		
Barbara Kemp-Harper		

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date**
Main Supervisor's Signature		**Date**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

General Introduction

This thesis focuses on the vasoprotective actions of nitroxyl (HNO) in the setting of vascular disease. A comprehensive review of the literature pertaining to the vasoprotective actions of HNO is provided in the accompanying article which was published in 2011 and authored by myself and colleagues (Bullen et al., 2011 Nitroxyl (HNO) as a vasoprotective signalling molecule, *Antioxidants & Redox Signaling* 14: 1675-1686). The following General Introduction provides an update to this review article and also addresses other areas central to this thesis including the general chemistry and biological targets of HNO, HNO donors, therapeutic actions of HNO, the pathogenesis of atherosclerosis and the role of Nox2 oxidase in vascular dysfunction.

ANTIOXIDANTS & REDOX SIGNALING
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FORUM REVIEW ARTICLE

Nitroxyl (HNO) as a Vasoprotective Signaling Molecule

Michelle L. Bullen,[1] Alyson A. Miller,[1] Karen L. Andrews,[2] Jennifer C. Irvine,[3] Rebecca H. Ritchie,[3] Christopher G. Sobey,[1] and Barbara K. Kemp–Harper[1]

Abstract

Nitroxyl (HNO), the one electron reduced and protonated form of nitric oxide (NO•), is rapidly emerging as a novel nitrogen oxide with distinct pharmacology and therapeutic advantages over its redox sibling. Whilst the cardioprotective effects of HNO in heart failure have been established, it is apparent that HNO may also confer a number of vasoprotective properties. Like NO•, HNO induces vasodilatation, inhibits platelet aggregation, and limits vascular smooth muscle cell proliferation. In addition, HNO can be putatively generated within the vasculature, and recent evidence suggests it also serves as an endothelium-derived relaxing factor (EDRF). Significantly, HNO targets signaling pathways distinct from NO• with an ability to activate K_V and K_{ATP} channels in resistance arteries, cause coronary vasodilatation in part via release of calcitonin-gene related peptide (CGRP), and exhibits resistance to scavenging by superoxide and vascular tolerance development. As such, HNO synthesis and bioavailability may be preserved and/or enhanced during disease states, in particular those associated with oxidative stress. Moreover, it may compensate, in part, for a loss of NO• signaling. Here we explore the vasoprotective actions of HNO and discuss the therapeutic potential of HNO donors in the treatment of vascular dysfunction. *Antioxid. Redox Signal.* 00, 000–000.

Introduction

THE BIOLOGICALLY ACTIVE GAS, nitric oxide (NO•) is well recognized as an important modulator of vascular homeostasis with vasorelaxant, anti-aggregatory, and anti-proliferative properties (55). NO• elicits its vasoprotective effects primarily through activation of the cytosolic enzyme soluble guanylyl cyclase (sGC) which catalyses the conversion of GTP to cGMP (40). An impairment in endogenous NO•-mediated vasoprotection is associated with a plethora of cardiovascular pathologies, arising as a consequence of a decrease in NO• synthesis, impaired NO• bioavailability and/or dysfunction at the level of its receptor (sGC) and its signaling pathways (31, 78). Such deficiencies in NO• signaling can be overcome in part by the use of NO• donors, such as the organic nitrate glyceryl trinitrate (GTN), which have been utilized for >100 years in the treatment of angina, heart failure, and acute hypertensive crises. However, the clinical efficacy of traditional NO• donors is limited due to their susceptibility to tolerance development with continued use and diminished vasoprotective actions under conditions of oxidative stress (41).

Interestingly, nitroxyl (HNO), the one electron reduced and protonated form of NO• is rapidly emerging as a novel redox sibling of NO• with distinct pharmacological actions and therapeutic advantages over NO• (35) (Table 1). In particular, its cardioprotective actions have received much attention with HNO, unlike NO•, increasing myocardial contractility (via thiol interaction) (8, 63, 79) and conferring protection in the setting of acute experimental heart failure (62). The concomitant ability of HNO to serve as a positive cardiac inotrope and to unload the heart (via vasodilatation) is of benefit in the treatment of heart failure. Thus, together with its myocardial effects, the vascular actions of HNO are also likely to be of importance and warrant further investigation.

Like NO•, HNO may be produced endogenously within the vasculature and has similar vasoprotective properties such as the ability to induce vasorelaxation (3, 19, 20, 33), inhibit platelet aggregation (5, 56), and inhibit vascular smooth muscle cell (VSMC) proliferation (80). In contrast to NO•, HNO targets distinct signaling pathways in the vasculature that include activation of voltage-sensitive K^+ channels (K_v) (19, 33) and the release of calcitonin-gene related peptide (CGRP) (20). In addition, HNO is resistant to scavenging by superoxide ($\cdot O_2^-$) (54) and is not susceptible to tolerance development (34, 37). As such, the vascular actions of HNO may be preserved under disease conditions, whereas those of NO• are compromised (*i.e.*, during oxidative stress). Thus, HNO

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Table 1. Distinct Pharmacology of HNO versus NO• in the Vasculature

Properties	*Nitroxyl (HNO)*	*Nitric oxide (NO•)*
General properties		
Thiol reactivity	High reactivity	No direct reactivity
Heme protein targets	Fe^{3+}-heme > Fe^{2+}-heme	Fe^{2+}-heme
Biomarker of activity	Plasma CGRP	Plasma cGMP
$^{\cdot}O_2^-$ reactivity	Resistant to scavenging by $^{\cdot}O_2^-$	Scavenged by $^{\cdot}O_2^-$
Pharmacological inhibitors	L-cysteine	Carboxy-PTIO
	N-acetyl-L-cysteine	Hydroxocobalamin
	Dithiothreitol	
Endogenous generation		
Sources	Coupled NOS	Coupled NOS
	Uncoupled NOS	S-Nitrosothiols
	NOS intermediates	Nitrite and nitrate
	S-Nitrosothiols	
	Reduction of NO•	
EDRF	Large and small arteries	Large and small arteries
EDHF (atypical)	Small arteries	Small arteries
Mediates spreading vasodilatation	Small arteries	Absent
Vasodilator activity		
Vasodepressor	Lowers blood pressure	Lowers blood pressure
Vasorelaxant	Large and small arteries	Large and small arteries
Tolerance	Resistant	Develops tolerance
sGC/cGMP	Large and small arteries	Large and small arteries
	Fe^{2+}-sGC	Fe^{2+}-sGC
	Conversion to NO• required?	Direct activation
K^+ channels	cGMP-dependent activation	cGMP-independent activation
• Rat mesenteric arteries	K_v channels	K_{Ca} channels
• Rat coronary arteries	K_{ATP}	K_{Ca} channels
• Mouse mesenteric arteries	K_v	Unknown
CGRP	Rat coronary arteries	Unknown
Vascular superoxide generation		
NADPH oxidase activity	Suppression following acute administration (*in vitro*)	Suppression following prolonged administration (*in vitro*)
Mechanism	cGMP-independent?	cGMP-independent
Cellular proliferation		
VSMC	Inhibition (high concentration)	Inhibition (high concentration)
	S-phase cell cycle arrest	G_0/G_1 cell cycle arrest
	Stimulation?	Stimulation (low concentration)
Endothelial cell	Inhibition?	Stimulation
Platelet activity	Anti-aggregatory	Anti-aggregatory
Signaling	sGC/cGMP	sGC/cGMP
	cAMP	cAMP

donors may offer a superior alternative to traditional nitrovasodilators for the treatment of vascular dysfunction. This review discusses the vasoprotective actions of HNO in the context of cardiovascular health and disease, exploring the role of HNO as an endogenous vascular signaling molecule, as well as the mechanisms via which it modulates vascular function and the therapeutic potential of HNO donors in the treatment of vascular disease.

Chemical and Biological Properties of HNO

Prior to discussing the vasoprotective actions of HNO, it is important to briefly consider the chemistry and biological targets of this nitrogen oxide. HNO has been shown to be a weak acid with a pK_a value of approximately 11.4 (4), suggesting that at physiological pH, HNO rather than the nitroxyl anion (NO^-) will predominate. HNO is highly reactive and undergoes rapid dimerization and dehydration to nitrous oxide (N_2O) (51). As such, HNO cannot be stored as a stable molecule and is typically studied using HNO donor compounds. Readers are referred to Miranda (51) for a comprehensive review on the chemistry of HNO.

HNO donors are essential tools to elucidate the biological actions of HNO, and in particular Angeli's salt, discovered over 100 years ago, has thus far been the mainstay of the HNO research field (35). Angeli's salt ($Na_2N_2O_3$) spontaneously decomposes to generate HNO and nitrite (NO_2^-) (32), however, its short half-life ($\sim$2.5 minutes) and concomitant release of NO_2^- confers limitations on its usefulness. Isopropylamine NONOate (IPA/NO), a primary amine diadiazeniumdiolate, has more recently been used to study HNO-induced effects in the cardiovascular system (52). Yet, whilst IPA/NO spontaneously decomposes at physiological pH to generate HNO and exerts similar physiological effects as Angeli's salt (52), it too has a short half life ($\sim$2.3 minutes), may also generate NO• (at pH < 7), and its nitrosamine byproduct may exert nonspecific effects (35). Ultimately, a pure and longer-lasting HNO donor is

required to further our understanding of the biological actions of HNO.

Despite the limitations of currently available HNO donors, they have enabled the pharmacology of this nitrogen oxide to be substantially delineated. Indeed, the biological activity of HNO is governed predominantly by its high reactivity with metallo- and thiol-containing proteins (22, 61). Thus, HNO reduces metals such as iron, copper, and manganese (22, 53, 58) and preferentially targets ferric (Fe^{3+}) versus ferrous (Fe^{2+}) heme groups in a number of proteins (53). In the vasculature, the heme-containing protein, sGC, represents a major cellular target of HNO. Moreover, the direct interaction of HNO with thiols underlies many of the distinct pharmacological actions of HNO versus NO• (35) and may direct the actions of HNO to thiol-containing receptors, ion channels, and enzymes in the blood vessel wall. Studies exploring the vasoprotective properties of HNO have exploited its high reactivity with thiols to distinguish its actions from those of NO• and provide evidence for its endogenous generation. Thus, high concentrations of thiols such as L-cysteine, *N*-acetyl-L-cysteine (NAC), and dithiothreitol will attenuate the actions of HNO but not those attributable to NO• (20, 33, 34, 63, 64). Conversely, NO• scavengers such as carboxy-PTIO ((2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxy-3-oxide)) and hydroxocobalamin will scavenge NO• but not HNO (18, 33, 34, 42, 83).

Employing HNO donors such as Angeli's salt and IPA/NO and thiol-based HNO scavengers, the capacity for HNO to modulate vascular tone, $^{\cdot}O_2^-$ generation, VSMC proliferation and platelet function has been examined.

HNO as an Endothelium-Derived Relaxing Factor

In 1980, Furchgott and Zawadzki identified that a factor was released from the endothelium that evoked VSMC relaxation (24). This endothelium-derived relaxing factor (EDRF) was subsequently identified as NO• (60) and its biosynthetic pathway rapidly elucidated (55). Within the endothelium, nitric oxide synthase (NOS) catalyses NADPH-dependent oxidation of L-arginine to form the unstable intermediate N-hydroxy-L-arginine (NOHA), into which O_2 is incorporated to yield NO• and L-citrulline (55). Following synthesis, NO• diffuses rapidly to the VSMC whereby it stimulates sGC to form cGMP that can interact with a number of downstream targets, including cGMP-dependent protein kinases (cGKs), phosphodiesterases (PDEs), and cGMP-modulated cation channels to cause vasorelaxation (40).

The importance of NO• as an endogenous regulator of vascular tone is irrefutable, yet over the last 10 years evidence has emerged that NO• may not be the sole endothelial-derived nitrogen oxide, with HNO likely to also play such a role (3, 23, 45, 83). Thus, like NO•, HNO elevates VSMC cGMP to mediate vasorelaxation (23, 34) and can potentially be generated via a number of biosynthetic pathways in the vasculature (Fig. 1). These include direct production of HNO from NOS itself, whereby HNO serves as an intermediate in the conversion of L-arginine to NO• (29, 72). In particular, superoxide dismutase (SOD) facilitates oxidation of HNO to NO•. Moreover, reduced levels of the NOS cofactor, tetrahydrobiopterin (BH_4), results in the partial uncoupling of NADPH oxidation and NO• synthesis to promote the production of HNO over NO• (71). HNO can also be formed after oxidative degradation of the NOS intermediate, NOHA (66). NOHA represents a feasible biosynthetic pathway for the generation of HNO *in vivo*, given it is detected in plasma (27) and produced by some cells (90). In addition, heme protein-mediated oxidation of hydroxylamine (NH_2OH), a product of cellular and NOS metabolism, leads to HNO generation (15). Additionally, HNO can be formed from non-NOS sources. Whilst a direct reduction of NO• to HNO is unlikely to occur spontaneously (4, 61), a number of enzymes catalyze this reaction, including mitochondrial cytochrome c, ubiquinol, hemoglobin, xanthine oxidase, and manganese SOD (35, 61). Finally, S-nitrosothiols have also been known to generate HNO via S-thiolation, a reaction between S-nitrosothiols and other thiol species (87).

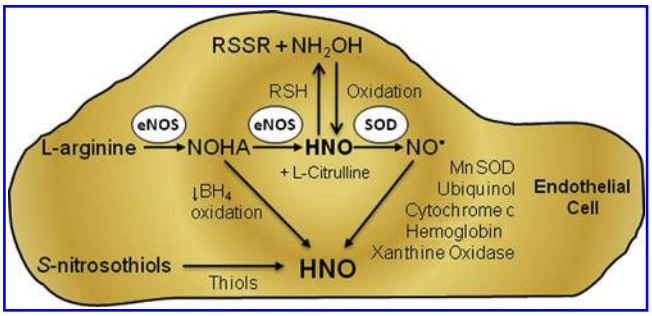

FIG. 1. Putative endogenous sources of nitroxyl (HNO) in the vascular endothelium. Biochemical studies indicate that HNO can be synthesised via nitric oxide (NO) synthase (eNOS)-dependent and -independent pathways. HNO may be formed directly from eNOS itself and subsequently oxidized to NO• by superoxide dismutase (SOD). A depletion in the eNOS cofactor, tetrahydrobiopterin (BH_4) or oxidation of the eNOS intermediate, N-hydroxy-L-arginine (NOHA) or by-product, hydroxylamine (NH_2OH) leads to HNO generation. HNO can also be formed directly from S-nitrosothiols or via enzymatic reduction of NO•.

Whilst definitive proof for the endogenous generation of HNO is absent due to the lack of direct and sensitive detection methods for HNO in mammalian cells, its role as an EDRF can be inferred from pharmacological studies. Thus, in large conduit arteries, the profile of the EDRF response resembles HNO more closely than NO•. For example, the HNO scavenger L-cysteine reduces HNO- and ACh-mediated vasorelaxation in rat (18) and mouse (83) aortae, yet responses to NO• gas are unchanged or enhanced in the presence of L-cysteine. We have shown evidence for a similar contribution of HNO to ACh-mediated vasorelaxation in resistance-like arteries, particularly following inhibition of endothelium-derived hyperpolarizing factor (EDHF) (3). Together, these findings suggest that HNO contributes, at least in part, to the EDRF response previously attributed to NO• and that these two redox siblings work in concert to mediate endothelium-dependent vasorelaxation.

It is likely that in the blood vessel wall, HNO is derived predominantly from endothelial NOS (eNOS), as the component of endothelium-dependent relaxation attributed to HNO is, for the most part, sensitive to the NOS inhibitor N-nitro-L-arginine methyl ester (L-NAME) (3). Interestingly, neuronal NOS (nNOS) may also serve as a source of HNO *in situ*, given the attenuation of nitrergic transmission by

L-cysteine (11, 42). Whether HNO can also be generated endogenously from non-NOS sources is unclear. Our finding in rat small mesenteric arteries that L-NAME does not abolish the component of endothelium-dependent relaxation attributed to HNO (3) may be indicative of the release of HNO from a non-NOS source such as preformed thiol stores (87) or, alternatively, incomplete inhibition of NOS by L-NAME.

Together with its ability to cause vasorelaxation, exogenous HNO induces VSMC hyperpolarization via opening of K_v channels (33). As such, endogenous HNO may also serve as an EDHF distinct from the classical EDHF, which is NOS-independent and targets calcium-sensitive K^+ channels (K_{Ca}) channels (17). Evidence in support of this concept comes from our recent findings in rat small mesenteric arteries where, following negation of EDHF (by K_{Ca} inhibition), a hyperpolarization response to ACh persisted which, like that of HNO, was sensitive to K_v channel inhibition (3). Similarly, in mouse mesenteric arteries, HNO-induced VSMC hyperpolarization contributes to ACh-mediated vasorelaxation (3). These findings highlight a role for endogenous HNO in local VSMC hyperpolarization and vasodilatation.

We have now advanced this concept further, with recent evidence that exogenous HNO can initiate, and endogenous HNO mediates (in response to ACh) spreading vasodilatation in pressurized rat small mesenteric arteries (88). Spreading vasodilatation is dependent upon VSMC hyperpolarization and is of physiological importance (16). Thus, the local action of a vasodilator is conducted upstream to ensure a significant drop in vascular resistance and thereby a sufficient increase in tissue perfusion. Whilst endothelium-derived HNO appears to initiate spreading vasodilatation, similar observations have not been made with NO• (86).

The identification of HNO as an EDRF and EDHF in the vasculature has heightened interest in this nitrogen oxide (45). Together, NO• and HNO appear to play an integral role in the control of vascular tone and we hypothesize that HNO production and/or bioavailability is preserved during oxidative stress and in disease. Thus, uncoupled NOS preferentially generates HNO over NO• (71), HNO is resistant to scavenging by $\cdot O_2^-$ (54), and disease-associated thiol depletion (6) may lead to a reduction in HNO scavenging. As such, HNO may compensate, at least in part, for a loss of endogenous NO• (3) and classical EDHF (17) under pathophysiological conditions. We eagerly await the future development of methods to detect HNO production in biological systems such that the endogenous generation of HNO can be proved conclusively.

Vasodilator Properties of HNO Donors

HNO donors are known to be potent vasodilators both *in vitro* and *in vivo* (35). The seminal work of Fukuto *et al.* (23) demonstrated an ability of the HNO donor, Angeli's salt, to induce relaxation of isolated rabbit aorta and bovine intrapulmonary artery. Such an effect of HNO was distinguished from that of NO• via the sensitivity of HNO-mediated vasorelaxation to scavenging by thiols (64). Subsequently, Angeli's salt has been shown to induce vasorelaxation in other large conduit (18, 34, 83) and small resistance-like arteries (3, 19, 33). Similarly, in the intact circulation, Angeli's salt dilates the rat coronary (20) and cat pulmonary (12) vascular beds and decreases mean arterial blood pressure in anaesthetized rabbits (44), conscious dogs (62, 63), and rats (37). Although HNO appears to be a preferential venodilator *in vivo* (63), such an effect is lost in the setting of heart failure with equivalent arterial and venous dilation observed (62). Importantly, there is now evidence that HNO is a potent vasodilator in the human vasculature, with Angeli's salt inducing vasorelaxation of human isolated radial arteries (Andrews *et al.*, unpublished, Fig. 2).

sGC/cGMP signaling

HNO signals predominantly via the sGC/cGMP pathway to mediate vasorelaxation (Fig. 3). Thus, Angeli's salt elicits an increase in vascular cGMP levels (23, 34) and its vasorelaxant responses are resistant to the NO• scavengers carboxy-PTIO and hydroxocobalamin (3, 18–20, 33, 83) and markedly attenuated by the sGC inhibitor 1H-(1,2,4)oxadiazole(4,3,-a)quinoxaline-1-one (ODQ) (3, 19, 20, 33, 34, 83). Interestingly, HNO-mediated vasorelaxation is more susceptible to inhibition by ODQ than vasodilator responses to NO gas or NO• donors such as diethylamine-NONOate (DEA/NO) (3, 19, 33, 34, 83). Together such findings indicate that HNO directly targets sGC, yet this concept remains a matter of contention.

Biochemical studies originally suggested that NO• was the only nitrogen oxide capable of activating sGC (14), with HNO

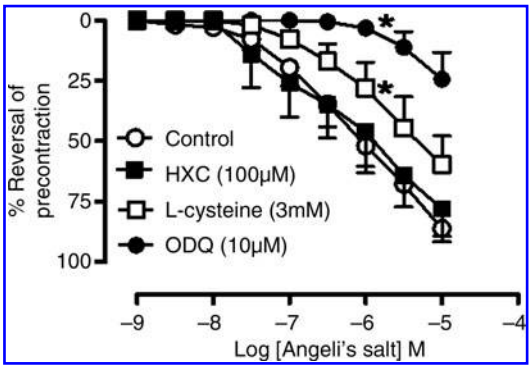

FIG. 2. HNO-mediated relaxation of human arteries. Concentration-dependent relaxation to the HNO donor, Angeli's salt, in radial arteries isolated from patients undergoing coronary artery bypass surgery. Responses were obtained in the absence (○, $n = 8$) and presence of the NO• scavenger, hydroxocobalamin (HXC, 100 μM, 15 min; ■, $n = 6$), HNO scavenger, L-cysteine (3 mM, 3 min; □, $n = 8$), and soluble guanylyl cyclase inhibitor, ODQ (10 μM, 30 min; ●, $n = 8$). In brief, arteries (2 mm segments) were mounted in organ baths, maintained in carbogen bubbled (95% O_2, 5% CO_2) Krebs' solution at 37°C and changes in isometric tension recorded (34). Following equilibration at an optimal resting tension of 2 *g*, arteries were maximally contracted with a K^+-depolarizing solution (124 mM K^+, KPSS). Subsequently, cumulative concentration-response curves to Angeli's salt were constructed in arteries precontracted to ~50% KPSS with endothelin-1 (10–100 nM). Maximal relaxation was ensured by using sodium nitroprusside (10 μM) at the conclusion of each concentration-response curve. Values are plotted as percentage reversal of the pre-contraction and expressed as mean ± s.e. mean, where n = number of donors. *$p < 0.05$; for treatment concentration-response curve vs untreated control (2-way ANOVA, Bonferroni post-hoc test).

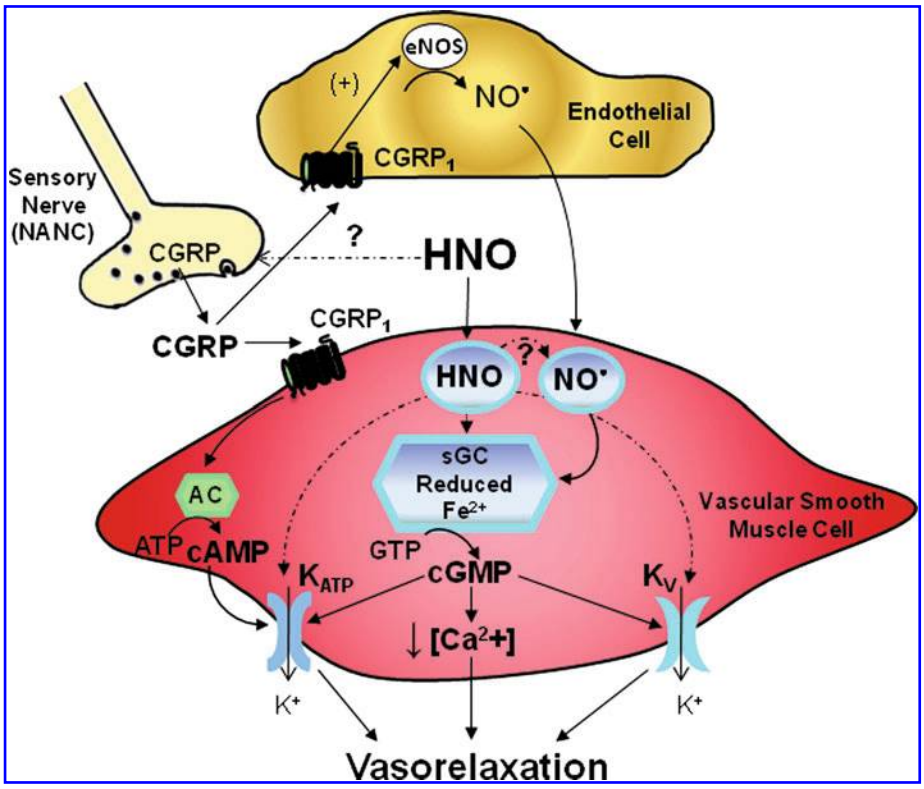

FIG. 3. Vasodilator actions of HNO. HNO induces vasorelaxation predominantly via stimulation of soluble guanylyl cyclase (sGC) and subsequent increase in cGMP concentration. Whether HNO requires intracellular conversion to NO• prior to activation of sGC remains unclear. HNO can also activate both voltage-gated (K_v) and ATP-sensitive (K_{ATP}) potassium channels via a cGMP-dependent mechanism. In the coronary vasculature, at least, HNO may also stimulate release of the vasodilator neuropeptide, calcitonin-gene related peptide (CGRP) from nonadrenergic noncholinergic (NANC) nerves. CGRP targets $CGRP_1$ receptors on the endothelium and vascular smooth muscle to release NO• and activate adenylate cyclase (AC), respectively. The subsequent increase in cAMP may lead to activation of K_{ATP} channels.

presumably requiring oxidation to NO• prior to sGC stimulation. However, those studies were performed in the presence of high concentrations of thiols, sufficient to scavenge HNO and possibly negate its effect. Recent studies that have re-examined the interaction of HNO with sGC have now yielded contrasting results (50, 89). Thus, in the absence of extracellular thiols and under anaerobic conditions, Miller and colleagues demonstrated an ability of the HNO donors, Angeli's salt and 1-nitrosocyclohexyl trifluroacetate (NCTFA), to directly stimulate purified bovine lung sGC (up to 60-fold), an effect independent of oxidation of HNO to NO• (50). In contrast, utilizing purified bovine lung sGC and cultured endothelial cells, Zeller and colleagues reported that Angeli's salt had no significant effect on sGC activity and cGMP levels in the absence of SOD (89). Given that Cu(II), Zn(II)-SOD (SOD1) has been shown to facilitate the conversion of HNO to NO• in a cell-free assay (58), those investigators proposed that Angeli's salt activates sGC predominantly via SOD-induced oxidation of HNO to NO• (89). Whether the intracellular concentration of SOD is sufficient to facilitate such a conversion in the intact cell remains to be determined. Clearly further work is required to elucidate the precise mechanisms by which HNO activates sGC.

Aside from the mechanism(s) of sGC activation by HNO, a number of other interesting observations have been made regarding its interaction with that heme-containing protein. First, high concentrations of Angeli's salt (0.1 m*M*) cause inhibition of sGC activity via an apparent oxidative modification of cysteine thiols (50). From a therapeutic standpoint, such an action of HNO may be beneficial in that it would limit excessive vasodilatation by HNO donors. Second, the preference of HNO for Fe^{3+} versus Fe^{2+} heme groups (53) suggests that HNO may target the oxidized (Fe^{3+}) rather than reduced (Fe^{2+}) form of sGC. Given the predominance of NO•-insensitive, oxidized sGC in the diseased vasculature (76), such a property of HNO may allow its vasorelaxant activity to be sustained and/or enhanced in the setting of disease whereas traditional NO• donors may be compromised. However, surprisingly recent studies failed to demonstrate an ability of HNO to stimulate oxidized sGC (50, 89) and like NO•, HNO was found to activate the reduced, ferrous heme on sGC. Such findings may be indicative of a kinetically slow interaction of HNO with the ferric form of sGC as compared with its ability to dimerize or react with thiols (50, 89). In addition, the presence of multiple cysteine residues on sGC may contribute to the resistance of ferric sGC to reductive nitrosylation by HNO (50).

cGMP-independent signaling

Whilst the cGMP-dependence of HNO induced vasorelaxation *in situ* is evident, the HNO donors, Angeli's salt and IPA/NO, do not elevate plasma cGMP following intravenous administration (52, 62, 63). Such discrepancies may be indicative of plasma cGMP levels not reflecting changes at the cellular level, differential sensitivity in the detection of plasma versus cellular cGMP, and/or HNO targeting sGC/cGMP-independent pathways *in vivo*. Indeed, administration of Angeli's salt and IPA/NO to conscious dogs leads to an elevation in plasma levels of the sensory neuropeptide, CGRP, an effect not observed with NO• donors (52, 62, 63). CGRP serves as a vasodilator, targeting CGRP1 receptors on the endothelium and VSMC to stimulate NOS and adenylate cyclase, respectively (Fig. 3) (7). Interestingly, CGRP appears to contribute, at least in part, to Angeli's salt-mediated vasorelaxation in the rat coronary vasculature (20). The

mechanisms underlying HNO-mediated release of CGRP from sensory neurons remain to be elucidated and its role *in vivo* is unclear given that vasodepressor responses to HNO donors are unchanged in the presence of the CGRP receptor antagonist $CGRP_{8-37}$ (63). Nevertheless, CGRP remains a valuable biomarker to distinguish between the effects of HNO and NO• in the circulation.

K^+ channel activation

Another important pathway involved in HNO-induced vasodilatation is through activation of K^+ channels (Fig. 3). Specifically, we have identified an ability of HNO to target K_v (3, 19, 20, 33) and ATP-sensitive K^+ channels (K_{ATP}) (20) in the resistance vasculature. For instance, Angeli's salt-induced relaxation of small mesenteric (rat and mouse) and coronary (rat) arteries is attenuated by the K_v channel inhibitor, 4-aminopyridine (4-AP) (3, 19, 33) and K_{ATP} channel inhibitor, glibenclamide (20), respectively. Importantly, electrophysiological studies have confirmed an ability of HNO to target K_v channels with Angeli's salt-induced VSMC hyperpolarisation abolished by 4-AP (19). Although NO• can also activate K_v channels *in vitro* and *in vivo* (75), this is not evident in rat small mesenteric arteries. Rather, in these vessels, NO• targets K_{Ca} channels via a cGMP-independent mechanism (65). Moreover, we have shown that HNO is more efficacious in eliciting VSMC hyperpolarization than NO• in resistance arteries (19). Together, these findings serve to further highlight the distinct vascular actions of NO• and HNO and indicate that the nature of HNO signaling (*i.e*, type of K^+ channel activated, role of CGRP) may be dependent on the vessel size and vascular bed.

Given the reactivity of HNO with thiols (35, 61), it is tempting to speculate that HNO directly modifies cysteine residues on K_v and K_{ATP} channels to modulate their activity. However, recent findings suggest that HNO primarily activates K^+ channels via a cGMP-dependent mechanism. Thus, in rat small mesenteric arteries, sGC inhibition (ODQ) virtually abolishes Angeli's salt-induced VSMC hyperpolarization (19). Furthermore, VSMC hyperpolarization in response to the cGMP elevating agent YC-1 (a NO•-independent stimulator of sGC) is attenuated by 4-AP (19). Together these findings indicate that HNO induces VSMC hyperpolarization via cGMP-dependent activation of K_v channels.

It is clear that the vascular actions of HNO and NO• differ with respect to the K^+ channels they target and their dependence upon sGC/cGMP signaling (*i.e,* HNO > NO•). However, given both HNO and NO• will lead to an elevation in vascular cGMP levels, it is difficult to reconcile the finding that HNO, but not NO•, targets K^+ channels via a cGMP-dependent mechanism. It is possible that the distinct chemistry of these two redox siblings confers such biological differences. Given that HNO is resistant to scavenging by $^{\cdot}O_2^-$, we predict that it has a longer intracellular half-life than NO•, potentially leading to alternate cellular targets and modes of activation. In addition, cellular thiol concentrations may compartmentalise the actions of HNO such that it targets membrane-bound molecules where the thiol concentration is low (85).

HNO's distinct pharmacology (Table 1), in conjunction with its vasodilator capacity, may confer therapeutic advantages over traditional NO• donors. A major limitation of currently used nitrovasodilators such as the organic nitrate, GTN, is that they develop tolerance with continued use. The mechanisms underlying tolerance development are likely to be multifactorial, involving reduced biotransformation of GTN, desensitization of sGC, increased activity of cGMP-degrading PDEs, or reduced NO• bioavailability (41). Importantly we have shown that unlike GTN, Angeli's salt does not develop tolerance following administration either acutely *in vitro* (34) or chronically *in vivo* (37). Moreover, cross-tolerance is not observed such that the vasodilator efficacy of HNO is sustained in animals rendered tolerant to GTN (34, 37). This is of particular relevance in a clinical setting as HNO donors may be of use in patients resistant to the effects of GTN and administered alone or in conjunction with traditional nitrovasodilators for the treatment of vascular pathologies such as angina and heart failure.

In addition to tolerance development, a loss of potency of NO•-based therapeutics *per se* is observed in a number of cardiovascular disorders (47, 70) as a consequence of the high degree of oxidative stress associated with these pathologies (21, 48). Thus, NO• bioavailability could be reduced due to scavenging by the reactive oxygen species (ROS), $^{\cdot}O_2^-$ generating the powerful oxidant, peroxynitrite ($ONOO^-$). $ONOO^-$ can also reduce NO• efficacy by oxidizing the NO• target sGC, to its NO-insensitive ferric or heme-free form (76). In contrast to NO•, HNO is resistant to scavenging by $^{\cdot}O_2^-$ (54), may target oxidized ferric heme-proteins, does not develop tolerance, and its bioavailability may be augmented in the face of disease-associated thiol depletion (35). As such, the efficacy of HNO donors may be preserved and/or enhanced under pathological conditions. Whilst this concept remains to be fully explored, evidence to date indicates that the vasodilator activity of HNO is sustained in heart failure. Thus, the vasodepressor action of Angeli's salt in conscious dogs (63) appears to be maintained in the setting of acute heart failure (62). Further work is needed to determine if the efficacy of HNO donors is similarly preserved in other cardiovascular disease states.

Importantly, HNO can induce relaxation of human arteries (Fig. 2), with a similar potency and efficacy as GTN. Coupled with its lack of tolerance development and potential for preserved bioavailability under conditions of oxidative stress, HNO donors may represent a realistic novel therapeutic approach to the treatment of vascular disorders such as angina, hypertension, and heart failure.

Anti-Aggregatory Properties of HNO Donors

NO• plays an important role in the prevention of platelet adhesion and aggregation (82). Like its redox sibling, HNO also modulates platelet function by exerting anti-aggregatory actions. Thus, Angeli's salt has been shown to inhibit aggregation of human platelets induced by adenosine diphosphate (ADP), arachidonic acid, adrenaline, thrombin, and collagen (5, 56). A role for HNO in these actions was confirmed by partial reversal of the anti-aggregatory effects of Angeli's salt by the HNO scavenger, L-cysteine (5). Moreover, HNO decreases markers of platelet activation (5) and has been found to modify cysteine residues in up to 10 platelet proteins, some of which are involved in cytoskeletal changes, metabolic processes, and platelet activation (30).

Currently, the mechanisms by which HNO inhibits platelet aggregation remain to be fully elucidated. Most of the inhib-

itory effects of NO• in platelets are via the sGC/cGMP pathway (82). Similarly, HNO appears to target platelet sGC, given that Angeli's salt increases platelet cGMP levels and its anti-aggregatory actions are partially reversed by the sGC inhibitor, ODQ (5). The effects of cGMP in the platelet are transduced predominantly via cGMP-PDEs and cGKs (82). Interestingly, despite an ability of Angeli's salt to elevate platelet cGMP, its anti-agreggatory effect in human platelets is resistant to a cGK inhibitor, yet reversed by a cAMP-dependent protein kinase (cAK) inhibitor (5). Similar observations in human platelets have been made with NO• donors (36, 46). Taken together, these findings are indicative of potential cross-talk between the cGMP and cAMP signaling pathways following platelet stimulation with HNO (Fig. 4), and PDE3 may serve as the link. Thus, binding of cGMP to PDE3 inhibits its hydrolysis of cAMP, which can then accumulate (36, 46) subsequently activating cAK and inhibiting platelet aggregation. Clearly, the potential role of cAMP/cAK in the regulation of platelet function by HNO warrants further investigation.

With an ability to inhibit platelet aggregation, HNO donors may be of use in the treatment of atherothrombotic syndromes and offer advantages over traditional nitrovasodilators. Thus, patients with cardiovascular diseases such as angina, ischemic heart disease, and diabetes often display resistance to the anti-aggregatory effects of NO• (9, 84). Such resistance is independent of prior exposure to organic nitrates and may arise as a consequence of decreased NO• efficacy due to scavenging by $^{\cdot}O_2^-$ and dysfunction of sGC (9). Given the resistance of HNO to scavenging by $^{\cdot}O_2^-$, it is anticipated that HNO donors will retain their anti-aggregatory properties under conditions of oxidative stress. Indeed, the anti-aggregatory effect of Angeli's salt is sustained in patients suffering from sickle cell disease (56), a condition associated with vascular oxidative stress (25). Furthermore, we have made similar observations in platelets from hypercholesterolemic mice (Bullen *et al.*, unpublished). Together, these results indicate that platelets do not develop resistance to HNO donors during disease and as such these compounds may serve as effective anti-aggregatory agents.

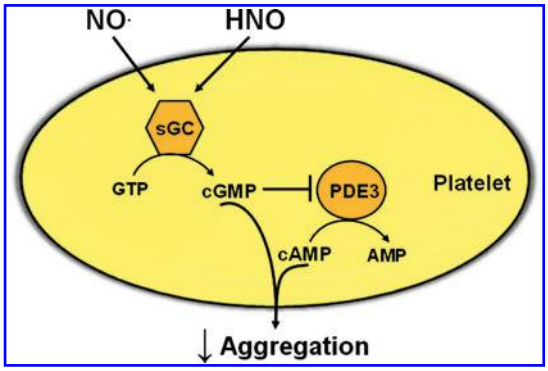

FIG. 4. Mechanisms via which HNO inhibits platelet aggregation. HNO inhibits human platelet aggregation via cGMP-dependent actions. Stimulation of platelet soluble guanylyl cyclase (sGC) by HNO leads to an increase in cGMP which may directly impair aggregation. In addition, binding of cGMP to the cAMP-degrading phosphodiesterase (PDE3) inhibits its activity leading to an increase in cAMP and attenuated aggregation. Note NO· can also target cGMP- and cAMP-dependent pathways to inhibit platelet aggregation.

Whilst it is well recognized that NO• also modulates the function of other blood cell types such as leukocytes (2), little is known with respect to the effects of HNO on leukocyte adhesion, rolling, and intravasation. To date, HNO has been shown to stimulate human neutrophil migration (81), and indirect evidence suggests that it may increase neutrophil accumulation during myocardial ischemia (44). Future studies exploring HNO-mediated modulation of leukocyte function will be of importance, particularly in light of recent findings that the innate and acquired immune systems are central to the pathology of vascular diseases such as hypertension (26).

$^{\cdot}O_2^-$ Suppressing Properties of HNO Donors

An augmentation of ROS production and/or impairment in ROS metabolism is thought to lead to vascular oxidative stress, which plays a pivotal role in the pathogenesis of numerous cardiovascular pathologies (21, 48). Indeed, increased levels of ROS such as $^{\cdot}O_2^-$, hydrogen peroxide (H_2O_2), hydroxyl ($OH^{\cdot}$), and $ONOO^-$ cause many of the vascular changes associated with cardiovascular disease, including endothelial dysfunction, vascular remodeling, and inflammation (78). Although there are several sources of pathological ROS, the family of enzymes called the NADPH-oxidases are emerging as strong candidates for the excessive ROS production that leads to oxidative stress (49, 74). As such, an ability to limit ROS production by NADPH-oxidase and/or other sources is a desirable trait of a vasoprotective drug.

Evidence exists that HNO limits oxidative stress via a number of mechanisms. For example, HNO may serve as a one-electron reductant via donation of its hydrogen atom. In nonvascular cells, HNO has been shown to inhibit membrane lipid peroxidation (43) and stimulate the expression and activity of the antioxidant protein, heme oxygenase-1 (56). Moreover, we have preliminary evidence that HNO suppresses activity of the $^{\cdot}O_2^-$ generating enzyme, NADPH oxidase, in neonatal rat cardiomyocytes (68). Based upon these observations, it is anticipated that HNO donors will also limit NADPH oxidase activity in the vasculature. We have previously shown that in human endothelial cells, prolonged treatment with the NO• donor DETA/NONOate leads to inhibition of NADPH oxidase-stimulated $^{\cdot}O_2^-$ production, possibly via S-nitrosylation of its regulatory cytosolic subunit p47phox (73). Pilot studies also indicate that the HNO donors, Angeli's salt and IPA/NO, rapidly attenuate $^{\cdot}O_2^-$ production by NADPH oxidase, in both the cerebral and peripheral vasculature (Miller et al., unpublished, Fig. 5). Such a property of HNO appears to be sGC/cGMP-independent and may arise as a consequence of post-translational modifications of reactive cysteine thiols within NADPH oxidase. By suppressing $^{\cdot}O_2^-$ generation, HNO donors may help to preserve the bioavailability of endogenous NO• and limit oxidation of sGC, thereby maintaining vascular NO•- and HNO-mediated signaling. Given that NADPH oxidase has been identified as a major contributor to oxidative burden in the vasculature (74), an ability of HNO to modulate its activity is of significant potential therapeutic benefit and the mechanisms underlying such actions warrant further investigation.

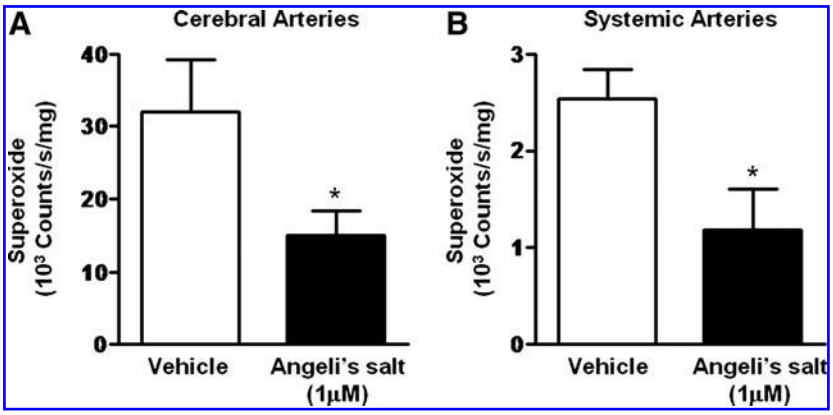

FIG. 5. Evidence for HNO-induced suppression of NAPDH-oxidase derived superoxide in the vasculature. The effect of acute administration of Angeli's salt (1 μM) upon angiotensin II (0.1 μM, activator of NADPH oxidase)-stimulated superoxide production in **(A)** cerebral (pooled basilar and middle cerebral) and **(B)** systemic (common carotid, 2–3 mm segments) arteries isolated from male C57Bl6/J mice. Following excision, arteries were placed in Krebs-HEPES solution and transferred to a 96-well Opti-plate containing lucigenin (5 μM) and angiotensin II (0.1 μM) in the absence and presence of Angeli's salt (1 μM). Lucigenin-enhanced chemiluminescence was measured over 30 min using a Plate Chameleon Luminescence Reader (38). Background counts were subtracted and superoxide production by each vessel segment was expressed as counts/s/mg dry tissue weight. Results are expressed as mean ± s.e. mean ($n = 6$–9 for both). *$p < 0.05$ versus vehicle (0.01 *M* NaOH), unpaired *t*-test.

Regulation of Growth and Proliferation of Vascular Cells by HNO Donors

Amongst its vasoprotective actions, NO• is known to regulate VSMC proliferation and migration with stimulatory and inhibitory effects observed at low and high concentrations, respectively (40). The antiproliferative actions of NO• have the potential to limit VSMC migration and mitogenesis in atherosclerosis (69). Conversely, NO• stimulates endothelial cell proliferation, leading to endothelial regeneration following vascular injury (1). Until recently, little was known with respect to HNO-mediated regulation of endothelial and VSMC proliferation and migration.

However, Tsihilis and colleagues (80) have recently shown that a high concentration of IPA/NO (1 m*M*) inhibits proliferation, but not migration, of VSMC and endothelial cells in culture. This effect of IPA/NO in VSMC was via S-phase cell cycle arrest, yet NO• induces G_0/G_1 cell cycle arrest in the same cell type (80). In addition, following topical application of IPA/NO powder (10 mg) to the periadventitial surface of carotid arteries immediately post balloon injury, a modest reduction in neointimal hyperplasia together with reduced VSMC proliferation and macrophage infiltration was observed 14 days later (80).

Whilst IPA/NO limited neointimal hyperplasia following vascular injury, it also appeared to attenuate endothelial regeneration (80). Although it is currently unclear if such effects were due to HNO itself or other components of IPA/NO decomposition (*i.e,* isopropylamine, isopropanol), such findings are indicative of a potential anti-angiogenic property of HNO. This notion is further supported by the observation that Angeli's salt reduces overall blood vessel density in mouse tumours with an associated trend for decreased serum levels of vascular endothelial growth factor (VEGF) (59). Such an anti-angiogenic activity may indicate potential for the use of HNO donors in the treatment of cancer, yet it also raises the question as to their ability to preserve endothelial integrity post vascular injury.

It is important to note that research on the vascular actions of HNO is in its infancy, and many important questions remain unanswered. Thus, whilst *in vivo* administration of IPA/NO led to a modest attenuation of neointimal hyperplasia and impaired endothelial regeneration, which was associated with high mortality (80), it remains to be determined if these effects were attributable to HNO itself or to the breakdown products of IPA/NO. Moreover, in the same study the mode of administration of IPA/NO (*i.e.,* topical application) prevents accurate determination of the effective concentration of HNO donor applied and may limit access of HNO across the blood vessel wall, as well as facilitating nonspecific effects such as a chemical interaction with the adjacent vagal nerve. Further investigation into the antiproliferative effects of HNO in the vasculature will be essential to ascertain the clinical feasibility of such an action of HNO donors.

Therapeutic Potential of HNO Donors

The therapeutic application of HNO-donating compounds is tenable given the HNO donor, cyanamide, is currently used in the treatment of chronic alcoholism with minimal adverse effects (13, 61). Attention is now being afforded to the potential use of HNO donors in the treatment of heart failure, given the unique ability of HNO to increase myocardial contractility and unload the heart (via vasodilatation) (62, 67). In fact, a pure, small molecule HNO donor, CXL-1020 developed by Cardioxyl Pharmaceuticals (Chapel Hill, NC), is currently being tested in a Phase I/IIA clinical trial in patients with stable heart failure and we await the outcomes of this trial with interest.

With respect to the vascular actions of HNO, its ability to induce vasodilatation, inhibit platelet aggregation, and suppress $^{\cdot}O_2^-$ generation, coupled with its resistance to scavenging by $^{\cdot}O_2^-$ and lack of tolerance development indicate that HNO donors may be of use in the treatment of vascular dysfunction associated with angina and atherothrombotic syndromes. The clinical efficacy of traditional nitrovasodilators, such as GTN, in these pathologies is limited by their susceptibility to tolerance development and potential resistance in platelets. As such, HNO donors may represent novel stand-alone or combination therapies (*i.e.,* with GTN), and be of particular use in those patients exhibiting tolerance and/or resistance to NO• treatment.

However, it should be recognized that the apparent therapeutic benefits of HNO may be tempered by possible nonspecific and toxic effects. Thus, toxic actions of HNO, albeit at high concentrations (2–4 m*M* Angeli's salt), have been reported in cells such as neurons (28) and involve DNA oxidation and thiol

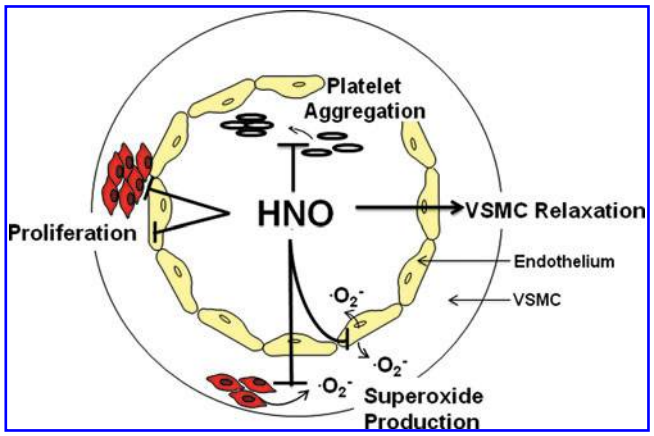

FIG. 6. Overview of the vasoprotective actions of HNO. HNO modulates several aspects of vascular function by causing vascular smooth muscle cell (VSMC) relaxation, inhibiting platelet aggregation, suppressing superoxide ($\cdot O_2^-$) generation, and potentially limiting VSMC and endothelial cell proliferation. Importantly, HNO appears to be resistant to both scavenging by $\cdot O_2^-$ and tolerance development.

loss. Moreover, Angeli's salt has been shown to exacerbate ischemia-reperfusion injury in both the brain (10) and heart (44). It remains to be determined if such deleterious effects of HNO are due to its ability to decrease blood pressure and potentially reduce organ perfusion, stimulate neutrophil migration (77), or directly modulate cellular function. Despite these potential adverse effects of HNO, the anti-alcoholism drug, cyanamide, has few reported side effects in man and the long-term administration of Angeli's salt in animals is well tolerated (LD_{50} > 130 mg/kg) with no observable carcinogenesis (39).

Clearly more work is required before the therapeutic utility of HNO donors can be fully assessed. Nevertheless the vasoprotective actions of HNO coupled with its distinct pharmacology, as compared with NO•, confer potential for the use of HNO donors in the treatment of vascular disease.

Perspectives

The redox siblings, NO• and HNO, have distinct biological and pharmacological properties which are readily apparent in the cardiovascular system (35) (Table 1). In recent years, considerable attention has been afforded to HNO as it has been demonstrated to increase myocardial contractility and decrease cardiac preload in the setting of heart failure. Likely to be of equal therapeutic importance is the action of HNO in the vasculature, with evidence emerging that endogenous and exogenous HNO target novel signaling pathways to confer a number of vasoprotective properties.

Whilst the role of HNO as an endogenous modulator of vascular function remains to be proven conclusively, we should no longer consider NO• as the sole endothelium-derived nitrogen oxide. Rather, with the potential to be generated from NOS-dependent sources, HNO appears to work in concert with NO• to mediate endothelium-dependent vasodilatation and it may compensate for a disease-associated reduction in NO• bioavailability. The field now awaits the development of new approaches to detect HNO selectively in the intact cell and thus confirm its endogenous generation.

In the vasculature, HNO shares some similar features with NO•, such as an ability to induce vasodilatation, limit VSMC proliferation and $\cdot O_2^-$ generation, and inhibit platelet aggregation (Fig. 6). However, often in contrast to traditional nitrovasodilators, HNO donors appear to activate distinct vascular signaling mechanisms (*i.e.*, K_v and K_{ATP} channels, CGRP release), are not scavenged by $\cdot O_2^-$ nor do they develop vascular tolerance. Intriguingly, these properties may allow the vasoprotective actions of HNO to be preserved under conditions of oxidative stress in which those to NO• are compromised.

Although it is clear that HNO donors offer considerable advantages over traditional nitrovasodilators, a number of important issues must be addressed before the therapeutic potential of HNO donors can be fully realized. Namely, the potential for HNO to inhibit endothelial regeneration and exert nonspecific effects due to its high thiol reactivity, requires further investigation. A comprehensive evaluation of the efficacy of HNO donors under disease and oxidative stress conditions is also needed. In addition, pure, longer-acting HNO donors are urgently required for experimental evaluation in order to advance the field through the study of long-term vascular effects of HNO.

In summary, the vasoprotective actions of HNO coupled with its lack of tolerance development and potential for preserved bioavailability under conditions of oxidative stress indicate that HNO donors may represent novel strategies for the treatment of vascular dysfunction associated with diseases such as angina, hypertension, and atherosclerosis. Undoubtedly, as research continues in this area, further novel properties and therapeutic applications of HNO will emerge.

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References

1. Ahanchi SS, Tsihlis ND, and Kibbe MR. The role of nitric oxide in the pathophysiology of intimal hyperplasia. *J Vasc Surg* 45: A64–73, 2007.
2. Ahluwalia A, Foster P, Scotland RS, McLean PG, Mathur A, Perretti M, Moncada S, and Hobbs AJ. Antiinflammatory activity of soluble guanylate cyclase: cGMP-dependent downregulation of P-selectin expression and leukocyte recruitment. *Proc Natl Acad Sci USA* 101: 1386–1391, 2004.
3. Andrews KL, Irvine JC, Tare M, Apostolopoulos J, Favaloro JL, Triggle CR, and Kemp-Harper BK. A role for nitroxyl (HNO) as an endothelium-derived relaxing and hyperpolarizing factor in resistance arteries. *Br J Pharmacol* 157: 540–550, 2009.

4. Bartberger MD, Liu W, Ford E, Miranda K, Switzer CH, and Fukuto JM. The reduction potential of nitric oxide (NO) and its importance to NO biochemistry. *Proc Natl Acad Sci U S A* 99: 10958–10963, 2002.
5. Bermejo E, Saenz DA, Alberto F, Rosenstein RE, Bari SE, and Lazzari MA. Effect of nitroxyl on human platelets function. *Thromb Haemost* 94: 578–584, 2005.
6. Berry C, Brosnan MJ, Fennell J, Hamilton CA, and Dominiczak AF. Oxidative stress and vascular damage in hypertension. *Curr Opin Nephrol Hypertens* 10: 247–255, 2001.
7. Brain SD and Grant AD. Vascular actions of calcitonin gene-related peptide and adrenomedullin. *Physiol Rev* 84: 903–934, 2004.
8. Cheong E, Tumbev V, Abramson J, Salama G, and Stoyanovsky DA. Nitroxyl triggers Ca^{2+} release from skeletal and cardiac sarcoplasmic reticulum by oxidizing ryanodine receptors. *Cell Calcium* 37: 87–96, 2005.
9. Chirkov YY, Holmes AS, Chirkova LP, and Horowitz JD. Nitrate resistance in platelets from patients with stable angina pectoris. *Circulation* 100: 129–134, 1999.
10. Choe CU, Lewerenz J, Fischer G, Uliasz TF, Espey MG, Hummel FC, King SB, Schwedhelm E, Boger RH, Gerloff C, Hewett SJ, Magnus T, and Donzelli S. Nitroxyl exacerbates ischemic cerebral injury and oxidative neurotoxicity. *J Neurochem* 110: 1766–1773, 2009.
11. Costa G, Labadia A, Triguero D, Jimenez E, and Garcia–Pascual A. Nitrergic relaxation in urethral smooth muscle: Involvement of potassium channels and alternative redox forms of NO. *Naunyn Schmiedebergs Arch Pharmacol* 364: 516–523, 2001.
12. De Witt BJ, Marrone JR, Kaye AD, Keefer LK, and Kadowitz PJ. Comparison of responses to novel nitric oxide donors in the feline pulmonary vascular bed. *Eur J Pharmacol* 430: 311–315, 2001.
13. DeMaster EG, Redfern B, and Nagasawa HT. Mechanisms of inhibition of aldehyde dehydrogenase by nitroxyl, the active metabolite of the alcohol deterrent agent cyanamide. *Biochem Pharmacol* 55: 2007–2015, 1998.
14. Dierks EA and Burstyn JN. Nitric oxide (NO), the only nitrogen monoxide redox form capable of activating soluble guanylyl cyclase. *Biochem Pharmacol* 51: 1593–1600, 1996.
15. Donzelli S, Espey MG, Flores–Santana W, Switzer CH, Yeh GC, Huang J, Stuehr DJ, King SB, Miranda KM, and Wink DA. Generation of nitroxyl by heme protein-mediated peroxidation of hydroxylamine but not N-hydroxy-L-arginine. *Free Radic Biol Med* 45: 578–584, 2008.
16. Dora KA. Coordination of vasomotor responses by the endothelium. *Circ J* 74: 226–32, 2010.
17. Edwards G, Feletou M, and Weston AH. Endothelium-derived hyperpolarising factors and associated pathways: A synopsis. *Pflugers Arch* 459: 863–879, 2010.
18. Ellis A, Li CG, and Rand MJ. Differential actions of L-cysteine on responses to nitric oxide, nitroxyl anions and EDRF in the rat aorta. *Br J Pharmacol* 129: 315–322, 2000.
19. Favaloro JL and Kemp–Harper B. Redox variants of NO (NO. and HNO) elict vasorelaxation of resistance arteries via distinct mechanisms. *Am J Physiol Heart Circ Physiol* 296: H1274–H1280, 2009.
20. Favaloro JL and Kemp–Harper BK. The nitroxyl anion (HNO) is a potent dilator of rat coronary vasculature. *Cardiovasc Res* 73: 587–596, 2007.
21. Forstermann U. Oxidative stress in vascular disease: Causes, defense mechanisms and potential therapies. *Nat Clin Pract Cardiovasc Med* 5: 338–349, 2008.
22. Fukuto JM, Bartberger MD, Dutton AS, Paolocci N, Wink DA, and Houk KN. The physiological chemisty and biological activity of nitroxyl (HNO): The neglected, misunderstood, and enigmatic nitrogen oxide. *Chem Res Toxicol* 18: 790–801, 2005.
23. Fukuto JM, Chiang K, Hszieh R, Wong P, and Chaudhuri G. The pharmacological activity of nitroxyl: A potent vasodilator with activity similar to nitric oxide and/or endothelium-derived relaxing factor. *J Pharmacol Exp Ther* 263: 546–551, 1992.
24. Furchgott RF and Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288: 373–376, 1980.
25. George A, Pushkaran S, Li L, An X, Zheng Y, Mohandas N, Joiner CH, and Kalfa TA. Altered phosphorylation of cytoskeleton proteins in sickle red blood cells: The role of protein kinase C, Rac GTPases, and reactive oxygen species. *Blood Cells Mol Dis* 45: 41–45, 2010.
26. Harrison DG, Vinh A, Lob H, and Madhur MS. Role of the adaptive immune system in hypertension. *Curr Opin Pharmacol* 10: 203–207, 2010.
27. Hecker M, Schott C, Bucher B, Busse R, and Stoclet JC. Increase in serum NG-hydroxy-L-arginine in rats treated with bacterial lipopolysaccharide. *Eur J Pharmacol* 275: R1–3, 1995.
28. Hewett SJ, Espey MG, Uliasz TF, and Wink DA. Neurotoxicity of nitroxyl: Insights into HNO and NO biochemical imbalance. *Free Radic Biol Med* 39: 1478–1488, 2005.
29. Hobbs AJ, Fukuto JM, and Ignarro LJ. Formation of free nitric oxide from L-arginine by nitric oxide synthase: Direct enhancement of generation by superoxide dismutase. *Proc Natl Acad Sci USA* 91: 10992–10996, 1994.
30. Hoffman MD, Walsh GM, Rogalski JC, and Kast J. Identification of nitroxyl-induced modifications in human platelet proteins using a novel mass spectrometric detection method. *Mol Cell Proteomics* 8: 887–903, 2009.
31. Huang PL. eNOS, metabolic syndrome and cardiovascular disease. *Trends Endocrinol Metab* 20: 295–302, 2009.
32. Hughes MN and Cammack R. Synthesis, chemistry, and applications of nitroxyl ion releasers sodium trioxodinitrate (Angeli's salt) and Piloty's acid. *Methods Enzymol* 310: 279–287, 1999.
33. Irvine JC, Favaloro JL, and Kemp-Harper BK. NO^{-} activates soluble guanylate cyclase and Kv channels to vasodilate resistance arteries. *Hypertension* 41: 1301–1307, 2003.
34. Irvine JC, Favaloro JL, Widdop RE, and Kemp–Harper BK. Nitroxyl anion donor, Angeli's salt, does not develop tolerance in rat isolated aortae. *Hypertension* 49: 885–892, 2007.
35. Irvine JC, Ritchie RH, Favaloro JL, Andrews KL, Widdop RE, and Kemp–Harper BK. Nitroxyl (HNO): The Cinderella of the nitric oxide story. *Trends Pharmacol Sci* 29: 601–608, 2008.
36. Jang EK, Azzam JE, Dickinson NT, Davidson MM, and Haslam RJ. Roles for both cyclic GMP and cyclic AMP in the inhibition of collagen-induced platelet aggregation by nitroprusside. *Br J Haematol* 117: 664–675, 2002.
37. Irvine JC, Kemp-Harper BK, and Widdop RE. Chronic administration of the HNO donor, Angeli's salt does not lead to tolerance, cross-tolerance or endothelial dysfunction: comparison with GTN and DEA/NO. *Antioxid Redox Signal* [Epub ahead of print], Sep 19, 2010.
38. Judkins CP, Diep H, Broughton BR, Mast AE, Hooker EU, Miller AA, Selemidis S, Dusting GJ, Sobey CG, and Drummond GR. Direct evidence of a role for Nox2 in superoxide production, reduced nitric oxide bioavailability, and early atherosclerotic plaque formation in ApoE-/- mice. *Am J Physiol Heart Circ Physiol* 298: H24–32, 2010.

39. Keefer LK, Anderson LM, Diwan BA, Driver CL, Haines DC, Maragos CM, Wink DA, and Rice JM. Experimental tests of the mutagenicity and carcinogenicity of nitric oxide and its progenitors. *Methods* 7: 121–130, 1995.
40. Kemp–Harper B and Schmidt HH. cGMP in the vasculature. *Handb Exp Pharmacol* 191: 447–467, 2009.
41. Klemenska E and Beresewicz A. Bioactivation of organic nitrates and the mechanism of nitrate tolerance. *Cardiol J* 16: 11–19, 2009.
42. Li CG, Karagiannis J, and Rand MJ. Comparison of the redox forms of nitrogen monoxide with the nitrergic transmitter in the rat anococcygeus muscle. *Br J Pharmacol* 127: 826–834, 1999.
43. Lopez BE, Shinyashiki M, Han TH, and Fukuto JM. Antioxidant actions of nitroxyl (HNO). *Free Radic Biol Med* 42: 482–491, 2007.
44. Ma XL, Gao F, Liu GL, Lopez BL, Christopher TA, Fukuto JM, Wink DA, and Feelisch M. Opposite effects of nitric oxide and nitroxyl on postischemic myocardial injury. *Proc Natl Acad Sci USA* 96: 14617–14622, 1999.
45. Martin W. Nitroxyl anion—The universal signalling partner of endogenously produced nitric oxide? *Br J Pharmacol* 157: 537–539, 2009.
46. Maurice DH and Haslam RJ. Molecular basis of the synergistic inhibition of platelet function by nitrovasodilators and activators of adenylate cyclase: Inhibition of cyclic AMP breakdown by cyclic GMP. *Mol Pharmacol* 37: 671–681, 1990.
47. Melichar VO, Kumar A, Behr–Roussel D, Zabel U, Uttenthal LO, Rodrigo J, Rupin A, Verbeuren TJ, and Schmidt HH. Activity and expression of signalling enzymes downstream of nitric oxide is decreased in hypercholesterolemia-induced atherosclerosis in rabbits. *Proc Natl Acad Sci USA* 101: 16671–16676, 2004.
48. Miller AA, Budzyn K, and Sobey CG. Vascular dysfunction in cerebrovascular disease: Mechanisms and therapeutic intervention. *Clin Sci (Lond)* 119: 1–17, 2010.
49. Miller AA, Drummond GR, and Sobey CG. Novel isoforms of NADPH-oxidase in cerebral vascular control. *Pharmacol Ther* 111: 928–948, 2006.
50. Miller TW, Cherney MM, Lee AJ, Francoleon NE, Farmer PJ, King SB, Hobbs AJ, Miranda KM, Burstyn JN, and Fukuto JM. The effects of nitroxyl (HNO) on soluble guanylate cyclase activity: Interactions at ferrous heme and cysteine thiols. *J Biol Chem* 284: 21788–21796, 2009.
51. Miranda K. The chemistry of nitroxyl (HNO) and implications in biology. *Coordination Chem Rev* 249: 433–455, 2005.
52. Miranda KM, Katori T, Torres de Holding CL, Thomas L, Ridnour LA, McLendon WJ, Cologna SM, Dutton AS, Champion HC, Mancardi D, Tocchetti CG, Saavedra JE, Keefer LK, Houk KN, Fukuto JM, Kass DA, Paolocci N, and Wink DA. Comparison of the NO and HNO donating properties of diazeniumdiolates: Primary amine adducts release HNO *in vivo*. *J Med Chem* 48: 8220–8228, 2005.
53. Miranda KM, Nims RW, Thomas DD, Espey MG, Citrin D, Bartberger MD, Paolocci N, Fukuto JM, Feelisch M, and Wink DA. Comparison of the reactivity of nitric oxide and nitroxyl with heme proteins. A chemical discussion of the differential biological effects of these redox related products of NOS. *J Inorg Biochem* 93: 52–60, 2003.
54. Miranda KM, Yamada K, Espey MG, Thomas DD, DeGraff W, Mitchell JB, Krishna MC, Colton CA, and Wink DA. Further evidence for distinct reactive intermediates from nitroxyl and peroxynitrite: effects of buffer composition on the chemistry of Angeli's salt and synthetic peroxynitrite. *Arch Biochem Biophys* 401: 134–144, 2002.
55. Moncada S and Higgs EA. The discovery of nitric oxide and its role in vascular biology. *Br J Pharmacol* 147: S193–S201, 2006.
56. Mondoro TH, Ryan BB, Hrinczenko BW, Schechter AN, Vostal JG, and Alayash AI. Biological action of nitric oxide donor compounds on platelets from patients with sickle cell disease. *Br J Haematol* 112: 1048–1054, 2001.
57. Naughton P, Foresti R, Bains SK, Hoque M, Green CJ, and Motterlini R. Induction of heme oxygenase 1 by nitrosative stress. A role for nitroxyl anion. *J Biol Chem* 277: 40666–40674, 2002.
58. Nelli S, Hillen M, Buyukafsar K, and Martin W. Oxidation of nitroxyl anion to nitric oxide by copper ions. *Br J Pharmacol* 131: 356–362, 2000.
59. Norris AJ, Sartippour MR, Lu M, Park T, Rao JY, Jackson MI, Fukuto JM, and Brooks MN. Nitroxyl inhibits breast tumor growth and angiogenesis. *Int J Cancer* 122: 1905–1910, 2008.
60. Palmer RM, Ferrige AG, and Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 327: 524–526, 1987.
61. Paolocci N, Jackson MI, Lopez BE, Miranda K, Tocchetti CG, Wink DA, Hobbs AJ, and Fukuto JM. The pharmacology of nitroxyl (HNO) and its therapeutic potential: Not just the Janus face of NO. *Pharmacol Ther* 113: 442–458, 2007.
62. Paolocci N, Katori T, Champion HC, St. John ME, Miranda K, Fukuto JM, Wink DA, and Kass DA. Positive inotropic and lusitropic effects of HNO/NO- in failing hearts: Independence from β-adrenergic signalling. *Proc Natl Acad Sci USA* 100: 5537–5542, 2003.
63. Paolocci N, Saavedra WF, Miranda KM, Martignani C, Isoda T, Hare JM, Espey MG, Fukuto JM, Feelisch M, Wink DA, and Kass DA. Nitroxyl anion exerts redox-sensitive positive cardiac inotropy *in vivo* by calcitonin gene-related peptide signaling. *Proc Natl Acad Sci USA* 98: 10463–10468, 2001.
64. Pino RZ and Feelisch M. Bioassay discrimination between nitric oxide (NO.) and nitroxyl (NO-) using L-cysteine. *Biochem Biophys Res Commun* 201: 54–62, 1994.
65. Plane F, Sampson LJ, Smith JJ, and Garland CJ. Relaxation to authentic nitric oxide and SIN-1 in rat isolated mesenteric arteries: Variable role for smooth muscle hyperpolarization. *Br J Pharmacol* 133: 665–672, 2001.
66. Pufahl RA, Wishnok JS, and Marletta MA. Hydrogen peroxide-supported oxidation of NG-hydroxy-L-arginine by nitric oxide synthase. *Biochemistry* 34: 1930–1941, 1995.
67. Ritchie RH, Irvine JC, Rosenkranz AC, Patel R, Wendt IR, Horowitz JD, and Kemp-Harper BK. Exploiting cGMP-based therapies for the prevention of left ventricular hypertrophy: $NO^{\bullet}$ and beyond. *Pharmacol Ther* 124: 279–300, 2009.
68. Ritchie RH, Lin EQ, Cao AH, Patel R, Kaye DM, and Kemp–Harper B. The NO redox sibling, nitroxyl (HNO), blocks cardiomyocyte hypertrophy via suppression of NADPH-oxidase. *Circulation* 116: S19–S20, 2007.
69. Rudijanto A. The role of vascular smooth muscle cells on the pathogenesis of atherosclerosis. *Acta Med Indones* 39: 86–93, 2007.
70. Ruetten H, Zabel U, and Schmidt HH. Down-regulation of soluble guanylate cyclase in young and ageing spontaneously hypertensive rats. *Circ Res* 85: 534–541, 1999.
71. Rusche KM, Spiering MM, and Marletta MA. Reactions catalyzed by tetrahydrobiopterin-free nitric oxide synthase. *Biochemistry* 37: 15503–15512, 1998.
72. Schmidt HH, Hoffman MD, Schindler U, Shutenko ZS, Cunningham DD, and Feelisch M. No ·NO from NO Synthase. *Proc Natl Acad Sci USA* 93: 14492–14497, 1996.
73. Selemidis S, Dusting GJ, Peshavariya H, Kemp–Harper B, and Drummond GR. Nitric oxide supresses NADPH

oxidase-dependent superoxide production by S-nitrosylation in human endothelial cells. *Cardiovasc Res* 75: 349–358, 2007.
74. Selemidis S, Sobey CG, Wingler K, Schmidt HH, and Drummond GR. NADPH oxidases in the vasculature: Molecular features, roles in disease and pharmacological inhibition. *Pharmacol Ther* 120: 254–291, 2008.
75. Sobey CG and Faraci FM. Inhibitory effect of 4-aminopyridine on responses of the basilar artery to nitric oxide. *Br J Pharmacol* 126: 1437–1443, 1999.
76. Stasch JP, Schmidt PM, Nedvetsky PI, Nedvetskaya TY, H SA, Meurer S, Deile M, Taye A, Knorr A, Lapp H, Muller H, Turgay Y, Rothkegel C, Tersteegen A, Kemp–Harper B, Muller–Esterl W, and Schmidt HH. Targeting the heme-oxidized nitric oxide receptor for selective vasodilatation of diseased blood vessels. *J Clin Invest* 116: 2552–2561, 2006.
77. Takahira R, Yonemura K, Fujise Y, and Hishida A. Dexamethasone attenuates neutrophil infiltration in the rat kidney in ischemia/reperfusion injury: The possible role of nitroxyl. *Free Radic Biol Med* 31: 809–815, 2001.
78. Thomas SR, Witting PK, and Drummond GR. Redox control of endothelial function and dysfunction: Molecular mechanisms and therapeutic opportunities. *Antioxid Redox Signal* 10: 1713–1765, 2008.
79. Tochetti CG, Wang W, Froehlich JP, Huke S, Aon MA, Wilson GM, Benedetto GD, O'Rourke B, Gao WD, Wink DA, Toscano JP, Zaccolo M, Bers DM, Valdivia HH, Cheng H, Kass DA, and Paolocci N. Nitroxyl improves cellular heart function by directly enhancing cardiac sarcoplasmic reticulum Ca^{2+} cycling. *Circ Res* 100: 96–104, 2007.
80. Tsihlis ND, Murar J, Kapadia MR, Ahanchi SS, Oustwani CS, Saavedra JE, Keefer LK, and Kibbe MR. Isopropylamine NONOate (IPA/NO) moderates neointimal hyperplasia following vascular injury. *J Vasc Surg* 51: 1248–1259, 2010.
81. Vanuffelen BE, Van Der Zee J, De Koster BM, Vansteveninck J, and Elferink JG. Intracellular but not extracellular conversion of nitroxyl anion into nitric oxide leads to stimulation of human neutrophil migration. *Biochem J* 330: 719–722, 1998.
82. Walter U and Gambaryan S. cGMP and cGMP-dependent protein kinase in platelets and blood cells. *Handb Exp Pharmacol* 191: 533–548, 2009.
83. Wanstall JC, Jeffery TK, Gambino A, Lovren F, and Triggle CR. Vascular smooth muscle relaxation mediated by nitric oxide donors: A comparison with acetylcholine, nitric oxide and nitroxyl ion. *Br J Pharmacol* 134: 463–472, 2001.
84. Willoughby SR, Stewart S, Holmes AS, Chirkov YY, and Horowitz JD. Platelet nitric oxide responsiveness: A novel prognostic marker in acute coronary syndromes. *Arterioscler Thromb Vasc Biol* 25: 2661–2666, 2005.
85. Wink DA, Miranda KM, Katori T, Mancardi D, Thomas DD, Ridnour L, Espey MG, Feelisch M, Colton CA, Fukuto JM, Pagliaro P, Kass DA, and Paolocci N. Orthogonal properties of the redox siblings nitroxyl and nitric oxide in the cardiovascular system: A novel redox paradigm. *Am J Physiol Heart Circ Physiol* 285: H2264–2276, 2003.
86. Winter P and Dora KA. Spreading dilatation to luminal perfusion of ATP and UTP in rat isolated small mesenteric arteries. *J Physiol* 582: 335–347, 2007.
87. Wong P, Hyun J, Fukuto JM, Shirota FN, DeMaster EG, Shoeman DW, and Nagasawa HT. Reaction between S-nitrosothiols and thiols: Generation of nitroxyl (HNO) and subsequent chemistry. *Biochemistry* 37: 5362–5371, 1998.
88. Yuill K, Yarova P, Kemp-Harper BK, Garland CJ, and Dora KA. A novel role of HNO in local and spreading vasodilatation in rat mesenteric resistance arteries. *Antioxid Redox Signal* [Epub ahead of print], Jul 8, 2010.
89. Zeller A, Wenzl MV, Beretta M, Stessel H, Russwurm M, Koesling D, Schmidt K, and Mayer B. Mechanisms underlying activation of soluble guanylate cyclase by the HNO donor Angeli's Salt. *Mol Pharmacol* 76: 1115–1122, 2009.
90. Zembowicz A, Hecker M, Macarthur H, Sessa WC, and Vane JR. Nitric oxide and another potent vasodilator are formed from NG-hydroxy-L-arginine by cultured endothelial cells. *Proc Natl Acad Sci USA* 88: 11172–11176, 1991.

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Abbreviations Used

4-AP = 4-aminopyridine
BH_4 = tetrahydrobiopterin
cAK = cAMP-dependent protein kinase
carboxy-PTIO = 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxy-3-oxide
cGK = cGMP-dependent protein kinase
CGRP = calcitonin-gene related peptide
DEA/NO = diethylamine-NONOate
EDHF = endothelium-derived hyperpolarizing factor
EDRF = endothelium-derived relaxing factor
eNOS = endothelial NOS
GTN = glyceryl trinitrate
HNO = nitroxyl
H_2O_2 = hydrogen peroxide
IPA/NO = isopropylamine NONOate
K_{ATP} = ATP-sensitive K^+ channel
K_{Ca} = calcium-sensitive K^+ channel
K_v = voltage-sensitive K^+ channel
L-NAME = N-nitro-L-arginine methyl ester
NCTFA = 1-nitrosocyclohexy-trifluoroacetate
NH_2OH = hydroxylamine
nNOS = neuronal NOS
$NO^\bullet$ = nitric oxide
NOHA = N-hydroxy-L-arginine
NOS = nitric oxide synthase
$\cdot O_2^-$ = superoxide
ODQ = 1H-(1,2,4)oxadiazole(4,3,-a) quinoxaline-1-one
$ONOO^-$ = peroxynitrite
PDEs = phosphodiesterases
ROS = reactive oxygen species
sGC = soluble guanylyl cyclase
SOD = superoxide dismutase
VEGF = vascular endothelial growth factor
VSMC = vascular smooth muscle cell

1.1 General Overview

The biologically active gas, nitric oxide ($NO^{\bullet}$) is well recognised as an important modulator of vascular homeostasis with vasorelaxant, anti-aggregatory and anti-proliferative properties (Moncada and Higgs 2006). $NO^{\bullet}$ elicits its vasoprotective effects primarily through activation of the cytosolic enzyme soluble guanylyl cyclase (sGC), which catalyses the conversion of GTP to cGMP (Kemp-Harper and Schmidt 2008). An impairment in endogenous $NO^{\bullet}$-mediated signalling is associated with a plethora of cardiovascular pathologies, arising as a consequence of decreased $NO^{\bullet}$ synthesis, increased $NO^{\bullet}$ inactivation and/or dysfunction at the level of its receptor (sGC) or downstream signaling pathways (Huang 2009; Thomas et al. 2008). Such deficiencies in $NO^{\bullet}$ signaling can be overcome in part, through the use of $NO^{\bullet}$ donors, such as the organic nitrate glyceryl trinitrate (GTN), which have been utilised for >100 years in the treatment of angina, heart failure and acute hypertensive crises. However, the clinical efficacy of traditional $NO^{\bullet}$ donors is limited due to their susceptibility to tolerance development with continued use and diminished vasoprotective actions under conditions of oxidative stress (Klemenska and Beresewicz 2009).

Thus, there is a need to develop novel therapies that have a similar vasoprotective profile as $NO^{\bullet}$ donors, but do not display the limitations associated with these drugs. Importantly, nitrogen oxide (NO) can exist in three different redox forms; as the uncharged free radical ($NO^{\bullet}$), nitroxyl anion (NO^{-}) and nitrosonium cation (NO^{+}). NO^{+} is unlikely to have a functional role in the vasculature, as it is only found in aqueous solution of very low pH (Hughes 1999). In contrast, NO^{-} exists in its protonated form at physiological pH, suggesting that nitroxyl (HNO) may have biological function in the vasculature (Miranda 2005). Indeed, over the last decade HNO has emerged as a viable therapeutic option, displaying a number of cardio- and vasoprotective actions. Moreover, HNO exhibits distinct pharmacological actions to that of $NO^{\bullet}$ (Irvine et al. 2008; Paolocci et al. 2007), as such, HNO-based therapies may have therapeutic advantages over clinically used $NO^{\bullet}$ donors.

1.2 Nitroxyl (HNO)

1.2.1 General Chemistry of HNO

Initially, HNO was reported to exist in its deprotonated state (NO^-) at physiological pH, with a pK_a value of ~4.7 (Grätzel et al. 1970). However, subsequent studies have proven HNO to be a weak acid with a pK_a value of ~11.4 (Bartberger et al. 2002; Shafirovich and Lymar 2002), indicating that at physiological pH, HNO rather than NO^- will predominate. Moreover, HNO and NO^- exist in different electronic ground states, HNO as a singlet and NO^- in a triplet ground state (Fukuto and Carrington 2011). As such, deprotonation of HNO to NO^- is limited by a spin-forbidden reaction (rate constant; k = 5 x 10^4 $M^{-1}s^{-1}$) (Fukuto and Carrington 2011). Additionally, direct reduction of $NO^\bullet$ to NO^- is unlikely to occur, due to the reaction being thermodynamically unfavorable (Miranda et al. 2003). Taken together, these findings suggest that HNO is the biologically active species, with little contribution by NO^- (Fukuto and Carrington 2011).

Importantly, HNO can readily cross the cellular membrane and is highly reactive, since it can serve as either an electrophile or nucleophile depending on environmental conditions (Fukuto and Carrington 2011). Thus, HNO can interact with a variety of biological targets, including thiols and metalloproteins. The fate of HNO is governed by the concentration of its target molecules and also its ability to rapidly dimerise (k = 8 x 10^6 $M^{-1}s^{-1}$) to yield hyponitrous acid ($H_2N_2O_2$), which can undergo spontaneous decomposition via dehydration to produce nitrous oxide (N_2O) (Miranda 2005). As such, HNO cannot be stored as a stable molecule and is typically studied using HNO donor compounds. It is important to note that although HNO can also interact with superoxide ($^{\cdot}O_2^-$) this reaction is very slow and as such is unlikely to impact upon the actions of HNO *in vivo* (Miranda et al. 2003).

1.2.2 Biological targets of HNO

HNO is gaining interest as a therapeutic alternative to clinically used $NO^\bullet$ donors, as it displays similar vasoprotective actions, but has a distinct pharmacological profile to that of $NO^\bullet$. These differences between redox species enable HNO to target distinct signalling pathways in the vasculature and circumvent many of the limitations surrounding clinically used $NO^\bullet$ donors. Indeed, the biological

activity of HNO is governed predominantly by its reactivity with metallo- and thiol-containing proteins (Fukuto et al. 2005; Paolocci et al. 2007).

Interaction with metalloproteins

HNO reduces metals such as iron (Fe), copper (Cu) and manganese (Fukuto et al. 2005; Miranda et al. 2003; Nelli et al. 2000) and preferentially targets ferric (Fe^{3+}) versus ferrous (Fe^{2+}) haem groups (Miranda et al. 2003). Specifically, HNO can interact with oxidised Fe^{3+} haem proteins through reductive nitrosylation to form a stable Fe^{2+}-nitrosyl complex (Reaction 1). In contrast, $NO^{\bullet}$ preferentially targets Fe^{2+} haem groups over Fe^{3+} haem groups, to generate the same Fe^{2+}-nitrosyl complex as HNO (Reaction 2; Miranda et al. 2003). These observations indicate that HNO may target oxidised haem groups in proteins such as haemoglobin, myoglobin, cytochromes and peroxidases (Miranda et al. 2003).

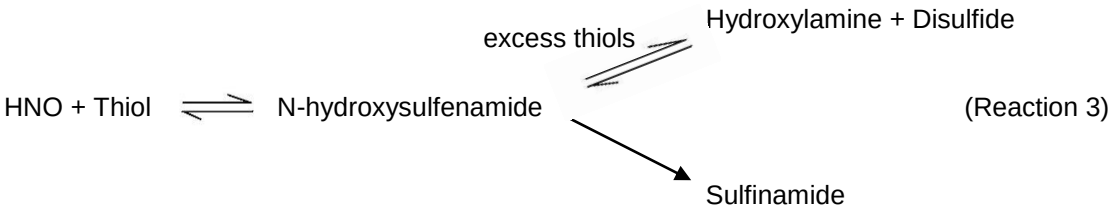
$$HNO + Fe^{3+}\text{-Heme} \rightarrow ON\text{-}Fe^{2+}\text{-Heme} + H^{+} \qquad \text{(Reaction 1)}$$

$$NO^{\bullet} + Fe^{2+}\text{-Heme} \rightarrow ON\text{-}Fe^{2+}\text{-Heme} \qquad \text{(Reaction 2)}$$

The most recognised heme-containing protein that both HNO and $NO^{\bullet}$ target is sGC. Indeed the vasoprotective actions of HNO and $NO^{\bullet}$ are predominantly mediated via sGC/cGMP signalling (Irvine et al. 2008; Moncada and Higgs 2006; Paolocci et al. 2007). Whilst HNO has previously been reported to mediate its actions through sGC, in its reduced (Fe^{2+}) state (Miller et al. 2009), it remains contentious whether HNO can interact directly with sGC or first requires conversion to $NO^{\bullet}$ (Zeller et al. 2009). Indeed, HNO can be converted to $NO^{\bullet}$, via reductive nitrosylation, by enzymes such as Cu(II) and zinc(II)-SOD (Nelli et al. 2000; Zeller et al. 2009). Whilst a recent study indicates that SOD-stimulated conversion of HNO to $NO^{\bullet}$ is required to activate sGC (Zeller et al. 2009), another study demonstrated that HNO can directly activate sGC (Miller et al. 2009). In addition, given HNO preferentially targets ferric heme groups, it has been proposed that HNO may stimulate oxidised (Fe^{3+}), rather than reduced, sGC, however studies to date have found no evidence for this interaction (Miller et al. 2009; Zeller et al. 2009).

Interaction with thiols

The electrophilic nature of HNO enables it to target many nucleophiles including thiols (Donzelli et al. 2006; Fukuto and Carrington 2011; Shen and English 2005). Due to its highly thiophilic nature, HNO may interact with thiol-containing receptors, ion channels and enzymes in the vasculature to modify their function. Depending on the reaction conditions the interaction between HNO and thiols can occur in either a reversible or irreversible manner. Both pathways require an initial nucleophilic attack of the thiol by HNO, leading to the formation of an unstable N-hydroxysulfenamide intermediate (Reaction 3; Donzelli et al. 2006; Fukuto and Carrington 2011). N-hydroxysulfenamide can react readily with additional thiols, in a reversible manner, to produce hydroxylamine and a disulfide. Alternatively, N-hydroxysulfenamide can undergo rearrangement resulting in the formation of a sulfinamide (Reaction 3; Donzelli et al. 2006; Fukuto and Carrington 2011), an irreversible process. $NO^{\bullet}$ also has the capacity to interact with thiols, however it first needs to be converted to a reactive NO species, which can lead to S-nitrosylation (Selemidis et al. 2007; Wink et al. 1994).



(Reaction 3)

Importantly, the direct interaction of HNO with thiols underlies many of the distinct biological actions of HNO versus $NO^{\bullet}$. Indeed, in non-vascular cells, HNO was shown to target critical thiol residues on enzymes such as aldehyde dehydrogenase (DeMaster et al. 1998; Shoeman et al. 2000), glyceraldehyde 3-phosphate dehydrogenase (Lopez et al. 2005), N-methyl-D-aspartate receptor (Kim et al. 1999), cysteine proteases (Vaananen et al. 2005; Vaananen et al. 2008) and mitochondrial respiratory complexes I and II (Shiva et al. 2004) to inhibit their activity. Moreover, the thiophilic nature of HNO underlies its unique pharmacology in the cardiovascular system. Specifically HNO, but not $NO^{\bullet}$ serves as a positive cardiac inotrope, mediating its inotropic and lusitropic effects through modification of cysteine residues on cardiac ryanodine receptors, ATP-dependent SR Ca^{2+}-ATPase (SERCA2a), the SERCA2a inhibitor phospholamban and myofilaments to improve myocardial contractility (Froehlich et al. 2008; Lancel et al. 2009; Tocchetti et al. 2007).

Given the propensity of HNO to interact with thiols, it is conceivable that this interaction may lead to non-specific actions. However, HNO has a minimal effect on glutathione (GSH) levels or the ratio between GSH and oxidised GSH, suggesting that HNO may target specific thiol-containing proteins (Hammond and Fry 1999; Lopez et al. 2005). Indeed, the affinity of HNO for certain protein thiols may be influenced by various reaction conditions such as the cellular concentrations of a specific protein thiol, its rate of reaction with HNO, whether the reaction is thermodynamically favorable and/or reversible (Fukuto and Carrington 2011; Lopez et al. 2005). Additionally, the electrophilic and hydrophobic nature of HNO may lend it to targeting thiolates over sulfhydryls and HNO may also interact more readily with membrane-bound molecules (Flores-Santana et al. 2011; Kemp-Harper 2011). Nevertheless, the interaction between HNO and thiols represents an important area for further research into the unique signaling actions of HNO in the vasculature.

1.2.3 Discriminating between HNO and $NO^{\bullet}$

Due to the unstable nature of HNO, there is a lack of direct and sensitive detections methods for this nitrogen oxide in mammalian cells. Rather, the distinct interaction of HNO with thiols has been used to discriminate between HNO and $NO^{\bullet}$. Indeed, high concentrations of thiols such as L-cysteine, *N*-acetyl-L-cysteine (NAC) and dithiothreitol (DTT) can attenuate the actions of HNO both *in vivo* and *in vitro* but not those attributable to $NO^{\bullet}$ (Favaloro and Kemp-Harper 2007; Irvine et al. 2003; Irvine et al. 2007; Paolocci et al. 2001; Pino and Feelisch 1994). Conversely, the use of $NO^{\bullet}$ scavengers such as carboxy-PTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxy-3-oxide) and hydroxocobalamin will lead to scavenging of $NO^{\bullet}$, but not HNO (Ellis et al. 2000; Irvine et al. 2003; Irvine et al. 2007; Li et al. 1999; Wanstall et al. 2001). Whilst these scavengers play a key role in differentiating between HNO and $NO^{\bullet}$, they provide little information with regard to the generation of HNO.

The most common method for measuring HNO in a cell free system is to assess one of the final reaction products of HNO dimerisation, N_2O (Fukuto et al. 1992). However, this technique has *in vitro* and *in vivo* limitations, as it is unlikely that dimerisation of HNO will account for all of HNO's interactions, as HNO can interact with thiols and N_2O can also be generated from non-HNO sources

(Reisz et al. 2011). Given measurement of N_2O is of limited use in biological systems, a number of other approaches have been taken to develop HNO sensors. The direct interaction of HNO with thiols (Donzelli et al. 2006; Shen and English 2005) leads to the formation of HNO-derived sulfinamides, which is thought to be unique to HNO (Donzelli et al. 2006). Recently, isotope-edited nuclear magnetic resonance has been used to detect HNO-derived sulfinamides (Keceli et al. 2013) and could be used as a means to distinguish between HNO and $NO^{\bullet}$ signaling. In addition to targeting thiols, HNO preferentially targets oxidised metalloproteinases such as metmyoglobin (Miranda et al. 2003) and manganese (III) porphyrins (Marti et al. 2005), leading to reductive nitrosylation and the formation of stable nitrosyl complexes. The formation of these nitrosyl complexes is then analysed via UV-visible spectroscopy or electron paramagnetic resonance to measure HNO and distinguish its effects from those of $NO^{\bullet}$ (Miranda et al. 2005; Paolocci et al. 2007). However, the use of this technique *in vivo* is not feasible since nitrosyl complexes can form via multiple signaling pathways (Reisz et al. 2011).

Recently, Cu^{2+}-based probes have been developed to act as near-infrared fluorescent sensors of HNO (Apfel et al. 2013; Rosenthal and Lippard 2010; Wrobel et al. 2014). In the presence of HNO, probes such as CuBOT1 and CuDHX1 are reduced from a Cu^{2+} to a Cu^{1+} complex, leading to the detection of a fluorescent signal (Rosenthal and Lippard 2010; Wrobel et al. 2014). Importantly, fluorescence can be measured in live cells and may also provide information with regard to the cellular localization of HNO (Rosenthal and Lippard 2010; Wrobel et al. 2014). Whilst probes are selective for HNO, hydrogen sulfide may also produce a fluorescent signal, although the intensity of this signal is ~2-fold less than HNO (Wrobel et al. 2014) and AS at a concentration of 50µM can be detected by these probes (Rosenthal and Lippard 2010). However, further development is required before these probes are useful in detecting HNO in mammalian cells.

Another promising class of HNO sensors are carbamates, which use a phosphine-based method for HNO detection (Reisz et al. 2011). Currently there are two carbamates that are available for commercial use; HNO detector 15a (4-Nitrophenyl(2-(diphenylphosphino)-ethyl)carbamate) and HNO detector 15b (Phenyl (2-(Diphenylphsophino)ethyl)carbamate). Upon HNO binding, these compounds undergo phosphine-ligation leading to the production of aza-ylides and subsequently the generation of HNO-derived urea, which can be assessed via mass spectrometry (Reisz et al. 2011). HNO detector

15a is also a colourmetric sensor as it yields ρ-nitrophenol and forms a yellow solution that can be analysed via UV-visible spectroscopy (Reisz et al. 2011). Although susceptible to oxidation and hydrolysis at pH>7 (Reisz et al. 2011), these HNO detectors are designed for both *in vitro* and *in vivo* use, thus warrant further investigation into their use as a direct method of HNO detection in mammalian cells.

1.2.4 HNO donors

Due to the rapid dimerization of HNO, it is unable to be stored as a stable molecule. Thus, there is a need to develop donor compounds to elucidate the biological actions of HNO. In recent times, the most commonly used donors for cardiovascular research include Angeli's salt (AS), primary amine diadiazeniumdiolates and acyloxy nitroso compounds and further discussion will be focused on these compounds (Table 1; DuMond and King 2011). It should be noted that HNO can also be generated via a number of other donors including, but not limited to, Piloty's acid and cyanamide (Table 1; DuMond and King 2011).

Angeli's salt

The mainstay of the HNO research field is AS (sodium trioxodinitrate; $Na_2N_2O_3$), a diazeniumdiolate which was discovered over 100 years ago (Irvine et al. 2008). The generation of HNO from AS is dependent upon first-order kinetics and influenced by pH. Whilst AS is relatively stable at high pH (above pH 8), it can spontaneously decompose to generate HNO and nitrite (NO_2^-) from pH 4-8 (Hughes and Cammack 1999). At physiological temperatures (37˚C), AS has a rate constant of 4-5 x 10^3 $M^{-1}s^{-1}$ and a half-life of ~2.5 minutes (DuMond and King 2011; Hughes and Cammack 1999; Maragos et al. 1991). Importantly, numerous experimental studies have observed that AS displays both cardioprotective and vasoprotective actions under non-disease conditions (Irvine et al. 2008; Paolocci et al. 2007; Tocchetti et al. 2011). However, the short half-life of AS impedes its use as a viable therapeutic treatment. In addition to generating HNO, AS co-releases NO_2^- (Demoncheaux et al. 2003) and acts exclusively as an $NO^\bullet$ donor at pH below 4 and at concentrations exceeding 10 µM (Irvine et al. 2008). Whilst both HNO and NO_2^-, can exert physiological effects in their own right, the concentrations of NO_2^- required for biological function (i.e. vasorelaxation) far exceed those of HNO

(>15,000 fold). Thus it is unlikely that NO_2^- contributes to the cardioprotective and vasoprotective actions of AS (Irvine et al. 2003).

Table 1 HNO donors and their properties

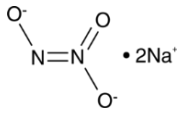
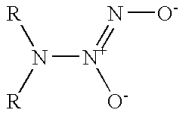
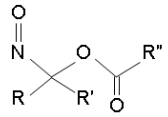
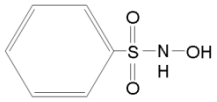
HNO Donor	Structure	Properties
Angeli's salt (sodium trioxodinitrate; $Na_2N_2O_3$; AS)	 (DuMond and King 2011)	• Dissociates at physiological pH and temperature to yield HNO • Half-life ~2.5 min (DuMond and King 2011; Hughes and Cammack 1999; Maragos et al. 1991) • $NO^{\bullet}$ donor at pH < 4 and high concentrations (>10µM) (Irvine et al. 2008) • Co-releases nitrite (Demoncheaux et al. 2003)
Primary amine diazeniumdiolate - Isopropylamine NONOate (IPA/NO)	 (Miranda et al. 2005)	• Dissociates at physiological pH and temperature to yield HNO • Half-life ~2.3 minutes (DuMond and King 2011; Maragos et al. 1991) • Donates $NO^{\bullet}$ at pH < 7 (DuMond and King 2011; Miranda et al. 2005) • No co-release of nitrite • Nitrosamine by-product (DuMond and King 2011; Miranda et al. 2005)
Acyloxy nitroso compounds - 1-nitrosocyclohexyl acetate (1-NCA)	 (Irvine et al. 2008; Sha et al. 2006)	• Undergoes hydrolysis to yield HNO (Sha et al. 2006) • Rate of HNO release dependent upon pH (Sha et al. 2006) • Neutral pH, half-life ~800-890 min (Sha et al. 2006) • Co-releases nitrite and $NO^{\bullet}$ (Sha et al. 2006) • Lower efficacy than AS and IPA/NO (Donzelli et al. 2013; Sha et al. 2006)

Table 1 HNO donors and their properties

HNO Donor	Structure	Properties
Piloty's acid	 (Irvine et al. 2008; Paolocci et al. 2007)	• Decomposes to release HNO • Rate of HNO release dependent upon pH (Hughes and Cammack 1999) • HNO donor only above physiological pH (Pino and Feelisch 1994; Zamora et al. 1995) • $NO^{\bullet}$ donor at physiological pH (Pino and Feelisch 1994; Zamora et al. 1995) • Co-releases benzensulfinate (Zamora et al. 1995)
Cyanamide (H_2NCN)	$H_2NC{\equiv}N$ (DuMond and King 2011)	• Enzymatic biotransformation to generate HNO (DeMaster et al. 1998) • Co-releases cyanide (DeMaster et al. 1998)

Acyloxy nitroso compounds

Recently, interest has been generated surrounding acyloxy nitroso compounds such as 1-nitrosocyclohexyl acetate (1-NCA), as they have the potential to serve as longer acting HNO donors. Indeed, at pH 7 1-NCA undergoes hydrolysis to generate HNO and a corresponding ketone, with a half-life between 13-15 hours (Sha et al. 2006). Yet under basic pH conditions (pH~12.4), the half-life of 1-NCA is significantly reduced to ~8 minutes (Sha et al. 2006). At present, the biological actions of 1-NCA have not been fully elucidated, although there is evidence to suggest that 1-NCA has cardioprotective (El-Armouche et al. 2010; Gao et al. 2012) and vasoprotective actions (Donzelli et al. 2013; Sha et al. 2006) and its vasorelaxant effects appear to be preserved under disease conditions (Donzelli et al. 2013). Despite having a lower efficacy than AS and IPA/NO (Donzelli et al. 2013; Sha et al. 2006), 1-NCA appears to be a more pure HNO donor, generating minimal amounts of $NO^{\bullet}$ (0.4-0.5%) and NO_2^- (3-4%) at neutral pH (Sha et al. 2006). Nevertheless, the prolonged actions of 1-NCA warrant an investigation into any potential off-target effects. Taken together, acyloxy nitroso compounds have the potential to be an important tool in translating the protective actions of HNO donors seen in the laboratory, into a more therapeutically relevant setting.

1.2.5 Actions of HNO in the cardiovascular system

Endogenous generation of HNO and its role as an endothelium-derived relaxation factor (EDRF)

There are a number of different biological pathways via which HNO can be generated endogenously and there is pharmacological evidence to suggest that HNO contributes, in part, to endothelium-dependent relaxation previously attributed solely to $NO^{\bullet}$. The potential biosynthetic pathways of HNO in the vasculature and the evidence in support of HNO as an EDRF are described in detail in the accompanying review article (see Bullen *et al.*, 2011; Antioxidants & Redox Signaling, 14: pages 1677-1678). Building upon this knowledge, it has recently been proposed that the ability of HNO to serve as an EDRF may be sustained and/or enhanced in disease states where oxidative stress is augmented. Indeed, recent evidence demonstrated a contribution of HNO to endothelium-dependent relaxation in diabetic and hypertensive mice (Leo et al. 2012; Wynne et al. 2012). This effect was augmented in diabetes (Leo et al. 2012) and could reflect increased HNO generation due to uncoupling of endothelial nitric oxide synthase (eNOS) or through eNOS-independent mechanisms (Ponnuswamy et al. 2012; Rusche et al. 1998). Alternatively, the bioavailability of HNO could be preserved due to its resistance to scavenging by $^{\cdot}O_2^-$ and/or improved in the face of disease-associated thiol depletion (Leo et al. 2012; Miller 2013; Miranda et al. 2002). Interestingly, these findings suggest that HNO contributes to endothelium-dependent relaxation in disease and may compensate for a loss of $NO^{\bullet}$ under conditions of oxidative stress.

Vasorelaxant properties of HNO

It has been well-characterised that AS can mediate vasorelaxation both *in vitro* and *in vivo*, primarily through activation of the sGC/cGMP pathway. In addition, it has been proposed that HNO can also target voltage- and ATP-sensitive potassium channels (K_v and K_{ATP}) and may elevate calcitonin gene related peptide (CGRP) levels to mediate its vasodilatory effects. Please refer to Bullen et al., 2011 (Antioxidants & Redox Signaling, 14: pages 1678-1680) for a detailed discussion of the vasodilator properties of HNO. In accordance with these studies, a recent report demonstrated that the vasorelaxant responses to 1-NCA are also mediated by HNO and display a sGC- and CGRP-dependent component (Donzelli et al. 2013). The majority of these studies have examined the vasodilatory capacity of HNO under physiological conditions. However given resistance of HNO to both tolerance development and direct scavenging by $^{\cdot}O_2^-$, it has been hypothesised that the efficacy

of HNO donors may be preserved and/or enhanced under pathological conditions. Indeed, recent studies in animal models of acute heart failure (Paolocci et al. 2003; Paolocci et al. 2001), atherosclerosis (Donzelli et al. 2013), hypertension (Irvine et al. 2013; Wynne et al. 2012) and diabetes (Leo et al. 2012) have demonstrated sustained vasorelaxant activity of HNO both *in vitro* and *in vivo*. As such, these studies indicate that HNO donors represent a viable strategy to improve vascular function in cardiovascular diseases.

Anti-aggregatory properties of HNO

A detailed overview of the anti-platelet effects of HNO is provided in Bullen *et al.*, 2011 (Antioxidants & Redox Signaling, 14: pages 1680-1681). However, since this was written, a recent report has demonstrated that 1-NCA can also serve as anti-aggregatory agents in platelets from healthy volunteers and its effects were maintained in the presence of the $NO^{\bullet}$ scavenger carboxy-PTIO but were partially reversed by the sGC inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (Donzelli et al. 2013). As such, the authors suggest that HNO is mediating the effects of 1-NCA by targeting sGC/cGMP signalling. Furthermore, it appears that the anti-platelet effects of HNO are conserved under disease conditions. Since the ability of IPA/NO to inhibit platelet aggregation and elevate platelet cGMP levels was maintained in platelets from patients with ischemic heart disease (Dautov et al. 2013). In contrast, the anti-platelet actions of sodium nitroprusside (SNP) were compromised in platelets from these patients (Dautov et al. 2013). Since these donors were administered acutely, it is possible that the reduced responses observed with SNP could be attributed to the development of platelet $NO^{\bullet}$ resistance.

Unlike tolerance, platelet $NO^{\bullet}$ resistance occurs in the absence of prior exposure to $NO^{\bullet}$ donors and is not exclusive to organic nitrates (Horowitz 2000). Previously, platelet $NO^{\bullet}$ resistance has been reported in platelets from patients with congestive heart failure, aortic stenosis, stable angina pectoris, acute coronary syndrome, type 2 diabetes and obesity (Anderson et al. 2004; Anfossi et al. 1998; Anfossi et al. 2004; Chirkov et al. 1996; Chirkov et al. 1999; Chirkov et al. 2004; Chirkov et al. 2002; Chirkov et al. 2001). Moreover, impaired responses to $NO^{\bullet}$ were associated with scavenging of $NO^{\bullet}$ by $^{\cdot}O_2^-$ (Chirkov et al. 1999; Lopez Farre and Casado 2001) and/or dysfunctional platelet sGC (Chirkov et al. 1999; Dautov et al. 2013) as signalling mechanisms downstream of cGMP appear to be conserved in platelets from patients with SAP (Chirkov et al. 1996; Chirkov et al. 1999). Given that

$NO^{\bullet}$ donors represent an important anti-platelet therapy in acute thrombotic events, it is important to develop new treatments that can circumvent this platelet $NO^{\bullet}$ resistance. Given the anti-aggregatory actions of IPA/NO were preserved in ischemic heart disease (Dautov et al. 2013), this suggests that HNO donors could represent a novel strategy in circumventing platelet $NO^{\bullet}$ resistance.

Superoxide ($^{\cdot}O_2^-$) supressing actions of HNO

Enhanced oxidative stress is a hallmark of many cardiovascular disorders, thus developing strategies to limit reactive oxygen species (ROS) generation is a desirable trait of any vasoprotective drug. Please refer to Bullen et al., 2011 (Antioxidants & Redox Signaling, 14: page 1682) for a detailed discussion examining the ROS limiting properties of HNO. Since this review was written, subsequent studies have demonstrate that AS and IPA/NO can supress angiotensin II (Ang II)-stimulated $^{\cdot}O_2^-$ generation in both neonatal rat cardiomyocytes (Lin et al. 2012; Ritchie et al. 2007) and mouse cerebral arteries (Miller 2013). Unlike $NO^{\bullet}$, the ability of HNO to limit $^{\cdot}O_2^-$ production was not through direct scavenging of $^{\cdot}O_2^-$ (Leo et al. 2012; Miller 2013). Instead, AS significantly reduced $^{\cdot}O_2^-$ levels (~2.5-fold) and mRNA expression of the Nox2 isoform of the pro-oxidant enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (~4-fold) in Ang II-stimulated cardiomyocytes (Lin et al. 2012). Furthermore, IPA/NO did not reduce $^{\cdot}O_2^-$ production in cerebral arteries from Nox2 oxidase deficient mice (Miller 2013). Collectively, these results suggest that HNO has the potential to directly modulate Nox2 oxidase expression and/or activity to limit $^{\cdot}O_2^-$ production. Interestingly, HNO limited $^{\cdot}O_2^-$ generation in a sGC/cGMP-dependent manner in cardiomyocytes, yet its effects in the vasculature appear to be independent of sGC/cGMP (Lin et al. 2012; Miller 2013). Since HNO is highly thiophilic and has previously been shown to target thiol residues to modulate its cardioprotective effects, it is possible that HNO modulates Nox2 oxidase in the vasculature through a similar mechanism (Tocchetti et al. 2011). Previously, prolonged treatment with the $NO^{\bullet}$ donor, diethylenetriamine NONOate has been shown to supress endothelial Nox2 oxidase activity via S-nitrosylation of thiol residues of the cytosolic subunit $p47^{phox}$ (Selemidis et al. 2007). Thus with an ability to act as a ROS limiting agent and inhibit Nox2-derived $^{\cdot}O_2^-$ generation, HNO donors represent a future therapeutic strategy in limiting oxidative stress.

Anti-inflammatory actions of HNO

Inflammation is a key driving factor in the pathogenesis of many cardiovascular disorders, however at present it is unclear the exact role HNO plays in modulating the immune system. Importantly, HNO reduced macrophage infiltration into the vascular wall after vascular injury (Tsihlis et al. 2010) and was recently reported to limit leukocyte adhesion to the endothelium [Andrews *et al.*, unpublished]. Similarly, HNO donors can reduce the ability of the cytokine, leukemia inhibitory factor to activate the signal transducer and activator of transcription 3 in cardiomyocytes and endothelial cells (Zgheib et al. 2012), leading to decreased expression of the pro-inflammatory molecules intracellular adhesion molecule 1 (ICAM-1) and CCAAT/enhancer binding protein delta (Zgheib et al. 2012). Suggesting that HNO can modulate leukocyte recruitment and the activation of inflammatory genes. This is supported by the fact that AS reduced the level of the pro-inflammatory cytokines tumour necrosis factor alpha (TNFα) and interleukin (IL)-1β in hyperalgesic rats (Zarpelon et al. 2013). However, it is important to note that AS increased neutrophil accumulation and ICAM-1 expression in renal ischemia-reperfusion (Takahira et al. 2001) and at high concentrations was cytotoxic to human lymphocytes (Augustyniak et al. 2013). As such there is a need for further investigation into the anti-inflammatory effects of HNO in order to fully elucidate the therapeutic applications of HNO donors.

Regulation of vascular smooth muscle cell (VSMC) growth and proliferation by HNO

With an ability to inhibit VSMC and endothelial cell proliferation, reduce neointimal hyperplasia and limited blood vessel density in mouse tumours, HNO has potential anti-proliferative and anti-angiogenic properties (Norris et al. 2008; Tsihlis et al. 2010). Please refer to Bullen et al., 2011 (Antioxidants & Redox Signaling, 14: page 1682-1683) for a full overview of the anti-proliferative actions of HNO.

Cardioprotective actions of HNO

Unlike $NO^{\bullet}$ donors, AS and 1-NCA display positive lusitropic and inotropic actions to improve myocardial contractility under non-disease conditions (Gao et al. 2012; Tocchetti et al. 2011). A brief overview of the cardioprotective effects of HNO was discussed in Bullen *et al.*, 2011 (Antioxidants & Redox Signaling, 14: pages 1683) and this General Introduction will expand upon this knowledge.

HNO improves excitation-contraction coupling within cardiac cells predominantly through enhanced sarcoplasmic reticulum (SR) calcium (Ca^{2+}) cycling (Tocchetti et al. 2011). In the heart, Ca^{2+} can enter

the SR through voltage-dependent Ca^{2+} channels, which promotes the release of Ca^{2+} from the SR via Ca^{2+}-gated channels (ryanodine receptors; RyR) (Tocchetti et al. 2011). This in turn enables Ca^{2+} to interact with myofilaments within the sarcomere to promote contraction of myocardial cells (Tocchetti et al. 2011). HNO can enhance Ca^{2+} release from the SR by targeting RyR's to increase the opening probability of these channels and the frequency of intracellular Ca^{2+} release (Ca2+ sparks; Cheong et al. 2005; Tocchetti et al. 2007). Additionally, HNO can influence SR Ca^{2+} reuptake by interacting with SERCA2a and the SERCA2a inhibitor phospholamban, yet has no effect on sodium-Ca^{2+} exchange (Froehlich et al. 2008; Lancel et al. 2009; Tocchetti et al. 2007).

The ability of HNO to serve as a cardioprotective agent appears to be independent of sGC/cGMP signalling as ODQ and a cGMP-dependent protein kinase inhibitor were unable to reverse the positive inotropic effects of AS (Chin et al. 2014; Tocchetti et al. 2007). Instead, HNO may target these proteins by thiol-modification as S-glutathiolation of SERCA2a was found to be increased in the presence of AS (Lancel et al. 2009). This idea was further supported by the fact that the reducing agent DTT impaired the ability of AS to enhance S-glutathiolation and activity of SERCA2a, RyR opening probability and Ca^{2+} spark frequency (Cheong et al. 2005; Lancel et al. 2009; Tocchetti et al. 2007). Similarly, the ability of HNO to target SERCA2a and phospholamban was lost when cysteine residues were mutated in critical regions of these proteins (Froehlich et al. 2008; Lancel et al. 2009). Finally, HNO was shown to directly target cysteine residues on myofilaments to enhance their sensitivity to Ca^{2+} (Dai et al. 2007; Gao et al. 2012). Taken together these findings indicate that HNO enhances myocardial contractility via a reversible thiol-sensitive mechanism. Importantly, these positive inotropic and lusitropic effects of AS are preserved in experimental heart failure (Paolocci et al. 2003). Thus it appears under disease conditions, HNO maintains its ability to improve myocardial contractility.

In addition to having a direct effect upon the myocardium, HNO is also beneficial in heart failure due to its ability to unload the heart via vasodilatation (Chin et al. 2014; Tocchetti et al. 2011). Numerous studies have demonstrated that HNO can mediate vasorelaxation systemically in both large conduit and small resistance arteries (as discussed previously). HNO also displays vasodepressor effects and can act as a preferential venodilator *in vivo* under non-disease conditions (Irvine et al. 2011; Irvine et al. 2013; Miranda et al. 2005; Paolocci et al. 2001). These actions appear to be mediated by both

sGC-dependent and –independent mechanisms as ODQ partially impaired relaxation responses to AS in rat coronary arteries and AS increased cGMP levels in rat aortae (Chin et al. 2014; Irvine et al. 2011). In contrast, AS appears to mediate both arterial and venous dilation in heart failure (Paolocci et al. 2003), yet maintained its vasodepressor actions in spontaneously hypertensive rats (Irvine et al. 2013). Taken together, these findings indicate that the ability of HNO to unload the heart contributes to the protective actions of HNO in heart failure. In fact, the cardioprotective actions of a pure, small molecule HNO donor, CXL-1020 have recently been tested in both a canine models of cardiac failure and in patients with decompensated heart failure (Sabbah et al. 2013). In accordance with previous studies, CXL-1020 exhibited positive inotropic and lusitropic actions and displayed a mild vasodepressor effect in an *in vivo* model of experimental heart failure (Sabbah et al. 2013). For the first time in humans, CXCL-1020 improved cardiac output and unloaded the heart in a heart rate independent manner (Sabbah et al. 2013). Importantly, these effects were maintained over a 6 hour infusion period and adverse reactions were minimal (Sabbah et al. 2013). Given $NO^{\bullet}$ donors does not display inotropic or lusitropic effects (Paolocci et al. 2003; Paolocci et al. 2001), this evidence indicates that HNO donors may be superior to traditional nitrovasodilators and represent a novel therapy for the treatment of heart failure.

1.2.6 Potential adverse effects of HNO

Whilst it is clear that HNO displays both vasoprotective and cardioprotective actions, it must be recognised that HNO has previously been shown to display cytotoxic actions at high concentrations and have deleterious actions in ischemia-reperfusion models. For a full overview of the potential adverse interactions of HNO please refer to Bullen et al., 2011 (Antioxidants & Redox Signaling, 14: page 1683).

1.2.7 Therapeutic potential of HNO as a vasoprotective agent

Notwithstanding potential toxicity of HNO, the therapeutic potential of this nitrogen oxide is clear, although further work is required to fully elucidate the clinical benefits of HNO donors. Specifically, the vasoprotective actions of endogenous and exogenously generated HNO need to be assessed in disease states associated with oxidative stress and reduced $NO^{\bullet}$ bioavailability. Moreover, with a lack of tolerance development and resistance to scavenging by $^{\cdot}O_2^-$, HNO donors have the potential to be more advantageous than traditional nitrovasodilators in patients where nitrate tolerance and/or platelet $NO^{\bullet}$ resistance has developed. Thus additional studies are needed to directly compare the vasoprotective actions of HNO donors to those of $NO^{\bullet}$ donors under disease states where endogenous $NO^{\bullet}$ bioavailability may be compromised.

Although not examined in this thesis, there is also a need to confirm that the protective actions of HNO seen in animal models of disease correlate with those observed in cardiovascular disease patients. Importantly, previous studies have indicate that the vasoprotective and cardioprotective effects of HNO were conserved in patients with decompensative heart failure (Sabbah et al. 2013), in arteries from patients with coronary artery disease Bullen *et al.* 2011, (Antioxidants & Redox Signaling, 14: page1678) and in platelets from patients with ischemic heart disease (Dautov et al. 2013).

Taken together, these findings indicate that HNO donors may also be protective in other disease states, such as atherosclerosis, which is associated with dyslipidaemia, enhanced oxidative stress, impaired endogenous $NO^{\bullet}$ bioavailability, endothelial dysfunction, vascular remodelling and inflammation (Libby et al. 2011). Whilst a recent report suggested that vasorelaxation responses to 1-NCA are maintained in atherosclerosis (Donzelli et al. 2013), additional studies are required to fully explore the vasoprotective actions of HNO in the setting of atherosclerosis.

1.3 Atherosclerosis

Cardiovascular disease is the leading cause of death in western society and accounts for ~30% of deaths worldwide, with atherosclerosis a key underlining cause (Libby et al. 2013). Current pharmacological interventions such as lipid-lowering statins, reduce the incidence of cardiovascular mortality by ~30%, however this leaves ~70% of the population at risk of experiencing a cardiovascular event (Mihaylova et al. 2012). Thus, there is a need to develop alternative therapeutic strategies to limit the burden of cardiovascular disease upon society.

Atherosclerosis is a chronic inflammatory disorder characterised by the formation of atherosclerotic lesions within the vessel wall. It is a major risk factor for a number of cardiovascular complications including myocardial infarction, unstable angina and stroke (Libby et al. 2013). Many of these complications arise from either abnormal narrowing of a blood vessel or plaque rupture leading to thrombus formation and vessel occlusion (see Figure 1; Libby et al. 2011). Thus from a therapeutic viewpoint, a reduction in lesion size and improvement in lesion stability are sought.

When considering the pathogenesis of atherosclerosis targeting oxidative stress and the immune system are emerging as viable strategies as they play a key role in the formation and progression of lesions in the arterial wall. Endothelial dysfunction and a loss of vasoprotective $NO^{\bullet}$, is a critical initiating step in the development of lesions and can be triggered by a number of factors including oxidative stress (Landmesser et al. 2004; Vasquez et al. 2012). Endothelial damage leads to the release of cytokines which induce activation of the endothelium leading to increased expression of adhesion molecules and the recruitment and infiltration of monocytes and T lymphocytes into the vessel wall (Fenyo and Gafencu 2013; Libby 2008; Libby 2012). Subsequent differentiation of monocytes into macrophages promotes the expression of cell surface scavenger receptors and enables macrophages to internalise low density lipoprotein (LDL) particles (Fenyo and Gafencu 2013; Libby et al. 2011), leading to lipid retention within macrophages and foam cell formation (Fenyo and Gafencu 2013; Libby 2008).

Whilst it is important to develop therapeutic strategies to limit lesion progression, it has become increasingly evident that promoting plaque stabilisation is also essential to reducing the burden of atherosclerosis. Indeed, lipids, inflammatory cells, VSMC and extracellular matrix molecules such as collagen and elastin are all important for lesion stability (Silvestre-Roig et al. 2014). In advanced

atherosclerosis, high levels of inflammatory cells within the lesion can promote macrophage and VSMC apoptosis (Silvestre-Roig et al. 2014). This in turn leads to the development of a large necrotic core, inflammation, thinning of the fibrous cap and reduced extracellular matrix deposition, resulting in unstable plaques that are prone to rupture (Silvestre-Roig et al. 2014). Whereas stable atherosclerotic lesions are associated with greater VSMC content within the lesion and at the fibrous cap (Silvestre-Roig et al. 2014). An increase in VSMC content can lead to the production of extracellular matrix molecules such as collagen and elastin, which can reinforce the fibrous cap, improve the structural integrity of the lesion and limit necrotic core formation (Silvestre-Roig et al. 2014). Thus, developing therapeutic strategies that promote plaque stability will reduce the likelihood of rupture and subsequently thrombosis. A current limitation of this field is that there are no animal models of plaque rupture that specifically mimic the effects of plaque rupture in human atherosclerosis (Libby et al. 2013; Silvestre-Roig et al. 2014). Although, murine models of hyperlipidaemia are commonly used to assess atherosclerotic lesion development as they display similar characteristics to human atherosclerosis, plaque rupture is rarely observed in these mice (Libby et al. 2013; Silvestre-Roig et al. 2014). Nevertheless, these models of hyperlipidaemia are still useful for evaluating the effect of different therapies on plaque stability.

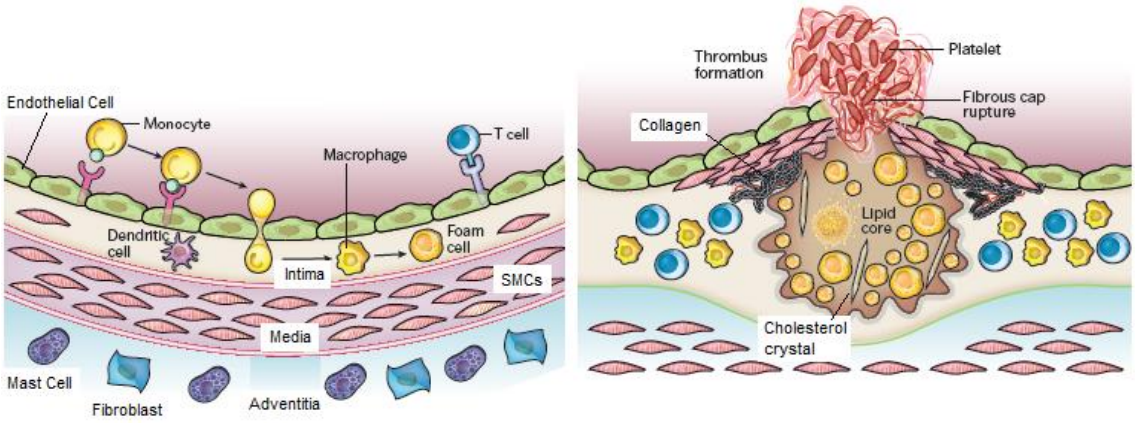


Figure 1. Atherosclerotic lesion development and rupture. *Left*: Cytokine-induced activation of endothelial cells leads increases the expression of adhesion molecules, which allows blood leukocytes to adhere and migrate into the vessel wall. Monocytes can then mature into macrophages, which can uptake low density lipoprotein particles and can form foam cells and further lesion development. *Right*: Atherosclerotic lesions are primarily comprised of lipid, collagen, macrophages and VSMCs, which are integral to lesion progression and stability. Unstable lesions often display a thin fibrous cap, large-necrotic core and the extracellular matrix is less developed, making these lesions more prone to rupture. A physical disruption to a lesion can result in rupture, as a fracture of the fibrous cap can trigger the formation of a thrombus, which can extend into the vessel lumen and limit blood flow. Adapted from (Libby et al. 2011).

1.3.1 Role of macrophages in atherosclerosis

Macrophages are important in lesion progression as they are a major source of cytokines, ROS and matrix metalloproteinases, which are important in extracellular matrix remodelling (Libby 2008; Libby 2012; Tavakoli and Asmis 2012). Previous studies have indicated a variety of roles for macrophages including recruitment of other inflammatory cells (T and B lymphocytes, granulocytes, mast cells), VSMC proliferation and apoptosis, removal of apoptotic cells (efferocytosis) and extracellular matrix remodelling (Fenyo and Gafencu 2013; Libby 2008; Libby 2012; Libby et al. 2013; Tavakoli and Asmis 2012). Additionally, macrophages exist as a heterogeneous population within atherosclerotic lesions and display different functional roles. The two most studied phenotypes are the M1, or classically activated, macrophages and the M2, or alternatively activated, macrophage (Fenyo and Gafencu 2013; Libby 2012; Tavakoli and Asmis 2012), although there is a certain degree of plasticity, as macrophages can switch phenotypes depending upon the local cytokine milieu (Khallou-Laschet et al.

2010; Leitinger and Schulman 2013). It appears that M1 macrophages may be more detrimental in atherosclerosis, as a greater accumulation of M1 macrophages was observed in a mouse model of advanced atherosclerosis (Khallou-Laschet et al. 2010) and patients with coronary artery disease (Hirata et al. 2011). Similarly, in atherosclerotic patients, M1 macrophages tend to accumulate in the necrotic core and the shoulder region of a plaque, which are the areas that are often prone to rupture (Chinetti-Gbaguidi et al. 2011; Stoger et al. 2012). M1 macrophages may promote plaque instability via secretion of pro-inflammatory cytokines (TNFα, IL-1β, IL-6 and IL-12) and are a major source of Nox2-derived ROS (Tavakoli and Asmis 2012), which can activate matrix metalloproteinases (MMPs) to degrade the fibrous cap and extracellular matrix (Leitinger and Schulman 2013; Papaharalambus and Griendling 2007; Tavakoli and Asmis 2012). Indeed, oxidative stress was previously demonstrated to be linked to M1 macrophage polarisation [44]. In contrast, M2 macrophages may be protective in atherosclerosis as they release anti-inflammatory cytokines (transforming growth factor beta; TGF-β, IL-4, IL-13, IL-10), produce less ROS than M1 macrophages, promote angiogenesis and collagen synthesis (Gordon and Martinez 2010; Khallou-Laschet et al. 2010; Leitinger and Schulman 2013; Tavakoli and Asmis 2012). Hence, a shift towards an M2 macrophage phenotype may be protective in atherosclerosis.

1.3.2 Role of ROS and NADPH oxidases in atherosclerosis

As indicated previously, inflammation and oxidative stress both play an integral role in atherogenesis. Indeed, augmented vascular $^{\cdot}O_2^-$ generation is observed in human atherosclerosis and experimental models of atherosclerosis, and is attributed primarily to the increased expression and activity the ROS generating enzymes, NAPDH oxidases (Drummond et al. 2011; Konior et al. 2013; Violi et al. 2009). The NADPH oxidase family consists of seven isoforms in mammals; Nox1-5 and Duox1-2, however at present, only Nox1, Nox2, Nox4 and Nox5 have been associated with atherosclerosis, which will be discussed in more detail (Gray et al. 2013; Guzik et al. 2008; Guzik et al. 2006; Judkins et al. 2010; Sheehan et al. 2011; Sorescu et al. 2002). Each isoform contains a core Nox catalytic subunit, however Nox1, Nox2 and Nox4-containing NADPH oxidases require up to five additional subunits, such as the membrane-bound subunit ($p22^{phox}$), the cytosolic regulatory subunits (NoxA1, $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$) and the GPTase Rac (see Figure 2; Drummond et al. 2011; Konior et al. 2013).

The activity of Nox4 oxidase also appears to be regulated by polymerase δ-interacting protein 2 (POLDIP2) and Nox4 has been shown to be constitutively active (Drummond et al. 2011; Konior et al. 2013). Activation of these enzymes requires the formation of the NADPH complex at the cellular membrane, which facilitates an electron transfer from NADPH leading to the generation of $^{\cdot}O_2^-$ and/or H_2O_2 (Drummond et al. 2011; Konior et al. 2013). In contrast, Nox5 does not appear to interact with any known NADPH oxidase regulatory subunits, although Nox5 oxidase is associated with four EF hand motifs and is activated by Ca^{2+} (see Figure 2; Drummond et al. 2011; Konior et al. 2013). Whilst Nox1 and Nox2 primarily generate $^{\cdot}O_2^-$, Nox4 produces H_2O_2 rather than $^{\cdot}O_2^-$ and Nox5 has been reported to generate both $^{\cdot}O_2^-$ and H_2O_2 (Drummond et al. 2011; Konior et al. 2013). Additionally, expression of Nox1 and Nox4 has only been reported in cells in the vascular wall (Drummond et al. 2011; Konior et al. 2013). Whereas Nox2 has been identified in the vascular wall and immune cells in atherosclerosis (Drummond et al. 2011; Konior et al. 2013) and there is evidence to suggest that Nox5 oxidase may also be expressed in both locations (Lewis et al., unpublished, Drummond et al. 2011; Konior et al. 2013). However, Nox5 oxidase is only expressed in primates and does not appear to be found in rodents (Drummond et al. 2011; Konior et al. 2013), which is a limitation when translating findings from mice to humans. Nevertheless, NADPH oxidases are an important source of ROS in blood vessels and immune cells and NADPH oxidase-dependent oxidative stress has been associated with a number of cardiovascular diseases including atherosclerosis, hypertension, restenosis after injury, pulmonary hypertension and cardiac failure (Drummond et al. 2011; Konior et al. 2013).

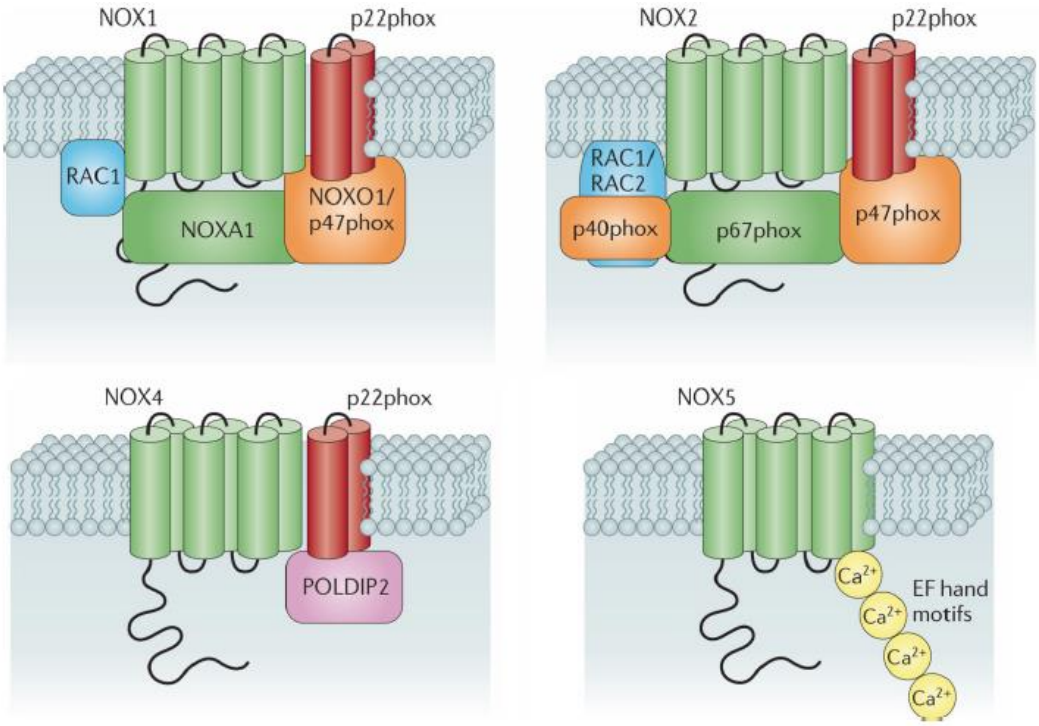


Figure 2. Structure of Nox1, Nox2, Nox4 and Nox5 NADPH oxidases. The Nox1-containing isoform consists of two membrane-bound subunits (Nox1 and p22phox), two regulatory subunits ($p47^{phox}$ and NoxA1) and the GTPase Rac. The structure of Nox2-containing NADPH is similar to the Nox1 isoform as it is comprised of two membrane-bound subunits (Nox2 and p22phox), three regulatory subunits ($p40^{phox}$, $p47^{phox}$ and $p67^{phox}$) and the GTPase Rac. Similarly the Nox4-containing isoform consists of two membrane-bound subunits (Nox4 and p22phox) and appears to be regulated by polymerase δ-interacting protein 2 (POLDIP2). In contrast, the Nox5-containing isoform does not appear to interact with known NADPH oxidase regulatory subunits, although Nox5 oxidase is associated with four EF hand motifs. Adapted from (Drummond et al. 2011).

In atherosclerosis, enhanced $^{\cdot}O_2^-$ production can lead to endothelial dysfunction and impaired endogenous $NO^{\bullet}$ bioavailability (Landmesser et al. 2004; Vasquez et al. 2012). $NO^{\bullet}$ can be generated in a eNOS-dependent manner, however under conditions of oxidative stress, eNOS can become uncoupled due to depletion of the eNOS co-factor tetrahydrobiopterin and the levels of the eNOS inhibitor asymmetric dimethyl-L-arginine may be elevated, leading to reduced $NO^{\bullet}$ generation from this enzyme (Ponnuswamy et al. 2012). Additionally, oxidative stress can reduce the efficacy of $NO^{\bullet}$ in atherosclerosis, through increased scavenging by $^{\cdot}O_2^-$ or due to a shift towards an $NO^{\bullet}$-insensitive form of sGC (Stasch et al. 2006). Impaired $NO^{\bullet}$ bioavailability may also contribute to endothelial dysfunction, as $NO^{\bullet}$ is an important mediator of endothelium-dependent vasorelaxation and a

deficiency in $NO^{\bullet}$ also leads to a proinflammatory and prothrombotic environment (Landmesser et al. 2004; Siegel-Axel et al. 2008). Indeed, relaxation responses to acetylcholine were impaired in aortas from atherosclerotic mice, which was attributed to increased scavenging by $^{\cdot}O_2^-$ (d'Uscio et al. 2001). Thus, targeting the $NO^{\bullet}$ system to improve $NO^{\bullet}$ bioavailability may have a protective effect in atherosclerosis.

In addition to impaired endogenous $NO^{\bullet}$ bioavailability, oxidative stress has also been implicated in lesional development. In experimental models of atherosclerosis, genetic deletion of either Nox2 or its cytosolic subunit, $p47^{phox}$, led to a reduction in lesion area along the length of the aorta (Barry-Lane et al. 2001; Judkins et al. 2010; Vendrov et al. 2007), although did not alter lesion size in the aortic sinus (Barry-Lane et al. 2001; Hsich et al. 2000; Kirk et al. 2000). Suggesting that the effects of Nox2 oxidase may be dependent upon the arterial site of the lesion. Nonetheless, Nox2-derived $^{\cdot}O_2^-$ may play a role in oxidation of LDL (Libby et al. 2013; Tavakoli and Asmis 2012), as a deficiency in macrophage Nox2 oxidase led to reduced plasma oxLDL levels and macrophage accumulation in early atherosclerosis (Vendrov et al. 2007). Furthermore, oxidative stress has also been implicated in endothelial cell activation, increased expression of adhesion molecules and cytokine release, leading to the infiltration and retention of inflammatory cells within the vascular wall (see Figure 1; Libby et al. 2013; Tavakoli and Asmis 2012). Specifically, a deficiency of Nox2 oxidase in the vessel wall was previously shown to limit endothelial cell activation and macrophage accumulation in early atherosclerosis (Vendrov et al. 2007), whereas overexpression of endothelial Nox2 oxidase increased macrophage recruitment (Douglas et al. 2012). Taken together these findings indicate that Nox2 oxidase plays an important role in the initiation and development of atherosclerotic lesions. However, at present it is unclear what effect Nox2 oxidase has upon plaque stability. Nonetheless Nox2 oxidase represents a viable therapeutic target, which may be beneficial in reducing the burden atherosclerosis.

Given current pharmacological interventions such as lipid-lowering statins are only beneficial in ~30% of the population, new therapies are sought that have anti-inflammatory actions together with an ability to induce vasorelaxation and limit thrombus formation. $NO^{\bullet}$ donors represent an alternative therapeutic strategy, as they display a number of vasoprotective actions. Specifically, $NO^{\bullet}$ donors were previously found to induce vasorelaxation, inhibit platelet aggregation, has anti-proliferative

properties and can modulate Nox2 oxidase activity to reduce $^{\cdot}O_2^-$ generation in human endothelial cells (Moncada and Higgs 2006; Selemidis et al. 2007). However, the susceptibility of clinically used $NO^{\bullet}$ donors to tolerance development and impaired efficacy under conditions of enhanced oxidative stress (Klemenska and Beresewicz 2009), may limit their effectiveness in atherosclerosis.

As discussed in Bullen *et al.* 2011, (Antioxidants & Redox Signaling, 14: pages 1675-1686), HNO donors demonstrate similar vasoprotective actions to those of $NO^{\bullet}$ donors. Additionally, HNO displays distinct pharmacological actions to that of $NO^{\bullet}$, which could enable its vasoprotective actions to be sustained and/or enhanced in atherosclerosis. As previously mentioned, atherosclerosis is associated with eNOS uncoupling, enhanced oxidative stress (Ponnuswamy et al. 2012) and depletion of cellular thiol levels (Park and Oh 2011). Since one of the proposed mechanisms for HNO generation is via uncoupled eNOS, there is potential for HNO generation to be augmented in atherosclerosis (Ponnuswamy et al. 2012; Rusche et al. 1998). Similarly, the bioavailability of HNO could be preserved as it is resistant to scavenging by $^{\cdot}O_2^-$ (Leo et al. 2012; Miller 2013), does not develop tolerance with continual use (Irvine et al. 2010; Irvine et al. 2007) and HNO donors are more efficacious in the absence of thiol scavengers (Wink et al. 1998). Importantly, previous studies have reported that the vasoprotective actions of HNO are sustained in other cardiovascular disease states (Dautov et al. 2013; Donzelli et al. 2013; Leo et al. 2012; Wynne et al. 2012). Thus, HNO donors may be more advantageous than traditional nitrovasodilators in the setting of atherosclerosis. However, at present, little is known about the vasoprotective actions of HNO in hypercholesterolemia or atherosclerosis.

1.3.3 The Apolipoprotein E-deficient (ApoE$^{-/-}$) mouse as a model of atherosclerosis

The ApoE$^{-/-}$ mouse model was developed concurrently by two laboratories in the United States in 1992 and targets the ApoE gene via homologous recombination of embryonic stem cells (Piedrahita et al. 1992; Plump et al. 1992). ApoE$^{-/-}$ mice were originally developed on either C57BL/6J, BALB/cJ or 129 mouse genetic backgrounds and as such these strains can be used as wild-type controls (Piedrahita et al. 1992; Plump et al. 1992). These mice lack apolipoprotein E which is involved in the transport of plasma lipoproteins to the liver for metabolism (Zhang et al. 1992). As a result, ApoE$^{-/-}$ mice are associated with hypercholesterolemia and spontaneous atherosclerotic lesion formation, making these mice an appropriate model of atherosclerosis (Zhang et al. 1992). Moreover, maintaining these mice on a high fat diet leads to an acceleration in atherosclerosis development (Meyrelles et al. 2011).

The development of atherosclerotic lesions in ApoE$^{-/-}$ mice occurs at a relatively young age between 8-10 weeks of age and these lesions are mainly comprised of smooth muscle and foam cells (Kolovou et al. 2008; Meyrelles et al. 2011). However, as the disease progresses, more advanced, fibrous plaques start to appear between 15-20 weeks of age (Kolovou et al. 2008; Meyrelles et al. 2011). Eventually these plaques lead to almost total occlusion of blood vessels at a very old age of ~70 weeks (Meyrelles et al. 2011). In addition to atherogenesis, lesional stability also plays a crucial role in the pathogenesis of atherosclerosis as an acute physical disruption of a lesion can trigger thrombosis, which can lead to a number of different vascular complications (Libby et al. 2013; Tavakoli and Asmis 2012). Whilst it is a matter of contention whether plaque rupture occurs in the ApoE$^{-/-}$ mouse model, advanced lesions do share many commonalities to human atherosclerosis (Bond and Jackson 2011; Getz and Reardon 2012; Meyrelles et al. 2011; Rosenfeld et al. 2008). As such, ApoE$^{-/-}$ mice can be used to assess how pharmacological interventions (Bea et al. 2002; Gaspari et al. 2013) and/or genetic mutations can affect plaque composition and stability.

Atherogenesis in patients and ApoE$^{-/-}$ mice is characterised by spontaneous atheroma development, enhanced oxidative stress, endothelial dysfunction and hypercholesterolemia (Kolovou et al. 2008; Meyrelles et al. 2011). Whereby augmented $^{\cdot}O_2^-$ production in the vasculature is attributed in part, to upregulation of Nox2 oxidase expression in human and animal models of atherosclerosis (Guzik et al. 2006; Judkins et al. 2010; Sorescu et al. 2002). As such, the ApoE$^{-/-}$ mouse model is an appropriate

model to study vasoprotective properties and the effects of oxidative stress upon plaque formation and stability.

1.4 Aims

The vasoprotective actions of HNO are well-established such that HNO **i)** can mediate vasorelaxation, inhibit platelet aggregation, suppress Nox2-derived $^{\cdot}O_2^-$ production and is resistant to tolerance development under non-disease conditions, **ii)** display distinct pharmacological actions that may confer advantages over traditional nitrovasodilators and **iii)** may be generated endogenously, serving as an EDRF. The vasoprotective actions of HNO in the setting of vascular disease are not well characterised and as such the specific aims of this thesis were to;

1) Determine if the vasoprotective actions of the HNO donor, IPA/NO are preserved in an ApoE$^{-/-}$ mouse model of hypercholesterolemia and subsequently compare its effects to the NO$^{\bullet}$ donor, GTN (Chapter 2)

2) Examine the vasoprotective actions of IPA/NO and elucidate the contribution of endogenous HNO to endothelium-dependent vasorelaxation in an ApoE$^{-/-}$ mouse model of advanced atherosclerosis and subsequently compare the effects of IPA/NO with those of the NO$^{\bullet}$ donors, GTN and diethylamine NONOate (DEA/NO; Chapter 3)

3) Establish a model of platelet NO$^{\bullet}$ resistance in ApoE$^{-/-}$ mice, such that impaired anti-aggregatory responses to the NO$^{\bullet}$ donor, SNP were apparent. Subsequently determine the potential mechanism of resistance and elucidate whether the NO$^{\bullet}$-independent sGC stimulators, IPA/NO and BAY 41-2272 can circumvent platelet NO$^{\bullet}$ resistance in this model (Chapter 4)

4) Examine what effect genetic deletion of Nox2 oxidase has upon vascular function, lesion size, lesion stability and macrophage phenotype in an ApoE$^{-/-}$ mouse model of advanced atherosclerosis (Chapter 5)

Monash University

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Performed all experiments except those for total plasma cholesterol, analysed results and wrote the manuscript	75%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Alyson Miller	Provided intellectual advice during experimental and manuscript preparation stages and assisted with the editorial process.	
Janahan Dharmarajah		5%
Grant Drummond		
Christopher Sobey		
Barbara Kemp-Harper		

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date**
Main Supervisor's Signature		**Date**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

Vasorelaxant and antiaggregatory actions of the nitroxyl donor isopropylamine NONOate are maintained in hypercholesterolemia

Michelle L. Bullen, Alyson A. Miller, Janahan Dharmarajah, Grant R. Drummond, Christopher G. Sobey and Barbara K. Kemp-Harper

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Bullen ML, Miller AA, Dharmarajah J, Drummond GR, Sobey CG, Kemp-Harper BK. Vasorelaxant and antiaggregatory actions of the nitroxyl donor isopropylamine NONOate are maintained in hypercholesterolemia. *Am J Physiol Heart Circ Physiol* 301: H1405–H1414, 2011. First published July 29, 2011; doi:10.1152/ajpheart.00489.2011.—Nitroxyl (HNO) displays pharmacological and therapeutic actions distinct from those of its redox sibling nitric oxide ($NO^{\bullet}$). It remains unclear, however, whether the vasoprotective actions of HNO are preserved in disease. The ability of the HNO donor isopropylamine NONOate (IPA/NO) to induce vasorelaxation, its susceptibility to tolerance development, and antiaggregatory actions were compared with those of a clinically used $NO^{\bullet}$ donor, glyceryl trinitrate (GTN), in hypercholesterolemic mice. The vasorelaxant and antiaggregatory properties of IPA/NO and GTN were examined in isolated carotid arteries and washed platelets, respectively, from male C57BL/6J mice [wild-type (WT)] maintained on either a normal diet (WT-ND) or high fat diet (WT-HFD; 7 wk) as well as apolipoprotein E-deficient mice maintained on a HFD ($ApoE^{-/-}$-HFD; 7 wk). In WT-ND mice, IPA/NO (0.1–30 μmol/l) induced concentration-dependent vasorelaxation and inhibition of collagen (30 μg/ml)-stimulated platelet aggregation, which was predominantly soluble guanylyl cyclase/cGMP dependent. Compared with WT-HFD mice, $ApoE^{-/-}$-HFD mice displayed an increase in total plasma cholesterol levels ($P < 0.001$), vascular ($P < 0.05$) and platelet ($P < 0.05$) superoxide ($O_2^{\cdot -}$) production, and reduced endogenous $NO^{\bullet}$ bioavailability ($P < 0.001$). Vasorelaxant responses to both IPA/NO and GTN were preserved in hypercholesterolemia, whereas vascular tolerance developed to GTN ($P < 0.001$) but not to IPA/NO. The ability of IPA/NO (3 μmol/l) to inhibit platelet aggregation was preserved in hypercholesterolemia, whereas the actions of GTN (100 μmol/l) were abolished. In conclusion, the vasoprotective effects of IPA/NO were maintained in hypercholesterolemia and, thus, HNO donors may represent future novel treatments for vascular diseases.

nitric oxide; vasorelaxation; platelet aggregation

THE THERAPEUTIC UTILITY of the nitric oxide ($NO^{\bullet}$)-soluble guanylyl cyclase (sGC) signaling pathway has long been recognized, with organic nitrates, such as glyceryl trinitrate (GTN), used in the treatment of angina pectoris and heart failure for over 100 yr. However, the clinical efficacy of such traditional nitrovasodilators is limited due to their susceptibility to tolerance development with continued used (4) and their impaired antiplatelet (3) and vasodilatory efficacy (26) during oxidative stress. Thus, there is a need for a new generation of novel $NO^{\bullet}$-like drugs.

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Nitroxyl (HNO), the one electron reduced and protonated congener of $NO^{\bullet}$, displays distinct pharmacological actions and may have therapeutic advantages over its redox sibling $NO^{\bullet}$ (2, 13). Unlike $NO^{\bullet}$, HNO appears to be resistant to scavenging by superoxide ($O_2^{\cdot -}$) (18, 20, 27), does not develop tolerance with continued use (10, 13), and serves as a positive cardiac inotrope (via thiol interaction) (22, 28). As such, HNO donors confer protection in the setting of acute experimental heart failure (22), where $NO^{\bullet}$ donors have negligible effects.

In addition to its cardioprotective properties, HNO displays vasoprotective actions (2). Using HNO donors, such as Angeli's salt and isopropylamine NONOate (IPA/NO), HNO has been shown to serve as a potent vasodilator in vitro (7, 9, 11, 12) and in vivo (10, 22, 23). Like $NO^{\bullet}$, HNO elicits vasorelaxation, at least in vitro, predominantly via the activation of sGC (8, 11) and subsequent generation of cGMP (9, 12). However, HNO can also signal through distinct mechanisms such as via the release of calcitonin gene-related peptide (CGRP) (8, 23) or activation of vascular voltage-dependent K^+ (K_v) channels (7, 11) and ATP-sensitive K^+ (K_{ATP}) channels (8). Moreover, Angeli's salt can also serve as an antiaggregatory agent, inhibiting human platelet aggregation in response to a variety of stimuli (1, 21), via a sGC-dependent mechanism (1). Thus, under nondisease conditions, HNO donors serve as potent vasodilators and antiaggregatory agents.

From a therapeutic perspective, it is important to determine if such vasoprotective actions of HNO are preserved in disease states associated with oxidative stress and compromised $NO^{\bullet}$ signaling. Given that HNO, unlike $NO^{\bullet}$, has been postulated to be resistant to scavenging by $O_2^{\cdot -}$, (18, 20, 27), does not develop tolerance under physiological conditions (10, 12), can target distinct signaling pathways in the vasculature (K_v channels, K_{ATP} channels, and CGRP), and its bioavailability may be augmented in the face of disease-associated thiol depletion, it may be anticipated that HNO donor efficacy will be preserved and/or enhanced under pathophysiological conditions. Indeed, a handful of studies to date have indicated, albeit indirectly, that the vasoprotective properties of HNO may be sustained in disease. Thus, Angeli's salt has been shown to lower blood pressure in the setting of acute experimental heart failure (22) and inhibit the aggregation of platelets from patients with sickle cell disease (21). In addition, IPA/NO has been reported to limit neointimal hyperplasia in rat carotid arteries after balloon injury (29).

The present study sought to fully explore the concept of preserved vasoactivity to HNO in disease and compare, for the first time, the vasoprotective actions of HNO with the clinically used nitrovasodilator GTN under conditions associated with oxidative stress. Using a model of hypercholesterolemia in

mice [apolipoprotein E-deficient ($ApoE^{-/-}$) mice placed on a high-fat diet (HFD) for 7 wk], which is characterized by increased plasma cholesterol, elevated vascular $O_2^{\cdot-}$ generation, and reduced endogenous $NO^{\bullet}$ bioavailablity (14), the vasorelaxant and antiaggregatory actions of the HNO donor IPA/NO and its susceptibility to tolerance development were compared with GTN.

METHODS

This study was approved by the School of Biomedical Sciences Animal Ethics Committee of Monash University (Clayton, Victoria, Australia) and conformed with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Experimental animals. Wild-type (WT) and $ApoE^{-/-}$ mice were obtained from the Animal Resources Centre (Canning Vale, WA, Australia). All mice studied were male and fully backcrossed to the C57BL/6J background. From 5 wk of age, C57BL6/J mice were either maintained on a normal diet of standard chow (WT-ND) or a HFD (WT-HFD; 21% fat and 0.15% cholesterol, Speciality Feeds) for 7 wk. $ApoE^{-/-}$ mice were also maintained on a HFD for 7 wk from 5 wk of age. For all experiments, mice were deeply anesthetized by isoflurane inhalation (Baxter Healthcare) before being euthanized by decapitation.

Measurement of total plasma cholesterol levels. Blood from WT-HFD and $ApoE^{-/-}$-HFD mice was collected from the inferior vena cava into heparinized tubes, and plasma was isolated via centrifugation (4,000 *g*, 4°C, 10 min). Plasma total cholesterol levels were then determined using a Roche MODULAR 917 (Roche Diagnostics, Castle Hill, NSW, Australia) enzymatic colorimetric array.

Histological experiments. Internal and common carotid arteries from WT-HFD and $ApoE^{-/-}$-HFD mice were examined for the presence of atherosclerotic lesions. Arteries were isolated, cut into 2- to 3-mm-long segments, mounted in an OCT Tissue-Tek mould, snap frozen in liquid nitrogen, and stored at −80°C. Arteries were sectioned (10 μm) and thaw mounted onto poly-L-lysine-coated microscope slides. Slides were fixed with paraformaldehyde (4%, 10 min) before being washed in 60% isopropyl alcohol (1.5 min) and left to dry. Sections were then stained with oil red O (0.5% in 60% isopropyl alcohol, 60 min). Excess stain was removed with 60% isopropyl alcohol (5 min) and distilled water (1 min), and sections were then counterstained with hematoxylin (25%, 2 min) as previously described (14, 17). Sections were viewed using an Olympus BX51 microscope (Olympus, Tokyo, Japan) equipped with a ×40 oil immersion lens. Images were digitized using a color OP70 Peltier cooled digital camera and captured with a data-acquisition system (Analysis LS Starter version 3.0, Olympus Soft Imaging Solutions, Munster, Germany).

Superoxide detection. L-012 (100 μmol/l)-enhanced chemiluminescence was used to measure basal and phorbol 12,13-dibutyrate (PDB; 10 μmol/l)-stimulated $O_2^{\cdot-}$ production by isolated common carotid artery segments or platelets from WT-HFD and $ApoE^{-/-}$-HFD mice, as previously described (14).

Carotid arteries were excised, cleaned, and cut into segments of ~2 mm in length. Background chemiluminescence signals were obtained over a 30-min period using a Plate Chameleon Luminescence Reader (Hidex) with separate wells of a white 96-well Opti-plate containing Krebs-HEPES [composed of (in mmol/l) 99 NaCl, 4.7 KCl, 1.2 $MgSO_4 \cdot 7H_2O$, 1.0 KH_2PO_4, 19.6 Na_2HCO_3, 20 Na-HEPES, 11.1 D-glucose, 2.5 $CaCl_2$, and 0.026 EDTA; pH 7.4] and L-012 (100 μmol/l). In semi-darkness, arteries were placed in separate wells, and L-012-enhanced chemiluminescence was measured every 2 min over a 30-min period. Experiments were performed in duplicate. $O_2^{\cdot-}$ production for each ring segment was calculated by subtracting the background chemiluminescence signal (in relative light units/s) from the signal in the presence of the artery and then normalized to dry tissue weight (in mg).

Platelets were isolated from whole blood, resuspended in Tyrode buffer [composed of (in mmol/l) 12 $NaHCO_3$, 10 HEPES, 137 NaCl, 2.7 KCl, and 5.5 D-glucose with 0.026 EDTA; pH 7.2–7.4, 5×10^7 platelets/ml] and equilibrated for 30 min at 37°C as described in *Platelet aggregation*. Background counts were obtained over a 20-min period in a 96-well OptiPlate containing Krebs-HEPES and L-012 (100 μmol/l). In semi-darkness, platelets (5×10^8 platelets/ml) were placed in separate wells, and basal chemiluminescence was measured every 2 min over a 20-min period. PDB (10 μmol/l) was then added to the wells, and chemiluminescence was measured every 2 min over a 40-min period. Experiments were performed in quintuplicate. Basal chemiluminescence was calculated by subtracting the background chemiluminescence signal from the basal platelet chemiluminescence signal. PDB-stimulated chemiluminescence was calculated by subtracting the basal platelet chemiluminescence signal from the PDB-stimulated platelet chemiluminescence signal.

Vascular function experiments. Common carotid arteries from WT-ND, WT-HFD, and $ApoE^{-/-}$-HFD mice were isolated, cleaned of fat and connective tissue, and cut into ~2-mm-long ring segments. Arteries were mounted in a Mulvany-style small vessel myograph (Danish Myo Technology, Skejbyparken, Denmark) for the measurement of isometric tension as previously described (11). Vessels were maintained in Krebs-bicarbonate solution [composed of (in mmol/l) 118 NaCl, 4.5 KCl, 0.5 $MgSO_4$, 1.0 KH_2PO_4, 25 $NaHCO_3$, 11.1 glucose, 2.5 $CaCl_2$ and 0.026 EDTA; pH 7.4] at 37°C and bubbled with carbogen (95% O_2-5% CO_2). After a 30-min equilibration period, arteries were stretched to a resting tension of 5 mN before being maximally contracted with U-46619 (1 μmol/l; F_{max}). To examine vasorelaxation responses, arteries were precontracted to ~50% F_{max} with titrated concentrations of U-46619 (3–30 nmol/l). In carotid arteries from WT-ND mice, cumulative concentration-response curves to the HNO donors IPA/NO (1 nmol/l–30 μmol/l) and Angeli's salt (1 nmol/l–30 μmol/l) were constructed in the absence or presence of the HNO scavenger L-cysteine (3 or 10 mmol/l, 5-min preincubation), the $NO^{\bullet}$ scavenger carboxy-[2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxy-3-oxide] (carboxy-PTIO; 200 μmol/l, 10-min preincubation), and the sGC inhibitor 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxaline-1-one (ODQ; 10 μmol/l, 30-min preincubation). Maximal relaxation was achieved using diethylamine NONOate (DEA/NO; 1 μmol/l) or levcromakalim (10 μmol/l) at the conclusion of each concentration-response curve. Only one concentration-response curve to any vasodilator was obtained for each vessel.

To assess the effect of hypercholesterolemia on vasorelaxant responses, cumulative concentration-response curves to IPA/NO (1 nmol/l–30 μmol/l), the $NO^{\bullet}$ donor GTN (1 nmol/l–30 μmol/l), and the endothelium-independent vasodilator papaverine (10 nmol/l–30 μmol/l) were constructed in external common carotid arteries from WT-HFD and $ApoE^{-/-}$-HFD mice as described above.

To investigate the potential development of tolerance to GTN or IPA/NO in hypercholesterolemia, carotid arteries from WT-HFD and $ApoE^{-/-}$-HFD mice were incubated in either the absence or presence of GTN (30 μmol/l) or IPA/NO (30 μmol/l) for a period of 60 min (12). Vessels were then washed thoroughly every 15 min for 60 min. Subsequently, arteries were precontracted to ~50% F_{max} with titrated concentrations of U-46619 (3–30 nmol/l) before concentration-response curves to GTN or IPA/NO were obtained.

Contractile responses to the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; 100 μmol/l) were measured as an indicator of NO bioavailability in external common carotid artery segments from WT-HFD and $ApoE^{-/-}$-HFD mice. Vessels were precontracted to ~20–30% F_{max} with titrated concentrations of U-46619 (4–20 nmol/l). Once the U-46619 contraction was stable, L-NAME (100 μmol/l) was added, and the contractile response was recorded once it reached a plateau.

Platelet aggregation. Platelets were isolated from whole blood collected from the inferior vena cava of WT-ND, WT-HFD, and $ApoE^{-/-}$-HFD mice and stimulated for aggregation experiments as

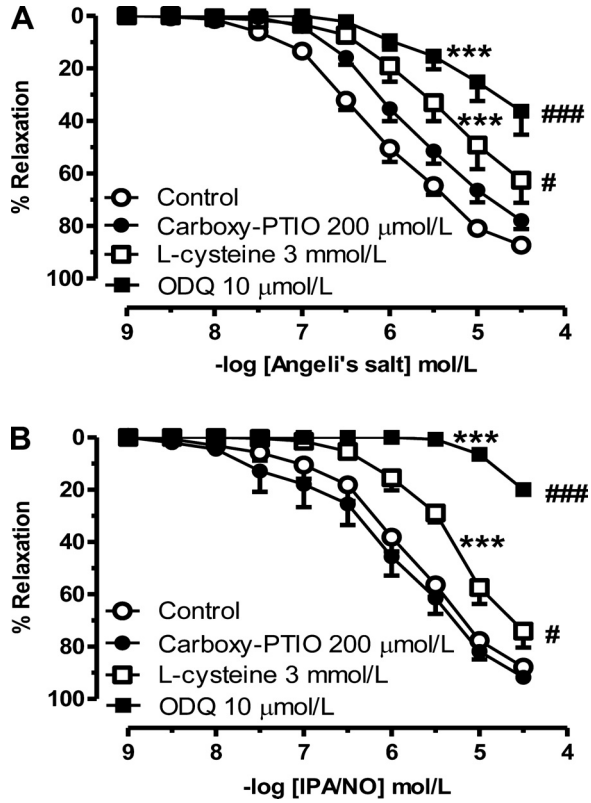

Fig. 1. Concentration-dependent vasorelaxation responses to Angeli's salt (*A*) and isopropylamine NONOate (IPA/NO; *B*) in common carotid arteries from wild-type (WT) mice on a normal diet (WT-ND) in the absence (control; $n = 10$) or presence of carboxy-[2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxy-3-oxide] (carboxy-PTIO; 200 μmol/l, $n = 6–10$), L-cysteine (3 mmol/l, $n = 6–7$), and 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxaline-1-one (ODQ; 10 μmol/l, $n = 6$). Values are expressed as the percent reversal of precontraction and are given as means ± SE, where n is the number of animals. ***$P < 0.001$ for the treatment concentration-response curve vs. the untreated control (two-way ANOVA, Tukey post hoc test); #$P < 0.05$ and ###$P < 0.001$ for the response at 30 μmol/l vs. the untreated control (one-way ANOVA, Dunnett modified *t*-test).

previously described (6). To prevent coagulation, blood was treated with a combination of low-molecular-weight heparin (clexane; 400 U/ml) and acid citrate dextrose buffer [composed of (in mmol/l) 85 trisodium citrate, 72.9 citric acid, and 110 D-glucose] immediately after collection. Blood was then washed with platelet wash buffer [composed of (in mmol/l) 4.3 K_2HPO_4, 4.3 Na_2HPO_4, 24.3 NaH_2PO_4, 113 NaCl, and 5.5 D-glucose with 10% BSA and 20 U/ml clexane; pH 6.5] and centrifuged at 200 *g* (37°C, 2 min). This was performed three times, and each time the platelet rich plasma was removed. After centrifugation of the pooled platelet-rich plasma (2,000 *g*, 1 min), the pellet was resuspended in Tyrode buffer to give a concentration of 5×10^7 platelets/ml. Platelets were added to siliconized cuvettes containing fibrinogen (2 mg/ml) and allowed to equilibrate at 37°C for 30 min before stimulation with collagen (30 μg/ml). Platelet aggregation was measured using a four-chamber turbidometric platelet aggregometer (AggRAM, Helena Laboratories) as a change in light transmission over 30 min under continuous stirring (600 rpm, 37°C).

To characterise the antiaggregatory actions of IPA/NO, platelets from WT-ND mice were treated with either vehicle (1 mmol/l NaOH, 2 min) or IPA/NO (0.1–3 μmol/l, 2 min) before stimulation with collagen (30 μg/ml). The effects of ODQ (10 μmol/l, 30 min) and the cGMP-dependent protein kinase (cGK) inhibitor Rp-8-pCPT-cGMPS (200 μmol/l, 10 min) on the ability of IPA/NO (1 μmol/l) to inhibit collagen-stimulated platelet aggregation were also examined.

To compare the antiaggregatory actions of HNO and NO• in hypercholesterolemia, platelets from WT-HFD and ApoE$^{-/-}$-HFD mice were preincubated in the presence of vehicle (1 mmol/l NaOH or 0.5% ethanol, 2 min), IPA/NO (3 μmol/l, 2 min), or GTN (100 μmol/l, 2 min) before stimulation with collagen (30 μg/ml).

Data and statistical analysis. Vasorelaxation responses were expressed as the percent reversal of U-46619 precontraction. Contractile responses to L-NAME were expressed as a percentage of the maximum response to U-46619 (1 μmol/l; F_{max}). Individual relaxation curves were fitted to a sigmoidal logistical equation (GraphPad Prism, version 5.0) to provide an estimate of the concentration of agonist causing a 50% relaxation (pEC_{50} value; in −log mol/l). Differences between mean pEC_{50} and maximum relaxation values were tested using either a Student's unpaired *t*-test or one-way ANOVA with either a Dunnett's or Bonferroni post hoc test. Where pEC_{50} values could not be obtained, concentration-response curves were compared by means of two-way ANOVA with a Tukey post hoc test (Sigma Stat 3.5).

Platelet aggregation responses were expressed as either total aggregation area under the curve (AUC; in arbitrary units) in percent increments of light transmission over the 30-min period after the addition of collagen or time to half-maximal aggregation (in s). Statistical comparisons were performed using one-way ANOVA with a Bonferroni post hoc test (GraphPad Prism, version 5.0). All results are expressed as means ± SE, with the number of animals denoted by *n*. *P* values of <0.05 were considered statistically significant.

Drugs and their sources. Drugs and their sources were as follows: clexane (enoxaparin sodium, Aventos), collagen (bovine, Helena Laboratories), GTN (Mayne), aquatex, isopentane, isopropyl alcohol, and paraformaldehyde (Merck), IPA/NO (a kind gift from K. Miranda, University of Arizona), L-012 (Wako), levcromakalim (Tocris Bioscience), Angeli's salt (sodium trioxodinitrate), carboxy-PTIO potassium salt, ODQ, and U-46619 (Sapphire Bioscience), and DEA/NO, fibrinogen-bovine serum type I, L-cysteine hydrochloride, L-

Table 1. *Effect of the NO• scavenger carboxy-PTIO, nitroxyl scavenger L-cysteine, and soluble guanylyl cyclase inhibitor ODQ on vasorelaxation responses to Angeli's salt and IPA/NO in common carotid arteries from WT mice on a normal diet*

	Angeli's Salt			IPA/NO		
Nitrovasodilator Treatment	*n*	pEC_{50}	R_{max}	*n*	pEC_{50}	R_{max}
Control	9	6.13 ± 0.15	88 ± 2	10	5.91 ± 0.13	88 ± 3
Carboxy-PTIO (200 μmol/l)	6	5.57 ± 0.28	80 ± 3	10	6.03 ± 0.24	92 ± 2
L-Cysteine (3 mmol/l)	6	ND	63 ± 9†	7	5.21 ± 0.04*	74 ± 6†
ODQ (10 μmol/l)	6	ND	34 ± 6‡	5	ND	20 ± 5‡

Values are mean ± SE; *n*, number of animals. pEC_{50} values are expressed as −log M. Maximum relaxation (R_{max}) values are expressed as the percent reversal of the level of precontraction in response to 30 μmol/l of the nitrovasodilator. ND, pEC_{50} value not determined as a plateau was not obtained. WT, wild type; NO•, nitric oxide radical; carboxy-PTIO, carboxy-[2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxy-3-oxide]; ODQ, 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxaline-1-one; IPA/NO, isopropylamine NONOate. *$P < 0.001$ for the treatment concentration-response curve vs. the untreated control (two-way ANOVA, Tukey post hoc test); †$P < 0.05$ and ‡$P < 0.001$ for the response at 30 μmol/l vs. the untreated control (one-way ANOVA, Dunnett's modified *t*-test).

NAME, oil red O, papaverine, and Rp-8-pCPT-cGMPS (Sigma). Clexane was prepared at 400 U/ml in saline (100%). Fibrinogen was prepared at 20 mg/ml in 0.9% saline, and all subsequent dilutions were in distilled water. GTN was prepared as a 10 mmol/l stock in 50% ethanol, and all subsequent dilutions were in distilled water. Angeli's salt, DEA/NO, and IPA/NO were prepared at 10 mmol/l in 0.01 mol/l NaOH, and all subsequent dilutions were in 0.01 mol/l NaOH. L-012 was prepared at 100 mmol/l in DMSO, and all subsequent dilutions were in Krebs-HEPES (pH 7.4) solution. Levcromakalim was prepared at 10 mmol/l in 100% methanol. ODQ was prepared at 10 mmol/l in 100% ethanol. Oil red O was prepared as a 0.5% stock solution in 100% isopropyl alcohol and then diluted to a 60% isopropyl alcohol working solution. All other drugs were dissolved and diluted in distilled water.

RESULTS

Vasorelaxant responses to HNO donors. The HNO donors Angeli's salt and IPA/NO elicited concentration-dependent relaxation in endothelium-intact carotid arteries from WT-ND mice that were of similar potency and efficacy (Fig. 1, *A* and *B*, and Table 1). Vasorelaxation to these donors was unchanged in the presence of the $NO^{\bullet}$ scavenger carboxy-PTIO, but their potencies were decreased by up to 10-fold ($P < 0.001$) by the HNO scavenger L-cysteine (3 mmol/l; Fig. 1, *A* and *B*, and Table 1). Increasing the concentration of L-cysteine to 10 mmol/l further attenuated IPA/NO-mediated vasorelaxation such that the response to 10 μmol/l was inhibited by 28.3 ± 7.7% ($n = 8$) versus 42.4 ± 6.5% ($n = 5$) in the presence of 3 and 10 mmol/l L-cysteine, respectively. In contrast, vasorelaxation to the $NO^{\bullet}$ donor GTN was unchanged in the presence of L-cysteine (3 mmol/l; data not shown). The sGC inhibitor ODQ markedly attenuated vasorelaxation to both Angeli's salt and IPA/NO ($P < 0.001$; Fig. 1, *A* and *B*, and Table 1). Given that Angeli's salt and IPA/NO displayed similar vasorelaxant potency, and because IPA/NO (unlike Angeli's salt) does not decompose to generate nitrite (13, 19), all subsequent experiments used IPA/NO.

Antiaggregatory actions of IPA/NO. IPA/NO caused concentration-dependent inhibition of collagen-stimulated aggregation of washed platelets from WT-ND mice (Fig. 2*A*), with concentrations of 1 and 3 μmol/l IPA/NO decreasing total aggregation by ~50% ($P < 0.05$; Fig. 2*B*). This was accompanied by a delay in the onset of platelet aggregation, which reached statistical significance at 3 μmol/l IPA/NO ($P < 0.05$; Fig. 2*C*).

Mechanisms underlying the antiaggregatory actions of IPA/NO. ODQ reversed the ability of IPA/NO (1 μmol/l) to inhibit collagen-stimulated aggregation of platelets from WT-ND mice ($P < 0.01$; Fig. 3, *A* and *B*). Similarly, the cGK inhibitor Rp-8-pCPT-cGMPS partially reversed ($P < 0.05$) the antiaggregatory effects of IPA/NO (1 μmol/l; Fig. 3, *C* and *D*). In platelets from a subset of mice, we confirmed that neither ODQ (total aggregation AUC: control 62,563 ± 4,178 vs. ODQ 50,353 ± 6,250, $n = 6$) nor Rp-8-pCPT-cGMPS (total aggregation AUC: control 47,468 ± 3,263 vs Rp-8-pCPT-cGMPS 48,728 ± 6,591, $n = 3$) alone significantly altered collagen-stimulated aggregation.

Plasma cholesterol, vascular morphology, vessel and platelet function in WT-HFD and ApoE$^{-/-}$-HFD mice. Total plasma cholesterol levels were approximately fourfold higher ($P < 0.001$) in ApoE$^{-/-}$-HFD than in WT-HFD mice (Table 2). Atherosclerotic lesions were apparent in the internal,

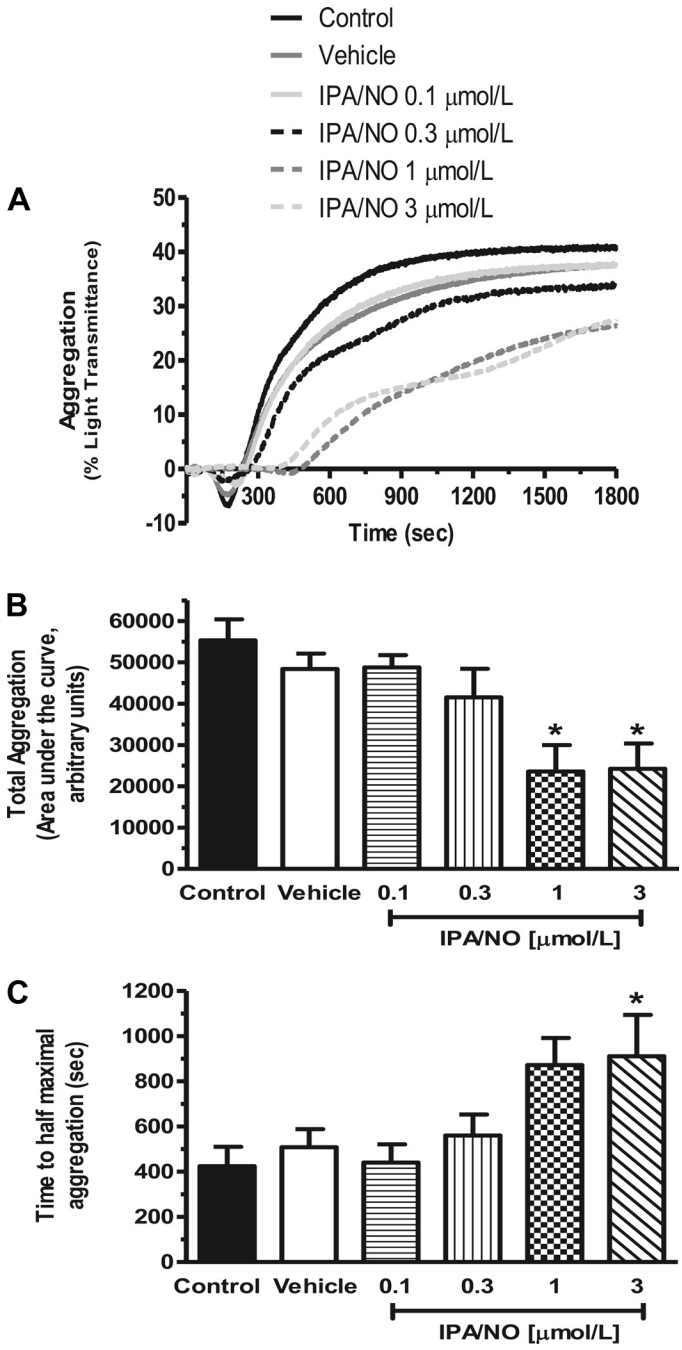

Fig. 2. *A*: average aggregation responses (expressed as a percentage of light transmission) of washed platelets from WT-ND mice after the addition of collagen (30 μg/ml) in the absence (control) or presence of either vehicle (1 mmol/l NaOH) or increasing concentrations of IPA/NO (0.1–3 μmol/l). Error bars are omitted for clarity. *B* and *C*: effect of IPA/NO on total aggregation (expressed as the area under the curve, in arbitrary units; *B*) and time to half-maximal aggregation (in s; *C*) in response to collagen. Values are given as means ± SE; $n = 5$–14 platelet samples from separate mice. *$P < 0.05$ for treatment vs. vehicle (one-way ANOVA, Bonferroni post hoc test).

but not common, carotid arteries of ApoE$^{-/-}$-HFD mice (Fig. 4, *B* and *D*). No lesions were detected in WT-HFD carotid arteries (Fig. 4, *A* and *C*). Despite the absence of lesions, basal $O_2^{\cdot -}$ production by common carotid arteries from ApoE$^{-/-}$-HFD mice was ~60% greater than levels generated by arteries from WT-HFD mice ($P < 0.05$; Table 2). Contractile re-

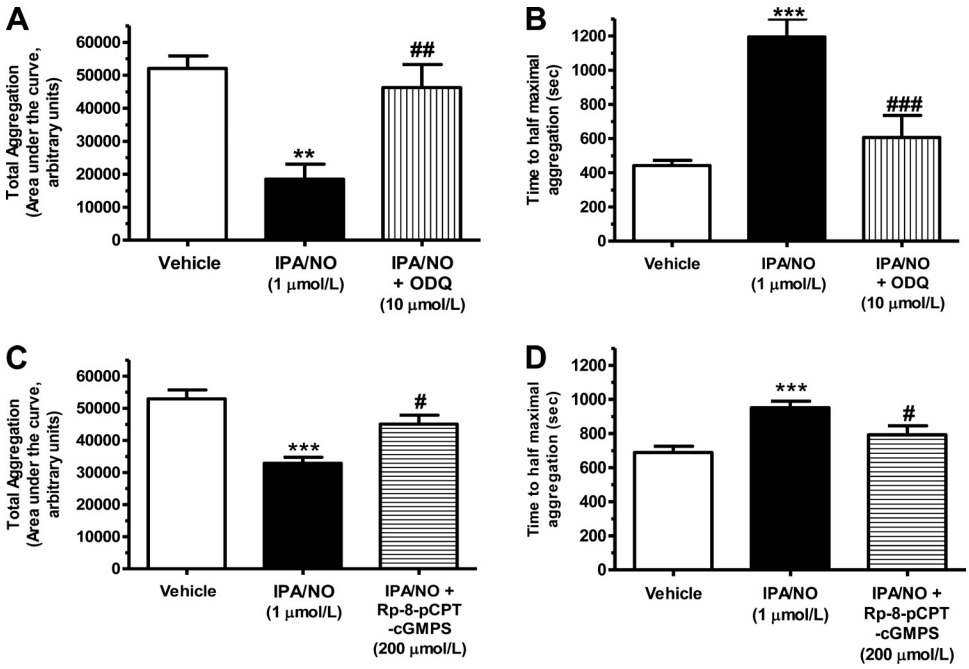

Fig. 3. Effect of IPA/NO (1 μmol/l) on collagen (30 μg/ml)-stimulated aggregation (vehicle; 1 mmol/l NaOH) of washed platelets from WT-ND mice in the absence (IPA/NO) or presence of ODQ (10 μmol/l; IPA/NO + ODQ; *A* and *B*) or Rp-8-pCPT-cGMPS (200 μmol/l; IPA/NO + Rp-8-pCPT-cGMPS; *C* and *D*). Aggregation responses are expressed as total aggregation (area under the curve, in arbitrary units; *A* and *C*) or time to half-maximal aggregation (in s; *B* and *D*) and are given as means ± SE; n = 7–13 platelet samples from separate mice. $^{**}P < 0.01$ and $^{***}P < 0.001$ for treatment vs. vehicle; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ for treatment vs. IPA/NO (one-way ANOVA, Bonferroni post hoc test).

sponses of common carotid arteries from ApoE$^{-/-}$-HFD mice to L-NAME (100 μmol/l) were markedly attenuated versus WT-HFD mice ($P < 0.001$; Table 2), indicative of decreased endogenous NO bioavailability. Basal $O_2^{\cdot-}$ production by platelets from WT-HFD and ApoE$^{-/-}$-HFD mice did not differ; however, PDB-stimulated $O_2^{\cdot-}$ production was ~80% greater in ApoE$^{-/-}$-HFD mice ($P < 0.05$; Table 2). Whereas total aggregation to collagen was similar between platelets from WT-HFD and ApoE$^{-/-}$-HFD mice, platelets from ApoE$^{-/-}$-HFD mice aggregated more rapidly ($P < 0.05$; Table 2).

Vasorelaxant responses to IPA/NO in ApoE$^{-/-}$-HFD mice. IPA/NO-induced vasorelaxation was preserved in carotid arteries from ApoE$^{-/-}$-HFD mice, with a small but significant 2.5-fold ($P < 0.01$) increase in sensitivity compared with WT-HFD arteries (Fig. 5*A*). Relaxant responses to the NO$^{\bullet}$ donor GTN and the NO-independent vasodilator papaverine were similar in carotid arteries from ApoE$^{-/-}$-HFD and WT-HFD mice (Fig. 5, *B* and *C*).

Pretreatment of carotid arteries from WT-HFD and ApoE$^{-/-}$-HFD mice with 30 μmo/l GTN for 60 min caused up to an ~16-fold ($P < 0.001$) decrease in sensitivity to the subsequent application of GTN (Fig. 6, *B* and *D*). In contrast, pretreatment of carotid arteries from WT-HFD and ApoE$^{-/-}$-HFD mice with IPA/NO (30 μmol/l, 60 min) had no effect on their subsequent vasorelaxation to IPA/NO (Fig. 6, *A* and *C*).

Antiaggregatory actions of IPA/NO in ApoE$^{-/-}$-HFD platelets. IPA/NO (3 μmo/l) inhibited collagen-induced aggregation in ApoE$^{-/-}$-HFD mice (32 ± 7% inhibition) to a similar extent as in WT-HFD platelets (39 ± 14% inhibition; Fig. 7*A*). In addition, IPA/NO caused a comparable increase

Table 2. *Plasma cholesterol, vascular morphology, and vessel and platelet function in 12-wk-old WT-HFD and ApoE$^{-/-}$-HFD mice*

Parameter	WT-HFD		ApoE$^{-/-}$-HFD	
	n	Means ± SE	n	Means ± SE
Total plasma cholesterol levels, mmol/l	7	5.7 ± 0.4	9	21.5 ± 2.2†
Common carotid artery				
Atherosclerotic lesions	3	Not apparent	3	Visible lesions in the internal carotid artery
Basal superoxide, RLU·s^{-1}·mg^{-1}	7	14,365 ± 4,525	7	24,387 ± 6,272*
L-NAME contraction, % U-46619 (1 μmol/l)	9	56 ± 5	10	24 ± 5*
Platelets				
Basal superoxide, RLU/s	6	259 ± 52	5	310 ± 39
PDB (10 μmol/l)-stimulated superoxide, RLU/s	6	5,453 ± 541	5	9,843 ± 1,667*
Collagen (30 μg/ml)-stimulated platelet aggregation				
Total aggregation (area under the curve), arbitrary units	10	48,888 ± 5,394	16	52,395 ± 4,199
Time to half-maximal aggregation, s	10	507 ± 82	16	335 ± 36*

Values are means ± SE; n, number of animals. WT and apolipoprotein E-deficient (ApoE$^{-/-}$) mice were maintained on a high-fat diet (HFD) from 5 wk of age. RLU, relative light units; L-NAME, N^{ω}-nitro-L-arginine methyl ester; PDB, phorbol 12,13-dibutyrate. *$P < 0.05$ and †$P < 0.001$ vs. WT-HFD (Student's unpaired t-test).

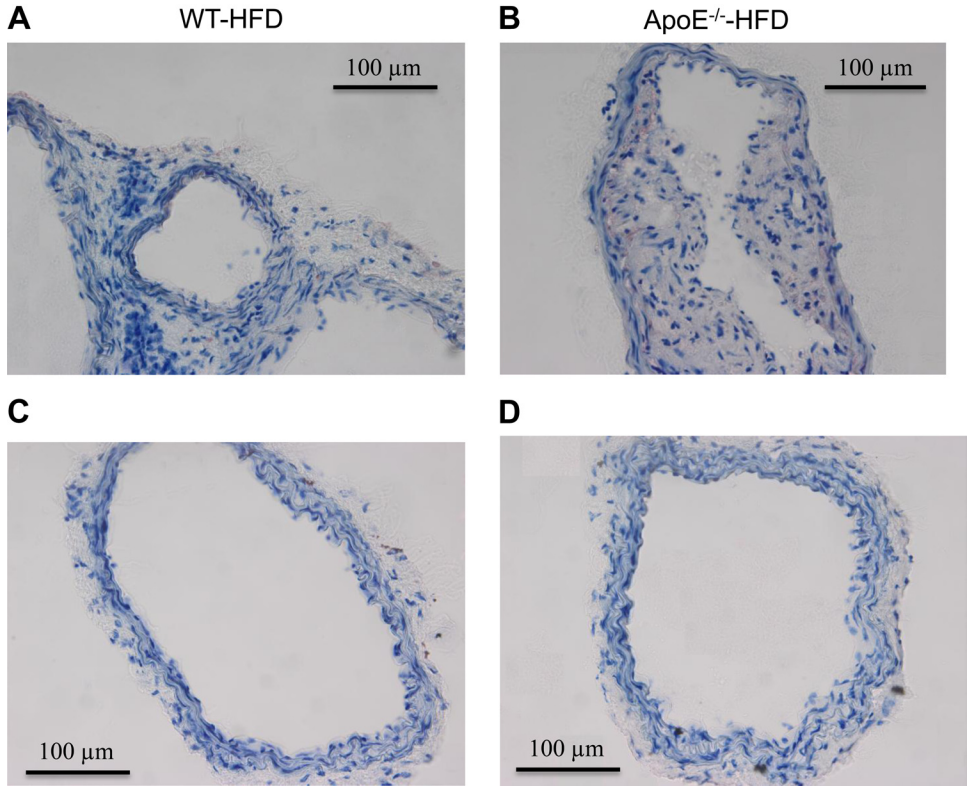

Fig. 4. Representative photomicrographs showing oil red O-stained sections of the internal (*A* and *B*) and common (*C* and *D*) carotid arteries from 12-wk-old WT mice and apolipoprotein E-deficient ($ApoE^{-/-}$) mice maintained on a high-fat diet (HFD) from 5 wk of age. Images are representative of $n = 3$ animals. Magnification: ×400. Scale bar = 100 μm.

in the time to half-maximal aggregation in $ApoE^{-/-}$-HFD and WT-HFD platelets (Fig. 7*B*). In contrast, the antiaggregatory actions of GTN (100 μmol/l, 40 ± 10% inhibition) were abolished in platelets from $ApoE^{-/-}$-HFD mice (Fig. 7, *C* and *D*).

DISCUSSION

This study demonstrated, for the first time, that the vasoprotective actions of HNO are equally effective in nondisease and disease conditions associated with elevated vascular and platelet $O_2^{\cdot-}$ generation and reduced endogenous $NO^{\bullet}$ bioavailability. Specifically, we found that the ability of the HNO donor IPA/NO to induce vasorelaxation, exhibit resistance to vascular tolerance development, and inhibit platelet aggregation was preserved in hypercholesterolemic mice. Furthermore, these findings contrast with those observed with the clinically used $NO^{\bullet}$ donor GTN, which developed vascular tolerance and exhibited an impaired ability to inhibit platelet aggregation in the setting of hypercholesterolemia. Together, these findings suggest that HNO donors may offer a therapeutic alternative to the currently used organic nitrates in the treatment of vascular disorders.

Before we examined the vasoprotective actions of HNO in hypercholesterolemia, it was important to fully characterise the vasorelaxant and antiaggregatory effects of this nitrogen oxide under nondisease conditions. This was achieved using the HNO donors Angeli's salt and IPA/NO, both of which caused relaxation of mouse common carotid arteries with a similar potency. Using the well-established HNO and $NO^{\bullet}$ scavengers L-cysteine (11, 12) and carboxy-PTIO (11, 12), respectively, we confirmed that HNO, and not $NO^{\bullet}$, mediates vasorelaxation in response to both Angeli's salt and IPA/NO. Moreover, in accordance with previous studies (7, 8, 11, 12) in large conduit and small resistance arteries, the sGC inhibitor ODQ markedly attenuated vasorelaxant responses to both Angeli's salt and IPA/NO in carotid arteries from nondiseased mice, indicating a predominant role for the sGC/cGMP pathway in HNO-mediated vasorelaxation. While both IPA/NO and Angeli's salt spontaneously decompose at physiological pH to donate HNO with similar half-lives (~2.5 min) (13, 19), subsequent experiments used IPA/NO to avoid the potentially confounding effect of nitrite, which is concomitantly released with HNO by Angeli's salt (13).

In addition to characterizing the vasodilator capacity of HNO, we demonstrated for the first time in mice that IPA/NO induces concentration-dependent inhibition of collagen-induced platelet aggregation. These findings are consistent with studies in human and mouse platelets, where Angeli's salt was shown to exert antiaggregatory properties in response to arachidonic acid, ADP, collagen, and thrombin (1, 21) as well as collagen (5), respectively. Furthermore, in accordance with these studies (1, 5), we found that HNO primarily inhibits platelet aggregation via a sGC/cGMP-dependent signaling pathway with the antiaggregatory effects of IPA/NO reversed by ODQ and attenuated, in part, by the cGK inhibitor Rp-8-pCPT-cGMPS.

While numerous studies, including the present one, have shown that HNO has vasoprotective actions under nondisease conditions, little is known about its efficacy during disease. Studies in support of preserved vascular actions of HNO under pathophysiological conditions include the finding that Angeli's salt-mediated inhibition of ADP-stimulated platelet aggregation was sustained in patients with sickle cell disease (21). IPA/NO has also been shown to limit neointimal hyperplasia in

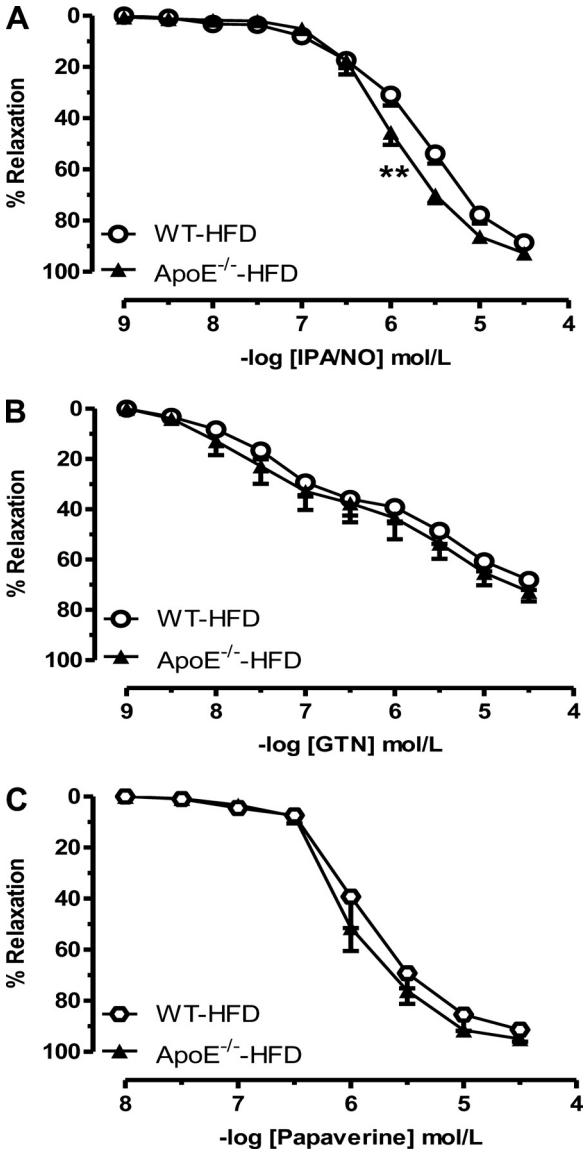

Fig. 5. Concentration-response curves to IPA/NO (n = 7; *A*), glyceryl trinitrate (GTN; n = 7–9; *B*) and papaverine (n = 5–6; *C*) in common carotid arteries from 12-wk-old WT and ApoE$^{-/-}$ mice maintained on a HFD from 5 wk of age. Values are expressed as the percent reversal of precontraction and are given as means ± SE, where n is the number of animals. $^{**}P < 0.01$ for pEC_{50} value vs. WT-HFD mice (Student's unpaired t-test).

rats (29), and Paolocci and colleagues (22) have indirectly shown that the hemodynamic actions of HNO are sustained in acute experimental heart failure. However, it remains unclear whether HNO donors offer advantages over traditional nitrovasodilators, such as GTN, in the treatment of vascular diseases where there is increased generation of ROS such as $O_2^{\cdot-}$ and reduced NO$^\bullet$ signaling. These are clinically relevant questions as the therapeutic efficacy of GTN is limited due to its propensity to induce tolerance to subsequent drug exposures (4), resistance of platelets to its antiaggregatory effects (3), and its reduced efficacy under conditions of elevated ROS generation (26) due to scavenging of NO$^\bullet$ by $O_2^{\cdot-}$ and dysfunction of sGC itself (16).

To begin to address these issues, this study used ApoE$^{-/-}$ mice maintained on a HFD for 7 wk as a model of hypercholesterolemia and early stage of disease. In agreement with previous studies (14, 15, 17), we found that these ApoE$^{-/-}$-HFD mice displayed elevated plasma cholesterol levels, increased vascular $O_2^{\cdot-}$ production, reduced vascular NO$^\bullet$ bioavailability, and atherosclerotic lesions in the internal carotid artery.

In addition to the vascular changes, we found that platelets from ApoE$^{-/-}$-HFD mice were able to aggregate more rapidly compared with those from WT-HFD. Moreover, consistent with findings of elevated $O_2^{\cdot-}$ generation in hypercholesterolemic patients (24, 25), $O_2^{\cdot-}$ production in response to PDB, an activator of PKC, was elevated in platelets from ApoE$^{-/-}$-HFD mice. As PKC can activate the ROS-generating enzyme NADPH oxidase, we hypothesize that increased activity of NADPH oxidase may account for the greater $O_2^{\cdot-}$ production in hypercholesterolemic mice. Taken together, these findings indicate that ApoE$^{-/-}$ mice maintained on a HFD for 7 wk display the hallmark characteristics associated with hypercholesterolemia.

Interestingly, in the setting of hypercholesterolemia, the vasodilator capacity of HNO was preserved with a small but significant increase in vasorelaxant potency to IPA/NO observed in carotid arteries from ApoE$^{-/-}$-HFD compared with normocholesterolemic WT-HFD mice. Similarly, vasorelaxant responses to the NO$^\bullet$ donor GTN and the sGC-independent vasodilator papaverine were unchanged in carotid arteries from ApoE$^{-/-}$-HFD versus normocholesterolemic mice. Our finding that vasorelaxant responses to GTN were maintained in hypercholesterolemia is not surprising given the relatively early stage of vascular disease. Indeed, oxidative stress and dysfunction of the NO-sGC-cGMP signaling pathway are more prominent in the later stages of hypercholesterolemia/atherosclerosis (16). Thus, future studies using ApoE$^{-/-}$ mice maintained on a HFD for a more prolonged period of time may unmask differential vasodilator effects of HNO versus NO$^\bullet$ donors.

Importantly, a further limitation of nitrovasodilators, such as GTN, is their susceptibility to tolerance development with continued use. Tolerance is a multifactorial process that may arise as a consequence of neurohumoral counterregulation, reduced mitochondrial biotransformation of organic nitrates, impaired sGC function, increased activity of cGMP-degrading phosphodiesterases, or increased $O_2^{\cdot-}$ generation (4). We have previously shown that, in contrast to GTN, the HNO donor Angeli's salt does not develop tolerance either in vitro (12) or when chronically administered in vivo (10). This resistance to tolerance development is likely due to the ability of Angeli's salt to spontaneously donate HNO, whereas GTN requires biotransformation to generate NO$^\bullet$. Here, we extended these observations, reporting for the first time that in a cardiovascular disease associated with increased $O_2^{\cdot-}$ generation, HNO donors, unlike GTN, are resistant to tolerance development in both normocholesterolemic and ApoE$^{-/-}$-HFD mice. Thus, preservation of the vasodilator efficacy of HNO, together with its lack of tolerance in disease, represents significant advantages over GTN.

Similarly, we found that the antiaggregatory actions of HNO are sustained in hypercholesterolemia with IPA/NO inhibiting collagen-induced platelet aggregation to a similar extent in

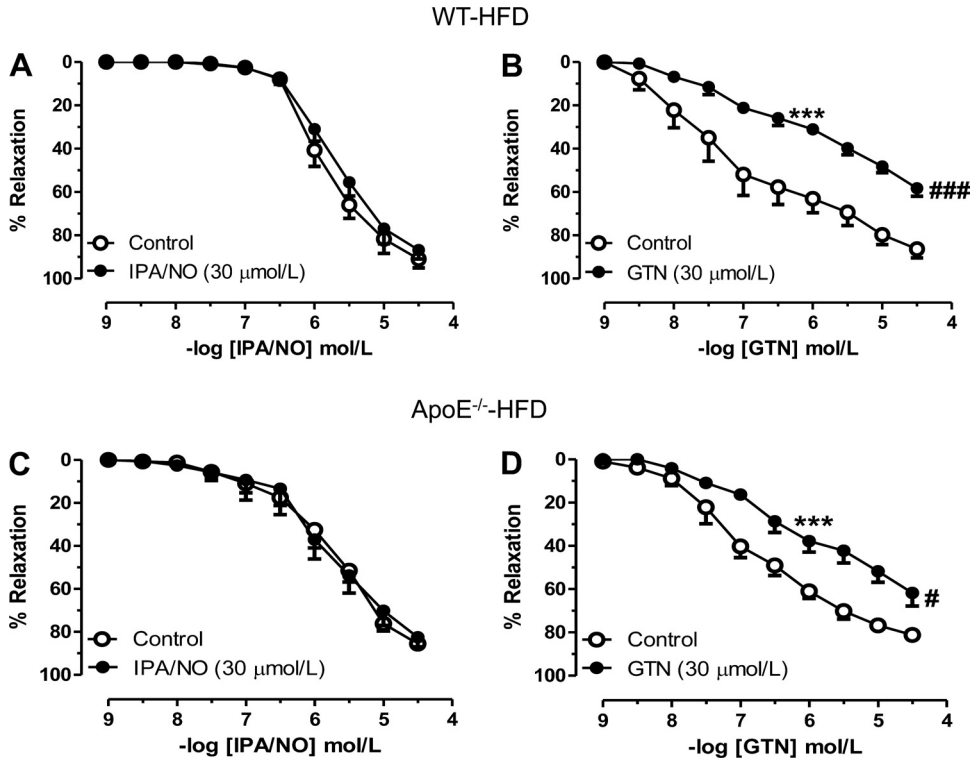

Fig. 6. Concentration-response curves to IPA/NO (*A* and *C*) and GTN (*B* and *D*) in common carotid arteries from 12-wk-old WT-HFD and ApoE$^{-/-}$-HFD mice after a 1-h incubation in either the absence (control; $n = 7$) or presence of 30 μmol/l IPA/NO ($n = 7$) or GTN ($n = 7$). Values are expressed as the percent reversal of precontraction and are given as means ± SE, where n is the number of animals. ***$P < 0.001$ for the treatment concentration-response curve vs. the untreated control (two-way ANOVA, Tukey post hoc test); #$P < 0.05$ and ###$P < 0.001$ for the response at 30 μmol/l vs. the untreated control (Student's unpaired *t*-test).

ApoE$^{-/-}$-HFD versus normocholesterolemic mice. These findings are consistent with a previous report (21) that showed that the antiaggregatory actions of Angeli's salt were preserved in platelets from patients with sickle cell disease. In contrast to HNO, GTN failed to inhibit platelet aggregation in ApoE$^{-/-}$-HFD mice. A loss of responsiveness to GTN has also been reported in platelets from patients with stable angina pectoris, acute coronary syndromes, and obesity (3), and this is commonly referred to as NO• resistance. This phenomenon of NO• resistance at the level of platelet aggregation has been attributed to scavenging of NO• by $O_2^{\cdot -}$ as well as to dysfunction of sGC (3). As discussed, we found that $O_2^{\cdot -}$ generation in response to PDB was elevated in platelets from ApoE$^{-/-}$ mice. Thus, we predict that the hyporesponsiveness to GTN in hypercholesterolemic mice may have arisen due to scavenging of NO• by $O_2^{\cdot -}$. In addition, an elevation of ROS may lead to

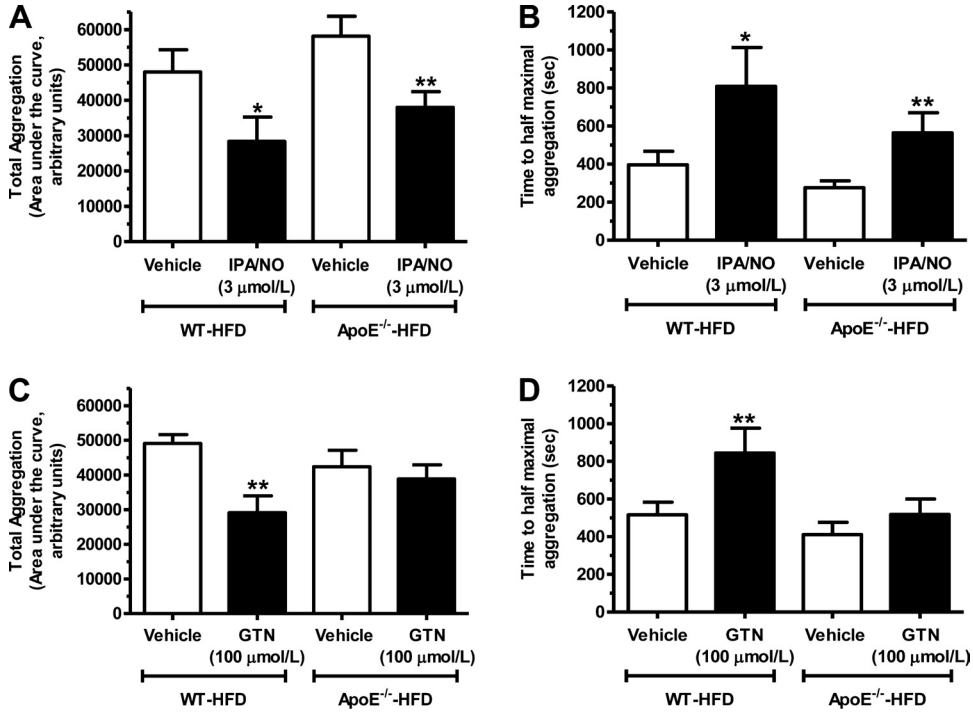

Fig. 7. Effect of IPA/NO (3 μmol/l, $n = 6–10$; *A* and *B*) and GTN (100 μmol/l, $n = 7–9$; *C* and *D*) on collagen (30 μg/ml)-stimulated aggregation of isolated washed platelets from 12-wk-old WT and ApoE$^{-/-}$ mice maintained on a HFD from 5 wk of age. Aggregation responses are expressed as total aggregation (area under the curve, in arbitrary units; *A* and *C*) or time to half-maximal aggregation (in s; *B* and *D*). Values are given as means ± SE; n is the number of platelet samples from separate mice. *$P < 0.05$ and **$P < 0.01$ for treatment vs. vehicle (Student's paired *t*-test). The vehicle in *A* and *B* was 1 mmol/l NaOH; the vehicle in *C* and *D* was 0.5% ethanol.

a reduced capacity of platelets to biotransform GTN to NO• (4). Based on our preliminary evidence showing that IPA/NO does not scavenge $O_2^{\cdot-}$ in a xanthine/xanthine oxidase cell-free assay (data not shown), we anticipate that the resistance of HNO to scavenging by $O_2^{\cdot-}$ may underlie its preserved anti-aggregatory actions in hypercholesterolemia.

In conclusion, this study has demonstrated, for the first time, that the vasodilator and antiaggregatory capacities of HNO are preserved in the early stages of vascular disease in which increased $O_2^{\cdot-}$ generation and compromised NO• signaling is evident. Importantly, HNO donors may circumvent the vascular tolerance development and platelet hyporesponsiveness to NO• observed with clinically used nitrovasodilators such as GTN. Thus, the novel cardioprotective actions of HNO (22), coupled with its vasoprotective properties (2), may offer significant advantages over traditional nitrovasodilators. Moreover, we predict that the vasoprotective actions of HNO, as demonstrated in this study, will be sustained in more advanced models of cardiovascular disease, a concept that is currently under investigation.

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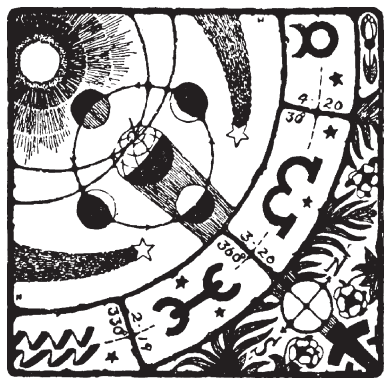
DISCLOSURES

B. K. Kemp-Harper has a collaborative research agreement with Cardioxyl Pharmaceuticals, a company that develops novel HNO donors, none of which were used in the present study.

REFERENCES

1. **Bermejo E, Saenz DA, Alberto F, Rosenstein RE, Bari SE, Lazzari MA.** Effect of nitroxyl on human platelets function. *Thromb Haemost* 94: 578–584, 2005.
2. **Bullen ML, Miller AA, Andrews KL, Irvine J, Ritchie RH, Sobey C, Kemp-Harper B.** Nitroxyl (HNO) as a vasoprotective signaling molecule. *Antioxid Redox Signal* 14: 1675–1686, 2011.
3. **Chirkov YY, Horowitz JD.** Impaired tissue responsiveness to organic nitrates and nitric oxide: a new therapeutic frontier? *Pharmacol Ther* 116: 287–305, 2007.
4. **Daiber A, Wenzel P, Oelze M, Munzel T.** New insights into bioactivation of organic nitrates, nitrate tolerance and cross-tolerance. *Clin Res Cardiol* 97: 12–20, 2008.
5. **Dangel O, Mergia E, Karlisch K, Groneberg D, Koesling D, Friebe A.** Nitric oxide-sensitive guanylyl cyclase is the only nitric oxide receptor mediating platelet inhibition. *J Thromb Haemost* 8: 1343–1352, 2010.
6. **Dharmarajah J, Arthur JF, Sobey CG, Drummond GR.** The anti-platelet effects of apocynin in mice are not mediated by inhibition of NADPH oxidase activity. *Naunyn Schmiedebergs Arch Pharmacol* 382: 377–384, 2010.
7. **Favaloro JL, Kemp-Harper B.** Redox variants of NO (NO• and HNO) elict vasorelaxation of resistance arteries via distinct mechanisms. *Am J Physiol Heart Circ Physiol* 296: H1274–H1280, 2009.
8. **Favaloro JL, Kemp-Harper BK.** The nitroxyl anion (HNO) is a potent dilator of rat coronary vasculature. *Cardiovasc Res* 73: 587–596, 2007.
9. **Fukuto JM, Chiang K, Hszieh R, Wong P, Chaudhuri G.** The pharmacological activity of nitroxyl: a potent vasodilator with activity similar to nitric oxide and/or endothelium-derived relaxing factor. *J Pharmacol Exp Ther* 263: 546–551, 1992.
10. **Irvine J, Kemp-Harper BK, Widdop RE.** Chronic administration of the HNO donor, Angeli's salt does not lead to tolerance, cross-tolerance or endothelial dysfunction: comparison with GTN and DEA/NO. *Antioxid Redox Signal* 14: 1615–1624, 2011.
11. **Irvine JC, Favaloro JL, Kemp-Harper BK.** NO^- activates soluble guanylate cyclase and Kv channels to vasodilate resistance arteries. *Hypertension* 41: 1301–1307, 2003.
12. **Irvine JC, Favaloro JL, Widdop RE, Kemp-Harper BK.** Nitroxyl anion donor, Angeli's salt, does not develop tolerance in rat isolated aortae. *Hypertension* 49: 885–892, 2007.
13. **Irvine JC, Ritchie RH, Favaloro JL, Andrews KL, Widdop RE, Kemp-Harper BK.** Nitroxyl (HNO): the Cinderella of the nitric oxide story. *Trends Pharmacol Sci* 29: 601–608, 2008.
14. **Judkins CP, Deip H, Broughton B, Mast AE, Hooker EU, Miller AA, Selemidis S, Dusting GJ, Sobey CG, Drummond GR.** Direct evidence of a role for Nox2 in superoxide production, reduced nitric oxide bioavailability and early atherosclerotic plaque formation in $ApoE^{-/-}$ mice. *Am J Physiol Heart Circ Physiol* 298: H24–H32, 2010.
15. **Kitayama J, Faraci FM, Lentz SR, Heistad DD.** Cerebral vascular dysfunction during hypercholesterolemia. *Stroke* 38: 2136–2141, 2007.
16. **Melichar VO, Behr-Roussel D, Zabel U, Uttenthal LO, Rodrigo J, Rupin A, Verbeuren TJ, Kumar HS, Schmidt HH.** Reduced cGMP signaling associated with neointimal proliferation and vascular dysfunction in late-stage atherosclerosis. *Proc Natl Acad Sci USA* 101: 16671–16676, 2004.
17. **Miller AA, De Silva TM, Judkins CP, Diep H, Drummond GR, Sobey CG.** Augmented superoxide production by Nox2-containing NADPH oxidase causes cerebral artery dysfunction during hypercholesterolemia. *Stroke* 41: 784–789, 2010.
18. **Miranda K, Paolocci N, Katori T, Thomas DD, Ford E, Bartberger MD, Espey MG, Kass DA, Feelisch M, Fukuto JM, Wink DA.** A biochemical rationale for the discrete behavior of nitroxyl and nitric oxide in the cardiovascular system. *Proc Natl Acad Sci USA* 100: 9196–9201, 2003.
19. **Miranda KM, Katori T, Torres de Holding CL, Thomas L, Ridnour LA, McLendon WJ, Cologna SM, Dutton AS, Champion HC, Mancardi D, Tocchetti CG, Saavedra JE, Keefer LK, Houk KN, Fukuto JM, Kass DA, Paolocci N, Wink DA.** Comparison of the NO and HNO donating properties of diazeniumdiolates: primary amine adducts release HNO in Vivo. *J Med Chem* 48: 8220–8228, 2005.
20. **Miranda KM, Yamada K, Espey MG, Thomas DD, DeGraff W, Mitchell JB, Krishna MC, Colton CA, Wink DA.** Further evidence for distinct reactive intermediates from nitroxyl and peroxynitrite: effects of buffer composition on the chemistry of Angeli's salt and synthetic peroxynitrite. *Arch Biochem Biophys* 401: 134–144, 2002.
21. **Mondoro TH, Ryan BB, Hrinczenko BW, Schechter AN, Vostal JG, Alayash AI.** Biological action of nitric oxide donor compounds on platelets from patients with sickle cell disease. *Br J Haematol* 112: 1048–1054, 2001.
22. **Paolocci N, Katori T, Champion HC, St. John ME, Miranda K, Fukuto JM, Wink DA, Kass DA.** Positive inotropic and lusitropic effects of HNO/NO^- in failing hearts: Independence from β-adrenergic signalling. *Proc Natl Acad Sci USA* 100: 5537–5542, 2003.
23. **Paolocci N, Saavedra WF, Miranda KM, Martignani C, Isoda T, Hare JM, Espey MG, Fukuto JM, Feelisch M, Wink DA, Kass DA.** Nitroxyl anion exerts redox-sensitive positive cardiac inotropy in vivo by calcitonin gene-related peptide signaling. *Proc Natl Acad Sci USA* 98: 10463–10468, 2001.
24. **Pignatelli P, Sanguigni V, Lenti L, Loffredo L, Carnevale R, Sorge R, Violi F.** Oxidative stress-mediated platelet CD40 ligand upregulation in patients with hypercholesterolemia: effect of atorvastatin. *J Thromb Haemost* 5: 1170–1178, 2007.
25. **Sanguigni V, Pignatelli P, Caccese D, Pulcinelli FM, Lenti L, Magnaterra R, Martini F, Lauro R, Violi F.** Increased superoxide anion production by platelets in hypercholesterolemic patients. *Thromb Haemost* 87: 796–801, 2002.
26. **Schachinger V, Britten MB, Zeiher AM.** Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. *Circulation* 101: 1899–906, 2000.

27. **Switzer CH, Flores-Santana W, Mancardi D, Donzelli S, Basudhar D, Ridnour L, Miranda K, Fukuto JM, Paolocci N, Wink DA.** The emergance of nitroxyl (HNO) as a pharmacological agent. *Biochim Biophys Acta* 1787: 835–840, 2009.
28. **Tochetti CG, Wang W, Froehlich JP, Huke S, Aon MA, Wilson GM, Benedetto GD, O'Rourke B, Gao WD, Wink DA, Toscano JP, Zaccolo M, Bers DM, Valdivia HH, Cheng H, Kass DA, Paolocci N.** Nitroxyl improves cellular heart function by directly enhancing cardiac sarcoplasmic reticulum Ca^{2+} cycling. *Circ Res* 100: 96–104, 2007.
29. **Tsihlis ND, Murar J, Kapadia MR, Ahanchi SS, Oustwani CS, Saavedra JE, Keefer LK, Kibbe MR.** Isopropylamine NONOate (IPA/NO) moderates neointimal hyperplasia following vascular injury. *J Vasc Surg* 51: 1248–1259, 2010.



Monash University

Declaration for Thesis Chapter 3

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Performed all experiments except those for total plasma cholesterol and some tissue harvesting, analysed all results and wrote the manuscript	75%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Alyson Miller	Provided intellectual advice during manuscript preparation stages and assisted with the editorial process.	
Henry Diep		
Christopher Sobey		
Grant Drummond		
Barbara Kemp-Harper		

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date**
Main Supervisor's Signature		**Date**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

The Vasoprotective Actions of Endogenous and Exogenously Generated Nitroxyl (HNO) are Preserved in Advanced Atherosclerosis

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Abstract

Atherosclerosis is a cardiovascular disease associated with impaired nitric oxide ($NO^{\bullet}$) bioavailability caused primarily by oxidative stress. As such, therapeutic strategies are required to overcome this vascular dysfunction. Nitroxyl (HNO), the redox sibling of $NO^{\bullet}$, is emerging as an endogenous regulator of vascular function with distinct pharmacological properties. Additionally, the vasoprotective properties of HNO donors are preserved in diseases that are risk factors for atherosclerosis (ie. hypertension, hypercholesterolemia). Thus, we sought to determine if HNO could compensate for impaired $NO^{\bullet}$ function in a mouse model of advanced atherosclerosis. Apolipoprotein E-deficient ($ApoE^{-/-}$) mice maintained on high fat diet for 21 weeks displayed atherosclerotic lesions, elevated total plasma cholesterol, reduced vascular NO• function and increased vascular superoxide ($^{\cdot}O_2^-$) generation compared to wild-type mice. The ability of HNO to serve as an endothelium-derived relaxation factor (EDRF) was enhanced, whereas NO• was compromised, in isolated common carotid arteries from $ApoE^{-/-}$ mice. Next, the vasorelaxant, anti-aggregatory and $^{\cdot}O_2^-$ suppressing actions of the HNO donor isopropylamine NONOate (IPA/NO) were compared with the NO• donors glyceryl trinitrate (GTN) and diethylamine NONOate (DEA/NO) in atherosclerosis. Vasorelaxant responses to IPA/NO, GTN and DEA/NO were preserved in common carotid arteries from $ApoE^{-/-}$ versus wild-type mice, although GTN developed vascular tolerance ($P<0.001$). Despite elevated $^{\cdot}O_2^-$ levels in carotid arteries from $ApoE^{-/-}$ mice ($P<0.05$), the $^{\cdot}O_2^-$ supressing capabilities of IPA/NO and DEA/NO, but not GTN were sustained ($P<0.01$). Similarly, the ability of IPA/NO and DEA/NO, but not GTN, to inhibit collagen-stimulated platelet aggregation was also maintained ($P<0.05$). In conclusion, this study demonstrates that endogenous HNO appears to compensate for impaired NO• function in a model of advanced atherosclerosis and shows that the vasoprotective actions of exogenous HNO are preserved.

Keywords: nitric oxide; nitroxyl; vasorelaxation; platelet aggregation; superoxide; atherosclerosis

Introduction:

Nitroxyl (HNO), the one electron reduced and protonated redox sibling of nitric oxide (NO•), is gaining interest as a novel therapeutic agent for the treatment of cardiovascular disorders (Tocchetti et al. 2011). In addition to serving as a positive cardiac inotrope and conferring protection in acute experimental heart failure (Paolocci et al. 2003; Tocchetti et al. 2011), HNO can regulate vascular homeostasis (Irvine et al. 2008; Paolocci et al. 2007). Indeed, the vasoprotective properties of HNO donors include an ability to cause vasorelaxation (Favaloro and Kemp-Harper 2009; Fukuto et al. 1992; Irvine et al. 2007), inhibit platelet aggregation (Donzelli et al. 2013; Mondoro et al. 2001), limit NADPH oxidase-derived superoxide ($^{\cdot}O_2^-$) generation (Lin et al. 2012; Miller 2013) and reduce vascular smooth muscle cell proliferation (Tsihlis et al. 2010). Whilst NO• also displays such vasoprotective properties, it is well recognised that NO• signalling (endogenous and exogenous) may be compromised in cardiovascular disease states associated with oxidative stress. Importantly, unlike NO•, HNO is not directly scavenged by $^{\cdot}O_2^-$ (Leo et al. 2012; Miller 2013; Miranda et al. 2002), does not develop tolerance with continual use (Irvine et al. 2010; Irvine et al. 2007) and can target distinct vascular signalling pathways (i.e calcitonin-gene related peptide release, voltage- and ATP-sensitive K^+ channels (Donzelli et al. 2013; Favaloro and Kemp-Harper 2009; Favaloro and Kemp-Harper 2007; Irvine et al. 2003). Thus, with the potential for preserved bioavailability and efficacy under conditions of oxidative stress, HNO donors represent a viable alternative to traditional nitrovasodilators in the treatment of vascular dysfunction.

Whilst much attention has been directed towards the vasoprotective actions of exogenously generated HNO, it appears that HNO may also serve as an endogenous regulator of vascular function. Indeed, it has been proposed that HNO can be generated via a number of biosynthetic pathways in the vasculature. HNO has the potential to be generated from endothelial nitric oxide synthase (eNOS) itself, as reduced levels of the NOS cofactor tetrahydrobiopterin can lead to the partial uncoupling, promoting the production of HNO over NO• (Ponnuswamy et al. 2012; Rusche et al. 1998). HNO can also be formed following oxidative degradation of the NOS metabolites N-hydroxy-L-arginine (Pufahl et al. 1995) and hydroxylamine (Donzelli et al. 2008). Additionally, HNO can be synthesised from non-NOS sources. Although direct reduction of NO• is unlikely to occur spontaneously (Bartberger et al. 2002), HNO generation via NO• can occur in the presence of a number of enzymes such as mitochondrial cytochrome c, ubiquinol, hemoglobin, xanthine oxidase

and manganese superoxide dismutase (Irvine et al. 2008; Paolocci et al. 2007). Finally, S-nitrosothiols have also been known to generate HNO via S-thiolation (Arnelle and Stamler 1995; Wong et al. 1998). Together, these biochemical studies provide support for the potential endogenous generation of HNO, however for definitive proof of its generation, techniques need to be developed for the direct detection of HNO. Despite an inability to directly detect HNO in the vasculature, a number of studies have inferred that HNO can serve as an endothelial-derived relaxing factor (EDRF). Indeed, the ability of HNO to act as an EDRF was observed in both large conduit (Ellis et al. 2000; Wanstall et al. 2001) and small resistance arteries (Andrews et al. 2009), by utilising scavengers for HNO (L-cysteine) and NO• (carboxy-PTIO and hydroxocobalamin). Additionally, spreading vasodilatation was found to be dependent upon generation of HNO, but not NO•, in resistance arteries (Yuill et al. 2010). Interestingly, in disease states where oxidative stress is enhanced and endogenous NO• is compromised, the effects of endogenous HNO may be preserved or enhanced. As HNO generation may be enhanced due to uncoupling of eNOS (Ponnuswamy et al. 2012; Rusche et al. 1998) and its bioavailability preserved due to its resistance to scavenging by $^{\cdot}O_2^-$ (Leo et al. 2012; Miller 2013; Miranda et al. 2002). This idea is supported by recent studies in the setting of hypertension (Wynne et al. 2012) and diabetes (Leo et al. 2012) where the ability of HNO to serve as an EDRF was sustained. Furthermore, if the bioavailability of HNO is conserved, there is potential for HNO to compensate, at least in part, for a reduction in endogenous NO• bioavailability. Thus, improving endogenous HNO bioavailability may also serve as a viable therapeutic option in disease states where endogenous NO• function is impaired.

In support of this concept, we and others have recently shown that the vasoprotective actions of HNO donors such as Angeli's salt, isopropylamine NONOate (IPA/NO) and 1-nitrosocyclohexyl acetate (1-NCA) are preserved in experimental models of vascular disease. Indeed, the vasodilatory capacity of HNO is sustained in acute experimental heart failure (Paolocci et al. 2003; Paolocci et al. 2001), atherosclerosis (Donzelli et al. 2013), hypertension (Irvine et al. 2011; Wynne et al. 2012) and diabetes (Leo et al. 2012). In addition, HNO donors maintain their ability to inhibit platelet aggregation in patients with sickle cell disease (Mondoro et al. 2001) and ischemic heart disease, where the anti-aggregatory actions of the NO• donor, sodium nitroprusside (SNP) were impaired (Dautov et al. 2013). Moreover, HNO donors do not develop tolerance under both physiological conditions (Irvine et

al. 2010; Irvine et al. 2007) and have been shown to limit neointimal hyperplasia in a carotid artery injury model (Tsihlis et al. 2010). However, little is known with regard to the vasoprotective role of HNO in more advanced cardiovascular disease states. This is of particular relevance, given disorders such as atherosclerosis are associated with enhanced oxidative stress, inflammation, reduced NO• bioavailability and cardiovascular remodelling (Libby et al. 2011). Thus the focus of the present study was to examine the vasoprotective role of endogenous and exogenously generated HNO in the setting of advanced atherosclerosis.

In apolipoprotein E-deficient ($ApoE^{-/-}$) mice maintained on high fat diet for 21 weeks, the relative contributions of endogenous HNO and NO• to endothelium-dependent relaxation were examined to determine if HNO can compensate for a loss in NO• bioavailability. Moreover, the vasoprotective properties of the HNO donor IPA/NO were compared to the spontaneous NO• donor diethylamine NONOate (DEA/NO), and the clinically used nitrovasodilator glyceryl trinitrate (GTN; undergoes biotransformation to release NO•). Given DEA/NO releases NO• with a similar kinetic profile as the release of HNO from IPA/NO, we were able to determine if any atherosclerosis-associated changes in the vasoprotective actions of nitrogen oxide donors were specific for the redox form of NO released or the mode of generation (i.e biotransformation vs. spontaneous release). Collectively, this study will provide important insight into the vasoprotective role of both endogenous and exogenously generated HNO in an advanced stage of vascular disease.

Methods

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

Experimental animals

Wild-type (WT) and Apolipoprotein E-deficient ($ApoE^{-/-}$) mice were obtained from Monash Animal Services (Clayton, Vic, Australia). All mice studied were male, backcrossed to a C57BL/6J background. From 5 weeks of age, all mice were maintained on a high-fat diet (21% fat, 0.15% cholesterol; Speciality Feeds, WA, Australia) for 21 weeks. In all experiments, mice were deeply anaesthetized by isoflurane inhalation (Baxter Healthcare) prior to being euthanized by decapitation.

Measurement of total plasma cholesterol levels

Blood from WT and $ApoE^{-/-}$ mice was collected from the inferior vena cava into heparinised tubes and plasma was isolated via centrifugation (4,000g, 4°C, 10 min). Plasma total cholesterol levels, high and low density cholesterol (HDL and LDL) and triglyceride levels were then determined using a Roche MODULAR 917 (Roche Diagnostics, Castle Hill, NSW, Australia) enzymatic colorimetric array.

Histological studies

Internal and common carotid arteries from WT and $ApoE^{-/-}$ mice were examined for the presence of atherosclerotic lesions as previously described (Judkins et al. 2010; Miller et al. 2010). As fibrotic lesion formation has been reported in vessels from $ApoE^{-/-}$ mice at a similar time point (15-20 weeks of age) (Meyrelles et al. 2011). Briefly, internal and common carotid arteries were mounted in an OCT Tissue-Tek mould and stored at -80°C. Arteries were then sectioned (10 µm) and thaw mounted onto poly-L-lysine-coated microscope slides. Sections were fixed with paraformaldehyde (4%, 10 min) and then stained with Oil Red O (0.5% in 60% isopropyl alcohol, 60 min), followed by counterstaining with haematoxylin (25%, 2 min). Sections were viewed using an Olympus BX51 microscope (Olympus, Tokyo, Japan) equipped with a 40× oil immersion lens. Images were digitized using a colour OP70 Peltier cooled digital camera and captured with a data acquisition system (Analysis LS Starter Version 3.0, Olympus Soft Imaging Solutions, Munster, Germany).

Vascular function studies

Tissue preparation

Common carotid arteries from WT and ApoE$^{-/-}$ mice were isolated, cleared of fat and connective tissue and cut into ~2mm long ring segments. Arteries were mounted in a Mulvany-style small vessel myograph (Danish Myo Technology A/S, Inc., Skejbyparken, Denmark) for the measurement of isometric tension as previously described (Irvine et al. 2003). Vessels were maintained in Krebs-Bicarbonate solution [composed of (in mmol/l) 118 NaCl, 4.5 KCl, 0.5 $MgSO_4$, 1.0 KH_2PO_4, 25 $NaHCO_3$, 11.1 glucose, 2.5 $CaCl_2$ and 0.026 EDTA; pH 7.4] at 37°C and bubbled with carbogen (95% O_2, 5% CO_2). Following a 30 min equilibration period, arteries were stretched to a resting tension of 5mN before being maximally contracted with the thromboxane A_2 mimetic, U46619 (1 µmol/l; F_{max}).

Vascular NO• function

Contractile responses to the NO• synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) were measured in common carotid arteries from WT and ApoE$^{-/-}$ as an indicator of basal NO• bioavailability as previously described (Irvine et al. 2003). Briefly, vessels were pre-contracted to ~20-30% F_{max} with titrated concentrations of U46619 (1-25 nmol/l). Once the U46619 contraction was stable, L-NAME (100 µmol/l) was added and the contractile response recorded once it reached a plateau.

Contribution of endogenous HNO and NO to endothelium-dependent relaxation

Responses to vasodilators were examined in arteries from WT and ApoE$^{-/-}$ mice precontracted to ~50% F_{max} with titrated concentrations of U46619 (3-100 nmol/l). The contribution of endogenous HNO and NO• to endothelium-dependent relaxation was assessed by constructing cumulative concentration-response curves to acetylcholine (ACh; 1 nmol/l - 30 µmol/l) in the absence and presence of (i) the HNO scavenger, L-cysteine (3 mmol/l; 5-min preincubation); (ii) the NO• scavenger, hydroxocobalamin (HXC 100 µmol/l; 15-min preincubation); (iii) HXC and L-cysteine in combination; (iv) L-NAME (100 µmol/l; 30-min preincubation) alone or in combination with (v) the sGC inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ 10 µmol/l; 30-min preincubation); (vi) L-cysteine or (vii) hydroxocobalamin.

Vasorelaxant effects of exogenously generated HNO and NO

Cumulative concentration-response curves to the HNO donor IPA/NO (1 nmol/l - 30 µmol/l), the NO• donors GTN (1 nmol/l - 30 µmol/l) or DEA/NO (1 nmol/l - 30 µmol/l) and the endothelium-independent vasodilator papaverine (10 nmol/l – 30 µmol/l) were constructed in common carotid arteries from WT and ApoE$^{-/-}$ mice to assess the effect of exogenously generated HNO and NO•.

Tolerance development in response to exogenously generated HNO and NO

To determine whether tolerance to IPA/NO, GTN or DEA/NO develops in advanced atherosclerosis, common carotid arteries from ApoE$^{-/-}$ mice were incubated in either the absence or presence of IPA/NO (30 µmol/l), GTN (30 µmol/l) or DEA/NO (30 µmol/l) for a period of 60 min (Irvine et al. 2007). Vessels were then washed thoroughly every 15 min for 60 min. Subsequently, arteries were precontracted to ~50% F_{max} with titrated concentrations of U46619 (5-26 nmol/l) and cumulative concentration-response curves to IPA/NO, GTN or DEA/NO were obtained. To ensure maximal relaxation, isoprenaline (1 µmol/l) and levcromakalim (10 µmol/l) were added at the end of each concentration-response curve.

Platelet aggregation

Platelets were isolated from whole blood collected from the inferior vena cava of WT and ApoE$^{-/-}$ mice and collagen-stimulated aggregation was measured as previously described (Dharmarajah et al. 2010). Briefly, to prevent coagulation, blood was treated with a combination of a low molecular weight heparin (clexane, 400 U/ml) and acid citrate dextrose buffer [composed of (in mmol/l) 85 trisodium citrate, 72.9 citric acid, 110 D-glucose] immediately following collection. Blood was then washed with platelet wash buffer [composed of (in mmol/l) 4.3 K_2HPO_4, 4.3 Na_2HPO_4, 24.3 NaH_2PO_4, 113 NaCl, 5.5 D-glucose, 10% BSA and 20 U/ml Clexane, pH 6.5] and centrifuged at 200×*g*, (37°C, 2 min). This was performed three times, and each time the platelet rich plasma (PRP) was removed and pooled. Following centrifugation of the pooled PRP (2000×*g*, 1 min), the pellet was resuspended in Tyrode's buffer to give a final concentration of 5×10^7 platelets/ml. Platelets were added to siliconised cuvettes containing fibrinogen (2 mg/ml) and were allowed to equilibrate at 37°C for 30 minutes prior to stimulation with collagen (30 µg/ml). Platelet aggregation was measured using a four-chamber

turbidometric platelet aggregometer (AggRAM™, Helena Laboratories, USA) as a change in light transmission over 30 minutes under continuous stirring (600 rpm, 37°C). To assess the anti-aggregatory actions of IPA/NO, GTN and DEA/NO in advanced atherosclerosis, platelets from WT and ApoE$^{-/-}$ mice were pre-incubated in the presence of vehicle (1 mmol/l NaOH or 0.5% EtOH, 2 min), IPA/NO (3 μmol/l, 2 min), GTN (100 μmol/l, 2 min) or DEA/NO (100μmol/l, 2 min) before stimulation with collagen (30 μg/ml).

Vascular and platelet superoxide detection

L-012 (100 μmol/l)-enhanced chemiluminescence was used to measure basal $\cdot O_2^-$ levels in common carotid arteries, and also basal and phorbol 12, 13-dibutyrate (PDB; 10 μmol/l)-stimulated $\cdot O_2^-$ levels in platelets from WT and ApoE$^{-/-}$ mice, as previously described (Judkins et al. 2010). Briefly, background chemiluminescence signals were obtained over a 20 minute period using a Plate Chameleon Luminescence Reader (Hidex), in separate wells of a white 96-well Opti-plate containing Krebs-HEPES [composed of (in mmol/l) 99 NaCl, 4.7 KCl, 1.2 $MgSO_4.7H_2O$, 1.0 KH_2PO_4, 19.6 Na_2HCO_3, 20 Na-HEPES, 11.1 D-Glucose, 2.5 $CaCl_2$, 0.026 EDTA, pH 7.4] and L-012 (100 μmol/l).

Carotid arteries were excised, cleaned and cut into segments of ~2mm in length. In semi-darkness, arteries were placed in separate wells and L-012-enhanced chemiluminescence was measured every minute over a 20 minute period and experiments were performed in duplicate.

Platelets were isolated from whole blood, resuspended in Tyrode's buffer to give a final concentration of 5 x 10^8 platelets/ml and equilibrated for 30 minutes at 37°C as described above. In semi-darkness platelets were added to separate wells and basal chemiluminescence was measured every minute over a 20 minute period. To measure $\cdot O_2^-$ levels in activated platelets, the protein kinase C activator PDB (10 μmol/l) was then added and chemiluminescence measured every minute over a 20 minute period. Experiments were performed in quintuplicate.

The effect of IPA/NO, GTN and DEA/NO on $\cdot O_2^-$ levels in common carotid arteries from ApoE$^{-/-}$ and WT mice was assessed using lucigenin (5 μmol/l)-enhanced chemiluminescence as previously described (Miller 2013; Miller et al. 2009; Miller et al. 2005). Briefly, angiotensin II (Ang II; 0.1 μmol/l)-stimulated $\cdot O_2^-$ production (in the presence of 100 μmol/l NADPH) by common carotid arteries from WT and ApoE$^{-/-}$ mice was measured in the presence of vehicle (1 mmol/l NaOH or 0.05% EtOH),

IPA/NO (3 μmol/l), GTN (10 μmol/l) or DEA/NO (1 μmol/l). Vehicle, HNO and NO• donors were added immediately prior to measuring $^{\cdot}O_2^-$ levels. In all experiments, $^{\cdot}O_2^-$ counts were measured every minute for 30 minutes and experiments were performed in duplicate.

Data and statistical analysis

All results are presented as mean ± SE and $P<0.05$ was considered statistically significant. Vasorelaxation responses were expressed as a percentage reversal of U46619 precontraction. Contractile responses to L-NAME were expressed as a percentage of the maximum response to U46619 (1 μmol/l; F_{max}). Individual relaxation curves were fitted to a sigmoidal logistical equation (GraphPad Prism, Version 5.0) to provide an estimate of the pEC_{50} value (concentration of agonist causing a 50% relaxation), expressed as –log mol/l and maximum relaxation (R_{max}). Differences between mean pEC_{50} and maximum relaxation values were tested using either a Student's unpaired t-test or 1-way ANOVA with a Bonferroni post hoc test. Where pEC_{50} values could not be obtained, concentration-response curves were compared by means of a 2-way ANOVA with a Bonferroni post-hoc test (Sigma Stat 3.5). Platelet aggregation responses were expressed as either total aggregation = area under the curve (AUC; arbitrary units) of percent increments of light transmission over the 30 minute period following collagen addition or time to half maximal aggregation (seconds). Statistical comparisons were performed using either a Student's unpaired t-test or one-way ANOVA with a Bonferroni post hoc test. Basal $^{\cdot}O_2^-$ and Ang II-stimulated $^{\cdot}O_2^-$ levels were determined by subtracting background counts from the chemiluminescence signal and expressed as either relative light units per second ($RLU{\cdot}s^{-1}$) or normalised to dry tissue weight ($RLU{\cdot}s^{-1}{\cdot}mg^{-1}$ tissue). PDB-stimulated $^{\cdot}O_2^-$ production was calculated by subtracting basal counts from the PDB-stimulated chemiluminescence signal and expressed as $RLU{\cdot}s^{-1}$. Statistical comparisons were performed using either a Student's unpaired or paired t-test.

Drugs and their sources

Drugs and there sources were: angiotensin II (Auspep, Australia); clexane (enoxaparin sodium; Aventos, New Zealand); collagen (bovine; Helena Laboratories, Australia); GTN (Mayne, United Kingdom); isopropyl alcohol and paraformaldehyde (Merck, Germany); IPA/NO (kind gift from K. Miranda; University of Arizona, USA); L-012 (Wako, Japan); levcromakalim (Tocris Bioscience, United Kingdom); carboxy-PTIO potassium salt, ODQ, DEA/NO and U46619 (Sapphire Bioscience, Australia) and all other drugs (Sigma, Australia). Angiotensin II was prepared at 10 mmol/l in 50 mmol/l acetic acid and all subsequent dilutions were in Krebs-HEPES (pH 7.4) solution. Clexane was prepared at 400 U/ml in saline (100%). GTN was prepared as a 10 mmol/l stock in 50% ethanol and all subsequent dilutions were in distilled water. IPA/NO and DEA/NO were prepared at 10 mmol/l in 0.01 mol/l NaOH and all subsequent dilutions were in 0.01 mol/l NaOH. L-012 was prepared at 100 mmol/l in dimethyl sulfoxide (DMSO) and all subsequent dilutions were in Krebs-HEPES (pH 7.4) solution. Levcromakalim was prepared at 10 mmol/l in 100% methanol and ODQ at 10 mmol/l in 100% ethanol. Fibrinogen was prepared at 20 mg/ml in 0.9% saline and all subsequent dilutions were in distilled water. Oil Red O was prepared as a 0.5% stock solution in 100% isopropyl alcohol then diluted to a 60% isopropyl alcohol working solution. All other drugs were dissolved and diluted in distilled water.

Results:

Plasma cholesterol, vascular morphology, vessel and platelet function in WT and ApoE$^{-/-}$ mice

Total plasma cholesterol and LDL cholesterol levels were ~7-fold (P<0.001) and ~18-fold (P<0.001) higher, respectively, in ApoE$^{-/-}$ compared to WT mice (Table I). Furthermore, HDL cholesterol levels were ~5-fold (P<0.001) lower in ApoE$^{-/-}$ and trigylceride levels were similar between ApoE$^{-/-}$ and WT mice (Table I). Atherosclerotic lesions were apparent in the internal and common carotid arteries from ApoE$^{-/-}$, but absent in arteries from WT mice (Table I, Figure 1). In ApoE$^{-/-}$ mice, common carotid artery basal $^{\cdot}O_2^-$ levels were ~2-fold higher when compared to WT mice (P<0.05; Table I), and this was associated with a marked reduction in the contractile response to L-NAME (100 µmol/l; basal NO• production) (P<0.01; Table I). Basal $^{\cdot}O_2^-$ levels in platelets from WT-HFD and ApoE$^{-/-}$ mice did not differ, however PDB-stimulated $^{\cdot}O_2^-$ levels in platelets were ~2-fold higher in ApoE$^{-/-}$ mice (P<0.05; Table I). Total aggregation and time to half maximal aggregation, in response to collagen, were similar between platelets from WT and ApoE$^{-/-}$ mice (Table I).

Contribution of endogenous HNO and NO• to endothelium-dependent vasorelaxation in advanced atherosclerosis

Relaxation responses to ACh in common carotid arteries from ApoE$^{-/-}$ were comparable to responses in arteries from WT mice (Table I, Figure 2). Pre-treatment with the NOS inhibitor, L-NAME (100 µmol/l) and the sGC inhibitor, ODQ (10 µmol/l) in combination virtually abolished ACh-mediated relaxation in WT and ApoE$^{-/-}$ mice (P<0.001; Figure 2a & 2c), indicating that endothelium-dependent relaxation was mediated predominantly by a nitrogen oxide species (NO• and/or HNO). To identify the redox form of NO responsible for endothelium-dependent relaxation in WT and ApoE$^{-/-}$ mice, NO• (HXC; 100 µmol/l) and HNO (L-cysteine; 3 mmol/l) scavengers were employed. In carotid arteries from WT mice, HXC reduced the sensitivity to ACh ~7-fold and suppressed the maximum response by 57 ± 6% (P<0.001, Figure 2a). By contrast, the effect of HXC on ACh-mediated relaxation was less pronounced in ApoE$^{-/-}$ mice with only a ~3-fold reduction in potency and a 32 ± 8% decrease in the maximum response obtained (P<0.001, Figure 2c). Whilst the contribution of NO• to endothelium-dependent relaxation was attenuated in atherosclerosis, the contribution of HNO appeared to be augmented. Thus, pre-treatment with L-cysteine lead to a greater reduction in the maximum response

to ACh in ApoE$^{-/-}$ mice (43 ± 8%, P<0.001; Figure 2c) when compared to WT mice (16 ± 8%, P<0.05; Figure 2a). As endothelium-dependent relaxation was primarily NO•-mediated (P<0.001, Figure 2c) in WT mice (Figure 2a), whereas NO• and HNO contributed equally to ACh-mediated relaxation in ApoE$^{-/-}$ mice (Figure 2c). Furthermore, the combination of L-cysteine and HXC inhibited ACh-mediated vasorelaxation to a similar extent as L-NAME and ODQ in combination in both WT and ApoE$^{-/-}$ mice (Figure 2a & 2c).

Next, we examined whether endogenous NO• and HNO generation was dependent upon eNOS. In the presence of L-NAME, the potency of ACh was reduced in both WT (P<0.001) and ApoE$^{-/-}$ mice (P<0.001) and the maximum response was impaired in WT mice (P<0.01, Figure 2b). However, L-NAME alone did not inhibit ACh-mediated vasorelaxation to the same extent as the combination of L-NAME and ODQ (Figure 2). Furthermore, the combination of L-NAME and HXC reduced the sensitivity of ACh (P<0.001) and inhibited maximum relaxation (P<0.05) to a greater extent than L-NAME alone in WT (Figure 2b), but not ApoE$^{-/-}$ mice (Figure 2d). In contrast, the combination of L-NAME and L-cysteine markedly reduced the potency of ACh (P<0.001) and maximum vasorelaxation responses to ACh in both WT (P<0.01, Figure 2b) and ApoE$^{-/-}$ mice (P<0.05, Figure 2d).

Vasorelaxant responses to IPA/NO, GTN and DEA/NO in advanced atherosclerosis

The HNO donor, IPA/NO, caused concentration-dependent vasorelaxation in common carotid arteries from WT and ApoE$^{-/-}$ mice, which was similar in potency and efficacy (Figure 3a, Table II). Likewise, relaxation responses to the NO• donors DEA/NO and GTN and the NO-independent vasodilator, papaverine, were comparable between ApoE$^{-/-}$ and WT mice (Figure 3b-d, Table II).

Susceptibility of IPA/NO, GTN and DEA/NO to tolerance development in advanced atherosclerosis

In common carotid arteries from ApoE$^{-/-}$ mice, pre-treatment with either IPA/NO (30 µmol/l, 60 min) or DEA/NO (30 µmol/l, 60 min) had no effect upon subsequent vasorelaxation responses to IPA/NO or DEA/NO, respectively (Figure 4a & 4b). In contrast, GTN was susceptible to vascular tolerance in ApoE$^{-/-}$ mice, as pre-treatment with GTN (30 µmol/l, 60 min) caused a significant right-ward shift in the subsequent concentration-response curve to GTN (P<0.001; Figure 4c).

Effect of IPA/NO, DEA/NO and GTN on vascular $^{\cdot}O_2^-$ levels in advanced atherosclerosis

Angiotensin II (0.1 µmol/l)-stimulated $^{\cdot}O_2^-$ levels were ~2.5-fold higher in common carotid arteries from ApoE$^{-/-}$ mice (10704±2040 RLU$^{\cdot}$s^{-1}$^{\cdot}$mg^{-1}, n=19; P<0.05) when compared to levels in arteries from WT mice (3971±490 RLU$^{\cdot}$s^{-1}$^{\cdot}$mg^{-1}, n=13). IPA/NO (3 µmol/l) attenuated $^{\cdot}O_2^-$ levels by 48 ± 7% (P<0.001) and 62 ± 5% (P<0.001) in arteries from WT and ApoE$^{-/-}$ mice, respectively (Figure 5a). Similarly, the ability of DEA/NO (1 µmol/l) to reduce $^{\cdot}O_2^-$ levels was maintained in arteries from ApoE$^{-/-}$ mice (42 ± 15% inhibition) as compared with WT mice (49 ± 11% inhibition; Figure 5b). By contrast, GTN (100 µmol/l) had no significant effect on $^{\cdot}O_2^-$ levels in arteries from ApoE$^{-/-}$ mice (Figure 5c) despite reducing $^{\cdot}O_2^-$ levels by 43 ± 15% (P<0.05) in arteries from WT mice.

Anti-aggregatory actions of IPA/NO, DEA/NO and GTN in advanced atherosclerosis

IPA/NO (3 µmol/l) inhibited collagen (30 µg/ml)-induced aggregation in ApoE$^{-/-}$ mice (36 ± 5%; P<0.001) to a similar extent as in WT mice (49 ± 5%; P<0.001) (Figure 6a). Similarly, the anti-aggregatory effects of DEA/NO (100 µmol/l) were comparable between WT (50 ± 9% inhibition; P<0.01) and ApoE$^{-/-}$ mice (48 ± 8% inhibition; P<0.05; Figure 6c). Whilst, GTN (100 µmol/l) inhibited platelet aggregation in WT mice by 29 ± 7% (P<0.05 Figure 6e), its anti-aggregatory actions were attenuated in platelets from ApoE$^{-/-}$ mice (10 ± 3%; Figure 6e).

Additionally, WT and ApoE$^{-/-}$ mice displayed a similar increase in time to half maximum response to IPA/NO and DEA/NO (Figure 6b, d). However the time to half maximum response for GTN was diminished in ApoE$^{-/-}$ mice when compared to WT mice (Figure 6f).

Table I. Plasma cholesterol, vascular morphology, and vessel and platelet function in 26-week-old WT and ApoE$^{-/-}$ mice maintained on a high fat diet (HFD) from 5 weeks of age.

Parameter	n	WT-HFD Mean ± SEM	n	ApoE$^{-/-}$-HFD Mean ± SEM
Total plasma cholesterol levels, mmol/l	8	4.5 ± 0.4	8	33.4 ± 2.8 ***
HDL cholesterol, mmol/l	8	2.3 ± 0.1	8	0.5 ± 0.1 ***
LDL cholesterol, mmol/l	8	1.8 ± 0.2	8	32.7 ± 3.1 ***
Triglycerides, mmol/l	8	1.5 ± 0.5	8	1.9 ± 0.3
Common Carotid Artery:				
- Atherosclerotic lesions	5	Not Apparent	5	Visible lesions in internal & common carotid artery
- Basal superoxide, RLU·s^{-1}·mg^{-1}	6	10,331 ± 3195	6	24,554 ± 5407*
- L-NAME contraction, % U46619 (1 µmol/l)	6	56 ± 6	6	31 ± 1**
- Acetylcholine relaxation · pEC$_{50}$, –log mol/l	8	7.5 ± 0.1	8	7.3 ± 0.1
· Max relaxation, % reversal of precontraction	8	80 ± 3	8	88 ± 2
Platelets:				
- Basal superoxide RLU·s^{-1}	6	139 ± 20	6	142 ± 16
- PDB (10 µmol/l)-stimulated superoxide RLU·s^{-1}	6	1,027 ± 147	6	2,346 ± 541*
Collagen (30 µg/ml)-stimulated platelet aggregation: - Total aggregation (AUC), arbitrary units	12	49,786 ± 2241	12	50,584 ± 4753
- Time to half-maximal aggregation, s	12	312 ± 26	12	320 ± 16

Values are given as mean ± SE, where n=number of animals.

HDL = high density lipoprotein; LDL = low density lipoprotein; RLU = Relative light units; AUC = area under the curve; L-NAME = N$^{\omega}$-nitro-L-arginine methyl ester; PDB = phorbol 12,13-dibutyrate; pEC$_{50}$ = concentration of agonist causing a 50% relaxation.

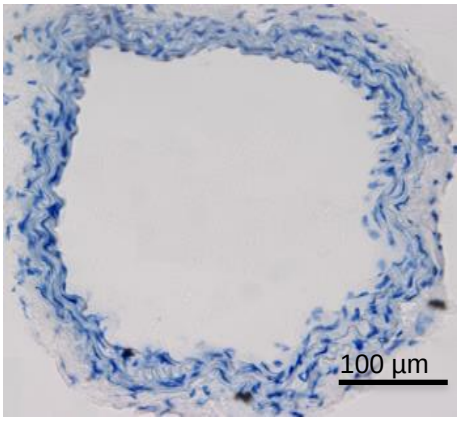
***P*<0.05, ****P*<0.01, *****P*<0.001 vs WT-HFD (Student's unpaired t-test).

Figure 1: Representative photomicrographs showing the presence or absence of atherosclerotic lesions (Oil Red-O staining) in the common (A, B) and internal (C, D) carotid arteries from 26 week-old wild-type (WT) and apolipoprotein E-deficient ($ApoE^{-/-}$) mice maintained on a high-fat diet (HFD) from 5 weeks of age. Images are representative of n=5 animals. Magnification: x20.

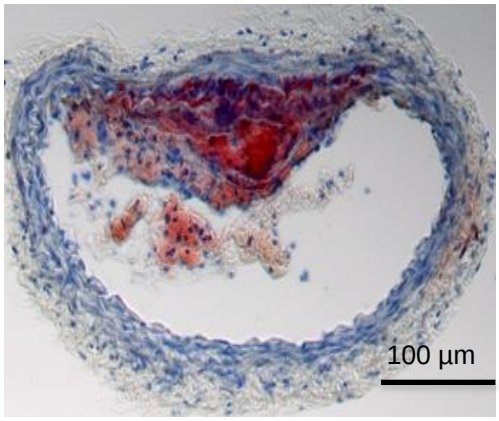
WT-HFD

ApoE$^{-/-}$-HFD

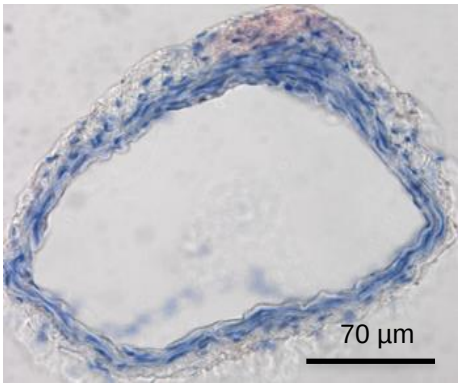
A



B



C



D

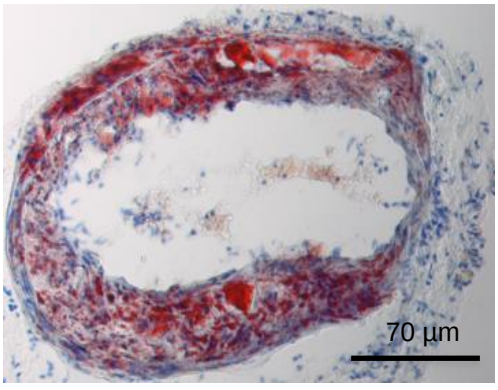
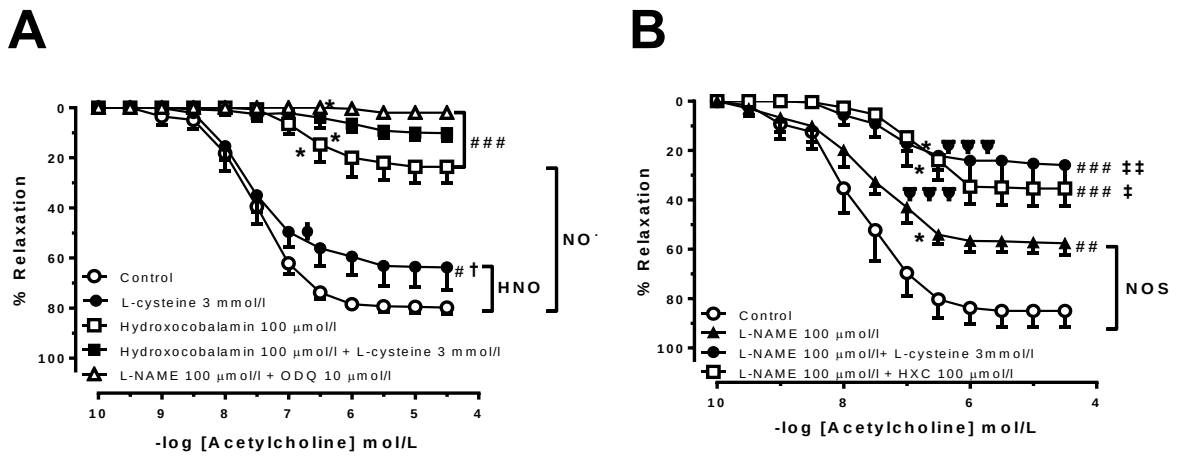


Figure 2: Concentration-dependent vasorelaxation responses in common carotid arteries from WT–HFD (A, B) and ApoE$^{-/-}$-HFD (C, D) mice. A and C: Relaxation responses to acetylcholine in the absence (○, n=7-8) or presence of hydroxocobalamin (HXC, 100 µmol/l; □, n=6-7), L-cysteine (3 mmol/l; ●, n=6-7), HXC and L-cysteine in combination (■, n=3-5) or L-NAME (100 µmol/l) and ODQ (10 µmol/l; ∆, n=3-5) in combination. B and D: Relaxation responses to acetylcholine in the absence (○, n=4-5) or presence of L-NAME (100 µmol/l; ▲, n=4-5) alone or in combination with either hydroxocobalamin (HXC, 100 µmol/l; □, n=4-5) or L-cysteine (3 mmol/l; ●, n=4-5). Values are expressed as percent reversal of precontraction and given as mean ± SE, where n=number of animals. **P*<0.001 for treatment curve vs control (2-way ANOVA, Tukey post hoc test), #*P*<0.05, ##*P*<0.01, ###*P*<0.001 for response at 30 µmol/l vs control, ϕ*P*<0.001 for treatment vs HXC alone, †*P*<0.001 for response at 30 µmol/l vs HXC alone, ΨΨΨ*P*<0.001 for treatment vs L-NAME alone, ‡*P*<0.05, ‡‡*P*<0.01 for response at 30 µmol/l vs L-NAME alone (1-way or 2-way ANOVA, Bonferroni or Tukey post-hoc test).

WT-HFD



ApoE$^{-/-}$-HFD

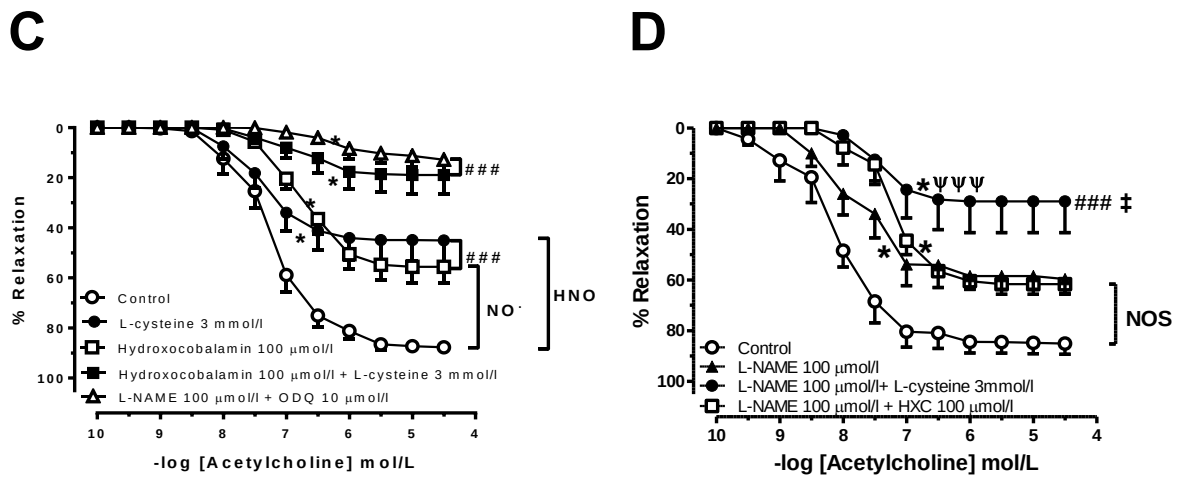


Figure 3: Concentration-response curves to IPA/NO (A; n=6-7), DEA/NO (B; n=5-7), GTN (C; n=6-8) and papaverine (D; n=6-8) in common carotid arteries from WT–HFD and ApoE$^{-/-}$-HFD mice. Values are expressed as the percent reversal of precontraction and given as mean ± SE, where n=number of animals.

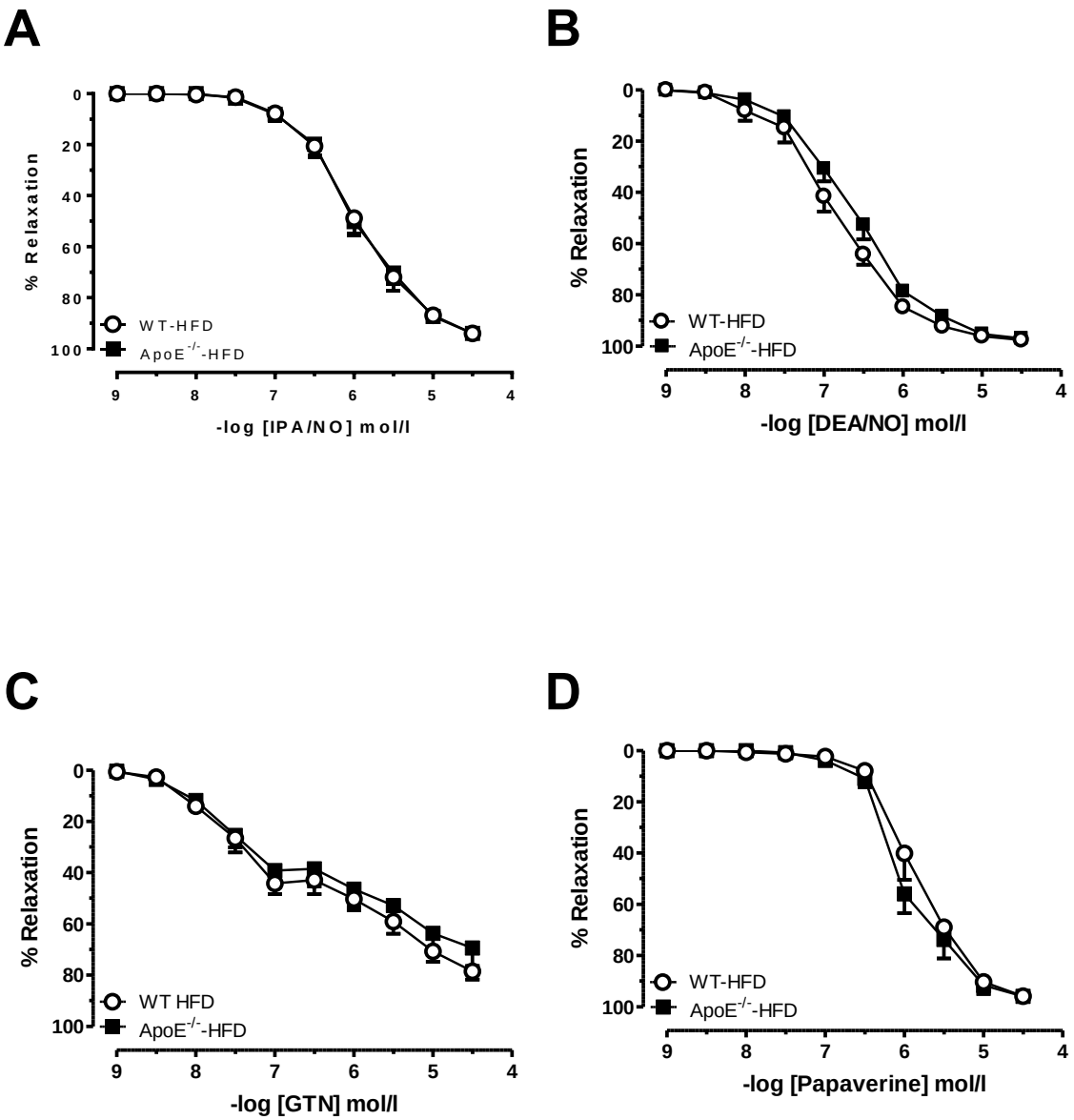

A
% Relaxation
0
20
40
60
80
100
WT-HFD
ApoE-/--HFD
9
8
7
6
5
4
-log [IPA/NO] mol/l
B
% Relaxation
0
20
40
60
80
100
WT-HFD
ApoE-/--HFD
9
8
7
6
5
4
-log [DEA/NO] mol/l
C
% Relaxation
0
20
40
60
80
100
WT HFD
ApoE-/--HFD
9
8
7
6
5
4
-log [GTN] mol/l
D
% Relaxation
0
20
40
60
80
100
WT-HFD
ApoE-/--HFD
9
8
7
6
5
4
-log [Papaverine] mol/l

Table II. Vasorelaxation responses to IPA/NO, DEA/NO, GTN and papaverine in common carotid arteries from 26 week old WT and ApoE$^{-/-}$ mice maintained on a high fat diet (HFD) from 5 weeks of age.

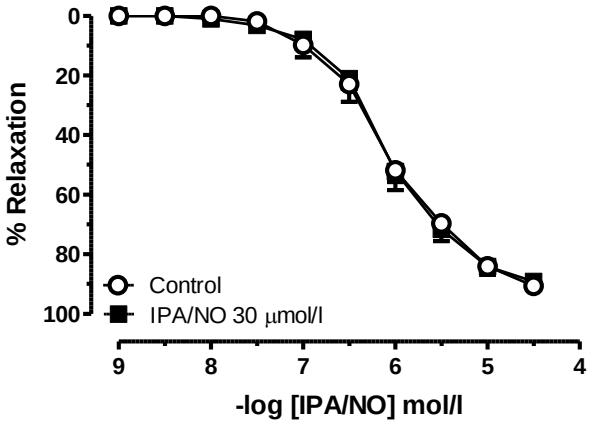
		WT-HFD			ApoE$^{-/-}$-HFD	
Vasodilator	**n**	**pEC_{50}**	**R_{max}**	**n**	**pEC_{50}**	**R_{max}**
IPA/NO	6	5.99 ± 0.10	94 ± 2	8	5.99 ± 0.09	95 ± 1
DEA/NO	5	6.82 ± 0.11	98 ± 1	8	6.73 ± 0.15	96 ± 1
GTN (*Phase 1*)	6	7.63 ± 0.08		8	7.61 ± 0.16	
GTN (*Phase 2*)	6	5.30 ± 0.12	78 ± 3	8	5.67 ± 0.18	69 ± 7
Papaverine	6	5.87 ± 0.10	96 ± 1	8	6.01 ± 0.11	96 ± 2

Values are given as mean ± SE, where n=number of animals.
IPA/NO = isopropylamine NONOate; DEA/NO = diethylamine NONOate; GTN = glyceryl trinitrate; pEC_{5O} = concentration of agonist causing 50% relaxation and is expressed as –log M. Maximum relaxation (R_{max}) = % reversal of the level of precontraction in response to 30 μmol/l of the vasodilator.

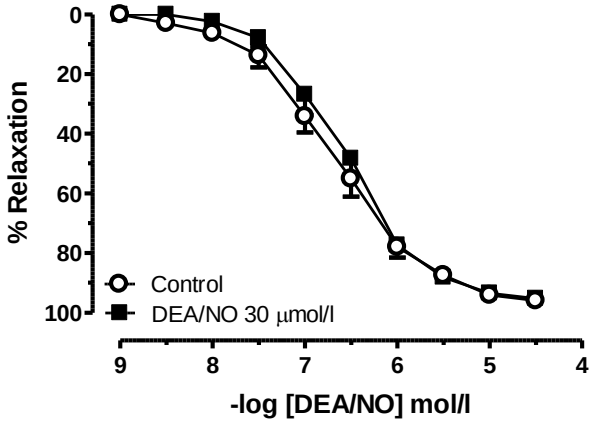
Figure 4: Concentration-response curves to IPA/NO (A), DEA/NO (B) and GTN (C) in common carotid arteries from ApoE$^{-/-}$-HFD mice after a 60 min incubation in the presence of either vehicle (0.01 mol/L NaOH or 0.05% EtOH; ○, n=5-6, Control) or 30 µmol/l IPA/NO (A; ■, n=5), DEA/NO (B; ■, n=6) or GTN (C; ■, n=5). Values are expressed as the percent reversal of precontraction and given as mean ± SE, where n=number of animals. ***P<0.001 for treatment curve vs control (2-way ANOVA, Bonferroni post-hoc test).

ApoE$^{-/-}$-HFD

A


% Relaxation
0
20
40
60
80
100
Control
IPA/NO 30 μmol/l
9
8
7
6
5
4
-log [IPA/NO] mol/l

B


% Relaxation
0
20
40
60
80
100
Control
DEA/NO 30 μmol/l
9
8
7
6
5
4
-log [DEA/NO] mol/l

C

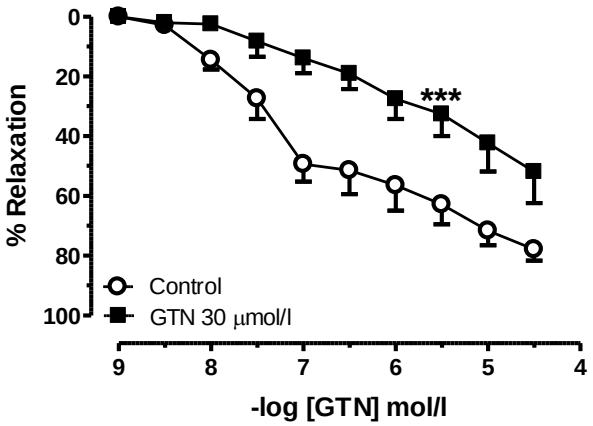

% Relaxation
0
20
40
60
80
100
***
Control
GTN 30 μmol/l
9
8
7
6
5
4
-log [GTN] mol/l

Figure 5: Effect of IPA/NO (3 µmol/l, n=6-8; A), DEA/NO (1 µmol/l, n=6-7; B) and GTN (100 µmol/l, n=5-7; C) upon angiotensin-II (0.1 µmol/l)-stimulated superoxide levels in common carotid artery segments from WT-HFD and ApoE$^{-/-}$-HFD mice, as measured by lucigenin (5 µmol/l)-enhanced chemiluminescence. Experiments were performed in the presence of NADPH (100 µmol/l). Values are expressed as % of levels in vehicle treated arteries, and are given as mean ± SE, where n= number of animals. *****P<0.05, ******P<0.01, *******P<0.001 for treatment vs vehicle (either 0.01 mol/l NaOH or 0.5% EtOH; Student's paired t-test).

Angiotensin II-stimulated superoxide

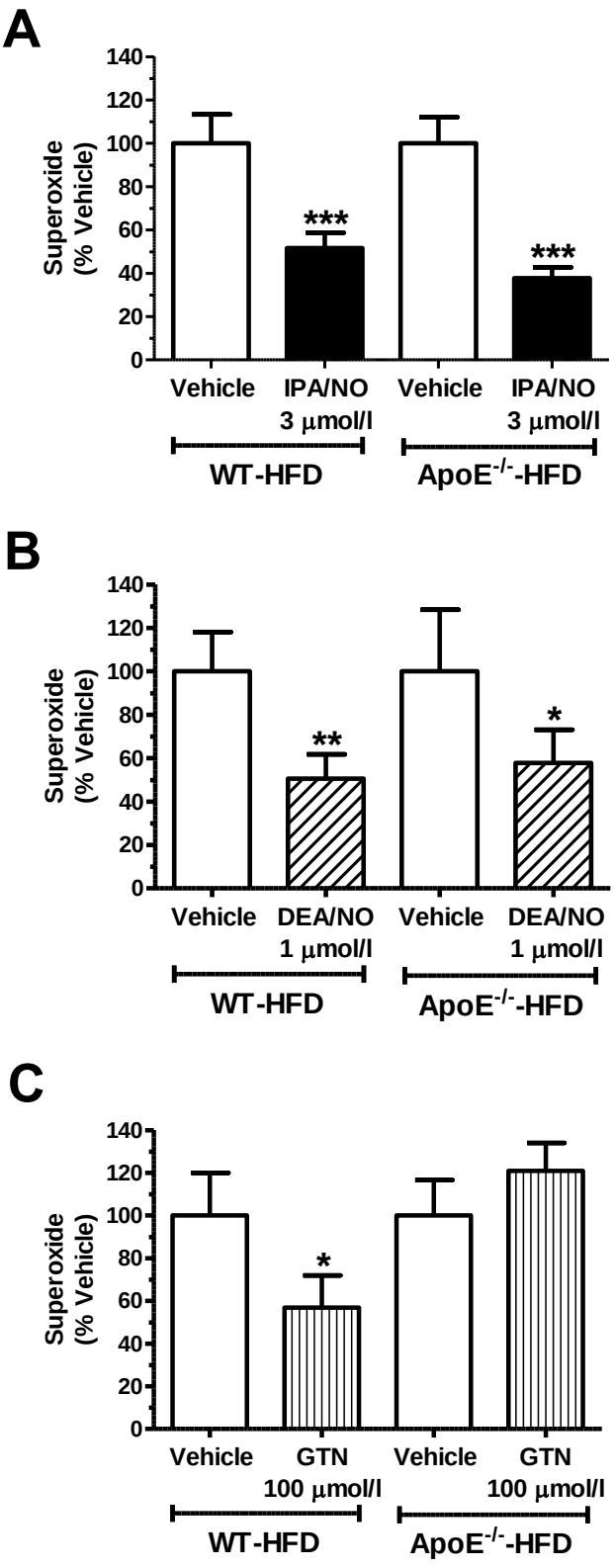
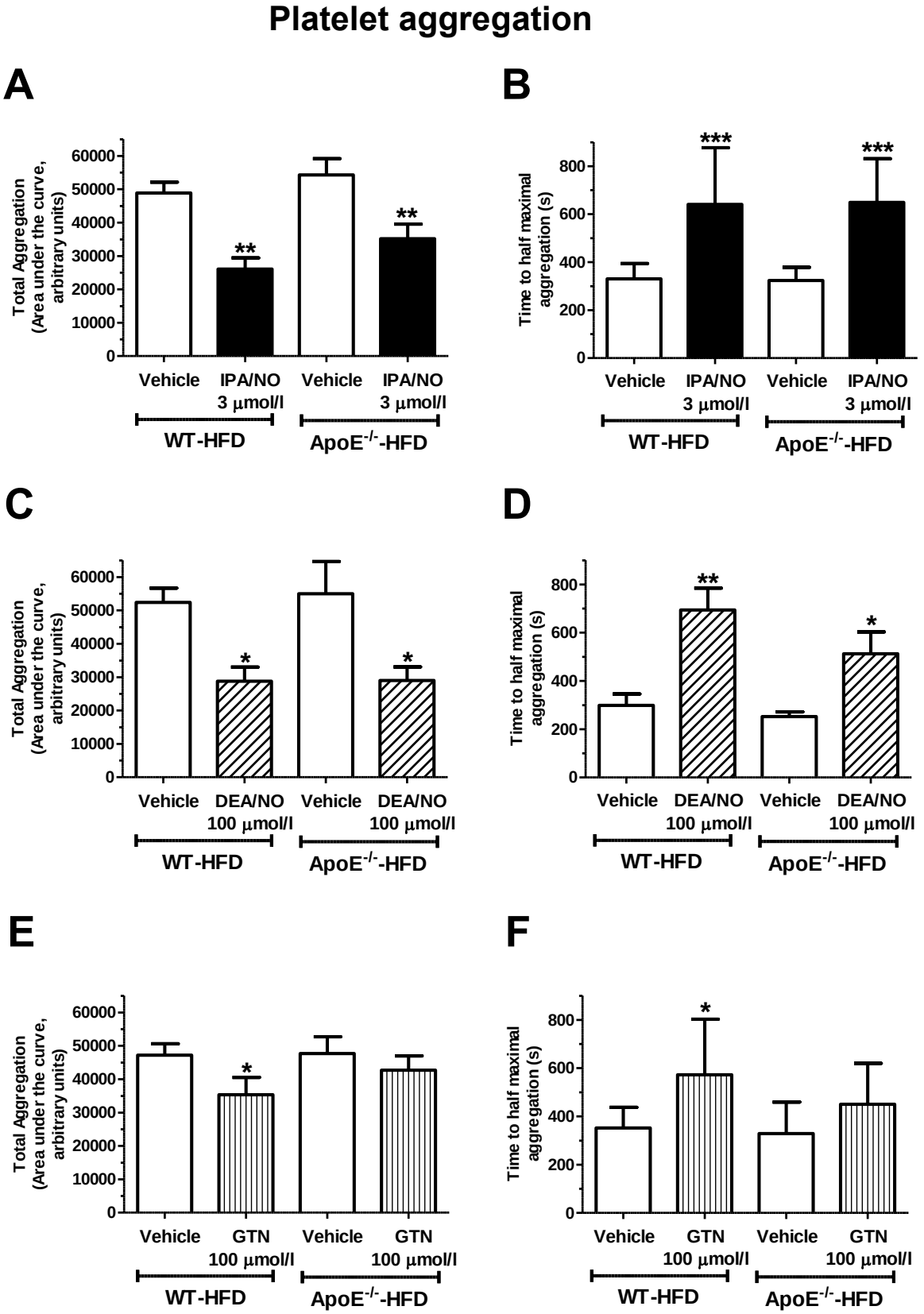


Figure 6: Effect of IPA/NO (A & B; 3 µmol/l, n=6-8), DEA/NO (C & D; 100 µmol/l, n=6) and GTN (E & F; 100 µmol/l, n=6-7) upon collagen (30 µg/ml)-stimulated aggregation in washed platelets from WT-HFD and ApoE$^{-/-}$-HFD mice. Aggregatory responses are expressed as either percent vehicle (area under the curve, arbitrary units; A, C, E) or time to half maximum response (B, D, F) and given as mean ± SE, where n= number of animals. *****P<0.05, ******P<0.01, *******P<0.001 for treatment vs vehicle (Student's paired t-test).


Platelet aggregation
A
Total Aggregation (Area under the curve, arbitrary units)
60000
50000
40000
30000
20000
10000
0
**
**
Vehicle
IPA/NO 3 µmol/l
Vehicle
IPA/NO 3 µmol/l
WT-HFD
ApoE-/--HFD
B
Time to half maximal aggregation (s)
800
600
400
200
0
***
***
Vehicle
IPA/NO 3 µmol/l
Vehicle
IPA/NO 3 µmol/l
WT-HFD
ApoE-/--HFD
C
Total Aggregation (Area under the curve, arbitrary units)
60000
50000
40000
30000
20000
10000
0
*
*
Vehicle
DEA/NO 100 µmol/l
Vehicle
DEA/NO 100 µmol/l
WT-HFD
ApoE-/--HFD
D
Time to half maximal aggregation (s)
800
600
400
200
0
**
*
Vehicle
DEA/NO 100 µmol/l
Vehicle
DEA/NO 100 µmol/l
WT-HFD
ApoE-/--HFD
E
Total Aggregation (Area under the curve, arbitrary units)
60000
50000
40000
30000
20000
10000
0
*
Vehicle
GTN 100 µmol/l
Vehicle
GTN 100 µmol/l
WT-HFD
ApoE-/--HFD
F
Time to half maximal aggregation (s)
800
600
400
200
0
*
Vehicle
GTN 100 µmol/l
Vehicle
GTN 100 µmol/l
WT-HFD
ApoE-/--HFD

Discussion:

This study has provided the first evidence for sustained vasoprotective actions of both endogenous and exogenously generated HNO in advanced atherosclerosis. Whilst a loss in endogenous NO• bioavailability was evident in atherosclerotic mice, the contribution of HNO to endothelium-dependent relaxation was augmented such that vasorelaxation to ACh was maintained. Moreover, the ability of the HNO donor IPA/NO, to induce vasorelaxation, limit $^{\cdot}O_2^-$ production and inhibit platelet aggregation was preserved in advanced atherosclerosis. Whilst these protective effects were similar to those seen with the NO• donor DEA/NO, we found that in the setting of atherosclerosis, GTN, a clinically used NO• donor, was unable to supress $^{\cdot}O_2^-$ levels or serve as an anti-aggregatory agent and was also susceptible to vascular tolerance development. Thus, it appears that HNO donors represent a therapeutic alternative to current nitrovasodilators in disease states such as atherosclerosis.

To investigate and compare the vasoprotective properties of HNO and NO• in advanced atherosclerosis, this study used ApoE$^{-/-}$ mice maintained on a HFD for 21 weeks. The ApoE$^{-/-}$ mouse is a well-established model of atherosclerosis and it has previously been shown that advanced fibrotic lesions are present at this time point (Meyrelles et al. 2011). Additionally, the ApoE$^{-/-}$ mouse model shares many commonalities with atherogenesis in patients, including spontaneous atheroma development, enhanced oxidative stress, endothelial dysfunction and hypercholesterolemia (Kolovou et al. 2008; Meyrelles et al. 2011). In accordance with previous reports (Judkins et al. 2010; Miller et al. 2010), we found that the ApoE$^{-/-}$ mice had elevated LDL and total plasma cholesterol levels, reduced HDL cholesterol and atherosclerotic lesions in carotid arteries. Moreover, both basal vascular and PDB-stimulated platelet $^{\cdot}O_2^-$ production was elevated and basal NO• bioavailability was reduced (measured via contraction to L-NAME) in atherosclerotic mice. Whilst NO• production has previously been shown to be elevated in atherosclerosis (Ponnuswamy et al. 2012), this does not necessarily correlate with NO• bioavailability. As this study also showed vascular $^{\cdot}O_2^-$ levels were elevated and there also appeared to be a partial uncoupling of eNOS (Ponnuswamy et al. 2012). Together, this suggests that despite an increase in NO• production, uncoupling of eNOS may contribute to elevated vascular $^{\cdot}O_2^-$ levels, which are in the proximity to directly scavenge NO• and impair its bioavailability.

Although we observed an impairment in basal NO• bioavailability, ACh-mediated vasorelaxation and collagen-stimulated platelet aggregation were unchanged in atherosclerotic mice. Impairment of ACh-

mediated vasorelaxation has previously been reported in carotid arteries from ApoE$^{-/-}$ mice maintained on a HFD for 26 weeks (d'Uscio et al. 2001; Matsumoto et al. 2003). However, it was not clear how well matched the level of precontraction with U46619 was between WT and ApoE$^{-/-}$ mice, as a greater level of contraction in ApoE$^{-/-}$ mice may lead to functional antagonism and an apparent reduction in ACh-mediated relaxation (Matsumoto et al. 2003). This study used titrated concentrations of U46619 to reach ~50% of the maximal contractile response, thereby eliminating any influence the level of pre-contraction has upon ACh-mediated vasorelaxation. Moreover, we have previously reported, that relaxation responses to ACh were sustained in carotid arteries from ApoE$^{-/-}$ mice, maintained on a HFD for 7 weeks (Chapter 2). Similar results have also been demonstrated in carotid arteries from aged ApoE$^{-/-}$ mice, maintained on a regular diet for 18 months (Crauwels et al. 2003).

Finally, endothelium-dependent relaxation may have been preserved in our study due to changes in the relative contribution of NO's under non-disease and disease conditions. Whilst NO• is traditionally considered the predominant EDRF under physiological conditions, recent pharmacological studies have identified that HNO may also contribute to endothelium-dependent relaxation in both large conduit (Ellis et al. 2000; Leo et al. 2012; Wanstall et al. 2001; Wynne et al. 2012) and small resistance vessels under physiological conditions (Andrews et al. 2009; Yuill et al. 2011). This study provides further evidence to support a role for HNO as an EDRF in large arteries under physiological conditions (i.e. in common carotid arteries from WT mice). However, the major novel finding of these experiments was that the contribution of HNO to endothelium-dependent relaxation appears to be augmented during atherosclerosis to compensate for a loss of endogenous NO•. Specifically, we found that whilst a small component of EDRF was attributed to HNO in vessels from WT mice, HNO had an equal contribution to that of NO• in ApoE$^{-/-}$ mice. These findings are in accordance to a previous report in diabetic rats, where the contribution of endogenous HNO as an EDRF was augmented and may account for the absence of endothelial dysfunction (Leo et al. 2012). Furthermore, HNO also serves as an EDRF in Ang II-induced hypertension, although HNO was unable to compensate for a marked reduction in ACh-mediated relaxation in this model (Wynne et al. 2012). The greater contribution of HNO to endothelium-dependent vasorelaxation in this model of atherosclerosis could reflect increased HNO synthesis and/or preserved bioavailability of HNO. Indeed, several biochemical pathways have been associated with HNO synthesis, including uncoupled NOS and oxidation of NOS intermediates (Irvine et al. 2007; Paolocci et al. 2007).

Atherosclerosis is associated with eNOS uncoupling and enhanced oxidative stress (Ponnuswamy et al. 2012), which may lead to enhanced HNO generation. Alternatively, the bioavailability of HNO could be preserved in atherosclerosis. As HNO can potentially be generated via uncoupled eNOS (Rusche et al. 1998) and its bioavailability is thought to be enhanced under condition of thiols depletion (Park and Oh 2011).

In addition to the enhanced contribution of HNO as an EDRF, we were also interested in the source of HNO, since HNO can be generated via both NOS-dependent and NOS-independent mechanisms. In atherosclerotic mice, pre-treatment with the NOS inhibitor, L-NAME led to partial inhibition of ACh-mediated relaxation, suggesting responses to ACh are mediated, in part through NOS-dependent mechanisms. When L-NAME was added in combination with HXC a similar ACh response was observed to that of L-NAME alone. In contrast, ACh-mediated relaxation was further impaired with the combination of L-NAME and L-cysteine. Taken together, this provides strong evidence in support of a NOS-independent source of HNO in atherosclerosis. A similar observation has also been reported in diabetic mice, where part of the EDRF response was attributed to non-NOS derived HNO (Leo et al. 2012). These NOS-independent sources of HNO may include direct enzymatic reduction of NO• itself (which is unlikely; Irvine et al. 2007; Paolocci et al. 2007) or through thiolation of S-nitrosothiols (Arnelle and Stamler 1995; Wong et al. 1998). Furthermore, under non-disease conditions, the addition of L-NAME in combination with either L-cysteine or HXC, led to a greater inhibition of ACh-mediate relaxation than L-NAME alone. Suggesting that both NO• and HNO mediate the response to ACh, in part through NOS-independent mechanisms. Indeed, it has been proposed that NO• can exist in pre-formed stores (Chauhan et al. 2003). However, these stores do not appear to contribute to ACh-mediated relaxation in atherosclerosis and this may account for HNO, but not NO• being upregulated in atherosclerosis.

After establishing a role for endogenous HNO in atherosclerosis, we next examined the vasoprotective actions of exogenous HNO. The effects of the HNO donor IPA/NO were compared to those of the well characterised NO• donors GTN, an organic nitrate which must first undergo biotransformation to release NO• and DEA/NO, a spontaneous NO• donor with a similar kinetic profile to IPA/NO. Interestingly, all three donors mediate their vasoprotective (i.e. vasorelaxant, anti-aggregatory) actions predominantly via the sGC/cGMP-dependent signalling pathway (Andrews et al.

2009; Bermejo et al. 2005; Dautov et al. 2013; Irvine et al. 2007). To determine if the specific NO species generated and/or the mode of NO generation are critical to maintaining the vasoprotective effects of these donors. In the present study, we found that vasorelaxant responses to IPA/NO, DEA/NO, GTN and the sGC-independent vasodilator, papaverine were preserved in common carotid arteries from atherosclerotic mice when compared to non-diseased vessels. These findings concur with our previous observations in hypercholesterolemic mice, where relaxation responses to IPA/NO and GTN were sustained (Chapter 2). Importantly, the vasodilatory actions of HNO donors have been shown to be preserved in other cardiovascular disease states such as experimental heart failure (Paolocci et al. 2003), hypertension (Ang II-induced and spontaneously hypertensive rats; Irvine et al. 2013; Wynne et al. 2012) and diabetes (Leo et al. 2012).

Whilst, endogenous NO• is commonly impaired under disease conditions, there are conflicting reports whether the vasorelaxant effects of NO• donors are diminished. For instance, vasorelaxation to GTN was preserved in the abdominal aorta, but not the aortic arch in hypercholesterolemic rabbits (Verbeuren et al. 1986). Similarly, in atherosclerotic mice relaxation responses to DEA/NO were compromised in aortic, but not carotid arteries (d'Uscio et al. 2001; d'Uscio et al. 2001). Moreover, atherosclerosis has also been associated with reduced sGC activity in rabbits (Melichar et al. 2004). In this study, we found that relaxation responses to DEA/NO and GTN were conserved in carotid arteries from atherosclerotic mice. Despite evidence for enhanced oxidative stress and reduced endogenous NO• bioavailability. Taken together, these reports indicate that responses to NO• donors during atherosclerosis may be dependent upon both the stage of the disease and the nature of the vessel studied.

Although we found the acute vasorelaxant effects of GTN to be preserved, a major clinical limitation surrounding its use is its susceptibility to tolerance development with continued use (Daiber et al. 2009). Indeed, we demonstrated that tolerance developed to GTN in common carotid arteries from atherosclerotic mice. By contrast, the vasorelaxant actions of both IPA/NO and DEA/NO were resistant to tolerance development. These results are in accordance with our previous *ex vivo* and *in vivo* experiments where Angeli's salt, IPA/NO and DEA/NO were found to be resistant to self-tolerance and cross-tolerance to GTN under non-disease conditions (Irvine et al. 2007; Irvine et al. 2010) and in hypercholesterolemia (Chapter 2). Thus in atherosclerosis, tolerance is not dependent

upon the NO species generated (i.e HNO or NO•) rather the nature of the nitrovasodilator utilised. IPA/NO and DEA/NO spontaneously decompose at physiological pH and temperature to generate HNO and NO•, respectively and were both found to be resistant to tolerance development (DuMond and King 2011). In contrast, GTN, which requires biotransformation to release NO•, is susceptible to tolerance development (Chen et al. 2002). Suggesting that tolerance in atherosclerosis is likely to due to impaired biotransformation of GTN. However, given tolerance is a multi-factorial process, we cannot discount that other mechanisms such as increased PDE activity that could play a role in the development of vascular tolerance (Daiber et al. 2009). Nevertheless, the resistance of HNO donors to tolerance development in advanced atherosclerosis has major clinical implications with regards to the chronic treatment in cardiovascular disorders such as coronary artery disease.

Another major advantage of HNO donors, is that they are resistant to scavenging by $^{\cdot}O_2^-$ (Leo et al. 2012; Miller 2013; Miranda et al. 2002) and recently it was identified that HNO limits vascular $^{\cdot}O_2^-$ generation in a Nox2 oxidase-dependent manner (Miller 2013). Nox2 oxidase is a member of the NADPH oxidase family and plays a central role in vascular oxidative stress and dysfunction in a number of cardiovascular disorders (Drummond et al. 2011). Indeed, we have previously reported increased expression of Nox2 oxidase in ApoE$^{-/-}$-HFD mice (Judkins et al. 2010; Miller et al. 2010). As such, it was important to determine if the ability of HNO to limit $^{\cdot}O_2^-$ levels was conserved in atherosclerosis. Importantly, this study provides the first demonstration that HNO can supress vascular $^{\cdot}O_2^-$ levels under disease conditions. Specifically, IPA/NO maintained its ability to limit angiotensin II-stimulated $^{\cdot}O_2^-$ generation in common carotid arteries from atherosclerotic mice. Given angiotensin II is a potent stimulator of Nox2 oxidase (Miller 2013), it appears that IPA/NO is mediating its $^{\cdot}O_2^-$ supressing actions by reducing Nox2 oxidase activity. Additionally, DEA/NO and GTN were found to limit $^{\cdot}O_2^-$ levels under non-disease conditions. Whilst, the NO• donor, diethylenetriamine NONOate has previously been shown to supress endothelial Nox2 oxidase-dependent $^{\cdot}O_2^-$ generation, a prolonged treatment period (6 hours) was required to achieve this effect (Selemidis et al. 2007). In the present study, DEA/NO and GTN were administered acutely, thus it is more likely that NO• is being directly scavenged by $^{\cdot}O_2^-$, as opposed to limiting its generation from Nox2 oxidase.

Interestingly, the $^{\cdot}O_2^-$ supressing actions of DEA/NO, but not GTN, were conserved in atherosclerosis. Under non-disease conditions, it is likely that NO• donors are limiting $^{\cdot}O_2^-$ levels via direct scavenging

of $^{\cdot}O_2^-$ by NO•. However, in atherosclerosis, where vascular $^{\cdot}O_2^-$ production is enhanced, this system could become overwhelmed. As such, the preserved actions of DEA/NO, as compared to GTN, could be attributed to a greater amount of NO• generated from this NO• donor. As 2 moles of NO• are released per mole of DEA/NO, whereas GTN generates the equivalent of 1 mole of NO• per parent compound (Miranda et al. 2005). Alternatively, the inability of GTN to limit vascular $^{\cdot}O_2^-$ levels in atherosclerosis may reflect impairment of biotransformation of this organic nitrate to NO•, as this has been reported in diabetic rats (Wang et al. 2011).

Nitrovasodilators are also well recognised for their anti-aggregatory actions and we have compared, for the first time, the ability of IPA/NO, DEA/NO and GTN to inhibit collagen-stimulated platelet aggregation in WT-HFD and ApoE$^{-/-}$-HFD mice. Consistent with previous reports (Chapter 2; Chirkov et al. 2001; Crane et al. 2005; Gudmundsdottir et al. 2005) all three donors displayed anti-aggregatory actions in platelets from WT-HFD mice, albeit higher concentrations of DEA/NO and GTN were required to inhibit aggregation to a similar extent as IPA/NO. Suggesting that IPA/NO could be a more potent anti-aggregatory agent. Given the anti-aggregatory actions of HNO are predominantly mediated by sGC/cGMP (Bermejo et al. 2005; Dautov et al. 2013), it is possible that the potency of IPA/NO could be attributed to an elevation in platelet cGMP. Indeed, we have found that IPA/NO elevates basal cGMP levels to a greater extent than the NO• donor, sodium nitroprusside (SNP) (Chapter 4) and Angeli's salt has previously been shown to increase cGMP levels to a greater extent that SNP in human platelets (Bermejo et al. 2005). Alternatively, the greater potency of IPA/NO may be due to HNO targeting unique signalling pathways, such as the modification of thiol residues on platelet proteins. As Angeli's salt has been shown to target key platelet surface receptors and cytoskeletal associated proteins that are involved in platelet activation (Hoffman et al. 2009), albeit, the impact of such modifications on platelet aggregation remains to be elucidated.

A further limitation of clinically used nitrovasodilators is that patients with stable angina, acute coronary syndrome and chronic heart failure can display reduced responsiveness to these donors (Chirkov et al. 2010; Chirkov et al. 1999; Chirkov et al. 2001). This phenomenon, known as platelet NO• resistance is distinct from tolerance, as resistance occurs without prior exposure to a nitrovasodilator (Horowitz 2000). Importantly, we observed that the anti-aggregatory actions of IPA/NO and DEA/NO were maintained in platelets from atherosclerotic mice, whereas the ability of

GTN to inhibit platelet aggregation was impaired. These results supports previous findings in hypercholesterolemia, showing the preservation of the anti-aggregatory actions of IPA/NO, but not GTN (Chapter 2). Suggesting that the reduced responsiveness to GTN could be attributed to platelet NO• resistance or an impairment in biotransformation of GTN. Where platelet NO• resistance may be the result of either reduced NO• bioavailability through scavenging of NO• by $^{\cdot}O_2^-$ or due to an impairment in sGC/cGMP signalling (Chirkov et al. 1999; Dautov et al. 2013). Albeit, the anti-aggregatory actions of DEA/NO were sustained, but this may reflect its ability to generate a larger amount of NO• (2 moles of NO• per parent compound) to overcome reduced NO• bioavailability. Whereas, the ability of HNO to serve as an anti-aggregatory agent in atherosclerosis is most likely due to its resistance to scavenging by vascular $^{\cdot}O_2^-$ or could act through sGC/cGMP-independent mechanisms.

By comparing the vasoprotective actions of a HNO donor with two NO• donors, that release NO• via different mechanisms (ie. spontaneous release vs. biotransformation), we have gained valuable insight into the mechanisms underlying diminished nitrovasodilator responsiveness in atherosclerosis. Under conditions of enhanced oxidative stress, diminished endogenous NO• bioavailability and vascular remodelling, the vasorelaxant, anti-aggregatory and $^{\cdot}O_2^-$ supressing actions of donors that spontaneously release either HNO or NO• were sustained. By contrast the vasoprotective actions of the NO• donor that needs to undergo biotransformation were compromised. Taken together this data indicates that it is not the nature of the NO species per se but possibly the mode of release and the amount of NO• generated that impacts the effectiveness of a donor in cardiovascular disease states.

Conclusion:

In conclusion, the findings of this study provide the first evidence that the vasoprotective actions of endogenous and exogenously generated HNO are preserved in an advanced model of atherosclerosis. Strikingly, our pharmacological evidence infers that endogenous HNO generation is augmented in atherosclerosis to compensate for a reduction in endogenous NO• production, thereby preserving endothelium-dependent relaxation. Importantly, in this advanced disease state, where oxidative stress was evident, the vasoprotective actions (i.e. vasorelaxant, anti-aggregatory, $^{\cdot}O_2^-$ suppressing) of exogenous HNO were also conserved. As such, HNO donors may have therapeutic advantages over the clinically used nitrovasodilator, GTN, which is susceptible to vascular tolerance and exhibits diminished anti-aggregatory and $^{\cdot}O_2^-$ limiting actions. Whilst DEA/NO also maintained its vasoprotective effects, the therapeutic application of this class of NO• donor is limited due its correlation with systemic hypotension and generation of hepatocarcinogens (Fathi et al. 2011; Keefer 2003). Consequently, the cardioprotective actions of HNO, together with its preserved bioavailability in advanced vascular disease, make HNO donors an appealing therapeutic option in the treatment of vascular dysfunction associated with diseases such as atherosclerosis.

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References:

Andrews KL, Irvine JC, Tare M, Apostolopoulos J, Favaloro JL, Triggle CR and Kemp-Harper BK (2009). A role for nitroxyl (HNO) as an endothelium-derived relaxing and hyperpolarizing factor in resistance arteries. *Br J Pharmacol* 157(4): 540-50.

Arnelle DR and Stamler JS (1995). NO^+, $NO^\cdot$, and NO^- donation of S-nitrosothiols: Implications for regulation of physiological functions by S-nitrosylation and aceleration of disulfide formation. *Arch Biochem Biophys* 318(2): 279-285.

Bermejo E, Saenz DA, Alberto F, Rosenstein RE, Bari SE and Lazzari MA (2005). Effect of nitroxyl on human platelets function. *Thromb Haemost* 94(3): 578-84.

Chauhan S, Rahman A, Nilsson H, Clapp L, MacAllister R and Ahluwalia A (2003). NO contributes to EDHF-like responses in rat small arteries: a role for NO stores. *Cardiovasc Res* 57(1): 207-16.

Chen Z, Zhang J and Stamler JS (2002). Identification of the enzymatic mechanism of nitroglycerin bioactivation. *Proc Natl Acad Sci U S A* 99(12): 8306-11.

Chirkov YY, De Sciscio M, Sverdlov AL, Leslie S, Sage PR and Horowitz JD (2010). Hydralazine does not ameliorate nitric oxide resistance in chronic heart failure. *Cardiovasc Drugs Ther* 24(2): 131-7.

Chirkov YY, Holmes AS, Chirkova LP and Horowitz JD (1999). Nitrate resistance in platelets from patients with stable angina pectoris. *Circulation* 100(2): 129-34.

Chirkov YY, Holmes AS, Willoughby SR, Stewart S, Wuttke RD, Sage PR and Horowitz JD (2001). Stable angina and acute coronary syndromes are associated with nitric oxide resistance in platelets. *J Am Coll Cardiol* 37(7): 1851-7.

Crane MS, Rossi AG and Megson IL (2005). A potential role for extracellular nitric oxide generation in cGMP-independent inhibition of human platelet aggregation: biochemical and pharmacological considerations. *Br J Pharmacol* 144(6): 849-59.

Crauwels HM, Van Hove CE, Holvoet P, Herman AG and Bult H (2003). Plaque-associated endothelial dysfunction in apolipoprotein E-deficient mice on a regular diet. Effect of human apolipoprotein AI. *Cardiovasc Res* 59(1): 189-99.

d'Uscio LV, Baker TA, Mantilla CB, Smith L, Weiler D, Sieck GC and Katusic ZS (2001). Mechanism of endothelial dysfunction in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol.* 21: 1017-1022.

d'Uscio LV, Smith LA and Katusic ZS (2001). Hypercholesterolemia impairs endothelium-dependent relaxations in common carotid arteries of apolipoprotein e-deficient mice. *Stroke* 32(11): 2658-64.

Daiber A, Wenzel P, Oelze M, Schuhmacher S, Jansen T and Munzel T (2009). Mitochondrial aldehyde dehydrogenase (ALDH-2)--maker of and marker for nitrate tolerance in response to nitroglycerin treatment. *Chem Biol Interact* 178(1-3): 40-7.

Dautov RF, Ngo DT, Licari G, Sverdlov AL, Ritchie RH, Kemp-Harper BK, Horowitz JD and Chirkov YY (2013). The nitric oxide redox sibling nitroxyl partially circumvents impairement of platelet nitric oxide responsiveness. *Nitric Oxide* 35: 72-78.

Drummond GR, Selemidis S, Griendling KK and Sobey CG (2011). Combating oxidative stress in vascular disease: NADPH oxidases as therapeutic targets. *Nat Rev Drug Discov* 10(6): 453-71.

DuMond JF and King SB (2011). The chemistry of nitroxyl-releasing compounds. *Antioxid Redox Signal* 14(9): 1637-48.

Ellis A, Li CG and Rand MJ (2000). Differential actions of L-cysteine on responses to nitric oxide, nitroxyl anions and EDRF in the rat aorta. *British Journal of Pharmacology* 129: 315-322.

Fathi AR, Bakhtian KD and Pluta RM (2011). The role of nitric oxide donors in treating cerebral vasospasm after subarachnoid hemorrhage. *Acta Neurochir Suppl* 110(Pt 1): 93-7.

Gudmundsdottir IJ, McRobbie SJ, Robinson SD, Newby DE and Megson IL (2005). Sildenafil potentiates nitric oxide mediated inhibition of human platelet aggregation. *Biochem Biophys Res Commun* 337(1): 382-5.

Hoffman MD, Walsh GM, Rogalski JC and Kast J (2009). Identification of nitroxyl-induced modifications in human platelet proteins using a novel mass spectrometric detection method. *Mol Cell Proteomics* 8(5): 887-903.

Horowitz JD (2000). Nitrovasodilators. Nitric oxide and the cardiovascular system. Loscalzo J and Vita JA. Totowa, New Jersey, Humana Press**:** 397-400.

Irvine JC, Favaloro JL, Widdop RE and Kemp-Harper BK (2007). Nitroxyl anion donor, Angeli's salt, does not develop tolerance in rat isolated aortae. *Hypertension* 49(4): 885-92.

Irvine JC, Kemp-Harper BK and Widdop RE (2010). Chronic administration of the HNO donor Angeli's salt does not lead to tolerance, cross-tolerance, or endothelial dysfunction: comparison with GTN and DEA/NO. *Antioxid Redox Signal* 14(9): 1615-24.

Irvine JC, Ravi RM, Kemp-Harper BK and Widdop RE (2013). Nitroxyl donors retain their depressor effects in hypertension. *Am J Physiol Heart Circ Physiol* 305(6): H939-45.

Judkins CP, Deip H, Broughton B, Mast AE, Hooker EU, Miller AA, Selemidis S, Dusting GJ, Sobey CG and Drummond GR (2010). Direct evidence of a role for Nox2 in superoxide production, reduced nitric oxide bioavailability and early atherosclerotic plaque formation in $ApoE^{-/-}$ mice. *Am J Physiol Heart Circ* 298(1): H24-32.

Keefer LK (2003). Progress toward clinical application of the nitric oxide-releasing diazeniumdiolates. *Annu Rev Pharmacol Toxicol* 43: 585-607.

Kolovou G, Anagnostopoulou K, Mikhailidis DP and Cokkinos DV (2008). Apolipoprotein E knockout models. *Curr Pharm Des* 14(4): 338-51.

Leo CH, Joshi A, Hart JL and Woodman OL (2012). Endothelium-dependent nitroxyl-mediated relaxation is resistant to superoxide anion scavenging and preserved in diabetic rat aorta. *Pharmacol Res* 66(5): 383-91.

Matsumoto T, D'Uscio L V, Eguchi D, Akiyama M, Smith LA and Katusic ZS (2003). Protective effect of chronic vitamin C treatment on endothelial function of apolipoprotein E-deficient mouse carotid artery. *J Pharmacol Exp Ther* 306(1): 103-8.

Melichar VO, Behr-Roussel D, Zabel U, Uttenthal LO, Rodrigo J, Rupin A, Verbeuren TJ, Kumar HSA and Schmidt HH (2004). Reduced cGMP signaling associated with neointimal proliferation and vascular dysfunction in late-stage atherosclerosis. *Proc Natl Acad Sci U S A* 101(47): 16671-6.

Meyrelles SS, Peotta VA, Pereira TM and Vasquez EC (2011). Endothelial dysfunction in the apolipoprotein E-deficient mouse: insights into the influence of diet, gender and aging. *Lipids Health Dis* 10: 211.

Miller AA, De Silva TM, Judkins CP, Diep H, Drummond GR and Sobey CG (2010). Augmented superoxide production by Nox2-containing NADPH oxidase causes cerebral artery dysfunction during hypercholesterolemia. *Stroke* 41(4): 784-9.

Miller AA, Maxwell, K.F., Bullen, M.L., Ku, J.M., De Silva, T.M., Selemidis, S., Hooker, E.U., Drummond, G.R., Sobey, C.G., Kemp-Harper, B.K., (2013). Nitroxyl (HNO) suppresses vascular Nox2 oxidase activity. *Free Radic Biol Med* 60: 264-71.

Miranda KM, Katori T, Torres de Holding CL, Thomas L, Ridnour LA, McLendon WJ, Cologna SM, Dutton AS, Champion HC, Mancardi D, Tocchetti CG, Saavedra JE, Keefer LK, Houk KN, Fukuto JM, Kass DA, Paolocci N and Wink DA (2005). Comparison of the NO and HNO donating properties of diazeniumdiolates: primary amine adducts release HNO in Vivo. *J Med Chem* 48(26): 8220-8.

Miranda KM, Yamada K, Espey MG, Thomas DD, DeGraff W, Mitchell JB, Krishna MC, Colton CA and Wink DA (2002). Further evidence for distinct reactive intermediates from nitroxyl and peroxynitrite: effects of buffer composition on the chemistry of Angeli's salt and synthetic peroxynitrite. *Arch Biochem Biophys* 401(2): 134-44.

Paolocci N, Jackson MI, Lopez BE, Miranda K, Tocchetti CG, Wink DA, Hobbs AJ and Fukuto JM (2007). The pharmacology of nitroxyl (HNO) and its therapeutic potential: not just the Janus face of NO. *Pharmacol Ther* 113(2): 442-58.

Paolocci N, Katori T, Champion HC, St. John ME, Miranda K, Fukuto JM, Wink DA and Kass DA (2003). Positive iontropic and lusitropic effects of HNO/NO- in failing hearts: Independence from β-adrenergic signalling. *Proc Natl Acad Sci U S A* 100: 5537-5542.

Park JG and Oh GT (2011). The role of peroxidases in the pathogenesis of atherosclerosis. *BMB Rep* 44(8): 497-505.

Ponnuswamy P, Schrottle A, Ostermeier E, Gruner S, Huang PL, Ertl G, Hoffmann U, Nieswandt B and Kuhlencordt PJ (2012). eNOS protects from atherosclerosis despite relevant superoxide production by the enzyme in apoE mice. *PLoS One* 7(1): e30193.

Rusche KM, Spiering MM and Marletta MA (1998). Reactions catalyzed by tetrahydrobiopterin-free nitric oxide synthase. *Biochemistry* 37: 15503-15512.

Selemidis S, Dusting GJ, Peshavariya H, Kemp-Harper B and Drummond GR (2007). Nitric Oxide supresses NADPH oxidase-dependent superoxide production by S-nitrosylation in human endothelial cells. *Cardiovasc Res* 75(2): 349-358.

Verbeuren TJ, Coene MC, Jordaens FH, Van Hove CE, Zonnekeyn LL and Herman AG (1986). Effect of hypercholesterolemia on vascular reactivity in the rabbit. II. Influence of treatment with dipyridamole on endothelium-dependent and endothelium-independent responses in isolated aortas of control and hypercholesterolemic rabbits. *Circ Res* 59(5): 496-504.

Wang J, Wang H, Hao P, Xue L, Wei S, Zhang Y and Chen Y (2011). Inhibition of aldehyde dehydrogenase 2 by oxidative stress is associated with cardiac dysfunction in diabetic rats. *Mol Med* 17(3-4): 172-9.

Wanstall JC, Jeffery TK, Gambino A, Lovren F and Triggle CR (2001). Vascular smooth muscle relaxation mediated by nitric oxide donors: a comparison with acetylcholine, nitric oxide and nitroxyl ion. *Br J Pharmacol* 134(3): 463-72.

Wong P, Hyun J, Fukuto JM, Shirota FN, DeMaster EG, Shoeman DW and Nagasawa HT (1998). Reaction between S-nitrosothiols and thiols: generation of nitroxyl (HNO) and subsequent chemistry. *Biochemistry* 37(16): 5362-5371.

Wynne BM, Labazi H, Tostes RC and Webb RC (2012). Aorta from angiotensin II hypertensive mice exhibit preserved nitroxyl anion mediated relaxation responses. *Pharmacol Res* 65(1): 41-47.

Yuill KH, Yarova P, Kemp XHBK, Garland CJ and Dora KA (2011). A Novel Role for HNO in Local and Spreading Vasodilatation in Rat Mesenteric Resistance Arteries. *Antioxid Redox Signal* 14(9): 1625-35.

Monash University

Declaration for Thesis Chapter 4

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Performed all experiments, analysed results and wrote the manuscript	80%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Alyson Miller	Provided intellectual advice during experimental and manuscript preparation stages and assisted with the editorial process.	
Grant Drummond		
Christopher Sobey		
Johannes-Peter Stasch		
Rebecca Ritchie		
Yuliy Chirkov		
John Horowitz		
Barbara Kemp-Harper		

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date**
Main Supervisor's Signature		**Date**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

Platelet Nitric Oxide Resistance in Hypercholesterolemia is Circumvented with the Nitroxyl (HNO) Donor Isopropylamine NONOate and the sGC Stimulator BAY 41-2272

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Abstract:

Nitric oxide ($NO^{\bullet}$) donors have a clinical role as acute anti-platelet therapies, yet in several cardiovascular disorders platelets can display "$NO^{\bullet}$ resistance" whereby the responsiveness to $NO^{\bullet}$ donors is diminished. This phenomenon is distinct from nitrate tolerance, as $NO^{\bullet}$ resistance occurs independently of prior nitrate therapy. Previous evidence indicates that resistance is attributed to scavenging of $NO^{\bullet}$ by superoxide or through an impairment of the soluble guanylyl cyclase (sGC)/cGMP signalling cascade. As such, there is a clinical need to develop anti-platelet agents that can circumvent this resistance. $NO^{\bullet}$–independent sGC stimulators display similar anti-aggregatory actions to $NO^{\bullet}$, but are not directly scavenged by superoxide, hence their effectiveness has the potential to be maintained in cardiovascular disorders where $NO^{\bullet}$ function is compromised. In this study, washed platelets were isolated from male wild-type (WT) and apolipoprotein E-deficient ($ApoE^{-/-}$) mice maintained on either a normal diet (ND) or high fat diet (HFD) for 7 weeks. The ability of the $NO^{\bullet}$ donor, sodium nitroprusside (SNP), the HNO donor, isopropylamine NONOate (IPA/NO) and the $NO^{\bullet}$-independent sGC stimulator, BAY 41-2272 to inhibit platelet aggregation and elevate intra-platelet cGMP accumulation was assessed. All three donors induced concentration-dependent inhibition of collagen (30 µg/ml)-stimulated platelet aggregation and increased intracellular cGMP levels in a time-dependent manner in platelets from WT-ND mice. However, the ability of SNP (10 µmol/l) to serve as an anti-aggregatory agent and elevate cGMP levels was compromised in platelets from $ApoE^{-/-}$-HFD mice. The anti-aggregatory effects of SNP were not improved in the presence of the antioxidant enzymes polyethylene glycol-catalase and polyethylene glycol-superoxide dismutase, yet were partially restored following the addition of the phosphodiesterase (PDE) 5 inhibitor, sildenafil (0.1 µmol/l). By contrast, the anti-aggregatory actions of IPA/NO (3 µmol/l; $P > 0.001$) and BAY 41-2272 (10 µmol/l; $P > 0.01$) were maintained, in $ApoE^{-/-}$-HFD mice. Similarly, the ability of IPA/NO and BAY 41-2272 to increase cGMP levels was sustained in $ApoE^{-/-}$-HFD mice. Thus, under conditions where anti-aggregatory responses to SNP were diminished, IPA/NO and BAY 41-2272 were able to circumvent $NO^{\bullet}$ resistance. As such, $NO^{\bullet}$-independent sGC stimulators may be useful anti-platelet agents in the setting of cardiovascular disease.

Introduction:

Platelets play an integral role in haemostasis, yet in several disease states abnormal platelet activation leads to vascular thrombosis and acute clinical events such as myocardial infarction and stroke. As such, anti-platelet agents are sought to prevent aggregation and thrombus formation. Current anti-platelet therapies involve chronic treatment with drugs such as aspirin and clopidogrel, which reduce platelet activation by inhibiting the aggregatory effects of thromboxane and ADP, respectively (Gouya et al. 2014). However, there is also interest in anti-aggregatory therapies such as nitric oxide ($NO^{\bullet}$) donors for the treatment of acute thrombotic events (Zhou and Frishman 2010).

In addition to its vasodilatory actions, $NO^{\bullet}$ is an effective anti-platelet agent as it can inhibit platelet-vessel wall interactions, aggregation and thrombosis formation (Gkaliagkousi et al. 2007; Rajendran and Chirkov 2008). These potent anti-aggregatory actions of $NO^{\bullet}$ are mediated predominantly via its ability to stimulate soluble guanylyl cyclase (sGC) and elevate cGMP (Gkaliagkousi et al. 2007). In turn, cGMP can activate cGMP-dependent protein kinases (cGKs) to prevent agonist-evoked mobilisation of calcium from intracellular stores and in part, prevent the entry of calcium into platelets (Rajendran and Chirkov 2008). Additionally, $NO^{\bullet}$ can indirectly increase cAMP levels through inhibition of phosphodiesterase (PDE) 3, allowing $NO^{\bullet}$ to target cAMP-dependent mechanisms to reduced aggregation (Gkaliagkousi et al. 2007). Consequently, downstream targets of cGK and cAMP-dependent protein kinases (cAKs) are primarily responsible for the anti-platelet actions of $NO^{\bullet}$ donors. Indeed, phosphorylation of the cGK and cAK substrate, vasodilator-stimulated phospho protein (VASP) has previously been shown to act as a negative regulator for integrin signalling and platelet adhesion (Rajendran and Chirkov 2008).

However, several studies have found that obese patients and patients with ischemic heart diseases such as congestive heart failure, aortic stenosis, stable angina pectoris, acute coronary syndrome and type 2 diabetes can display reduced responsiveness to the anti-aggregatory actions of $NO^{\bullet}$ donors (Anderson et al. 2004; Anfossi et al. 1998; Anfossi et al. 2004; Chirkov et al. 1996; Chirkov et al. 1999; Chirkov et al. 2004; Chirkov et al. 2002; Chirkov et al. 2001). This phenomenon termed "platelet $NO^{\bullet}$ resistance" can be distinguished from nitrate tolerance, as resistance is independent of any prior

nitrate therapy (Horowitz 2000). Furthermore $NO^{\bullet}$ resistance is independent of the nature of the $NO^{\bullet}$ donor as opposed to tolerance, which is exclusive to organic nitrates (Horowitz 2000).

Impaired anti-platelet responses to $NO^{\bullet}$ donors such as glyceryl trinitrate (GTN) and sodium nitroprusside (SNP) in patients with chronic heart failure, stable angina pectoris and acute coronary syndrome have previously been attributed to the increased oxidative stress associated with these diseases (Chirkov et al. 1999; Lopez Farre and Casado 2001). Under these conditions, NO• bioavailability is diminished due to the scavenging of $NO^{\bullet}$ by superoxide ($^{\cdot}O_2^-$) (Chirkov et al. 1999; Worthley et al. 2007). Furthermore, evidence from patients with stable angina pectoris indicates that platelet $NO^{\bullet}$ resistance may also reflect a desensitisation of platelet sGC to $NO^{\bullet}$ (Chirkov et al. 1999; Dautov et al. 2013). Interestingly, the effects downstream of cGMP appear to be maintained (Chirkov et al. 1996; Chirkov et al. 1999), suggesting that impaired $NO^{\bullet}$ signalling may be associated with either a decrease in $NO^{\bullet}$ bioavailability or dysfunction of sGC. As such, a viable treatment option to ameliorate $NO^{\bullet}$ resistance could be to restore sGC/cGMP signalling. Indeed, chronic treatment with angiotensin-converting enzyme (ACE) inhibitors, perhexiline or stains aim to improve platelet responsiveness to $NO^{\bullet}$ by limiting oxidative stress in patients with ischemic heart diseases, hypercholesterolemia and type 2 diabetes (Rajendran and Chirkov 2008; Willoughby et al. 2012). Thus statins have previously been shown to inhibit the production of NAPDH oxidase-derived $^{\cdot}O_2^-$ in platelets thereby reducing $NO^{\bullet}$ scavenging by $^{\cdot}O_2^-$ (Violi and Pignatelli 2014). Similarly, in patients with chronic heart failure, chronic treatment with ACE inhibitors enhanced anti-platelet responses to SNP, as a result of diminished plasma $^{\cdot}O_2^-$ levels and improved $NO^{\bullet}$ bioavailability (Chirkov et al. 2004). However, for acute thrombotic complications such as acute myocardial infarction, there is a need for alternative therapies which can rapidly circumvent $NO^{\bullet}$ resistance. One such strategy is the use of $NO^{\bullet}$-independent stimulators of sGC, which could potentially maintain their anti-platelet actions under conditions where $NO^{\bullet}$ function is compromised.

Nitroxyl (HNO), the redox sibling of $NO^{\bullet}$ displays similar vasoprotective properties to $NO^{\bullet}$ including an ability to inhibit platelet aggregation (Bermejo et al. 2005). Additionally, the anti-aggregatory actions of HNO are also predominantly mediated via activation of the sGC/cGMP signalling pathway (Bermejo et al. 2005; Dautov et al. 2013), although HNO also has the potential to interact directly with thiols (Fukuto and Carrington 2011; Fukuto et al. 2013) to modify platelet proteins (Hoffman et al. 2009).

Moreover, unlike $NO^{\bullet}$, HNO is not directly scavenged by $^{\cdot}O_2^-$ (Leo et al. 2012; Miller 2013; Miranda et al. 2002), nor is it susceptible to tolerance development or cross-tolerance with organic nitrates (Irvine et al. 2007; Irvine et al. 2010). Thus it is anticipated that HNO donors can circumvent $NO^{\bullet}$ resistance and maintain their anti-aggregatory actions in the face of oxidative stress and sGC dysfunction. Indeed, it was recently reported that HNO can partially circumvent platelet $NO^{\bullet}$ resistance in patients with ischemic heart disease (Dautov et al. 2013). Indeed, the anti-platelet effects of the HNO donor, isopropylamine NONOate (IPA/NO) were shown to be conserved in platelets where responses to SNP were compromised (Dautov et al. 2013). However, the mechanisms via which HNO donors circumvent platelet $NO^{\bullet}$ resistance remain to be fully elucidated and the efficacy of HNO donors as oppose to $NO^{\bullet}$-independent sGC stimulators has not yet been compared.

In addition to HNO donors, $NO^{\bullet}$-independent sGC stimulators such as BAY 41-2272 may represent novel strategies to overcome platelet $NO^{\bullet}$ resistance (Stasch et al. 2001). Like $NO^{\bullet}$, BAY 41-2272 stimulates sGC in a heme-dependent manner and is an effective anti-aggregatory agent, in both platelet-rich plasma and washed platelets (Hobbs and Moncada 2003; Roger et al. 2010; Stasch et al. 2001), and can also prolong tail-bleeding time in rats (Stasch et al. 2001). Additionally, BAY 41-2272 can act as an allosteric modulator, to increase the sensitivity of sGC to both $NO^{\bullet}$ and prostacyclin thereby augmenting the anti-platelet actions of these agents (Hobbs and Moncada 2003; Roger et al. 2010; Stasch et al. 2001). However, little is known with regard to the ability of BAY 41-2272 to inhibit platelet aggregation under disease conditions. Interestingly, a recent study reported that BAY 41-2272 served as an anti-aggregatory agent in obese rats, however, like SNP, its efficacy was reduced when compared to rats on a standard diet (Monteiro et al. 2012). Given BAY 41-2272 targets the reduced (Fe^{2+}) form of sGC (Stasch et al. 2001), such an observation may be indicative of oxidation of sGC into the $NO^{\bullet}$-insensitive (Fe^{3+}) state in obesity. These findings highlight the need to assess and compare the antiaggregatory actions of $NO^{\bullet}$-independent sGC stimulators, such as BAY 41-2272 and HNO donors under disease conditions in order to fully elucidate their potential to circumvent platelet $NO^{\bullet}$ resistance. In the present study we first sought to establish, and characterise, a model of platelet $NO^{\bullet}$ resistance in hypercholesterolemic mice. Subsequently we aimed to evaluate the antiaggregatory efficacy of the HNO donor, IPA/NO and $NO^{\bullet}$-independent sGC stimulator, BAY 41-2272 as a means to circumvent platelet $NO^{\bullet}$ resistance.

Methods:

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

Experimental Animals

Wild-type (WT) and apolipoprotein E deficient ($ApoE^{-/-}$) mice were obtained from the Animal Resources Centre (Canning Vale, WA, Australia). All mice studied were male and fully backcrossed to the C57BL/6J background. From 5 weeks of age, mice were either maintained on a normal diet of standard chow (ND) or high-fat diet (HFD; 21% fat, 0.15% cholesterol; Speciality Feeds, WA, Australia) for 7 weeks. For all experiments, mice were deeply anaesthetized by isoflurane inhalation (Baxter Healthcare), prior to being euthanized by decapitation.

Platelet Aggregation

Platelets were isolated from whole blood collected from the inferior vena cava of WT-ND, WT-HFD and $ApoE^{-/-}$-HFD mice and stimulated for aggregation studies as previously described (Dharmarajah et al. 2010). To prevent coagulation, blood was treated with a combination of a low molecular weight heparin (clexane, 400 U/ml) and acid citrate dextrose buffer [composed of (in mmol/l) 85 trisodium citrate, 72.9 citric acid, 110 D-glucose] immediately following collection. Blood was then washed with platelet wash buffer [composed of (in mmol/l) 4.3 K_2HPO_4, 4.3 Na_2HPO_4, 24.3 NaH_2PO_4, 113 NaCl, 5.5 D-glucose, 10% BSA and 20 U/ml Clexane, pH 6.5] and centrifuged at 200×*g*, (37°C, 2 min). This was performed three times to achieve maximum yield, and each time the platelet rich plasma (PRP) was removed. Following centrifugation of the pooled PRP (2000×*g*, 1 min), the pellet was resuspended in Tyrode's buffer [composed of (in mmol/l) 12 $NaHCO_3$, 10 HEPES, 137 NaCl, 2.7 KCl, 5.5 D-glucose and 20 mg/ml EDTA, pH 7.2-7.4] at 1×10^7 platelets/ml. Platelets (5×10^8 platelets/ml) were added to siliconised cuvettes containing fibrinogen (2 mg/ml), which is required for platelet-platelet interactions and were allowed to equilibrate at 37°C for 30 min prior to stimulation with collagen (30 µg/ml). Platelet aggregation was measured using a four-chamber turbidometric platelet aggregometer (AggRAM™, Helena Laboratories, USA) as a change in light transmission over 30 min under continuous stirring (600 rpm, 37°C).

Washed platelets from WT-ND mice were treated with either vehicle (dH_2O, 1 mmol/l NaOH or 5% DMSO), SNP (0.1-1 mmol/l), IPA/NO (0.1-3 µmol/l) or BAY 41-2272 (0.1-10 µmol/l) 2 min prior to stimulation with collagen (30 µg/ml) to induce aggregation. To characterise the anti-aggregatory effects of SNP (10 µmol/l), IPA/NO (3 µmol/l) and BAY 41-2272 (10 µmol/l), platelets were treated with either the $NO^{\bullet}$ scavenger, hydroxocobalamin (HXC; 30 µmol/l, 10 min), the sGC inhibitor, 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxaline-1-one (ODQ; 10 µmol/l, 30 min), the cGMP-dependent protein kinase inhibitor, Rp-8-pCPT-cGMPs (cGK inhibitor; 200 µmol/l, 30 min) or the cAMP-dependent protein kinase inhibitor, Rp-8-pCPT-cAMPs (cAK inhibitor; 100 µmol/l, 30 min) prior to addition of the anti-aggregatory agents.

To examine the anti-aggregatory actions of SNP, IPA/NO and BAY 41-2272 in hypercholesterolemia, washed platelets from WT-HFD and $ApoE^{-/-}$-HFD mice were incubated in the presence of vehicle (dH_2O, 1 mmol/l NaOH or 5% DMSO), SNP (10 µmol/l or 1 mmol/l), IPA/NO (3 µmol/l) or BAY 41-2272 (10 µmol/l) for 2 min before stimulation with collagen (30 µg/ml). The effect of the phosphodiesterase 5 (PDE5) inhibitor, sildenafil (0.1 µmol/l, 30 min) or the combination of polyethylene glycol-catalase (PEG-CAT; 1000 U/ml, 30 min) and polyethylene glycol-superoxide dismutase (PEG-SOD; 300 U/ml, 30 min) upon the anti-aggregatory actions of SNP (10 µmol/l) was also assessed in platelets from $ApoE^{-/-}$-HFD mice.

Extraction and measurement of cGMP

Platelets were isolated from whole blood collected from the inferior vena cava of WT-ND, WT-HFD and $ApoE^{-/-}$-HFD mice as previously described in *Platelet Aggregation* and re-suspended in Tyrode's buffer at 1 x 10^7 platelets/ml. Intra-platelet cGMP was extracted and measured with an enzyme immunoassay (EIA) kit, as previously described (Monteiro et al. 2012; Morganti et al. 2010).

Platelets (5×10^8 platelets/ml) were allowed to equilibrate at 37°C for 30 min in the presence of the PDE inhibitor, 3-isobutyl-1-methyl-xanthine (IBMX; 2 mmol/l). After the equilibration period, samples were incubated with either vehicle (dH_2O, 1 mmol/l NaOH or 5% DMSO), SNP (10 µmol/l or 1 mmol/l), IPA/NO (3 µmol/l) or BAY 41-2272 (10 µmol/l) for 2, 5, 10 or 30 min. The reaction was stopped by the addition of cold-acidified absolute ethanol (67%, vol/vol) and samples were vortexed for 30 sec. Samples were then centrifuged (4000 *g*, 30 min at 4˚C), the supernatant dried at 55-60˚C under a gentle stream of nitrogen before being snap frozen in liquid nitrogen and stored at -80˚C. cGMP levels

were assessed by using an EIA kit (standard non-acetylation protocol) from Cayman Chemicals and measured with a spectrophotometer at a wavelength of 405 nm (Versa Max Micro Plate Reader; Molecular Devices, USA). Experiments were performed in triplicate. The limit of cGMP detection for this kit is 1 pmol/ml.

Data and Statistical Analysis

Platelet aggregation responses were expressed as either: 1) total aggregation = area under the curve (AUC; arbitrary units) of percent increments of light transmission over the 30 min period following collagen addition; or 2) time to half maximal aggregation (seconds). Intra-platelet cGMP concentrations (pmol per 5×10^8 platelets/ml) were obtained using set standards and a linear regression analysis (GraphPad Prism Version 5.02; GraphPad Software Inc, USA). The relationship between platelet responsiveness to SNP and IPA/NO or SNP and BAY 41-2272 was examined using linear regression. Statistical comparisons were performed using either a one-way or two-way ANOVA with Bonferroni post-hoc test, or an unpaired t-test, with Mann-Whitney U post hoc test (GraphPad Prism). All results are expressed as either dot plots or mean ± standard error of mean (SEM), with the number of platelet samples from separate mice denoted by n*, P* < 0.05 was considered statistically significant.

Drugs and their Sources

Drugs and there sources were: BAY 41-2272 (kind gift from J-P. Stasch, Bayer HealthCare, Germany), clexane (enoxaparin sodium, Aventos, New Zealand); collagen (Bovine, Helena Laboratories, Australia); IPA/NO (kind gift from K. Miranda, University of Arizona, Arizona, USA); ODQ (Sapphire Bioscience, Australia); all other drugs were purchased from (Sigma, Australia).

BAY 41-2272 was prepared at 10 mmol/l in 100% DMSO, diluted to 1 mmol/l in 50% DMSO and all subsequent dilutions in distilled water. Clexane was prepared at 400 U/ml in saline (100%). Fibrinogen was prepared at 20 mg/ml in 0.9% saline and all subsequent dilutions in distilled water. IBMX was prepared at 100 mmol/l in 100% DMSO and all subsequent dilutions in distilled water. IPA/NO was prepared at 10 mmol/l in 0.01 mol/l NaOH and all subsequent dilutions were in 0.01 mol/l NaOH. ODQ was prepared at 10 mmol/l in 100% ethanol and all subsequent dilutions in distilled water. All other drugs were dissolved and diluted in distilled water.

Results:

Anti-aggregatory actions of SNP, IPA/NO and BAY 41-2272

The addition of collagen (30 µg/ml) induced aggregation (total aggregation = 61591 ± 2552 area under the curve, arbitrary units; time to half-maximum aggregation 415 ± 28 seconds, n = 25) in platelets from WT-ND mice. SNP, IPA/NO and BAY 41-2272 all induced concentration-dependent inhibition of collagen-stimulated aggregation in washed platelets from WT-ND mice (Figure 1A, D and G). Total aggregation was reduced by 47 ± 7% with SNP (10 µmol/l, $P < 0.001$, n = 5-7), 37 ± 11% with IPA/NO (3 µmol/l, $P < 0.001$, n = 7-9) and 55 ± 6% with BAY 41-2272 (10 µmol/l, $P < 0.001$, n = 5-9). This was accompanied by an increase in time to half maximum aggregation ($P < 0.05$; Figure 1B, E and H).

Effect of SNP, IPA/NO and BAY 41-2272 on platelet cGMP accumulation

In platelets from WT-ND mice, SNP (10 µmol/l) and BAY 41-2272 (10 µmol/l) caused a time-dependent increase in cGMP accumulation with significant increases observed 30 minutes after addition of either SNP (5.4 ± 0.1 to 13.9 ± 1.7 pmol/5x10^8 platelets/ml, $P < 0.001$, n = 3; Figure 1C) or BAY 41-2272 (5.3 ± 0.6 to 22.5 ± 5.9 pmol/5x10^8 platelets/ml, $P < 0.01$, n = 3; Figure 1I). In contrast, IPA/NO (3 µmol/l) induced a more rapid response such that a significant elevation in cGMP levels was observed at 2 minutes (5.4 ± 1.3 to 19.4 ± 3.7 pmol/5x10^8 platelets/ml; $P < 0.05$, n =3) and sustained over the ensuing 30 minute period (25.2 ± 3.8 pmol/5x10^8 platelets/ml, $P < 0.01$, n = 3; Figure 1F).

Mechanisms underlying the anti-aggregatory actions of SNP, IPA/NO and BAY 41-2272

The anti-aggregatory actions of SNP (10 µmol/l) were abolished by the $NO^{\bullet}$ scavenger, hydroxocobalamin (HXC, $P < 0.01$, n = 5; Figure 2A, B), yet responses to IPA/NO (3 µmol/l, n = 7) and BAY 41-2272 (10 µmol/l, n = 7) were sustained (Figure 2C-F). The sGC inhibitor, ODQ and cGK inhibitor, Rp-8-pCPT-cGMPs reversed the ability of SNP ($P < 0.01$, n =5-10; Figure 3A, B) and IPA/NO ($P < 0.01$, n = 5-12; Figure 3C, D) to inhibit platelet aggregation. In contrast, the anti-aggregatory actions of BAY 41-2272 were unchanged in the presence of ODQ, yet abolished in the present of the cGK inhibitor (n = 5-9; Figure 3E, F). The cAK inhibitor, Rp-8-pCPT-cAMPs had no significant effect on the anti-aggregatory actions of SNP, IPA/NO or BAY 41-2272 (Figure 3).

Additionally, the presence of HXC, ODQ, cGK and cAK inhibitors alone had minimal effect on platelet aggregation (Table I).

Platelet function in WT-HFD and ApoE$^{-/-}$-HFD mice

Total aggregation to collagen (30 µg/ml) was significantly reduced by ~11% in platelets from ApoE$^{-/-}$-HFD when compared to WT-HFD mice ($P< 0.05$, n = 24-32; Table II). Yet, the time to half maximal aggregation was similar between WT-HFD and ApoE$^{-/-}$-HFD mice (n = 24-32; Table II).

Effect of SNP on platelet aggregation and cGMP accumulation in ApoE$^{-/-}$-HFD mice

SNP (10 µmol/l) inhibited collagen-induced aggregation in WT-HFD platelets (54 ± 10% inhibition, $P < 0.001$, n = 10), yet this effect was markedly reduced in platelets from ApoE$^{-/-}$-HFD mice (14 ± 4% inhibition, $P < 0.05$, n =13; Figure 4A). The ability of SNP to increase cGMP accumulation, over a 30 minute period, was also impaired in platelets from ApoE$^{-/-}$-HFD mice ($P < 0.01$, n = 3; Figure 4B). Increasing the concentration of SNP to 1 mmol/l did not overcome resistance to its anti-aggregatory actions in platelets from ApoE$^{-/-}$-HFD mice. Specifically, 1 mmol/l SNP inhibited aggregation by 20 ± 10% versus 40 ± 4% (n = 7; Table III) in platelets from ApoE$^{-/-}$-HFD and WT-HFD mice, respectively. Additionally, the ability of 1mmol/l SNP to increase platelet cGMP levels in ApoE$^{-/-}$-HFD mice was also significantly impaired at 2 ($P < 0.01$, n =4) and 30 minutes ($P < 0.001$, n = 4; Table III) after treatment.

Mechanism underlying platelet NO resistance in ApoE$^{-/-}$-HFD mice

The antioxidant enzymes PEG-CAT (1000 U/ml) and PEG-SOD (300 U/ml), were unable to restore the anti-aggregatory effects of SNP (10 µmol/l) in platelets from ApoE$^{-/-}$-HFD mice (n = 5; Table III). In contrast, the PDE5 inhibitor, sildenafil (0.1 µmol/l) augmented the anti-aggregatory actions of SNP (10 µmol/l) in WT-HFD and ApoE$^{-/-}$-HFD mice such that platelet aggregation was inhibited by 78 ± 7% and 59 ± 10%, respectively ($P < 0.001$, n = 3; Table III).

Circumventing platelet NO resistance with IPA/NO and BAY 41-2272

In contrast to SNP, the anti-aggregatory actions of both IPA/NO (3 µmol/l; 39 ± 8% inhibition) and BAY 41-2272 (10 µmol/l; 43 ± 8% inhibition) were sustained in platelets from ApoE$^{-/-}$-HFD mice (IPA/NO: 29 ± 4% inhibition; BAY 41-2272: 31 ± 5% inhibition, n = 5-9; Figure 4C and E). Additionally, the anti-aggregatory actions of IPA/NO (3 µmol/l) were partially reversed by ODQ (Figure 4C), whilst responses to BAY 41-2272 were unchanged in platelets from ApoE$^{-/-}$-HFD mice (Figure 4E). The ability of IPA/NO (3 µmol/l) to elevate platelet cGMP levels was impaired after 2 minutes in ApoE$^{-/-}$-HFD mice ($P < 0.001$, n = 3; Figure 4D). Yet 30 minutes following the addition of IPA/NO, platelet cGMP levels were similar to that of WT-HFD mice (n = 3; Figure 4D). BAY 41-2272 also maintained its ability to elevated platelet cGMP levels in ApoE$^{-/-}$-HFD mice (n = 3; Figure 4F).

Relationship between the anti-aggregatory responses to SNP and IPA/NO

The relationship between the anti-aggregatory actions of SNP versus IPA/NO in WT-HFD and ApoE$^{-/-}$-HFD mice was further explored via fitting the data to a hyperbolic function and comparing the IPA/NO:SNP response ratios in each group. Such analysis indicated that platelets from ApoE$^{-/-}$-HFD mice were consistently more responsive to IPA/NO than SNP (n = 9-18; Figure 5A) with a significant increase in the IPA/NO:SNP response ratio ($P < 0.01$; Figure 5B).

Figure 1. Concentration-dependent effect of SNP (0.1-10 µmol/l; A and B), IPA/NO (0.1-3 µmol/l; D and E) and BAY 41-2272 (0.1-10 µmol/l; G and H) on total aggregation (expressed as % vehicle; left panel) and time to half maximal aggregation (in sec; middle panel) in response to collagen (30 µg/ml) in isolated washed platelets from WT-ND mice Values are given as mean ± SE; n = 5-9 platelet samples from separate mice. Also shown is time course of cGMP accumulation (Right panel) in isolated washed platelets from WT-ND mice after a single addition of either SNP (10 µmol/l; C), IPA/NO (3 µmol/l; F) or BAY 41-2272 (10 µmol/l; I). cGMP concentrations are given as pmol per 5 x 10^8 platelets/ml and expressed as mean ± SE; n = 3 platelet samples from separate mice. ***P* < 0.05, ****P* < 0.01, *****P* < 0.001 for treatment vs. vehicle (1-way or 2-way ANOVA, Bonferroni post hoc test). The vehicle in A, B and C was dH_2O; the vehicle in D, E and F was 1 mmol/l NaOH; the vehicle in G, H and I was 5% DMSO.

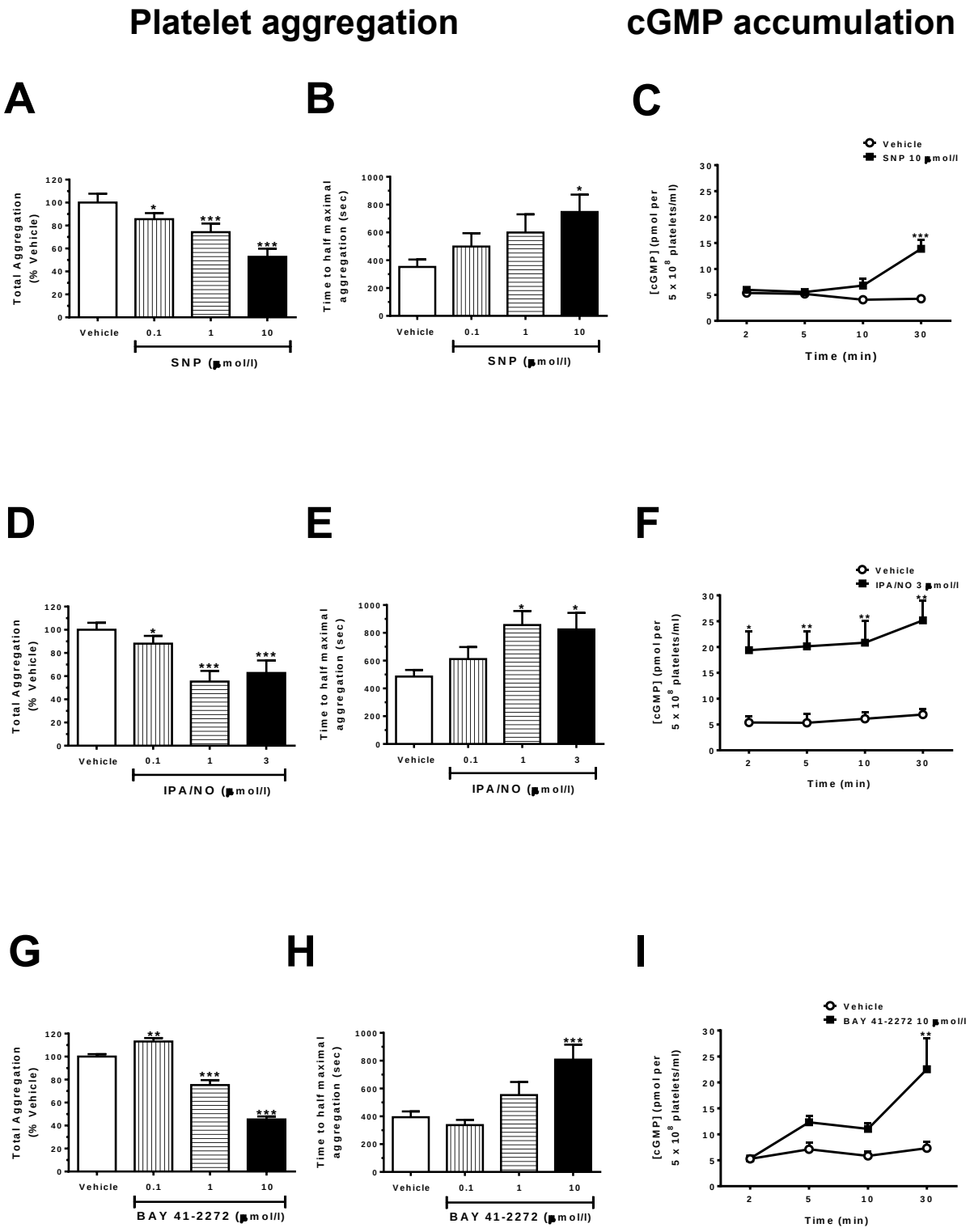

Platelet aggregation
cGMP accumulation
A
B
C
Total Aggregation (% Vehicle)
Vehicle
0.1
1
10
SNP (µmol/l)
Time to half maximal aggregation (sec)
[cGMP] (pmol per 5 x 10^8 platelets/ml)
Vehicle
SNP 10 µmol/l
Time (min)
D
E
F
IPA/NO (µmol/l)
3
IPA/NO 3 µmol/l
G
H
I
BAY 41-2272 (µmol/l)
BAY 41-2272 10 µmol/l

Figure 2. Effect of SNP (10 µmol/l; A and B), IPA/NO (3 µmol/l; C and D) and BAY 41-2272 (10 µmol/l; E and F) upon collagen (30 µg/ml)-stimulated aggregation of isolated washed platelets from WT-ND mice in the absence or presence of hydroxocobalamin (30 µmol/l; HXC). Aggregation responses are expressed as total aggregation (% vehicle; left panel) or time to half maximal aggregation (in sec; right panel) and given as mean ± SE; n = 5-7 platelet samples from separate mice. ********P* < 0.01, *********P* < 0.001 for treatment vs. vehicle, ##*P* < 0.01 vs. SNP alone (one-way ANOVA, Bonferroni post hoc test). The vehicle in A and B was dH_2O; the vehicle in C and D was 1 mmol/l NaOH; the vehicle in E and F was 5% DMSO.

Platelet aggregation

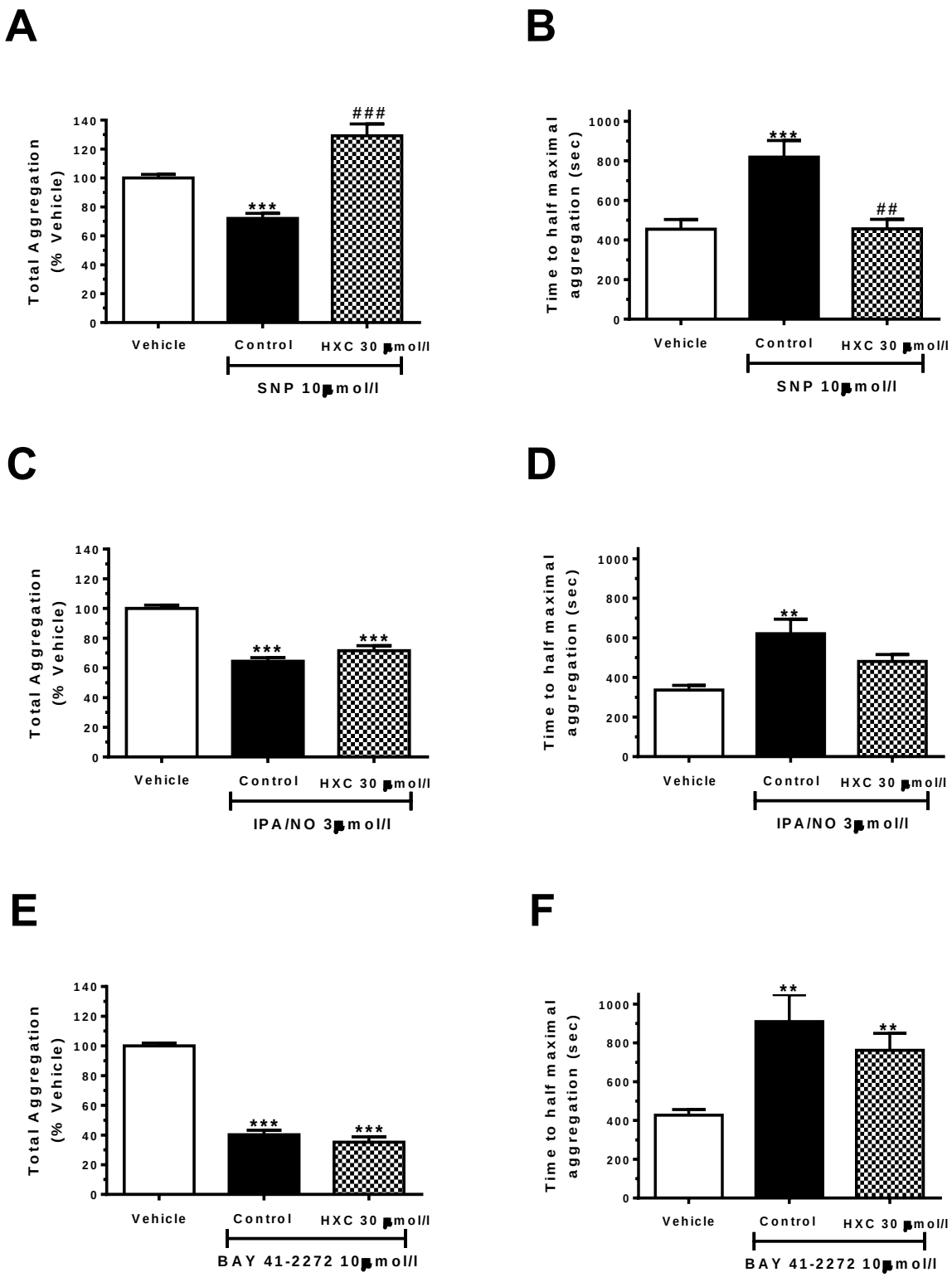


Figure 3. Effect of SNP (10 µmol/l; A and B), IPA/NO (3 µmol/l; C and D) and BAY 41-2272 (10 µmol/l; BAY 41; E and F) upon collagen (30 µg/ml)-stimulated aggregation of isolated washed platelets from WT-ND mice in the absence or presence of ODQ (10 µmol/l), Rp-8-pCPT-cGMPs (200 µmol/l; cGK inhibitor) or Rp-8-pCPT-cAMPs (100 µmol/l; cAK inhibitor). Aggregation responses are expressed as total aggregation (% vehicle; left panel) or time to half maximal aggregation (in sec; right panel) and given as mean ± SE; n = 5-12 platelet samples from separate mice. ***P* < 0.01, ****P* < 0.001 for treatment vs. vehicle, #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 vs. SNP, IPA/NO or BAY41-2272 alone (one-way ANOVA, Bonferroni post hoc test). The vehicle in A and B was dH_2O; the vehicle in C and D was 1 mmol/l NaOH; the vehicle in E and F was 5% DMSO.

Platelet aggregation

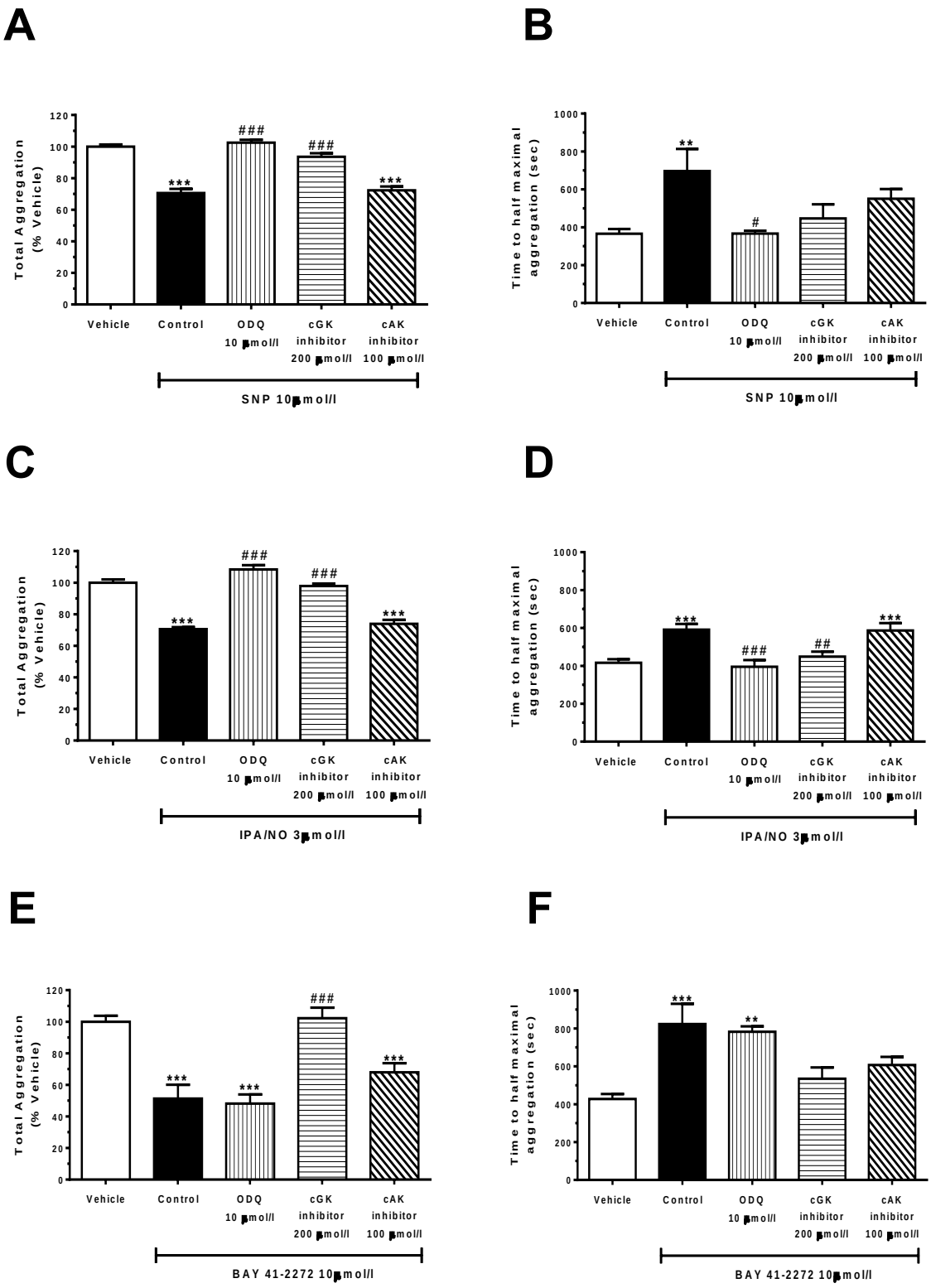


Table I. Effect of HXC, ODQ, cGK inhibitor and cAK inhibitor on collagen (30µg/ml)-stimulated platelet aggregation in washed platelets from 12 week old WT-HFD and ApoE$^{-/-}$-HFD mice

		Total aggregation (AUC, arbitrary units)	Time to half maximum aggregation (seconds)
WT-ND	**n**	**Mean ± SE**	**Mean ± SE**
Vehicle (dH_2O)	11	68564 ± 3196	386 ± 21
HXC 30µmol/l		71853 ± 4645	384 ± 31
Vehicle (EtOH)	8	59112 ± 4076	422 ± 21
ODQ 10µmol/l		50529 ± 4750	483 ± 52
Vehicle (dH_2O)	5	52595 ± 3568	400 ± 21
Rp-8-pCPT-cGMPs 200µmol/l		46403 ± 2679	439 ± 13
Vehicle (dH_2O)	4	81867 ± 3774	403 ± 37
Rp-8-pCPT-cAMPs 100µmol/l		75221 ± 3837	427 ± 28

Aggregation responses are expressed as total aggregation (area under the curve, AUC, in arbitrary units) or time to half maximum aggregation (in seconds).

Values are given as mean ± SE, where n=platelet samples from separate animals.

Wild-type (WT) and apolipoprotein E-deficient (ApoE$^{-/-}$) mice were maintained on a high-fat diet (HFD) from 5 weeks of age.

HXC = hydroxocobalamin, ODQ = 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, cGK = cGMP-dependent protein kinase, cAK = cAMP-dependent protein kinases where Rp-8-pCPT-cGMPs is a cGK inhibitor and Rp-8-pCPT-cAMPs is a cAK inhibitor

Table II. Platelet aggregation in response to collagen (30µg/ml) in washed platelets from 12 week old WT-HFD and ApoE$^{-/-}$-HFD mice

		Total aggregation (AUC, arbitrary units)	Time to half maximum aggregation (seconds)
	n	Mean ± SE	Mean ± SE
WT-HFD	24	57048 ± 2586	354 ± 20
ApoE$^{-/-}$-HFD	32	50565 ± 1128*	311 ± 19

Aggregation responses are expressed as total aggregation (area under the curve, AUC, in arbitrary units) or time to half maximum aggregation (in seconds).

Values are given as mean ± SE, where n=platelet samples from separate animals.

Wild-type (WT) and apolipoprotein E-deficient (ApoE$^{-/-}$) mice were maintained on a high-fat diet (HFD) from 5 weeks of age.

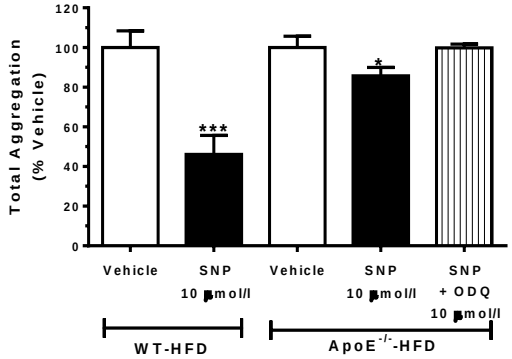
***P** < 0.05 vs WT-HFD (Student's unpaired t-test).

Figure 4. Left panel: Effect of SNP (10 µmol/l; A), IPA/NO (3 µmol/l; C) and BAY 41-2272 (10 µmol/l; BAY 41; E) upon collagen (30 µg/ml)-stimulated aggregation of isolated washed platelets from 12-wk-old WT and ApoE$^{-/-}$ mice maintained on a HFD from 5-wk of age in the absence or presence of ODQ (10 µmol/l). Aggregation responses are expressed as total aggregation (% vehicle) and given as mean ± SE; n = 5-10 platelet samples from separate mice. Right panel: Time course of cGMP accumulation in washed platelets from WT-HFD (open symbols) and ApoE$^{-/-}$-HFD mice (closed symbols) after a single addition of SNP (10 µmol/l; B), IPA/NO (3 µmol/l; D) or BAY 41-2272 (10 µmol/l; BAY 41; F). cGMP concentrations are given as pmol per 5 x 10^8 platelets/ml and given as mean ± SE; n = 3 platelet samples from separate mice. ***P* < 0.05, ****P* < 0.01, *****P* < 0.001 for treatment vs. vehicle, #*P* < 0.05 for ApoE$^{-/-}$-HFD vs. WT-HFD vs., ϕ*P* < 0.05 for IPA/NO + ODQ vs. IPA/NO (two-way ANOVA, Bonferroni post hoc test). The vehicle in A and B was dH_2O; the vehicle in C and D was 1 mmol/l NaOH; the vehicle in E and F was 5% DMSO.

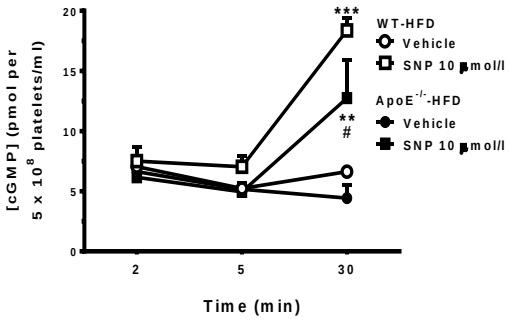
Platelet aggregation

cGMP accumulation

A



B



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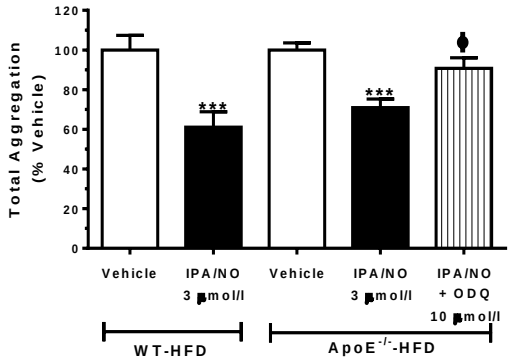

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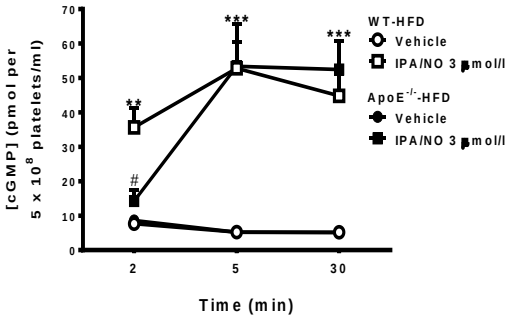

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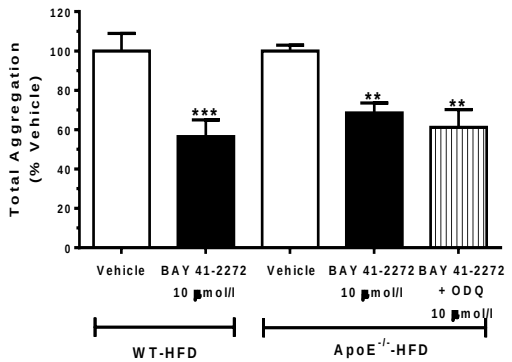

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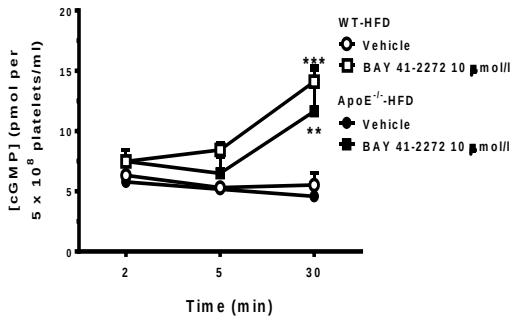


Table III. Effect of SNP on collagen (30µg/ml)-stimulated platelet aggregation and cGMP accumulation in washed platelets from 12 week old WT-HFD and ApoE$^{-/-}$-HFD mice

	Total aggregation (% Vehicle)		cGMP accumulation (pmol per 5 x 10^8 platelets/ml)	
	n	Mean ± SE	n	Mean ± SE
WT-HFD				
Vehicle	14	100 ± 5.1	7	6.6 ± 0.3
SNP 10µmol/l	7	59.8 ± 2.5***	3	18.4 ± 1.1***
SNP 10µmol/l + Sildenafil 0.1µmol/l	7	22.4 ± 7.3***###		
SNP 1mmol/l	7	60 ± 4.2***	4	53.2 ± 7.0***
ApoE$^{-/-}$-HFD				
Vehicle	15	100 ± 6.2	7	4.8 ± 0.7
SNP 10µmol/l	8	84.7 ± 5.2*†	3	12.7 ± 3.2**†
SNP 10µmol/l + Sildenafil 0.1µmol/l	3	41.3 ± 9.8***###		ND
SNP 10µmol/l + PEG-CAT (1000 U/ml) + PEG-SOD (300 U/ml)	5	101.4 ± 7.3##		ND
SNP 1mmol/l	7	79.7 ± 10.0**†	4	21.7 ± 1.9**†††

Total aggregation was calculated as area under the curve (AUC, arbitrary units) and expressed as a percentage of the response to collagen in the presence of the vehicle (dH_2O).

cGMP accumulation was measured at 30min after the addition of SNP.

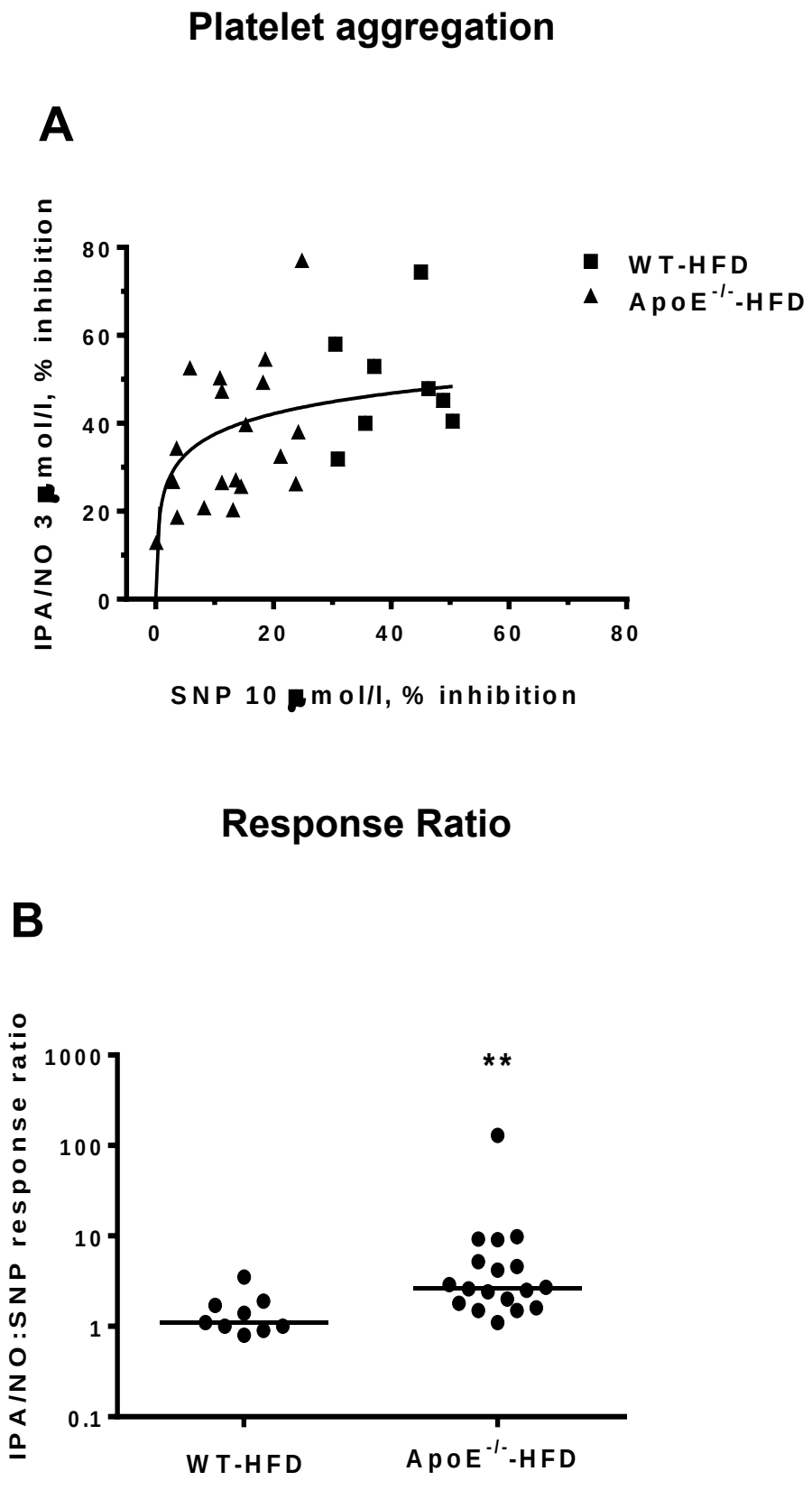
Values are given as mean ± SE, where n=platelet samples from separate animals.

Wild-type (WT) and apolipoprotein E-deficient (ApoE$^{-/-}$) mice were maintained on a high-fat diet (HFD) from 5 weeks of age.

SNP = sodium nitroprusside; PEG-CAT = polyethylene glycol-catalase; PEG-SOD = polyethylene glycol-superoxide dismutase; ND = not determined

*P < 0.05, **P < 0.01, ***P < 0.001 vs Vehicle (2-way ANOVA), ##P < 0.01, ###P < 0.001 vs SNP (2-way ANOVA), †P < 0.05, †††P < 0.001 vs WT-HFD (2-way ANOVA).

Figure 5. The relationship between platelet responsiveness to SNP (10 µmol/l) and IPA/NO (3 µmol/l) in isolated washed platelets from 12-wk-old WT (■) and ApoE$^{-/-}$ (▲) mice maintained on a HFD from 5-wk of age. Aggregation responses are expressed as either percentage inhibition of total aggregation to collagen (30 µg/ml; % inhibition; A) or as a ratio of the response (% inhibition) between IPA/NO:SNP (B). Results are given as a scatter dot plot with the median response, where n = 9-18 platelet samples from separate mice. ***P* < 0.01 for ApoE$^{-/-}$-HFD vs. WT-HFD (unpaired t-test, Mann-Whitney U post hoc).


Platelet aggregation
A
IPA/NO 3 µmol/l, % inhibition
80
60
40
20
0
0
20
40
60
80
SNP 10 µmol/l, % inhibition
WT-HFD
ApoE-/--HFD
Response Ratio
B
IPA/NO:SNP response ratio
1000
100
10
1
0.1
**
WT-HFD
ApoE-/--HFD

Discussion:

This study has demonstrated that ApoE$^{-/-}$ mice, maintained on a HFD, display platelet NO$^{\bullet}$ resistance. Specifically, we found that the anti-aggregatory actions of SNP in ApoE$^{-/-}$-HFD mice were reduced, together with a decrease in intracellular cGMP generation. Whilst NO$^{\bullet}$ resistance was not overcome by the antioxidants PEG-SOD and PEG-CAT, inhibition of PDE5 partially restored the anti-aggregatory actions of SNP. Moreover, the anti-aggregatory actions of the NO$^{\bullet}$-independent sGC stimulators IPA/NO and BAY 41-2272 were, for the most part, sustained in ApoE$^{-/-}$-HFD mice, indicative of an ability of these agents to circumvent platelet NO$^{\bullet}$ resistance. As such, NO$^{\bullet}$-independent sGC stimulators represent an alternative approach in the management of acute atherothrombotic events.

Before comparing the anti-aggregatory actions of SNP and NO$^{\bullet}$-independent sGC stimulators in platelets from ApoE$^{-/-}$ mice, we first characterised the signalling pathway via which SNP, IPA/NO and BAY 41-2272 exerted their anti-aggregatory actions under non-disease conditions. All three agents were able to inhibit platelet aggregation and increase platelet cGMP levels in platelets from WT mice. By using the well-characterised NO$^{\bullet}$ scavenger, hydroxocobalamin (Leo et al. 2012; Miller 2013; Wanstall et al. 2001), we confirmed that the effects of SNP, but not IPA/NO or BAY 41-2272 were mediated via NO$^{\bullet}$. However, we were unable to confirm that the response to IPA/NO was HNO-dependent, as the HNO scavengers, L-cysteine and N-acetyl-L-cysteine alone exert direct anti-aggregatory effects (Anfossi et al. 2001; Lale et al. 2003). Nevertheless, given the anti-aggregatory actions of IPA/NO were unaffected by hydroxocobalamin, together with previous a report that IPA/NO mediates its vascular effects (e.g. vasodilatation) via HNO (Irvine et al. 2013), suggests that in this study the effects of IPA/NO were also mediated by HNO, rather than NO$^{\bullet}$. BAY 41-2272 has also been reported to inhibit platelet aggregation in an NO$^{\bullet}$-independent manner (Hobbs and Moncada 2003; Roger et al. 2010).

This study also provided evidence for a predominant role of the sGC/cGMP/cGK signalling pathway in the anti-aggregatory actions of SNP and IPA/NO. Thus both SNP and IPA/NO elevated intracellular cGMP levels and inhibition of sGC (via ODQ) and cGK (via Rp-8-pCPT-cGMPs) abolished their anti-aggregatory responses. This is consistent with previous findings in human platelets, in which inhibition

of aggregation by SNP, GTN, IPA/NO and Angeli's salt is sensitive to ODQ and associated with an increase in intracellular cGMP (Bermejo et al. 2005; Dautov et al. 2013). Whilst the effects of cGMP are transduced by cGMP-PDE's and cGKs, it is important to note, that in human platelets both $NO^{\bullet}$ (ie. SNP and 3-morpholinosydnonimine) and HNO donors (i.e Angeli's salt) have also been shown to target cAMP-dependent pathways to inhibit platelet aggregation (Bermejo et al. 2005; Maurice and Haslam 1990). Indicating there is potential for cross-talk between the cGMP and cAMP signalling pathways, as cGMP can bind to PDE3 to inhibit the hydrolysis of cAMP, leading to cAK-dependent inhibition of platelet aggregation (Jang et al. 2002; Maurice and Haslam 1990). However, we found no evidence of cross-talk between cGMP/cAMP signalling pathways in mouse platelets as responses to SNP, IPA/NO and BAY 41-2272 were unchanged in the presence of the cAK inhibitor, Rp-8-pCPT-cAMPs. These differences in platelet signalling between humans and mice is a limitation of the current study and the use of murine platelets to characterise the anti-aggregatory effects of $NO^{\bullet}$ and HNO. Nevertheless, in human platelets cGK and cAK have previously been reported to activate similar downstream targets, such as phosphorylated VASP (Li et al. 2003), and as such $NO^{\bullet}$ and HNO may converge on the same target irrespective of cGK or cAK activation. An advantage of exploring platelet responses in mice is that patients with cardiovascular diseases are often on a variety of drug therapies that may affect platelet responses, making it difficult to assess the advantages of individual therapies. There is also the added benefit of being able to genetically modify mouse platelets, to gain a better understanding of signalling mechanisms. As such, our mouse model of hypercholesterolemia represents a useful tool in exploring the mechanisms of platelet $NO^{\bullet}$ resistance.

In contrast to the findings with regard to SNP and IPA/NO, we observed that the antiaggregatory actions of BAY 41-2272 were resistant to the inhibitory effects of ODQ, but associated with an increase in intraplatelet cGMP and abolished in the presence of the cGK inhibitor, Rp-8-pCPT-cGMPs. The resistance of BAY 41-2272 to the inhibitory actions of ODQ is somewhat surprising and may be indicative of an ability of BAY 41-2272 to either target oxidised/heme-free sGC, overcome the inhibitory actions of ODQ or elevate platelet cGMP via sGC-independent mechanisms. Given ODQ binds to, and oxidises, the heme group of sGC, leading to $NO^{\bullet}$-insensitive Fe^{3+} and heme-free states (Schrammel et al. 1996), a sustained anti-aggregatory action of BAY 41-2272 in the presence of ODQ may reflect an ability of this compound to target sGC in its oxidised form. Whilst there is little evidence

to support such an action of BAY 41-2272 at oxidised sGC, previous studies have shown that ODQ does not abolish the ability of BAY 41-2272 to stimulate purified sGC (Stasch et al. 2001) or exert anti-aggregatory and cGMP elevating actions in rat platelets (Roger et al. 2010). Such observations may reflect an ability of high concentrations of BAY 41-2272 to overcome the inhibitory actions of ODQ, as has been observed for the $NO^{\bullet}$ donor, DEA/NO in platelets from WT mice (Lies et al. 2013). Specifically, the ability of ODQ to reduce VASP phosphorylation was overcome with elevated concentrations of DEA/NO (Lies et al. 2013). Moreover, it has been proposed that intracellular reducing agents may convert oxidised heme to its reduced form, thus overcoming the inhibitory effects of ODQ (Lies et al. 2013). However this is unlikely to account for the sustained actions of BAY 41-2272 under our experimental conditions, as both SNP and IPA/NO were unable to inhibit platelet aggregation in the presence of ODQ. Alternatively, BAY 41-2272 could elevate cGMP and inhibit platelet aggregation via a secondary sGC-independent pathway. Indeed, BAY 41-2272 may increase cGMP levels via inhibition of the cGMP degrading enzyme, PDE5. Thus a previous report by Mullerhausen and colleagues observed that BAY 41-2272 had the ability to inhibit PDE5 activity and augment cGMP accumulation in cultured HEK293 cells and in human platelets, when sGC was maximally activated with the $NO^{\bullet}$ donor S-nitrosoglutathione (Mullershausen et al. 2004). By contrast, BAY 41-2272 failed to inhibit purified recombinant human PDE5 and had no effect on the activity of other PDE's (Bischoff and Stasch 2004). As such, the impact of BAY 41-2272 on PDE5 activity remains to be resolved and future studies in murine platelets should determine if BAY 41-2272 modulates PDE5. Nevertheless, we observed that BAY 41-2272 elevated cGMP levels, resulting in inhibition of platelet aggregation and this effect was not mediated by $NO^{\bullet}$. Together, these findings indicate that BAY 41-2272 is exerting its anti-aggregatory actions via a $NO^{\bullet}$-independent cGMP-dependent signalling mechanism.

Having characterised the mechanisms via which the HNO donor, IPA/NO and the $NO^{\bullet}$-independent sGC stimulator, BAY 41-2272 exert their anti-aggregatory actions under physiological conditions we next sought to identify an experimental model of platelet $NO^{\bullet}$ resistance. This is of particular importance given the phenomenon of platelet $NO^{\bullet}$ resistance has is observed in patients with cardiovascular diseases. Indeed, it has previously been reported that the efficacy of SNP and GTN are impaired in platelets from patients suffering from cardiovascular diseases such as stable angina

pectoris, acute coronary syndrome, type 2 diabetes and ischemic heart disease, when compared to healthy subjects (Chirkov et al. 1996; Chirkov et al. 1999; Chirkov et al. 2001; Worthley et al. 2007). Additionally, reduced efficacy of $NO^{\bullet}$ donors has been observed in platelets from obese patients and lipid lowering therapies can improve the anti-aggregatory actions of $NO^{\bullet}$ by reducing oxidative stress in patients with hypercholesterolemia (Anfossi et al. 1998; Anfossi et al. 2004; Stepien et al. 2003). Taken together these observations indicate that platelet $NO^{\bullet}$ resistance is associated with cardiovascular risk factors and established disease. Furthermore, patients with mild hypercholesterolemia displayed platelet hyperactivity when compared with normocholesterolemic patients (Haramaki et al. 2007; Stepien et al. 2003). To address platelet $NO^{\bullet}$ resistance, this study examined the anti-platelet actions of the $NO^{\bullet}$ donor, SNP in $ApoE^{-/-}$ mice maintained on a HFD for 7 weeks. Previously, we have demonstrated that these mice exhibit an ~4-fold elevation in total plasma cholesterol, increased phorbol 12,13-dibutyrate (PDB)-stimulated platelet $^{\cdot}O_2^-$ production (~80%), a more rapid aggregatory response to collagen and the presence of atherosclerotic lesions in the carotid artery (Chapter 2), modelling a state of hypercholesterolemia and associated atherosclerosis.

In the current study, we found that the anti-aggregatory actions of SNP were impaired in platelets from $ApoE^{-/-}$-HFD mice and this was associated with reduced cGMP accumulation. Importantly, resistance was not overcome by increasing the concentration of SNP to 1 mmol/l. Previous studies have indicated that oxidative stress, and inactivation of NO• by $^{\cdot}O_2^-$, is associated with reduced anti-aggregatory responses of NO• donors (GTN and SNP) in patients with stable angina pectoris and diabetes (Chirkov et al. 1999; Worthley et al. 2007). Additionally, $^{\cdot}O_2^-$ can be decompose to hydrongen peroxide (H_2O_2), which can amply collagen-induced platelet aggregation (Pignatelli et al. 1998). In the current study, we observed that the combination of the antioxidant enzymes polyethylene glycol-superoxide dismutase and polyethylene glycol-catalase did not restore the anti-aggregatory actions of SNP in platelets from $ApoE^{-/-}$-HFD mice, which suggests that inactivation of NO• by $^{\cdot}O_2^-$ did not contribute to platelet NO• resistance. Similarly, a recent report in patients with ischemic heart disease found no significant relationship between reactive oxygen species generation and SNP responses (Dautov et al. 2013), suggesting that the underlying cause of resistance may differ between disease models.

Another potential contributor to platelet NO• resistance is dysfunction at the level of sGC. Indeed, sGC dysfunction has previously been linked with platelet NO• resistance in patients with ischemic heart disease (Dautov et al. 2013) and stable angina pectoris (Chirkov et al. 1999). As discussed above, SNP demonstrated an impaired ability to increase cGMP levels in platelets from ApoE$^{-/-}$-HFD mice. However, the levels of cGMP achieved with SNP in ApoE$^{-/-}$-HFD mice should be sufficient to mediate its anti-aggregatory effects. Indeed, in platelets from WT-HFD mice we observed ~40-50% inhibition of aggregation at relatively low concentrations of cGMP (~14-20 pmol/5x10^8 platelets/ml). Moreover, in platelets from ApoE$^{-/-}$-HFD mice, 1 mmol/l SNP elevated cGMP levels to a similar level (~20 pmol/5x10^8 platelets/ml), yet its anti-aggregatory effects remained impaired. Together, these findings indicate that dysfunction at the level of sGC and/or the downstream targets of cGMP (ie. cGK, cGMP-metabolising PDEs) may contribute to platelet NO• resistance.

In platelets, cGMP is degraded predominantly via PDE5 and its enhanced activity may contribute to platelet NO• resistance. Interestingly, the anti-aggregatory actions of SNP in ApoE$^{-/-}$-HFD mice were partially restored in the presence of the PDE5 inhibitor, sildenafil. However, enhanced PDE5 activity did not account entirely for NO resistance in our mouse model as sildenafil did not restore the anti-aggregatory responses of SNP to the levels observed in platelets from WT-HFD mice. In addition, in ApoE$^{-/-}$-HFD mice, intraplatelet cGMP accumulation in response to SNP, remained impaired despite the assay being performed in the presence of the non-selective PDE inhibitor, IBMX. These findings support previous studies in which PDE activity was found to be unchanged in platelets from patients with stable angina pectoris (Chirkov et al. 1999) and in platelet cytosol fractions (Chirkov et al. 1996). Taken together these findings suggest that in the murine model of hypercholesterolemia employed in the current study, platelet NO• resistance was likely to be associated with sGC dysfunction.

Currently, platelet NO• resistance can be overcome through chronic treatment with angiotensin-converting enzyme inhibitors, perhexiline and stains. While these agents have no direct anti-aggregatory actions, they improve the responsiveness of platelets to NO• donors and improve NO• bioavailability by reducing oxidative stress. However, in an acute/emergency setting, more effective anti-platelet treatments are needed to circumvent NO• resistance. Although the anti-aggregatory actions of IPA/NO and BAY 41-2272 appeared to be slightly diminished (~30% inhibition) as compared to (~40% inhibition) these changes did not reach statistical significance. Additionally, we

observed that cGMP accumulation to IPA/NO and BAY 41-2272 was conserved in platelets from NO•-resistant mice, although we saw a slight delay in cGMP accumulation in ApoE$^{-/-}$ mice. Thus unlike SNP, IPA/NO and BAY 41-2272 circumvented NO• resistance. These findings are in agreement with those in patients with ischemic heart disease in which IPA/NO, but not SNP, maintained its ability to inhibit platelet aggregation and elevate platelet cGMP levels (Dautov et al. 2013). Similarly, the ability of BAY 41-2272 to inhibit platelet anti-aggregation (~30% inhibition) and elevate platelet cGMP levels was fairly well maintained in obese rats (Monteiro et al. 2012). Providing further evidence that IPA/NO and BAY 41-2272 can circumvent platelet NO• resistance in disease.

It is also important to note that intraplatelet cGMP accumulation in response to IPA/NO, in both WT and ApoE$^{-/-}$-HFD mice, occurred more rapidly and reached higher levels than either SNP or BAY 41-2272. Such findings suggest that HNO donors may display greater potency and efficacy than NO• donors as antiplatelet agents. This concept is further supported by studies in patients with ischemic heart disease, in which IPA/NO was found to inhibit platelet aggregation and increased cGMP levels to a greater extent than SNP in NO• resistant subjects (Dautov et al. 2013). Given the greater efficacy of IPA/NO as compared to SNP, it is possible that IPA/NO may also target unique signalling pathways. In this study, we observed that ODQ did not abolish the anti-aggregatory effects of IPA/NO in platelets from ApoE$^{-/-}$ mice, suggesting that the anti-aggregatory actions of IPA/NO were not completely dependent upon sGC/cGMP signalling. Similarly, in human platelets ODQ was only able to partially reverse the anti-aggregatory responses of IPA/NO and Angeli's salt (Bermejo et al. 2005; Dautov et al. 2013). In addition to directly interacting with ferrous (Fe^{2+}) heme sGC, a previous report demonstrated that a high concentration of Angeli's salt can modify thiols on sGC, leading to inhibition of sGC activity (Miller et al. 2009). However, it remains to be determined if HNO, at lower concentrations can modify cysteine residues to potentially enhance its function. Additionally, given the preference of HNO for ferric (Fe^{3+}) groups, it is possible that HNO may target oxidised sGC and thus displaying resistance to ODQ. Although two recent studies have refuted an ability of HNO to target oxidised sGC, these studies were performed using purified sGC and cultured cells and may not reflect the intact platelet (Miller et al. 2009; Zeller et al. 2009). Furthermore, a recent study utilising platelets from healthy humans observed that Angeli's salt can interact with 10 different cysteine-containing platelet proteins leading to dose-dependent, reversible sulfinamide modifications (Hoffman et al. 2009). Many of these proteins are important to platelet activation and have a range of functions from

metabolism, cytoskeletal rearrangement and signal transduction (Hoffman et al. 2009). However it is not known what effect these HNO-modifications will have on platelet function. Nevertheless, there is potential for HNO to mediate its anti-platelet effects through thiol-modification and this may account for its preserved actions in NO•-resistant platelets.

Like IPA/NO, BAY 41-2272 maintained its ability to inhibit platelet aggregation and elevate cGMP levels in NO•-resistant platelets, and thus may represent a viable anti-platelet therapy over NO• donors. In addition to stimulating sGC in an NO•-independent manner (Boerrigter and Burnett 2007; Stasch et al. 2001), BAY 41-2272 can also synergise with NO• donors, prostacyclin and carbon monoxide (Hobbs and Moncada 2003; Martin et al. 2005; Roger et al. 2010; Stasch et al. 2001), which could enable BAY 41-2272 to stimulate sGC under disease conditions where NO• bioavailability is compromised. Moreover, this study and others have found that ODQ does not completely inhibit the ability of BAY 41-2272 to stimulate sGC (Roger et al. 2010; Stasch et al. 2001), indicating that BAY 41-2272 may also stimulate oxidised or heme-free sGC, which is predominate under conditions of enhanced oxidative stress, overcome sGC inhibition by ODQ or have sGC-independent actions such as inhibition of PDE5 activity (Mullershausen et al. 2004). Thus, it will be important to study the anti-aggregatory actions of a heme-free NO•-independent sGC stimulator such as BAY 58-2667 in disease states associated with platelet NO• resistance. Nonetheless, these findings indicate that NO•-independent sGC stimulators may have clinical use as a novel therapy to circumvent the phenomenon of platelet NO• resistance.

Conclusion:

In conclusion, this study has established a murine model of platelet NO• resistance which displays many of the characteristics of platelet NO• resistance in man. Thus placing ApoE$^{-/-}$ mice on a HFD for 7 weeks lead to an impaired ability of the NO• donor, SNP to inhibit platelet aggregation and elevate intraplatelet cGMP levels. These mice also displayed similar NO•/sGC/cGMP dysfunction to that seen in man, although we found no evidence that elevated ROS contributed to platelet NO• resistance. As such, hypercholesterolemic mice represent a robust and useful tool to study platelet NO• resistance. Additionally, we have provided the first comparisons of the ability of HNO donors and NO•-independent sGC stimulators to circumvent platelet NO• resistance. Moreover, the ability of IPA/NO to limit vascular $^{\cdot}O_2^-$ generation (Miller 2013), could be useful in reducing oxidative stress, which has previously been shown to be an underlying cause of platelet NO• resistance in stable angina pectoris and diabetes (Chirkov et al. 1999; Worthley et al. 2007). As such, we believe that HNO donors and NO•-independent sGC stimulators such as BAY 41-2272 represent attractive anti-platelet therapies in an emergency situation where NO• function is compromised.

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References:

Anfossi G, Mularoni EM, Burzacca S, Ponziani MC, Massucco P, Mattiello L, Cavalot F and Trovati M (1998). Platelet resistance to nitrates in obesity and obese NIDDM, and normal platelet sensitivity to both insulin and nitrates in lean NIDDM. *Diabetes Care* 21(1): 121-6.

Anfossi G, Russo I, Massucco P, Mattiello L, Cavalot F and Trovati M (2001). N-acetyl-L-cysteine exerts direct anti-aggregating effect on human platelets. *Eur J Clin Invest* 31(5): 452-61.

Anfossi G, Russo I, Massucco P, Mattiello L, Doronzo G, De Salve A and Trovati M (2004). Impaired synthesis and action of antiaggregating cyclic nucleotides in platelets from obese subjects: possible role in platelet hyperactivation in obesity. *Eur J Clin Invest* 34(7): 482-9.

Bermejo E, Saenz DA, Alberto F, Rosenstein RE, Bari SE and Lazzari MA (2005). Effect of nitroxyl on human platelets function. *Thromb Haemost* 94(3): 578-84.

Bischoff E and Stasch JP (2004). Effects of the sGC stimulator BAY 41-2272 are not mediated by phosphodiesterase 5 inhibition. *Circulation* 110(12): e320-1; author reply e320-1.

Boerrigter G and Burnett JC, Jr. (2007). Nitric oxide-independent stimulation of soluble guanylate cyclase with BAY 41-2272 in cardiovascular disease. *Cardiovasc Drug Rev* 25(1): 30-45.

Chirkov YY, Chirkova LP and Horowitz JD (1996). Suppressed anti-aggregating and cGMP-elevating effects of sodium nitroprusside in platelets from patients with stable angina pectoris. *Naunyn Schmiedebergs Arch Pharmacol* 354(4): 520-5.

Chirkov YY, Holmes AS, Chirkova LP and Horowitz JD (1999). Nitrate resistance in platelets from patients with stable angina pectoris. *Circulation* 100(2): 129-34.

Chirkov YY, Holmes AS, Willoughby SR, Stewart S, Wuttke RD, Sage PR and Horowitz JD (2001). Stable angina and acute coronary syndromes are associated with nitric oxide resistance in platelets. *J Am Coll Cardiol* 37(7): 1851-7.

Dautov RF, Ngo DT, Licari G, Sverdlov AL, Ritchie RH, Kemp-Harper BK, Horowitz JD and Chirkov YY (2013). The nitric oxide redox sibling nitroxyl partially circumvents impairement of platelet nitric oxide responsiveness. *Nitric Oxide* 35: 72-78.

Haramaki N, Ikeda H, Takenaka K, Katoh A, Sugano R, Yamagishi S, Matsuoka H and Imaizumi T (2007). Fluvastatin alters platelet aggregability in patients with hypercholesterolemia: possible improvement of intraplatelet redox imbalance via HMG-CoA reductase. *Arterioscler Thromb Vasc Biol* 27(6): 1471-7.

Hobbs AJ and Moncada S (2003). Antiplatelet properties of a novel, non-NO-based soluble guanylate cyclase activator, BAY 41-2272. *Vascul Pharmacol* 40(3): 149-54.

Hoffman MD, Walsh GM, Rogalski JC and Kast J (2009). Identification of nitroxyl-induced modifications in human platelet proteins using a novel mass spectrometric detection method. *Mol Cell Proteomics* 8(5): 887-903.

Irvine JC, Ravi RM, Kemp-Harper BK and Widdop RE (2013). Nitroxyl donors retain their depressor effects in hypertension. *Am J Physiol Heart Circ Physiol* 305(6): H939-45.

Jang EK, Azzam JE, Dickinson NT, Davidson MM and Haslam RJ (2002). Roles for both cyclic GMP and cyclic AMP in the inhibition of collagen-induced platelet aggregation by nitroprusside. *Br J Haematol* 117(3): 664-75.

Lale A, Herbert JM and Savi P (2003). The antiaggregating activity of clopidogrel is not affected by N-acetyl L-cysteine. *Thromb Haemost* 90(5): 839-43.

Leo CH, Joshi A, Hart JL and Woodman OL (2012). Endothelium-dependent nitroxyl-mediated relaxation is resistant to superoxide anion scavenging and preserved in diabetic rat aorta. *Pharmacol Res* 66(5): 383-91.

Li Z, Ajdic J, Eigenthaler M and Du X (2003). A predominant role for cAMP-dependent protein kinase in the cGMP-induced phosphorylation of vasodilator-stimulated phosphoprotein and platelet inhibition in humans. *Blood* 101(11): 4423-9.

Lies B, Groneberg D, Gambaryan S and Friebe A (2013). Lack of effect of ODQ does not exclude cGMP signalling via NO-sensitive guanylyl cyclase. *Br J Pharmacol* 170(2): 317-27.

Martin E, Czarnecki K, Jayaraman V, Murad F and Kincaid J (2005). Resonance Raman and infrared spectroscopic studies of high-output forms of human soluble guanylyl cyclase. *J Am Chem Soc* 127(13): 4625-31.

Maurice DH and Haslam RJ (1990). Molecular basis of the synergistic inhibition of platelet function by nitrovasodilators and activators of adenylate cyclase: inhibition of cyclic AMP breakdown by cyclic GMP. *Mol Pharmacol* 37(5): 671-81.

Miller AA, Maxwell, K.F., Bullen, M.L., Ku, J.M., De Silva, T.M., Selemidis, S., Hooker, E.U., Drummond, G.R., Sobey, C.G., Kemp-Harper, B.K., (2013). Nitroxyl (HNO) suppresses vascular Nox2 oxidase activity. *Free Radic Biol Med* 60: 264-71.

Miller TW, Cherney MM, Lee AJ, Francoleon NE, Farmer PJ, King SB, Hobbs AJ, Miranda KM, Burstyn JN and Fukuto JM (2009). The effects of nitroxyl (HNO) on soluble guanylate cyclase activity: interactions at ferrous heme and cysteine thiols. *J Biol Chem* 284(33): 21788-21796.

Monteiro PF, Morganti RP, Delbin MA, Calixto MC, Lopes-Pires ME, Marcondes S, Zanesco A and Antunes E (2012). Platelet hyperaggregability in high-fat fed rats: a role for intraplatelet reactive-oxygen species production. *Cardiovasc Diabetol* 11: 5.

Mullershausen F, Russwurm M, Friebe A and Koesling D (2004). Inhibition of phosphodiesterase type 5 by the activator of nitric oxide-sensitive guanylyl cyclase BAY 41-2272. *Circulation* 109(14): 1711-3.

Pignatelli P, Pulcinelli FM, Lenti L, Gazzaniga PP and Violi F (1998). Hydrogen peroxide is involved in collagen-induced platelet activation. *Blood* 91(2): 484-90.

Roger S, Badier-Commander C, Paysant J, Cordi A, Verbeuren TJ and Feletou M (2010). The anti-aggregating effect of BAY 41-2272, a stimulator of soluble guanylyl cyclase, requires the presence of nitric oxide. *Br J Pharmacol* 161(5): 1044-58.

Schrammel A, Behrends S, Schmidt K, Koesling D and Mayer B (1996). Characterization of 1-H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one as a heme-site inhibitor of nitric oxide-sensitive guanylate cyclase. *Molecular Pharmacology* 50: 1-5.

Stasch JP, Becker EM, Alonso-Alija C, Apeler H, Dembowsky K, Feurer A, Gerzer R, Minuth T, Perzborn E, Pleiss U, Schroder H, Schroder W, Stahl E, Steinke W, Straub A and Schramm M (2001). NO-independent regulary site on soluble guanylate cyclase. *Nature* 410(212-215).

Stepien JM, Prideaux RM, Willoughby SR, Chirkov YY and Horowitz JD (2003). Pilot study examining the effect of cholesterol lowering on platelet nitric oxide responsiveness and arterial stiffness in subjects with isolated mild hypercholesterolaemia. *Clin Exp Pharmacol Physiol* 30(7): 507-12.

Wanstall JC, Jeffery TK, Gambino A, Lovren F and Triggle CR (2001). Vascular smooth muscle relaxation mediated by nitric oxide donors: a comparison with acetylcholine, nitric oxide and nitroxyl ion. *Br J Pharmacol* 134(3): 463-72.

Worthley MI, Holmes AS, Willoughby SR, Kucia AM, Heresztyn T, Stewart S, Chirkov YY, Zeitz CJ and Horowitz JD (2007). The deleterious effects of hyperglycemia on platelet function in diabetic patients with acute coronary syndromes mediation by superoxide production, resolution with intensive insulin administration. *J Am Coll Cardiol* 49(3): 304-10.

Zeller A, Wenzl MV, Beretta M, Stessel H, Russwurm M, Koesling D, Schmidt K and Mayer B (2009). Mechanisms underlying acivation of soluble guanylate cyclase by the HNO donor Angeli's Salt. *Molecular Pharmacology* 76(5): 1115-1122.

Monash University

Declaration for Thesis Chapter 5

Declaration by candidate

In the case of Chapter 5, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Performed all experiments except those for total plasma cholesterol and some tissue harvesting, analysed all results and wrote the manuscript	70%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Alyson Miller	Provided intellectual advice during experimental and manuscript preparation stages and assisted with the editorial process.	
Henry Diep		
Jeffery Moore		5%
Courtney Judkins		
Bradley Broughton		
Tracey Gaspari		
Christopher Sobey		
Grant Drummond		
Barbara Kemp-Harper		

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date**
Main Supervisor's Signature		**Date**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

Nox2 Oxidase Deficiency Improves Plaque Stability in Advanced Atherosclerosis

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Abstract

Atherosclerosis is a chronic disease, yet many of its complications (e.g. myocardial infarction, ischemic stroke) are the result of an acute, physical disruption of a lesion leading to thrombosis. Consequently, the stabilisation of atherosclerotic plaques represents an important preventive therapeutic strategy. Enhanced oxidative stress, which occurs largely as a result of enhanced activity of the superoxide ($^{\cdot}O_2^-$) generating enzyme Nox2 oxidase, has been implicated in early atherogenesis. However, it is not clear whether Nox2 oxidase influences plaque stability. Thus, this study aimed to determine if limiting $^{\cdot}O_2^-$ generation, via genetic deletion of Nox2 oxidase, is protective in a mouse model of advanced atherosclerosis. To achieve this aim we studied wild-type, apolipoprotein E-deficient ($ApoE^{-/-}$) and $Nox2^{-/y}ApoE^{-/-}$ mice that were maintained on a high fat diet for 21 weeks. $ApoE^{-/-}$ mice displayed dyslipidaemia ($P<0.001$), enhanced vascular $^{\cdot}O_2^-$ generation ($P<0.05$), reduced endogenous nitric oxide ($NO^{\bullet}$) bioavailability ($P<0.05$) and marked atherosclerotic lesions. The absence of Nox2 oxidase (i.e. in $Nox2^{-/y}ApoE^{-/-}$ mice) resulted in an ~70% reduction in vascular $^{\cdot}O_2^-$ production ($P<0.001$) but had minimal effect upon plasma cholesterol levels and endogenous $NO^{\bullet}$ bioavailability. Similarly, Nox2 deletion did not reduce lesional area, but was associated with changes in plaque composition that improved plaque stability. Specifically, collagen and α-SMC actin accumulation were increased in $Nox2^{-/y}ApoE^{-/-}$ mice by ~2.4-fold ($P<0.01$) and ~1.9-fold ($P<0.05$) respectively, and lipid deposition was reduced by ~40% ($P<0.01$). Whilst lesional macrophage content was unchanged, Nox2 deletion appeared to promote an anti-inflammatory M2 macrophage phenotype. In conclusion, this study indicates that a deficiency in Nox2 oxidase was associated with reduced vascular $^{\cdot}O_2^-$ production and improved plaque stability. Thus, targeting Nox2 oxidase may protect against cardiovascular complications associated with atherosclerosis.

Keywords: atherosclerosis; plaque stability; superoxide; macrophage polarisation

Introduction:

Reactive oxygen species (ROS) are required for a number of redox-dependent signalling processes within the vasculature, yet excessive generation of ROS contributes to the pathogenesis of several cardiovascular disorders (Drummond et al. 2011). Indeed, excess levels of ROS, derived largely from both vascular and inflammatory cells, plays a key role in atherosclerosis (Guzik et al. 2006; Judkins et al. 2010; Sorescu et al. 2002). Specifically, ROS can lead to a decrease in vasoprotective nitric oxide ($NO^{\bullet}$) and increased expression of adhesion molecules on the endothelium (Libby et al. 2013; Tavakoli and Asmis 2012). This in turn, leads to infiltration and retention of inflammatory cells within the vessel wall and the release of pro-inflammatory cytokines, resulting in lesional development (Libby et al. 2013; Tavakoli and Asmis 2012). Additionally, oxidation of low density lipoproteins (ox-LDL) by ROS was reported to generate cytotoxic by-products [25, 40]. Moreover, ox-LDL can be scavenged by macrophages and lead to foam cell formation (Libby et al. 2013; Tavakoli and Asmis 2012). ROS also have the potential to affect the structural integrity of atheromas, by promoting vascular smooth muscle cell (VSMC) proliferation, migration and apoptosis and oxidising matrix metalloproteinases (MMPs) (Tavakoli and Asmis 2012). Taken together, this evidence suggests that ROS may play an important role in the initiation, development and instability of atherosclerotic plaques.

NADPH oxidases (or Nox oxidases) are a family of multi-subunit enzyme complexes, which generate superoxide ($^{\cdot}O_2^-$) via the transfer of electrons from NADPH to molecular oxygen via a membrane-bound catalytic subunit (Nox 1-5; Drummond et al. 2011). Importantly, increasing evidence indicates that Nox1, Nox2, Nox4 and Nox5 oxidases are major sources of ROS in the vasculature. Of particular interest is the Nox2 oxidase isoform, as its expression is upregulated in endothelial and inflammatory cells within human plaques (Guzik et al. 2006; Sorescu et al. 2002) and in an animal model of atherosclerosis (Judkins et al. 2010). Indeed the importance of Nox2 in the development of atherosclerosis is highlighted in studies utilising $ApoE^{-/-}$ mice in which genetic deletion of either Nox2 or its cytosolic subunit, $p47^{phox}$, leads to a reduction in lesion area along the length of the aorta (Barry-Lane et al. 2001; Judkins et al. 2010; Vendrov et al. 2007). Whilst Nox2 contributes to early atherosclerotic plaque development in the descending aorta, Nox2 and $p47^{phox}$ deletion does not alter lesion size in the aortic sinus of $ApoE^{-/-}$ mice (Barry-Lane et al. 2001; Hsich et al. 2000; Kirk et al. 2000). Together these findings suggest that the ability of Nox2 oxidase to modulate atherogenesis may be dependent upon the arterial site of the lesion. Whilst limiting lesion size per se, is an important

therapeutic strategy, it has become increasingly evident that plaque stability also plays a key role in atherosclerosis.

Plaque stabilisation is an important aspect in the therapeutic management of atherosclerosis, as many vascular complications (myocardial infarction, unstable angina and ischemic stroke) arise from an acute physical disruption of the atherosclerotic plaque (Libby et al. 2013; Tavakoli and Asmis 2012). Lesions are primarily comprised of a lipid-rich necrotic core, inflammatory cells, VSMC and an extracellular matrix comprising of collagen, elastin, which are integral to the structural integrity of an atherosclerotic plaque (Silvestre-Roig et al. 2014). Plaques that are prone to rupture often display a thin fibrous cap and are composed of a large lipid-rich necrotic core, where the extracellular matrix is less developed and contains fewer VSMC (Libby et al. 2013; Tavakoli and Asmis 2012). Whilst a number of mechanisms are associated with plaque instability a key driving force is oxidative stress. Indeed, it has been proposed that ROS may promote inflammation through activation of nuclear factor-κB (NF-KB) to release pro-inflammatory cytokines (Drummond et al. 2011). ROS also oxidises MMPs, leading to their activation and breakdown of the extracellular matrix (Papaharalambus and Griendling 2007; Tavakoli and Asmis 2012; Violi et al. 2009). Similarly, inhibition of Nox2 oxidase has previously been shown to reduce MMP activity in cultured mouse macrophages (Kim et al. 2010). Additionally, Nox2-derived ROS was previously found to be associated with endoplasmic reticulum stress, apoptosis of macrophages and may contribute to necrotic core formation (Laurindo et al. 2014; Li et al. 2010). Finally, macrophages exist as a heterogeneous population, with the two most studied phenotypes being M1 and M2 macrophages. M1 or classically activated macrophages have been shown to be an important source of ROS (Pelegrin and Surprenant 2009) and can release a number of pro-inflammatory cytokines (Khallou-Laschet et al. 2010; Leitinger and Schulman 2013). Conversely, ROS generation is lower in M2 or alternatively activated macrophages (Balce et al. 2011; Pelegrin and Surprenant 2009) and these macrophages express a number of anti-inflammatory cytokines to promote angiogenesis and vascular remodelling (Gordon and Martinez 2010; Khallou-Laschet et al. 2010; Leitinger and Schulman 2013). Given macrophage polarisation is dependent upon environmental stimuli (Leitinger and Schulman 2013), it is possible that elevated Nox2-derived $^{\cdot}O_2^-$ from vascular and immune cells could promote M1 macrophage polarisation in atherosclerosis. Thus, developing therapeutic strategies that shift macrophages towards an M2 phenotype might lead

to improved plaque stability and an atheroprotective environment. However to date the role of Nox2 oxidase in macrophage polarisation has not been elucidated in atherosclerosis.

We hypothesised that Nox2 oxidase contributes to plaque instability in advanced atherosclerosis, via generation of vascular $^{\cdot}O_2^-$, decreasing lesional collagen content and promoting the inflammatory M1 macrophage phenotype. To test this hypothesis, vascular $^{\cdot}O_2^-$ generation, endogenous $NO^{\bullet}$ bioavailability and plaque formation and stability were assessed in aortae and innominate arteries from wild-type (WT), apolipoprotein E-deficient ($ApoE^{-/-}$) and $Nox2^{-/y}/ApoE^{-/-}$ double knockout mice maintained on high fat diet (HFD) for 21 weeks.

Methods

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

Experimental animals

Nox2-deficient ($Nox2^{-/-}$) mice were originally generated in the laboratory of Professor Mary Dinauer (Pollock et al. 1995) and bred at Ozgene (Bentley DC, WA, Australia). WT and $ApoE^{-/-}$ mice were obtained from the Animal Resources Centre (Canning Vale, WA, Australia). All mice studied were male and fully backcrossed to the C57BL/6J background. $Nox2^{-/-}$ mice were bred with $ApoE^{-/-}$ mice to generate a $Nox2^{-/y}ApoE^{-/-}$ double knockout colony and a genetically related $ApoE^{-/-}$ single knockout colony, as previously described (Judkins et al. 2010). Genotypes were determined by PCR amplification of tail DNA. From 5 weeks of age, mice were maintained on a HFD (21% fat, 0.15% cholesterol; Speciality Feeds, WA, Australia) for 21 weeks. Mice were deeply anaesthetized by isoflurane inhalation (Baxter Healthcare) prior to being euthanized by decapitation.

Measurement of plasma lipids

Blood from WT, $ApoE^{-/-}$ and $Nox2^{-/y}/ApoE^{-/-}$ mice was collected from the inferior vena cava into heparinised tubes and plasma was isolated via centrifugation (4,000g, 4°C, 10 min). Plasma total cholesterol levels, high density lipoprotein (HDL) cholesterol, LDL cholesterol and triglyceride levels were then determined using a Roche MODULAR 917 enzymatic colorimetric array (Roche Diagnostics, Castle Hill, NSW, Australia).

Assessment of atherosclerotic lesion size

Atherosclerotic lesion burden in aorta and innominate arteries from WT, $ApoE^{-/-}$ and $Nox2^{-/y}/ApoE^{-/-}$ mice was assessed as previously described (Bullen et al. 2011; Gaspari et al. 2013; Judkins et al. 2010; Miller et al. 2010). Whole aorta was assessed as a comparison to our previous study in whole aorta from $ApoE^{-/-}$ and $Nox2^{-/y}/ApoE^{-/-}$ mice at 12 and 19 weeks (Judkins et al. 2010) and innominate arteries were chosen as the represent a site of established unstable plaque formation (Bond and Jackson 2011). In brief, aortas were dissected in their entirety from just distal (~1 mm) to the coronary sinus to just proximal (~1 mm) to the iliac bifurcation and cut open longitudinally along the ventral

surface. Arteries were stained with Oil Red O (0.5% saturated oil red O, 30 min) and excess stain was removed with 60% isopropyl alcohol. Innominate arteries were mounted in an OCT Tissue-Tek mould and stored at -80°C. Arteries were then sectioned (10 µm) and thaw mounted onto poly-L-lysine-coated microscope slides. Three sections (100 µm apart) per mouse were stained with Oil Red O (0.5% saturated oil red O, 60 min) and counterstained with haematoxylin (25%, 2 min). Sections were viewed using a using an Olympus BX51 microscope (Olympus, Tokyo, Japan) and images digitized using a colour OP70 Peltier cooled digital camera and captured with a data acquisition system (Analysis LS Starter Version 3.0, Olympus Soft Imaging Solutions, Munster, Germany). Atherosclerotic plaque size was expressed as either an intima-to-media ratio (IMR) or as a percentage of the total luminal area.

Assessment of plaque stability

Plaque stability was evaluated via assessment of lipid, collagen, α-smooth muscle cell actin (α-SMC actin) and macrophage content of the plaque. Cryocut cross-sections (10 µm) of the innominate artery were prepared and three sections (100 µm apart) per mouse were either: (1) stained with Oil Red O (0.5% saturated oil red O, 60 min) and counterstained with haematoxylin (25%, 2 min) for lipid deposition and imaged under bright-field light, (2) stained with picrosirius red (0.05% saturated picorsirius red, 60 min) for collagen content and imaged under a polarised light or (3) incubated overnight at 4˚C with either a rabbit polyclonal antibody against α-SMC actin (1:1000; Dako, Glostrup, Denmark), rat monoclonal antibody against monocytes/macrophages (F4/80, 1:100; Serotec, Raleigh, NC, USA) or antibody diluent (negative control). The following day sections were washed in a phosphate buffer saline solution (PBS) and incubated for 3 hours at 4˚C with either the fluorescence labelled secondary antibody Alexa594 (goat anti-rabbit IgG; 1:1000), Alexa488 (goat anti-rat IgG; 1:500; Invitrogen, Carlsbad, CA, USA) or PBS (negative control). Sections were washed in PBS, coverslipped using a Vectorshield mounting medium for fluorescence (Vector Laboratories, Burlingame, CA, USA) before being viewed under a fluorescence microscope (BX51, Olympus) at x20 magnification where images were taken (OP70 Peltier cooled digital camera). All sections were coded so that images could be analysed in a blinded fashion. The percentage of immunofluorescence in the atherosclerotic plaque was quantified using ImageJ 1.46 software (Java, NIH, Bethesda, MD, USA), and averaged from the three artery sections per animal. Plaque stability was represented as a plaque

stability score as previously described (Gaspari et al. 2013; Mitani et al. 2003; Naghavi et al. 2003). Plaque stability score was calculated using the formula (collagen + α-SMC actin positive area)/(macrophage + lipid positive area).

Superoxide detection

Basal $^{\cdot}O_2^-$ production was assessed in segments (~2 mm length) of thoracic aortae from WT, ApoE$^{-/-}$ and Nox2$^{-/y}$/ApoE$^{-/-}$ mice via L-012 (100 μmol/l)-enhanced chemiluminescence, as previously described (Bullen et al. 2011; Judkins et al. 2010). Briefly, background chemiluminescence signals were firstly obtained over a 20 minute period using a Plate Chameleon Luminescence Reader (Hidex), in separate wells of a white 96-well Opti-plate containing Krebs-HEPES [composed of (in mmol/l) 99 NaCl, 4.7 KCl, 1.2 $MgSO_4.7H_2O$, 1.0 KH_2PO_4, 19.6 Na_2HCO_3, 20 Na-HEPES, 11.1 D-Glucose, 2.5 $CaCl_2$, 20 mg/ml EDTA, pH 7.4] and L-012 (100 μmol/l). In semi-darkness, arteries were placed in separate wells and L-012-enhanced chemiluminescence was then measured every minute over a 30 minute period. Experiments were performed in duplicate, as two vascular segments were obtained per animal. Basal $^{\cdot}O_2^-$ production for each ring segment was determined by subtracting the background chemiluminescence signal (in relative light units/s; RLU s^{-1}) from the signal in the presence of the artery and then normalised to dry tissue weight ($RLU{\cdot}s^{-1}{\cdot}mg^{-1}$).

Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qPCR) was used to assess mRNA expression of the macrophage marker, CD68 and markers of both M1 (Tumour necrosis factor alpha; TNFα, Chemokine (C-X-C motif ligand 2; Cxcl2) and M2 (Arginase 1; Arg1, Resistin-like molecule alpha 1; Fizz1) macrophages in thoracic aorta and the aortic arch of WT, ApoE$^{-/-}$ and Nox2$^{-/y}$/ApoE$^{-/-}$ mice as previously described (Chan et al. 2012). Gene expression, relative to levels in WT mice, was calculated using the comparative C_T method (Schmittgen and Livak 2008). In brief, RNA was extracted from aortae using a RNeasy Micro Kit-RNA (Qiagen, Venlo, Netherlands), quantified using a Nanodrop 1000D spectrophotometer (Thermo Scientific, Waltham, MA, USA) and reverse transcribed into complementary DNA (cDNA) using a RT^2 First Strand Kit (Qiagen). The resultant cDNA was then used as a template in qPCR to measure the genes of interest relative to the house-keeping gene β-actin. cDNA (20-100 ng) was loaded, in triplicate, into the wells of a 96-well plate together with a TaqMan Universal Master Mix (Applied Biosystems, USA) and predesigned Taqman primers and

probes for Arg1 (Mm00475988_m1), CD68 (Mm03047340_m1), Cxcl2 (MIP-2, Mm00436450_m1), Fizz1 (Retnla, Mm00445109_m1), Nox2 (Cybb, Mm01287743_m1), TNFα (Mm00443260_g1) and β-actin (ACTB, 4352341E; Applied Biosystems, USA). Real-time PCR was performed using a CFX96 Touch Real-Time PCR Detection machine (Bio-Rad, Hercules, CA, USA).

Vascular function studies

Ring segments from the abdominal aortae (~2 mm long) of WT, ApoE$^{-/-}$ and Nox2$^{-/y}$/ApoE$^{-/-}$ mice were mounted in a Mulvany-style small vessel myograph (Danish Myo Technology A/S, Inc., Skejbyparken, Denmark) for the measurement of isometric tension as previously described (Bullen et al. 2011; Irvine et al. 2003). Vessels were maintained in Krebs-Bicarbonate solution [composed of (in mmol/l) 118 NaCl, 4.5 KCl, 0.5 $MgSO_4$, 1.0 KH_2PO_4, 25 $NaHCO_3$, 11.1 glucose and 2.5 $CaCl_2$, pH 7.4] at 37°C and bubbled with carbogen (95% O_2, 5% CO_2). Following a 30 min equilibration period, arteries were stretched to a resting tension of 5 mN before being maximally contracted with the thromboxane A_2 mimetic, U46619 (1 µmol/l; F_{max}).

Contractile responses to phenylephrine (Phe; 1 nmol/l - 30 µmol/l) were obtained in aortae from WT, ApoE$^{-/-}$ and Nox2$^{-/y}$/ApoE$^{-/-}$ mice. Ring segments were then washed with Krebs-Bicarbonate solution before contractile responses to the NO$^{\bullet}$ synthase inhibitor, N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME, 100 µmol/l) were measured as an indicator of basal NO$^{\bullet}$ bioavailability as previously described (Irvine et al. 2003). Briefly, vessels were pre-contracted to ~20-30% F_{max} with titrated concentrations of U46619 (1-25 nmol/l). Once the U46619 contraction was stable, L-NAME (100 µmol/l) was added and the contractile response recorded once it reached a plateau (~30 minutes).

Responses to vasodilators were examined in aortic rings from WT, ApoE$^{-/-}$ and ApoE$^{-/-}$Nox2$^{-/y}$ mice precontracted to ~50% F_{max} with titrated concentrations of U46619 (3-100 nmol/l). Cumulative concentration-response curves to acetylcholine (ACh; 1 nmol/l - 30 µmol/l) were constructed to assess endothelium-dependent vasorelaxation. Ring segments were then washed with Krebs-Bicarbonate solution, precontracted with U46619 (~50% F_{max}) and endothelium-independent vasorelaxation to the NO$^{\bullet}$ donor, diethylamine NONOate (DEA/NO; 1 nmol/l - 30 µmol/l) was examined. To ensure maximal relaxation, the β-agonist, isoprenaline (1 µmol/l) and the ATp-sensitive potassium channel opener, levcromakalim (10 µmol/l) were added at the end of each curve.

Data and statistical analysis

All results are presented as mean ± SE, with the number of animals denoted by *n*. *P*<0.05 was considered statistically significant. Statistical comparisons were performed using either a Student's unpaired t-test or a 1-way ANOVA with a Bonferroni post hoc test using GraphPad Prism, Version 5.0 (GraphPad Software Inc).

Vasorelaxation responses were expressed as a percentage reversal of U46619 precontraction. Contractile responses to PE and L-NAME were expressed as a percentage of the maximum response to U46619 (1 µmol/l; F_{max}). Individual contraction and relaxation curves were fitted to a sigmoidal logistical equation to provide an estimate of the pEC_{50} value (concentration of agonist causing a 50% response), expressed as –log mol/l (GraphPad Prism). Differences between mean pEC_{50} and maximum contraction or relaxation values were determined.

Drugs and their sources

Drugs and there sources were: isopropyl alcohol and paraformaldehyde (Merck, Germany); L-012 (Wako, Japan); levcromakalim (Tocris Bioscience, United Kingdom); DEA/NO and U46619 (Sapphire Bioscience, Australia) and all other drugs (Sigma, Australia). DEA/NO were prepared at 10 mmol/l in 0.01 mol/l NaOH and all subsequent dilutions were in 0.01 mol/l NaOH. L-012 was prepared at 100 mmol/l in dimethyl sulfoxide (DMSO) and all subsequent dilutions were in Krebs-HEPES (pH 7.4) solution. Levcromakalim was prepared at 10 mmol/l in 100% methanol. Oil Red O was prepared as a 0.5% stock solution in 100% isopropyl alcohol then diluted to a 60% isopropyl alcohol working solution. Picrosirius Red was prepared as a 0.05% stock solution in 100% picric acid. All other drugs were dissolved and diluted in distilled water.

Results:

Plasma lipid profiles in WT, ApoE$^{-/-}$ and Nox2$^{-/y}$/ApoE$^{-/-}$ *mice*

Total plasma cholesterol levels were elevated ~7-fold (P<0.001) in ApoE$^{-/-}$ compared to WT mice (Table I). This change in plasma cholesterol was associated with a ~22-fold (P<0.001) increase and ~4-fold (P<0.001) decrease in LDL and HDL cholesterol, respectively. Plasma triglyceride levels were also elevated ~2-fold (P<0.05) in ApoE$^{-/-}$ versus WT mice (Table I). Plasma lipid profiles did not differ between ApoE$^{-/-}$ and Nox2$^{-/y}$/ApoE$^{-/-}$ mice (Table I).

Effect of Nox2 deletion on vascular superoxide generation, NO$^{\bullet}$ bioavailability and function in aorta from ApoE$^{-/-}$ mice.

Aortic Nox2 mRNA expression was elevated ~15-fold (P<0.001) in ApoE$^{-/-}$ mice as compared to WT (Figure 1A). This increase in Nox2 expression in ApoE$^{-/-}$ mice was associated with an ~2-fold (P<0.01) increase in thoracic aortic $^{\cdot}O_2^-$ (Figure 1B) and a reduction in basal NO$^{\bullet}$ bioavailability in the abdominal aorta (Figure 1C), as assayed via the contractile response to the NOS inhibitor, L-NAME. The loss of Nox2 oxidase led to impaired aortic $^{\cdot}O_2^-$ generation in Nox2$^{-/y}$/ApoE$^{-/-}$ mice, such that levels were similar to those observed in WT mice, yet did not improve endogenous NO$^{\bullet}$ bioavailability and aortic Nox2 mRNA expression was negligible in WT and Nox2$^{-/y}$/ApoE$^{-/-}$ mice (Figure 1). Contractile responses to phenylephrine did not differ between aortic segments from WT, ApoE$^{-/-}$ and Nox2$^{-/y}$/ApoE$^{-/-}$ mice (Figure 2A). Similarly, endothelium-dependent and –independent vasorelaxation by ACh (Figure 2B) and the NO$^{\bullet}$ donor, DEA/NO (Figure 2C), respectively remained unaltered in aortic segments from all study groups.

Effect of Nox2 deletion on atherosclerotic plaque development and stability in ApoE$^{-/-}$ mice

Atherosclerotic lesions were evident in whole aortae and innominate artery segments from ApoE$^{-/-}$ and Nox2$^{-/y}$/ApoE$^{-/-}$, but not WT mice (Figure 3). Nox2 deletion did not alter the degree of lesion development in either whole aortae (Figure 3A) or innominate arteries as assessed via the

measurement of lesion size (Figure 3E) and intima-to-media ratio (Figure 3F). Although Nox2 deletion did not influence lesion size, the lipid content of plaque was reduced in Nox2$^{-/y}$/ApoE$^{-/-}$ mice as compared to ApoE$^{-/-}$ mice (P<0.01; Figure 4A & 4C), whilst macrophage content remained unchanged (Figure 4B & 4D). In contrast, both collagen (P<0.01; Figure 5A & 5C) and α-SMC actin (P<0.05; Figure 5B & 5D) content were elevated in plaques from Nox2$^{-/y}$/ApoE$^{-/-}$ mice when compared to ApoE$^{-/-}$. Collagen and α-SMC actin were also predominant on the fibrous cap of the lesion. As such, the deletion of Nox2 in innominate arteries from ApoE$^{-/-}$ mice lead to a significant increase in the plaque stability score (quantified as the ratio of collagen and α-SMC actin content to lipid and macrophage content; P<0.01; Figure 5E).

Effect of Nox2 deletion on macrophage polarisation in ApoE$^{-/-}$ mice

Although macrophage content was similar in plaques from ApoE$^{-/-}$ and Nox2$^{-/y}$/ApoE$^{-/-}$ mice (Figure 4D), we wished to determine if Nox2 deletion had an effect on macrophage phenotype within the plaque. Quantitative reverse transcription polymerase chain reaction demonstrated that thoracic aortae from ApoE$^{-/-}$ mice had significantly higher mRNA levels of the macrophage marker, CD68 (~9-fold increase; P<0.01) as compared to WT mice (Figure 6A). In addition, mRNA for TNFα and Cxcl2, markers of pro-inflammatory M1 macrophages, was significantly increased (up to 5-fold; P<0.05) in ApoE$^{-/-}$ mice (Figure 6B & 6C). By contrast, expression of the anti-inflammatory M2 markers, Arg1 and Fizz1 were not altered in ApoE$^{-/-}$ versus WT (Figure 6D & 6E). Whilst Nox2 deletion did not alter aortic macrophage content per se in ApoE$^{-/-}$ mice (Figure 6A), a change in macrophage phenotype was apparent. Specifically, a deficiency in Nox2 lead to a reduction in mRNA expression of the M1 macrophage marker, TNFα in ApoE$^{-/-}$ (P<0.05, Figure 6B), such that levels were comparable to that in WT mice. Moreover, expression of the M2 macrophage marker, Arg1 was elevated ~5-fold (P<0.05, Figure 5D) in aortae from Nox2$^{-/y}$/ApoE$^{-/-}$ versus ApoE$^{-/-}$ mice. However, Nox2 deletion did not modulate the expression of the M1 marker, Cxcl2 or M2 marker Fizz1 in ApoE$^{-/-}$ mice.

Table I. Plasma lipid profiles in 26 week-old wild-type (WT), apolipoprotein E-deficient ($ApoE^{-/-}$) and $Nox2^{-/y}ApoE^{-/-}$ mice maintained on a high fat diet from 5 weeks of age.

		WT		$ApoE^{-/-}$		$Nox2^{-/y}ApoE^{-/-}$
Parameter	**n**	**Mean ± SE**	**n**	**Mean ± SE**	**n**	**Mean ± SE**
Total Plasma Cholesterol (mmol/l)	9	4.7 ± 0.3	11	32.2 ± 3.3***	12	27.8 ± 3.1***
HDL Cholesterol (mmol/l)	9	2.2 ± 0.2	11	0.5 ± 0.1***	12	0.3 ± 0.1***
LDL Cholesterol (mmol/l)	9	1.5 ± 0.1	11	32.1 ± 3.1***	12	26.8 ± 3.0***
Triglycerides (mmol/l)	9	1.1 ± 0.1	11	1.9 ± 0.3*	12	1.3 ± 0.3

Values are given as mean ± SE, where n= number of animals.
***P* < 0.05, ****P* < 0.001 vs WT (1-way ANOVA, Bonferroni post-hoc test).

Figure 1: A: mRNA expression of Nox2 oxidase in aortic arch, B: basal superoxide production in aortic segments as measured by L-012 (100 µmol/L)-enhanced chemiluminescence (n=8) and C: contractile response to L-NAME (100 µmol/L) in aortic segments from 26 week-old wild-type (WT), apolipoprotein E deficient ($ApoE^{-/-}$) and $Nox2^{-/y}ApoE^{-/-}$ mice maintained on a high fat diet from 5 weeks of age. Values are expressed as mean ± SE, where n= number of animals. *****P<0.05, *******P<0.001 vs WT mice, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 vs $ApoE^{-/-}$ mice (1-way ANOVA, Bonferroni post-hoc test).

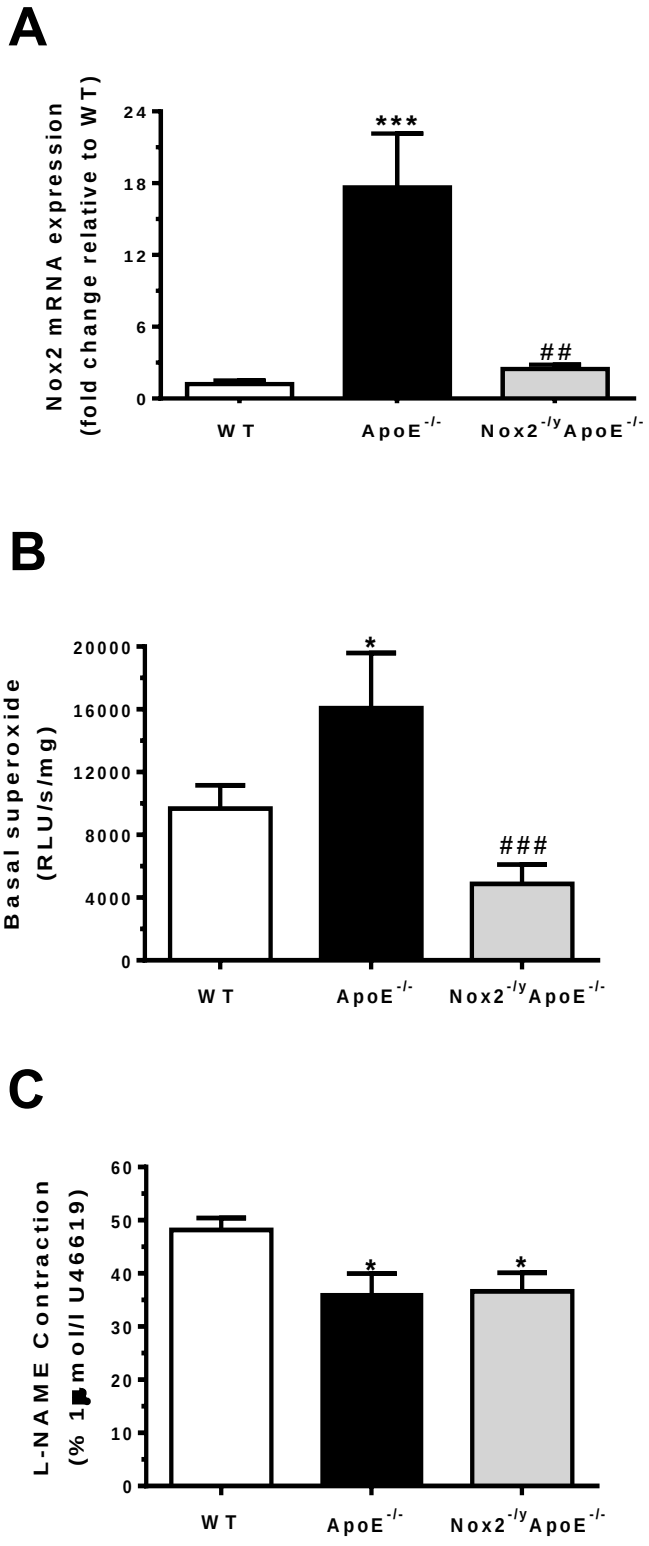

A
Nox2 mRNA expression
(fold change relative to WT)
24
18
12
6
0
***
##
WT
ApoE-/-
Nox2-/yApoE-/-
B
Basal superoxide
(RLU/s/mg)
20000
16000
12000
8000
4000
0
*
###
WT
ApoE-/-
Nox2-/yApoE-/-
C
L-NAME Contraction
(% 1µmol/l U46619)
60
50
40
30
20
10
0
*
*
WT
ApoE-/-
Nox2-/yApoE-/-

Figure 2: Concentration-response curves to phenylephrine (A; n=7-8), acetylcholine (ACh; B; n=7-8) or DEA/NO (C; n=7-8) in aortic segments from 26 week-old WT, $ApoE^{-/-}$ and $Nox2^{-/y}ApoE^{-/-}$ mice. Values are expressed as either a percentage of the contractile response to 1 µmol/L U46619 (A) or the percent reversal of precontraction (B and C) and given as mean ± SE, where n= number of animals.

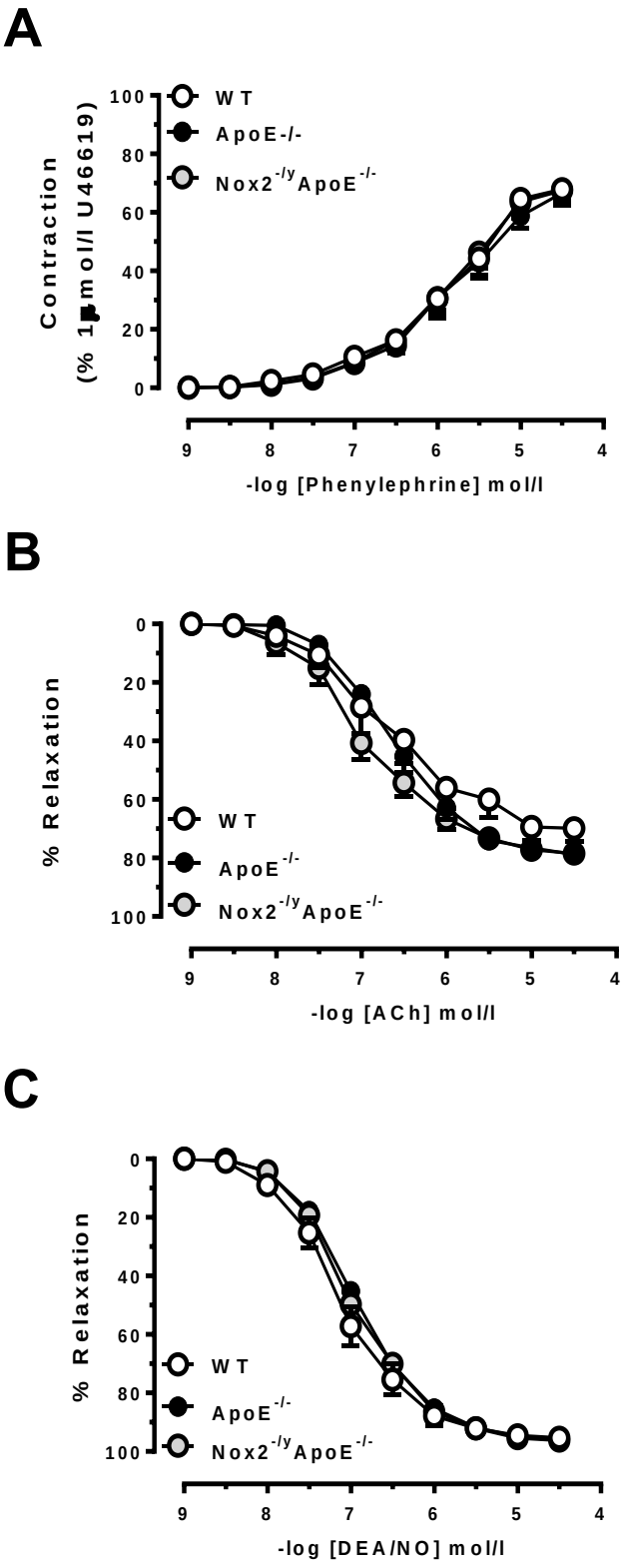

A
Contraction
(% 1μmol/l U46619)
100
80
60
40
20
0
WT
ApoE-/-
Nox2-/yApoE-/-
9
8
7
6
5
4
-log [Phenylephrine] mol/l
B
% Relaxation
0
20
40
60
80
100
WT
ApoE-/-
Nox2-/yApoE-/-
9
8
7
6
5
4
-log [ACh] mol/l
C
% Relaxation
0
20
40
60
80
100
WT
ApoE-/-
Nox2-/yApoE-/-
9
8
7
6
5
4
-log [DEA/NO] mol/l

Figure 3: A: En face lesion coverage of the aorta from 26 week-old ApoE$^{-/-}$ and Nox2$^{-/y}$ApoE$^{-/-}$ mice (n=5-7). B-D: Atherosclerotic lesions are present in innominate arteries from 26 week-old ApoE$^{-/-}$ and Nox2$^{-/y}$ApoE$^{-/-}$ mice, but absent in WT mice (n=6-9). Effect of Nox2 oxidase deletion on lesion size (E) and intima:media ratio (F) in innominate arteries from 26 week-old ApoE$^{-/-}$ and Nox2$^{-/y}$ApoE$^{-/-}$ mice maintained on a high fat diet from 5 weeks of age. Values are expressed as mean ± SE, where n= number of animals.

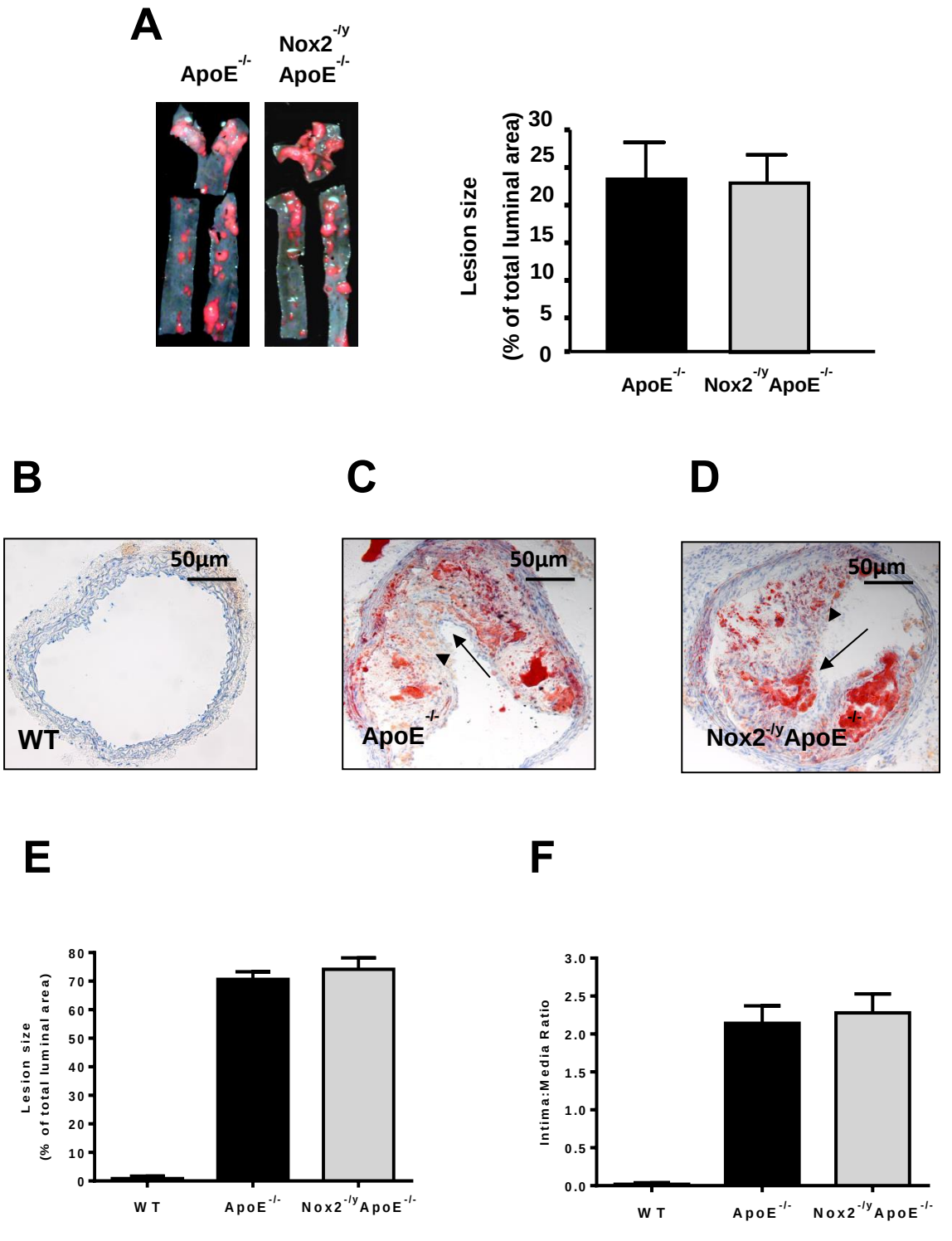
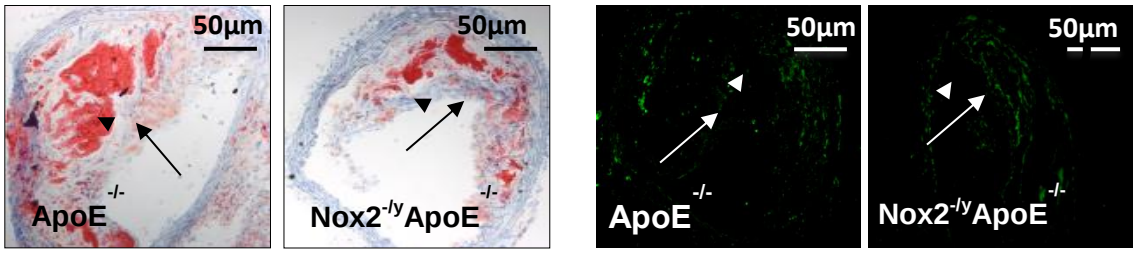

A
ApoE-/-
Nox2-/y ApoE-/-
Lesion size (% of total luminal area)
30
25
20
15
10
5
0
ApoE-/-
Nox2-/yApoE-/-
B
C
D
50µm
WT
ApoE-/-
Nox2-/yApoE-/-
E
F
Lesion size (% of total luminal area)
80
70
60
50
40
30
20
10
0
WT
ApoE-/-
Nox2-/yApoE-/-
Intima:Media Ratio
3.0
2.5
2.0
1.5
1.0
0.5
0.0

Figure 4: A and B: Representative photomicrographs of lipid deposition (Oil Red-O staining; A) and macrophage content (B) as highlighted by arrows, in innominate arteries from 26 week-old ApoE$^{-/-}$ and Nox2$^{-/y}$ApoE$^{-/-}$ mice maintained on a high-fat diet from 5 weeks of age. C and D: Group data (mean ± SE) of lipid (C) and macrophage (D) content. Images representative of n=9 animals and values are expressed as a percentage of the total plaque area. Magnification: x20. ***P*<0.01 vs ApoE$^{-/-}$ mice (Student's unpaired t-test).

Lipid content **Macrophage content**

A B



C D

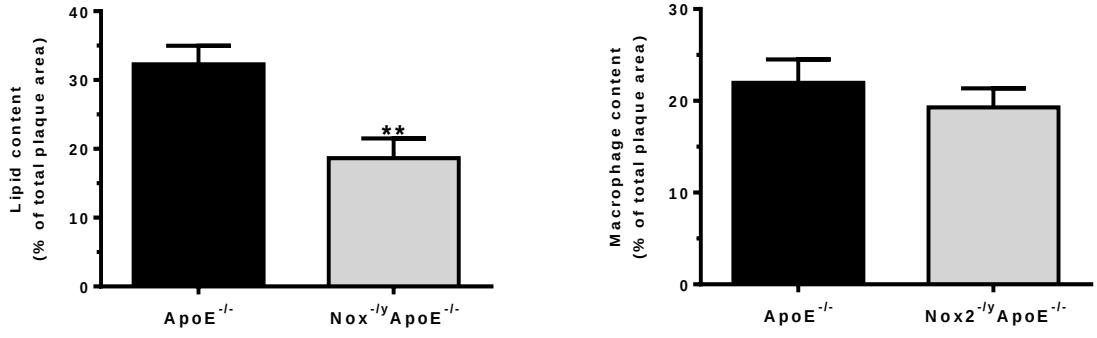


Figure 5: A and B: Representative photomicrographs of collagen (A) and α-SMC actin (B) content as highlighted by arrows, in innominate arteries from 26 week-old ApoE$^{-/-}$ and Nox2$^{-/y}$ApoE$^{-/-}$ mice maintained on a high-fat diet from 5 weeks of age. C and D: Group (mean ± SE) data of collagen (C) and α-SMC actin (D) deposition. Images representative of n=9 animals and values are expressed as a percentage of the total plaque area. Magnification: x20. E: Plaque stability score in innominate arteries from ApoE$^{-/-}$ and Nox2$^{-/y}$ApoE$^{-/-}$ mice. The plaque stability score was calculated using the formula (collagen + α-SMC actin positive area)/(macrophage + lipid positive area). Values are expressed as mean ± SE, where n= number of animals. *P<0.05, **P<0.01, ***P<0.001 vs ApoE$^{-/-}$ mice (Student's unpaired t-test).

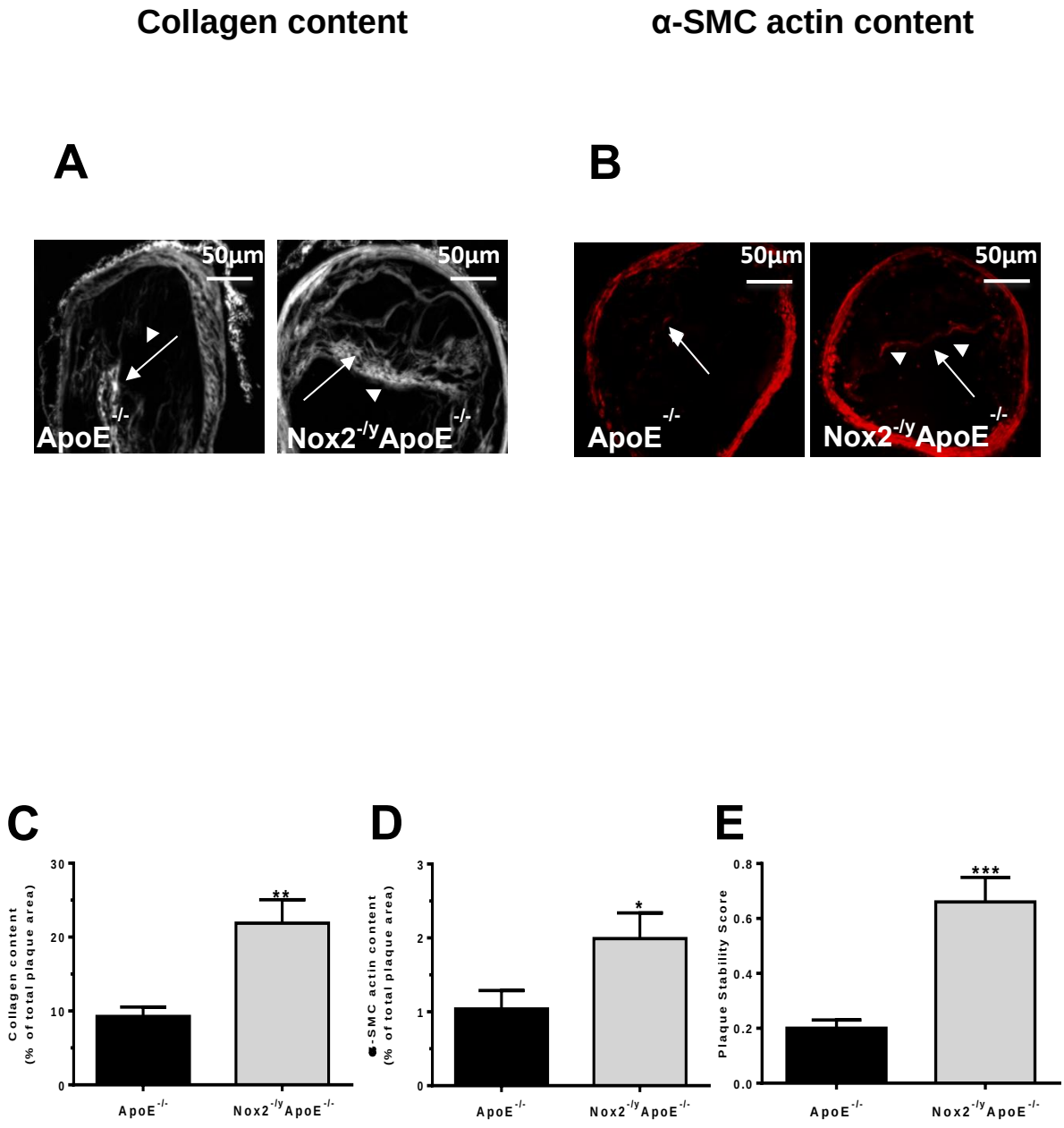
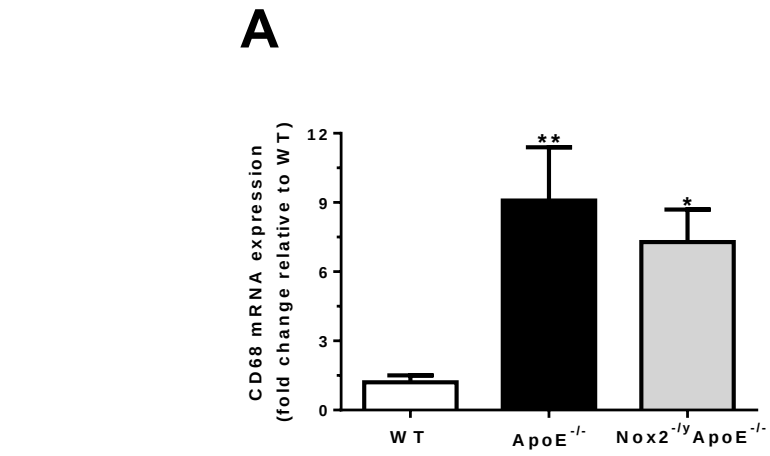
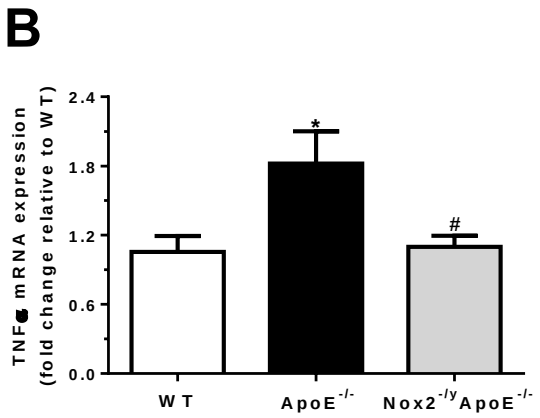
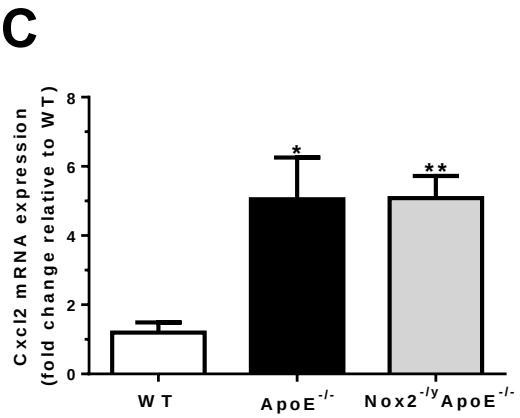
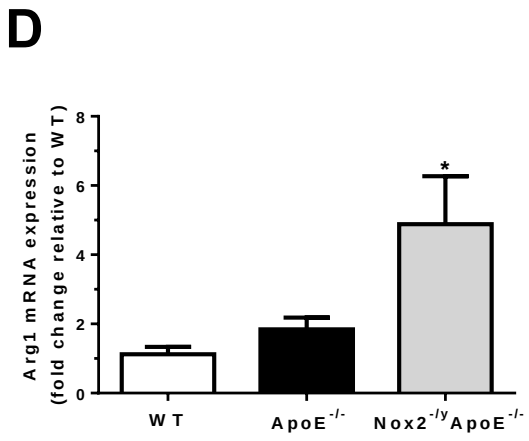

Collagen content
α-SMC actin content
A
B
50µm
50µm
50µm
50µm
ApoE-/-
Nox2-/yApoE-/-
ApoE-/-
Nox2-/yApoE-/-
C
D
E
Collagen content (% of total plaque area)
α-SMC actin content (% of total plaque area)
Plaque Stability Score
30
20
10
0
3
2
1
0
0.8
0.6
0.4
0.2
0.0
**
*
***
ApoE-/-
Nox2-/yApoE-/-
ApoE-/-
Nox2-/yApoE-/-
ApoE-/-
Nox2-/yApoE-/-

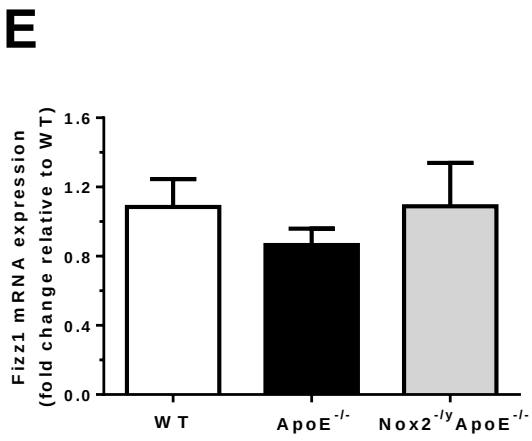
Figure 6: A-E: mRNA expression of the macrophage marker CD68 (A; n=7), pro-inflammatory M1 macrophage markers TNFα (B; n=6-7), Cxcl2 (C; n=7) and the M2 macrophage markers Arg1 (D; n=6-7) and Fizz1 (E; n=7) in thoracic aorta from 26 week-old WT, $ApoE^{-/-}$ and $Nox2^{-/y}ApoE^{-/-}$ mice maintained on a high-fat diet from 5 weeks of age. Values are expressed as mean ± SE, where n= number of animals. *****P<0.05, ******P<0.01 vs WT mice, $^{\#}P$<0.05 vs $ApoE^{-/-}$ mice (1-way ANOVA, Bonferroni post-hoc test).


A
CD68 mRNA expression
(fold change relative to WT)
12
9
6
3
0
**
*
WT
ApoE-/-
Nox2-/yApoE-/-


B
TNFα mRNA expression
(fold change relative to WT)
2.4
1.8
1.2
0.6
0.0
*
#
WT
ApoE-/-
Nox2-/yApoE-/-


C
Cxcl2 mRNA expression
(fold change relative to WT)
8
6
4
2
0
*
**
WT
ApoE-/-
Nox2-/yApoE-/-


D
Arg1 mRNA expression
(fold change relative to WT)
8
6
4
2
0
*
WT
ApoE-/-
Nox2-/yApoE-/-


E
Fizz1 mRNA expression
(fold change relative to WT)
1.6
1.2
0.8
0.4
0.0
WT
ApoE-/-
Nox2-/yApoE-/-

Discussion:

This study has demonstrated for the first time that the absence of Nox2 oxidase is associated with reduced vascular $^{\cdot}O_2^-$ production and improved plaque stability in advanced atherosclerosis. Although, Nox2 oxidase deletion had minimal impact on plaque size, Nox2$^{-/y}$ApoE$^{-/-}$ mice displayed reduced lipid deposition and increased lesional α-SMC actin and collagen accumulation when compared to ApoE$^{-/-}$ mice. Whilst macrophage content was similar between ApoE$^{-/-}$ and Nox2$^{-/y}$ApoE$^{-/-}$ mice, Nox2 oxidase deletion was associated with a shift towards an anti-inflammatory M2 macrophage phenotype, raising the possibility that Nox2-derived ROS may influence macrophage polarisation. Taken together, these results suggest that Nox2 oxidase contributes to plaque instability in advanced atherosclerosis.

Previous studies have demonstrated that Nox2 oxidase expression and activity is increased in vascular and immune cells within atherosclerotic lesions from patients with end-stage heart failure (Sorescu et al. 2002) and coronary artery disease (Guzik et al. 2006). Similarly, Nox2 oxidase was observed to be upregulated in aortic endothelium and lesional macrophages from atherosclerotic mice (Judkins et al. 2010). Additionally, overexpression of endothelial Nox2 oxidase led to increased vascular $^{\cdot}O_2^-$ production and macrophage recruitment in the aortic root, when compared to ApoE$^{-/-}$ mice (Douglas et al. 2012). In accordance with these reports, we found that Nox2 oxidase mRNA expression was increased in aortae from 26-week old ApoE$^{-/-}$ mice and this was associated with an increase in basal $^{\cdot}O_2^-$ production. Conversely, a deficiency in Nox2 oxidase limited basal $^{\cdot}O_2^-$ generation in Nox2$^{-/y}$ApoE$^{-/-}$ mice. Thus, these suggest indicate that Nox2 activity is not only elevated in the early stages of the atherosclerotic disease process (Judkins et al. 2010), but is maintained in more advanced stages of atherosclerosis. In the current study, we did not confirm the identity of cell types which display increased Nox2 expression within these aortic lesions. However, given that endothelial cells and macrophages are key sites of Nox2 expression in 19-week old ApoE$^{-/-}$ mice (Judkins et al. 2010), it is likely that Nox2 expression is also elevated in these cells types in 26-week old ApoE$^{-/-}$ mice.

Unlike earlier stages of atherosclerosis (Judkins et al. 2010; Miller et al. 2010), Nox2-derived $^{\cdot}O_2^-$ did not appear to contribute a reduction in endogenous NO$^{\bullet}$ bioavailability. Although contractions to the NOS inhibitor L-NAME were attenuated in aorta from ApoE$^{-/-}$ mice (indicative of reduced NO$^{\bullet}$ bioavailability) they were not augmented in aorta from Nox2$^{-/y}$ApoE$^{-/-}$ mice. These findings suggest

that the impaired bioavailability of endogenous $NO^{\bullet}$ in advanced atherosclerosis is not due to direct scavenging of $NO^{\bullet}$ by Nox2-derived $^{\bullet}O_2^-$ nor ROS-mediated dysfunction of eNOS itself such as depletion of the key eNOS co-factor tetrahydrobiopterin. Therefore, other mechanisms appear to be involved in impaired $NO^{\bullet}$ bioavailability during advanced atherosclerosis. Indeed, elevated levels of the endogenous eNOS inhibitor, asymmetric dimethyl-L-arginine were observed in patients with coronary artery disease (Antoniades et al. 2009; Forstermann and Sessa 2012). In atherosclerotic mice, eNOS activation has been reported to be impaired, due to a ROS-independent reduction in store-operated Ca^{2+} entry (Forstermann and Sessa 2012; Prendergast et al. 2014). Hypercholesterolemia has also been reported to promote upregulation of caveolin in bovine endothelial cells, which in turn can inhibit eNOS-derived $NO^{\bullet}$ generation (Feron et al. 1999). Although not directly examined in atherosclerosis, eNOS can also undergo S-glutathionylation to reduce $NO^{\bullet}$ production (Chen et al. 2010). Alternatively, a reduction in endogenous $NO^{\bullet}$ bioavailability could be attributed to impaired signalling at the level of its receptor or beyond. However, this is unlikely as relaxation responses to the $NO^{\bullet}$ donor, DEA/NO were preserved in aorta from $ApoE^{-/-}$ mice indicating intact sGC/cGMP signalling. Together these findings suggest that reduced $NO^{\bullet}$ bioavailability may be associated with ROS-independent inactivation of eNOS, leading to decreased $NO^{\bullet}$ generation, although further studies are needed to confirm this hypothesis.

Despite a reduction in endogenous $NO^{\bullet}$ bioavailability in aorta of 26 week-old $ApoE^{-/-}$ mice, we found that ACh-mediated relaxation was preserved. These findings are in agreement with our previous observations in carotid arteries from 26 week-old $ApoE^{-/-}$ mice (Chapter 3). Such findings may be indicative of upregulation of other endothelium-derived relaxing factors to compensate for a loss in $NO^{\bullet}$. Indeed nitroxyl (HNO), the protonated redox congener of $NO^{\bullet}$ has recently been shown to serve as an endothelium-dependent relaxation factor in hypertension and diabetes (Leo et al. 2012; Wynne et al. 2012). Furthermore, we have evidence that HNO generation may be augmented in advanced atherosclerosis and thus compensate for a loss in $NO^{\bullet}$ (Chapter 3).

We next sought to assess the contribution of Nox2 oxidase to atherosclerotic lesion development and instability. Enhanced Nox2 oxidase activity appears to contribute to atherogenesis in the early stages of the disease process. Indeed, lesions size has previously been reported to be reduced in aorta from both 12 and 19 week old $Nox2^{-/y}ApoE^{-/-}$ mice (Barry-Lane et al. 2001; Judkins et al. 2010). However,

in our model of advanced atherosclerosis (26 weeks), genetic deletion of Nox2 oxidase in ApoE$^{-/-}$ mice did not reduce lesion size along the length of the aorta. Together these findings suggest that the contribution of Nox2 oxidase to lesional progression may be dependent upon the stage of development, as other mechanisms could also contribute to atherogenesis. Indeed, previous studies have identified that expression of Nox1, Nox4 and Nox5 were upregulated in human and experimental models of atherosclerosis (Gray et al. 2013; Guzik et al. 2008; Guzik et al. 2006; Sheehan et al. 2011; Sorescu et al. 2002). As such, Nox1 and Nox4 may be contributing to lesional development in our model of advanced atherosclerosis, since Nox5 does not appear to be present in rodents (Drummond et al. 2011; Konior et al. 2013). Furthermore, a deficiency in Nox2 oxidase does not influence lesion size in the aortic sinus in early atherosclerosis (ApoE-/- mice; Hsich et al. 2000; Kirk et al. 2000). Similar findings have also been observed in mice that were deficient in the Nox2 cytosolic regulatory subunit, p47^{phox} (Barry-Lane et al. 2001; Hsich et al. 2000). In this study we observed that Nox2 deletion has minimal effect on lesion size in innominate arteries from ApoE$^{-/-}$ and Nox2$^{-/y}$ApoE$^{-/-}$ mice. Like the aortic sinus, the innominate artery has previously been identified as a site of plaque instability (Bond and Jackson 2011). Whilst other mechanisms including Nox1 and Nox4 may influence lesion size (Gray et al. 2013; Guzik et al. 2006; Sheehan et al. 2011; Sorescu et al. 2002), lesional development was previously demonstrated to occur at an accelerated rate in the aortic sinus when compared to the aortae and this may impact on the role of Nox2 oxidase in these arteries (Barry-Lane et al. 2001). Taken together, these studies highlight that the ability of Nox2 oxidase to modulate atherogenesis is dependent upon both the arterial site of the lesion and the stage of lesional development.

Whilst limiting lesion development can reduce the burden of disease associated with atherosclerosis, many cardiovascular complications arise from a physical disruption of a lesion. Thus, stabilisation of atherosclerotic lesions may also play a role in reducing the clinical sequela of atherosclerosis, such as myocardial infarction and stroke. In this study, plaque stability was determined using a plaque stability score, by balancing lipid and macrophage content versus α-SMC actin and collagen content in the lesion. Given more stable lesions are often associated with a thick fibrous cap, a small lipid-rich necrotic core and a well developed extracellular matrix, comprising of collagen and VSMC (Libby et al. 2013; Tavakoli and Asmis 2012). Additionally, a reduction in pro-inflammatory M1 macrophages together with a shift towards an M2 anti-inflammatory macrophage phenotype may also lead to

improved plaque stability (Khallou-Laschet et al. 2010; Leitinger and Schulman 2013). Importantly, this study has identified, for the first time, that the absence of Nox2 oxidase significantly improved plaque stability. Specifically, innominate arteries from Nox2$^{-/y}$ApoE$^{-/-}$ mice displayed increased α-SMC actin and collagen content both throughout the plaque and at the fibrotic cap. Additionally, plaque lipid content was significantly reduced in Nox2$^{-/y}$ApoE$^{-/-}$ mice. Whilst it remains a matter of contention whether plaque rupture occurs in the ApoE$^{-/-}$ mouse model (Bond and Jackson 2011; Getz and Reardon 2012; Meyrelles et al. 2011; Rosenfeld et al. 2008), this study clearly implicates a role for Nox2 oxidase in plaque instability. Raising the possibility that limiting Nox2 oxidase could be a efficacious approach to reduce lesion size, promote plaque stability and may reduce the likelihood of plaque rupture during atherosclerosis.

Nox2-derived ROS has the potential to promote plaque instability through a variety of mechanisms. Oxidative modification of MMPs by ROS, can lead to degradation of VSMC and collagen within the lesion to compromise the structural integrity of a plaque (Papaharalambus and Griendling 2007; Tavakoli and Asmis 2012). In this study, we observed that a deficiency in Nox2 oxidase led to increased VSMC and collagen content, as such future experiments should examine the activity of MMP's in ApoE$^{-/-}$ and Nox2$^{-/y}$ApoE$^{-/-}$ mice. Additionally, ROS can stimulate VSMC growth and migration, which are important for the maintenance of the fibrous cap and plaque morphology, however excessive ROS generation leads to increased VSMC apoptosis (Papaharalambus and Griendling 2007; Tavakoli and Asmis 2012). Oxidative stress is also a key driving force of endoplasmic reticulum stress, which in turn promotes apoptosis and the formation of a larger necrotic core (Li et al. 2010; Tavakoli and Asmis 2012). In this study we did not quantify the area of necrotic core in innominate arteries, thus further experiments examining apoptotic markers in atherosclerotic lesions from Nox2$^{-/y}$ApoE$^{-/-}$ mice could provide further evidence for plaque stabilisation in these mice. Collectively, inhibition of Nox2-derived $\cdot O_2^-$ generation may facilitate plaque stability by reducing thinning of the fibrous cap and improving the structural integrity within the lesion to limit extracellular matrix degradation and necrotic core formation.

Although macrophage content *per se,* was similar between ApoE$^{-/-}$ and Nox2$^{-/y}$ApoE$^{-/-}$ mice, we observed differences in lesional macrophage phenotype in advanced atherosclerosis. Specifically, aortic expression of the M1 macrophage markers TNFα and Cxcl2, but not the M2 macrophage

markers Arg1 and Fizz1, were elevated in atherosclerotic mice. Both M1 and M2 macrophages have recently been reported to be elevated in human atherosclerotic lesions (Stoger et al. 2012). Moreover, M1 macrophages were found to accumulate in the necrotic core and the shoulder region of a plaque, which is an area that is often prone to rupture (Chinetti-Gbaguidi et al. 2011; Stoger et al. 2012). Conversely, M2 macrophages tend to accumulate in the adventitia and other more stable regions of a plaque (Chinetti-Gbaguidi et al. 2011; Stoger et al. 2012). In $ApoE^{-/-}$ mice M2 macrophages were more prevalent than M1 macrophages in early atherogenesis, but this shifted towards a greater accumulation of M1 macrophages in advanced atherosclerosis (Khallou-Laschet et al. 2010). As such, macrophages exhibit a certain degree of plasticity and can switch phenotypes depending upon the local cytokine milieu (Khallou-Laschet et al. 2010; Leitinger and Schulman 2013). In addition, M2 macrophages which generate anti-inflammatory cytokines, VSMC and adventitial fibroblast proliferation, which may promoted plaque stability (Gordon 2003; Khallou-Laschet et al. 2010; Leitinger and Schulman 2013).

Importantly, in this study, we observed that in the absence of Nox2 oxidase, expression of the M2 macrophage marker Arg1 was elevated in aortae from $Nox2^{-/y}ApoE^{-/-}$ mice. Conversely, genetic deletion of Nox2 oxidase led to decreased expression of the M1 macrophage marker TNFα to similar levels as seen in WT mice. Since M1 macrophages are a major source of Nox2-derived ROS production (Tavakoli and Asmis 2012) and oxidative stress is associated with M1 macrophage polarisation (Zhang et al. 2013), limiting Nox2-derived ROS, could reduce the environmental stimuli driving lesional macrophages towards a M1 phenotype rather than promote M2 macrophages in advanced atherosclerosis. As discussed, promoting an M2 macrophage phenotype may contribute to an improvement in plaque stability. Indeed, increased α-SMC actin and collagen content of lesions in $Nox2^{-/y}ApoE^{-/-}$ mice together with reduced expression of the pro-inflammatory cytokine TNFα is likely to reflect the actions of M2 macrophages. Thus M2 macrophages may promote extracellular matrix deposition and an anti-inflammatory environment within the lesion to improve plaque stability and reducing the likelihood of rupture.

Future experiments are required to confirm our findings with regard to the effect Nox2 deletion has upon M1 and M2 macrophage polarisation in atherosclerosis. In this study we examined aortic mRNA expression of the M1 macrophage markers TNFα and CxCl2 and the M2 macrophage markers Arg1

and Fizz1. Given that Nox2 deletion only modified aortic mRNA expression of one M1 and M2 macrophage marker, other pro-inflammatory (IL-6, IL-1β IL-12) and anti-inflammatory (interferon gamma; IFN-γ, IL-4 and IL-10) cytokines associated with macrophage polarisation could also be assessed (Gordon and Martinez 2010; Martinez et al. 2006). In addition to confirming these results at the level of gene expression, future experiments could also examine protein expression of M1 and M2 macrophage markers in aortae from $ApoE^{-/-}$ and $Nox2^{-/y}ApoE^{-/-}$ mice. As previous studies have utilised immunohistochemistry to assess the expression of M1 and M2 macrophages in arteries from patients with carotid stenosis, limb ischemia and coronary artery disease (Hirata et al. 2011; Shaikh et al. 2012). Furthermore, we have recently published an experimental protocol utilising flow cytometric analysis to determine macrophage accumulation in the aortic wall of hypercholesterolemic mice (Moore et al. 2013) and it may be possible to modify this protocol to assess macrophage polarisation using M1 and M2 macrophage markers such as CD11c and CD206 (Fujii et al. 2013).

Conclusion:

In conclusion, this study has provided the novel finding that Nox2 oxidase contributes to plaque instability in advanced atherosclerosis. As such, developing strategies to target Nox2 oxidase may lead to reduced vascular $^{\cdot}O_2^-$ generation and improved plaque stability. We observed that the absence of Nox2 oxidase was associated with decreased lipid deposition, increased collagen and VSMC content and a shift in macrophage phenotype towards anti-inflammatory M2 macrophage. These findings suggest that inhibiting Nox2 oxidase could lead to increased generation of ECM molecules and strengthening of the lesion and fibrous cap, making plaques less likely to rupture. Thus, employing such a therapeutic approach, in conjunction with a lipid lowering therapy such as statins, may confer protection against many of the cardiovascular complications associated with atherosclerosis.

References:

Antoniades C, Shirodaria C, Leeson P, Antonopoulos A, Warrick N, Van-Assche T, Cunnington C, Tousoulis D, Pillai R, Ratnatunga C, Stefanadis C and Channon KM (2009). Association of plasma asymmetrical dimethylarginine (ADMA) with elevated vascular superoxide production and endothelial nitric oxide synthase uncoupling: implications for endothelial function in human atherosclerosis. *Eur Heart J* 30(9): 1142-50.

Barry-Lane PA, Patterson C, van der Merwe M, Hu Z, Holland SM, Yeh ET and Runge MS (2001). p47phox is required for atherosclerotic lesion progression in ApoE(-/-) mice. *J Clin Invest* 108(10): 1513-22.

Bond AR and Jackson CL (2011). The fat-fed apolipoprotein E knockout mouse brachiocephalic artery in the study of atherosclerotic plaque rupture. *J Biomed Biotechnol* 2011: 379069.

Chen CA, Wang TY, Varadharaj S, Reyes LA, Hemann C, Talukder MA, Chen YR, Druhan LJ and Zweier JL (2010). S-glutathionylation uncouples eNOS and regulates its cellular and vascular function. *Nature* 468(7327): 1115-8.

Chinetti-Gbaguidi G, Baron M, Bouhlel MA, Vanhoutte J, Copin C, Sebti Y, Derudas B, Mayi T, Bories G, Tailleux A, Haulon S, Zawadzki C, Jude B and Staels B (2011). Human atherosclerotic plaque alternative macrophages display low cholesterol handling but high phagocytosis because of distinct activities of the PPARgamma and LXRalpha pathways. *Circ Res* 108(8): 985-95.

Douglas G, Bendall JK, Crabtree MJ, Tatham AL, Carter EE, Hale AB and Channon KM (2012). Endothelial-specific Nox2 overexpression increases vascular superoxide and macrophage recruitment in ApoE(-)/(-) mice. *Cardiovasc Res* 94(1): 20-9.

Drummond GR, Selemidis S, Griendling KK and Sobey CG (2011). Combating oxidative stress in vascular disease: NADPH oxidases as therapeutic targets. *Nat Rev Drug Discov* 10(6): 453-71.

Feron O, Dessy C, Moniotte S, Desager JP and Balligand JL (1999). Hypercholesterolemia decreases nitric oxide production by promoting the interaction of caveolin and endothelial nitric oxide synthase. *J Clin Invest* 103(6): 897-905.

Forstermann U and Sessa WC (2012). Nitric oxide synthases: regulation and function. *Eur Heart J* 33(7): 829-37, 837a-837d.

Fujii M, Inoguchi T, Batchuluun B, Sugiyama N, Kobayashi K, Sonoda N and Takayanagi R (2013). CTLA-4Ig immunotherapy of obesity-induced insulin resistance by manipulation of macrophage polarization in adipose tissues. *Biochem Biophys Res Commun* 438(1): 103-9.

Getz GS and Reardon CA (2012). Animal models of atherosclerosis. *Arterioscler Thromb Vasc Biol* 32(5): 1104-15.

Gordon S (2003). Alternative activation of macrophages. *Nat Rev Immunol* 3(1): 23-35.

Gordon S and Martinez FO (2010). Alternative activation of macrophages: mechanism and functions. *Immunity* 32(5): 593-604.

Gray SP, Di Marco E, Okabe J, Szyndralewiez C, Heitz F, Montezano AC, de Haan JB, Koulis C, El-Osta A, Andrews KL, Chin-Dusting JP, Touyz RM, Wingler K, Cooper ME, Schmidt HH and Jandeleit-Dahm KA (2013). NADPH oxidase 1 plays a key role in diabetes mellitus-accelerated atherosclerosis. *Circulation* 127(18): 1888-902.

Guzik TJ, Chen W, Gongora MC, Guzik B, Lob HE, Mangalat D, Hoch N, Dikalov S, Rudzinski P, Kapelak B, Sadowski J and Harrison DG (2008). Calcium-dependent NOX5 nicotinamide adenine dinucleotide phosphate oxidase contributes to vascular oxidative stress in human coronary artery disease. *J Am Coll Cardiol* 52(22): 1803-9.

Guzik TJ, Sadowski J, Guzik B, Jopek A, Kapelak B, Przybylowski P, Wierzbicki K, Korbut R, Harrison DG and Channon KM (2006). Coronary artery superoxide production and nox isoform expression in human coronary artery disease. *Arterioscler Thromb Vasc Biol* 26(2): 333-9.

Hirata Y, Tabata M, Kurobe H, Motoki T, Akaike M, Nishio C, Higashida M, Mikasa H, Nakaya Y, Takanashi S, Igarashi T, Kitagawa T and Sata M (2011). Coronary atherosclerosis is associated with macrophage polarization in epicardial adipose tissue. *J Am Coll Cardiol* 58(3): 248-55.

Hsich E, Segal BH, Pagano PJ, Rey FE, Paigen B, Deleonardis J, Hoyt RF, Holland SM and Finkel T (2000). Vascular effects following homozygous disruption of p47(phox) : An essential component of NADPH oxidase. *Circulation* 101(11): 1234-6.

Judkins CP, Deip H, Broughton B, Mast AE, Hooker EU, Miller AA, Selemidis S, Dusting GJ, Sobey CG and Drummond GR (2010). Direct evidence of a role for Nox2 in superoxide production, reduced nitric oxide bioavailability and early atherosclerotic plaque formation in ApoE$^{-/-}$ mice. *Am J Physiol Heart Circ* 298(1): H24-32.

Khallou-Laschet J, Varthaman A, Fornasa G, Compain C, Gaston AT, Clement M, Dussiot M, Levillain O, Graff-Dubois S, Nicoletti A and Caligiuri G (2010). Macrophage plasticity in experimental atherosclerosis. *PLoS One* 5(1): e8852.

Kirk EA, Dinauer MC, Rosen H, Chait A, Heinecke JW and LeBoeuf RC (2000). Impaired superoxide production due to a deficiency in phagocyte NADPH oxidase fails to inhibit atherosclerosis in mice. *Arterioscler Thromb Vasc Biol* 20(6): 1529-35.

Konior A, Schramm A, Czesnikiewicz-Guzik M and Guzik TJ (2013). NADPH oxidases in vascular pathology. *Antioxid Redox Signal* 20(17): 2794-814.

Leitinger N and Schulman IG (2013). Phenotypic polarization of macrophages in atherosclerosis. *Arterioscler Thromb Vasc Biol* 33(6): 1120-6.

Leo CH, Joshi A, Hart JL and Woodman OL (2012). Endothelium-dependent nitroxyl-mediated relaxation is resistant to superoxide anion scavenging and preserved in diabetic rat aorta. *Pharmacol Res* 66(5): 383-91.

Li G, Scull C, Ozcan L and Tabas I (2010). NADPH oxidase links endoplasmic reticulum stress, oxidative stress, and PKR activation to induce apoptosis. *J Cell Biol* 191(6): 1113-25.

Libby P, Lichtman AH and Hansson GK (2013). Immune effector mechanisms implicated in atherosclerosis: from mice to humans. *Immunity* 38(6): 1092-104.

Martinez FO, Gordon S, Locati M and Mantovani A (2006). Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J Immunol* 177(10): 7303-11.

Meyrelles SS, Peotta VA, Pereira TM and Vasquez EC (2011). Endothelial dysfunction in the apolipoprotein E-deficient mouse: insights into the influence of diet, gender and aging. *Lipids Health Dis* 10: 211.

Miller AA, De Silva TM, Judkins CP, Diep H, Drummond GR and Sobey CG (2010). Augmented superoxide production by Nox2-containing NADPH oxidase causes cerebral artery dysfunction during hypercholesterolemia. *Stroke* 41(4): 784-9.

Moore JP, Sakkal S, Bullen ML, Kemp-Harper BK, Ricardo SD, Sobey CG and Drummond GR (2013). A flow cytometric method for the analysis of macrophages in the vascular wall. *J Immunol Methods* 396(1-2): 33-43.

Papaharalambus CA and Griendling KK (2007). Basic mechanisms of oxidative stress and reactive oxygen species in cardiovascular injury. *Trends Cardiovasc Med* 17(2): 48-54.

Prendergast C, Quayle J, Burdyga T and Wray S (2014). Atherosclerosis affects calcium signalling in endothelial cells from apolipoprotein E knockout mice before plaque formation. *Cell Calcium* 55(3): 146-54.

Rosenfeld ME, Averill MM, Bennett BJ and Schwartz SM (2008). Progression and disruption of advanced atherosclerotic plaques in murine models. *Curr Drug Targets* 9(3): 210-6.

Shaikh S, Brittenden J, Lahiri R, Brown PA, Thies F and Wilson HM (2012). Macrophage subtypes in symptomatic carotid artery and femoral artery plaques. *Eur J Vasc Endovasc Surg* 44(5): 491-7.

Sheehan AL, Carrell S, Johnson B, Stanic B, Banfi B and Miller FJ, Jr. (2011). Role for Nox1 NADPH oxidase in atherosclerosis. *Atherosclerosis* 216(2): 321-6.

Sorescu D, Weiss D and Lasse`gue B (2002). Superoxide production and expression of nox family proteins in human atherosclerosis. *Circulation* 105(1429-1435).

Stoger JL, Gijbels MJ, van der Velden S, Manca M, van der Loos CM, Biessen EA, Daemen MJ, Lutgens E and de Winther MP (2012). Distribution of macrophage polarization markers in human atherosclerosis. *Atherosclerosis* 225(2): 461-8.

Tavakoli S and Asmis R (2012). Reactive oxygen species and thiol redox signaling in the macrophage biology of atherosclerosis. *Antioxid Redox Signal* 17(12): 1785-95.

Wynne BM, Labazi H, Tostes RC and Webb RC (2012). Aorta from angiotensin II hypertensive mice exhibit preserved nitroxyl anion mediated relaxation responses. *Pharmacol Res* 65(1): 41-7.

Zhang SX, Khalyfa A, Wang Y, Carreras A, Hakim F, Neel BA, Brady MJ, Qiao Z, Hirotsu C and Gozal D (2013). Sleep fragmentation promotes NADPH oxidase 2-mediated adipose tissue inflammation leading to insulin resistance in mice. *Int J Obes (Lond)* Epub ahead of print.

Chapter 6: General Discussion

6.1 General Overview

The therapeutic utility of the $NO^{\bullet}$/sGC/GMP signaling pathway has long been recognised. Indeed, $NO^{\bullet}$ donors have been used extensively to treat heart failure, angina and acute hypertensive crises. However the use of these donors is limited due to their susceptibility to tolerance development with continual use. Furthermore, $NO^{\bullet}$ can be inactivated by $^{\cdot}O_2^-$ (Klemenska and Beresewicz 2009) and can display reduced efficacy under conditions of oxidative stress. Given the limitations of $NO^{\bullet}$ donors, interest has turned towards $NO^{\bullet}$-like drugs such as HNO donors. Like $NO^{\bullet}$, HNO displays a number of vasoprotective actions including an ability to mediate vasorelaxation (Andrews et al. 2009; Ellis et al. 2000; Favaloro and Kemp-Harper 2009; Fukuto et al. 1992; Irvine et al. 2003; Irvine et al. 2007; Sha et al. 2006; Wanstall et al. 2001), limit vascular $^{\cdot}O_2^-$ production (Leo et al. 2012; Miller 2013) and serves as an anti-aggregatory agent (Bermejo et al. 2005; Donzelli et al. 2013) under non-disease conditions. Additionally, HNO exhibits distinct pharmacological actions, which have the potential to overcome many of the limitations associated with $NO^{\bullet}$ donors. Specifically, previous studies have shown that unlike $NO^{\bullet}$, HNO is resistant to scavenging by $^{\cdot}O_2^-$ (Leo et al. 2012; Miller 2013), does not develop vascular tolerance (Irvine et al. 2007; Irvine et al. 2010), may preferentially target ferric haem proteins (Miranda et al. 2003), is highly thiophilic (Donzelli et al. 2006; Shen and English 2005) and can target unique signaling pathways in the vasculature (i.e calcitonin-gene related peptide release, voltage- and ATP-sensitive potassium channels; K_v and K_{ATP} (Donzelli et al. 2013; Favaloro and Kemp-Harper 2009; Favaloro and Kemp-Harper 2007; Irvine et al. 2003; Miller 2013)). Thus, there is potential for the vasoprotective actions of both endogenous and exogenous HNO to be maintained under conditions of oxidative stress and in acute coronary disease states where patients are susceptible to the development of nitrate tolerance and/or platelet $NO^{\bullet}$ resistance. Importantly, the HNO donor, CXL1020 has recently been reported to have cardioprotective actions in patients with congestive heart failure (Sabbah et al. 2013). However, we need to determine if HNO can maintain its vasoprotective actions in vascular diseases before the therapeutic potential of HNO donors can be fully realised. As such, the aim of this project was to elucidate the mechanisms of action and the efficacy of HNO in animal models of vascular disease and to directly compare the effects of HNO

donors to those of NO• donors. Specifically, we choose to examine their efficacy in murine models of hypercholesterolemia and advanced atherosclerosis, two important risk factors for cardiovascular events such as acute myocardial infarction and stroke.

6.2 The vasoprotective actions of HNO donors are maintained in cardiovascular disorders

In this thesis we observed that vascular and platelet $^{\cdot}O_2^-$ production was elevated in murine models of hypercholesterolemia and advanced atherosclerosis (Chapters 2 and 3). This increase in O_2^- production was associated with a reduction in endogenous NO• bioavailability. (Chapters 2 and 3). Under these conditions, the clinically used NO• donor, GTN maintained its ability to induce vasorelaxation, however its anti-aggregatory and $^{\cdot}O_2^-$ limiting actions were compromised in hypercholesterolemia and advanced atherosclerosis (Chapter 2 and 3). Previous studies have also observed that the ability of GTN to limit vascular $^{\cdot}O_2^-$ levels in diabetes (Wang et al. 2011), mediate vasorelaxation in hypercholesterolemic rabbits (Melichar et al. 2004; Verbeuren et al. 1986) and inhibit platelet aggregation is impaired in patients with stable angina pectoris, acute coronary syndrome, type 2 diabetes and obesity (Anderson et al. 2005; Anfossi et al. 1998; Anfossi et al. 2004; Chirkov et al. 1999; Chirkov et al. 2001). Additionally, we observed that GTN was susceptible to vascular tolerance development in hypercholesterolemia and advanced atherosclerosis after acute treatment *in vitro* (Chapters 2 and 3). This correlates with previous studies, which demonstrated that GTN developed vascular tolerance following either acute administration *in vitro* or chronic *in vivo* administration (Irvine et al. 2007; Irvine et al. 2010).

Importantly, this thesis clearly demonstrates that the ability of the HNO donor, IPA/NO to mediate vasorelaxation, inhibit platelet aggregation and suppress $^{\cdot}O_2^-$ generation was preserved in mouse models of hypercholesterolemia (Chapter 2 and 4) and advanced atherosclerosis (Chapter 3). Additionally, IPA/NO did not develop vascular tolerance in these disease settings (Chapters 2 and 3). Our findings are in accordance with a recent report, which found that the vasorelaxant actions of the HNO donor, 1-NCA are preserved in $ApoE^{-/-}$ mice (Donzelli et al. 2013). Similarly, the vasorelaxant and vasodepressor effects of Angeli's salt and IPA/NO have been shown to be maintained in a mouse model of hypertension (Irvine et al. 2013; Wynne et al. 2012). Having confirmed that the acute

vasoprotective actions of HNO donors are sustained in animal models of cardiovascular disorders, it will be vital to determine if these findings translate into the clinic. Thus far, research from our laboratory has found that relaxation responses to Angeli's salt are sustained in radial arteries from patients undergoing coronary bypass surgery (Bullen *et al.* 2011, General Introduction). Moreover, the anti-aggregatory actions of IPA/NO are maintained in patients with ischemic heart disease (Dautov et al. 2013). Thus, whilst future studies are necessary, these findings indicate that the protective actions of HNO are sustained in human disease, and thus highlight a potential therapeutic utility for HNO donors in the treatment of cardiovascular disorders.

Interestingly, the $NO^{\bullet}$ donor, DEA/NO were maintained in advanced atherosclerosis and was also shown to be resistant to vascular tolerance development (Chapter 3). When we compared the vasoprotective effects of GTN, IPA/NO and DEA/NO it was apparent that impaired responses to nitrovasodilators are not necessarily dependent upon the redox form of nitrogen oxide ($NO^{\bullet}$ vs HNO) donated, rather the nature of the donor itself. Whilst organic nitrates such as GTN must first undergo biotransformation to generate $NO^{\bullet}$, DEA/NO spontaneously releases $NO^{\bullet}$ (Daiber et al. 2008; Miranda et al. 2005). However, it is unlikely that reduced biotransformation of GTN is entirely responsible for the impaired anti-aggregatory and $^{\bullet}O_2^-$ limiting actions of this nitrovasodilator, as vasorelaxation responses to GTN were preserved in hypercholesterolemia and advanced atherosclerosis (Chapters 2 and 3). Rather, the preserved actions of DEA/NO could be attributed to the rapid release and greater amount of $NO^{\bullet}$ generated from this $NO^{\bullet}$ donor. Indeed, 2 moles of $NO^{\bullet}$ are released per mole of DEA/NO, compared with 1 mole of $NO^{\bullet}$ per parent GTN molecule (Miranda et al. 2005). Thus, the generation of more $NO^{\bullet}$ by DEA/NO (vs. GTN) could ultimately mean a greater level of $NO^{\bullet}$ bioavailability and hence a preservation of the vasoprotective actions of DEA/NO.

In this thesis we wanted to further explore the vasoprotective role of HNO donors and determine if these donors could circumvent platelet $NO^{\bullet}$ resistance. Platelet $NO^{\bullet}$ resistance differs from tolerance, as platelets display reduced efficacy to $NO^{\bullet}$ donors without prior exposure and the phenomenon is not exclusive to organic nitrates (Horowitz 2000). Indeed, anti-aggregatory responses to the $NO^{\bullet}$ donors GTN and SNP are impaired in platelets from obese patients and patients with congestive heart failure, aortic stenosis, stable angina pectoris, acute coronary syndrome and type 2 diabetes responses, indicating platelet $NO^{\bullet}$ resistance (Anderson et al. 2004; Anfossi et al. 1998; Anfossi et al. 2004;

Chirkov et al. 1996; Chirkov et al. 1999; Chirkov et al. 2004; Chirkov et al. 2002; Chirkov et al. 2001). As such, we firstly aimed to develop a murine model of platelet $NO^{\bullet}$ resistance to compare the efficacy of HNO and $NO^{\bullet}$ donors. We utilised hypercholesterolemic mice, as these mice displayed impaired anti-aggregatory responses to the $NO^{\bullet}$ donors, SNP and GTN (Chapter 2 and 4). Additionally, intraplatelet cGMP accumulation in response to SNP was also reduced in $ApoE^{-/-}$ mice when compared to WT mice, indicative of platelet $NO^{\bullet}$ resistance (Chapter 4).

It has previously been suggested that diminished responses to $NO^{\bullet}$ donors could be attributed to direct interaction with $^{\cdot}O_2^-$ and/or desensitisation of platelet sGC in patients with diabetes and stable angina pectoris (Chirkov et al. 1999; Worthley et al. 2007). Additionally, a recent report observed that sGC dysfunction may be associated with platelet $NO^{\bullet}$ resistance in patients with ischemic heart disease, yet increased $^{\cdot}O_2^-$ accumulation did not appear to contribute to resistance (Dautov et al. 2013). Taken together these findings suggest that the mechanisms for platelet $NO^{\bullet}$ resistant may be dependent on the nature of the cardiovascular disease. In our model, we demonstrated that intracellular platelet $^{\cdot}O_2^-$ levels are elevated (Chapter 2) and it has previously been shown that $^{\cdot}O_2^-$ can be decompose to H_2O_2 to amply collagen-induced platelet aggregation (Pignatelli et al. 1998). However, the anti-platelet effects of SNP were not restored with the ROS scavenging enzymes PEG-SOD and PEG-catalase, indicating that inactivation of $NO^{\bullet}$ by ROS did not contribute to platelet $NO^{\bullet}$ resistance (Chapter 4). Additionally, the PDE5 inhibitor, sildenafil was able to partially restore the anti-aggregatory actions of SNP in hypercholesterolemic mice, yet cGMP accumulation in response to SNP was impaired in the presence of the PDE inhibitor, IBMX (Chapter 4), indicating that increased PDE5 activity does not solely contribute to platelet $NO^{\bullet}$ resistance. Although further studies are required to confirm whether increased PDE5 activity contributes to platelet $NO^{\bullet}$ resistance in hypercholesterolemic mice. Moreover, increasing the concentration of SNP lead to increased cGMP accumulation in platelets from hypercholesterolemic mice, yet anti-aggregatory responses to SNP were still impaired (Chapter 4). Since the level of cGMP accumulation should have been sufficient to restore the anti-aggregatory effects of SNP, this indicates that dysfunction at the level of cGMP may be contributing to platelet $NO^{\bullet}$ resistance in hypercholesterolemic mice (Chapter 4). Taken together, these findings suggest that dysfunction at the level of sGC and/or downstream targets of cGMP appear to be the most likely causes of platelet $NO^{\bullet}$ resistance in hypercholesterolemic mice.

Additionally studies are required to confirm these mechanisms of platelet $NO^{\bullet}$ resistance hypercholesterolemic mice and to extrapolate these findings into hypercholesterolemic patients.

The sGC stimulators, IPA/NO and BAY 41-2272 modulate sGC/cGMP activity independently of $NO^{\bullet}$ and may target sGC/cGMP-independent signaling pathways in platelets (Hoffman et al. 2009; Mullershausen et al. 2004). As such, we hypothesised that these agents may circumvent platelet $NO^{\bullet}$ resistance. Indeed, IPA/NO and BAY 41-2272 inhibited platelet aggregation to a similar extent in platelets from WT and hypercholesterolemic mice (Chapter 4). Additionally, the degree of cGMP accumulation in response to IPA/NO and BAY 41-2272 was preserved in hypercholesterolemia (Chapter 4). Such findings indicate that IPA/NO and BAY 41-2272 are able to circumvent platelet $NO^{\bullet}$ resistance. IPA/NO has previously been shown to maintain its anti-aggregatory actions in platelets from patients with ischemic heart disease (Dautov et al. 2013), whilst the anti-platelet actions of BAY 41-2272 were fairly well maintained in obese rats (Monteiro et al. 2012). Furthermore, the anti-aggregatory actions of IPA/NO were partially inhibited in the presence of ODQ, yet ODQ had no effect on anti-aggregatory responses to BAY 41-2272 in hypercholesterolemic mice (Chapter 4). These findings suggest that both IPA/NO and BAY 41-2272 may be targeting sGC-independent signaling pathways to maintain their anti-aggregatory actions in hypercholesterolemia. Indeed, there is evidence to suggest that HNO donors can interact with platelet proteins via thiol modification, although it is currently not known what effect these interactions will have on platelet aggregation (Hoffman et al. 2009). Additionally, BAY 41-2272 has the potential to stimulate oxidised sGC and could inhibit PDE5 activity leading to increased cGMP accumulation (Mullershausen et al. 2004; Roger et al. 2010; Stasch et al. 2001). Future experiments are needed to confirm if IPA/NO and BAY 41-2272 display sGC/cGMP-independent actions via these mechanisms. Additionally, sGC dysfunction may be occurring as a result of increase oxidation of sGC to a $NO^{\bullet}$-insensitive Fe^{3+} or heme-free sGC, thus future experiments could also examine the anti-aggregatory actions of $NO^{\bullet}$- and heme-independent sGC activators like BAY 58-2267 in this model. Nevertheless, the anti-aggregatory actions of IPA/NO and BAY 41-2272 appeared to be greater than those of SNP in hypercholesterolemia. As such, HNO donors and other $NO^{\bullet}$-independent sGC stimulators represent viable targets for the future development of drugs that could circumvent platelet $NO^{\bullet}$ resistance.

Whilst IPA/NO and DEA/NO both maintained their vasoprotective actions in advanced atherosclerosis, HNO donors may have advantages over nitrovasodilators in general. One of the current limitations of nitrovasodilators is that they can display reduced efficacy under conditions of oxidative stress due to a direct interaction between $NO^{\bullet}$ and $^{\cdot}O_2^-$ (Leo et al. 2012). Although we did not observe any impairment to DEA/NO signalling in advanced atherosclerosis (Chapter 3), the interaction of $NO^{\bullet}$ and $^{\cdot}O_2^-$ leads to the formation of peroxynitrite ($ONOO^-$), a damaging oxidant that has previously been associated with vascular dysfunction (Thomas et al. 2008). In contrast, HNO is resistant to direct scavenging by $^{\cdot}O_2^-$ (Leo et al. 2012; Miller 2013), thus the bioavailability of HNO donors will be preserved under conditions of oxidative stress. Another issue surrounding the clinical application of NONOates, such as DEA/NO is its propensity to induce systemic hypotension and the formation of hepatocarcinogens (Fathi et al. 2011; Keefer 2003). However, systemic hypotension may not be a problem for HNO donors, as high concentrations of HNO can target thiol groups on sGC to reduce its activity (Miller et al. 2009), indicating that the actions of HNO may be self-limiting. Additionally, HNO can target distinct signalling pathways to that of HNO that may confer protection in cardiovascular diseases. Indeed, HNO targets a major source of oxidative stress, the $^{\cdot}O_2^-$ generating enzyme Nox2 oxidase, potentially through modification of critical thiols to reduce its activity (Miller 2013). In this study, we observed that IPA/NO limited $^{\cdot}O_2^-$ generation in advanced atherosclerosis, potentially via the same mechanism. Whilst the $NO^{\bullet}$ donor, diethylenetriamine was previously shown to reduce Nox2 oxidase activity via S-nitrosylation of critical thiols on the $p47^{phox}$ cytosolic subunit, this action is not immediate like HNO (Selemidis et al. 2007). Taken together these findings suggest that HNO donors may be more beneficial than DEA/NO as a novel therapy in cardiovascular disorders.

HNO donors also have the potential to be more potent and efficacious anti-platelet agents than $NO^{\bullet}$ donors, such as DEA/NO and SNP. In this thesis we observed that a ~33-fold higher concentration of DEA/NO was required to inhibit collagen-stimulated platelet aggregation to a similar extent as IPA/NO in platelets from WT and atherosclerotic mice (Chapter 3). Although we did not examine the effects of DEA/NO upon platelet cGMP accumulation, IPA/NO also elevated cGMP levels ~2-fold higher than those observed with SNP at a similar concentration (Chapter 4). IPA/NO also produced a rapid increase in platelet cGMP levels that was sustained over an extended time period, whereas cGMP accumulation in response to SNP was delayed (Chapter 4). Taken together these findings suggest

that HNO donors are more advantageous anti-platelet agents than NO• donors and could serve as a novel therapy in patients that have developed platelet NO• resistance or tolerance.

Moreover, IPA/NO targets unique signalling pathways that may lead to additional benefits over DEA/NO. As highlighted previously, HNO has sGC-independent actions that are particularly prevalent in the heart. Indeed, HNO can modify critical thiol residues on ryanodine receptors, SERCA2a, the SERCA2a inhibitor phospholamban and myofilaments, leading to positive inotropic and lusitropic effects that enhance myocardial contractility (Chen et al. 2010; Cheong et al. 2005; Dai et al. 2007; Froehlich et al. 2008; Gao et al. 2012; Lancel et al. 2009; Tocchetti et al. 2007). Therefore, it is possible that HNO could also mediate some of its vasoprotective actions by targeting distinct signalling pathways. Indeed, HNO can interact with K_v and K_{ATP} channels, although this interaction is sGC/cGMP-dependent, as such could be impaired in the face of sGC dysfunction (Andrews et al. 2009; Favaloro and Kemp-Harper 2009; Favaloro and Kemp-Harper 2007; Irvine et al. 2003). Additionally, HNO can elevate calcitonin-gene related peptide levels, which could be sustained if sGC-dependent signalling is compromised (Donzelli et al. 2013; Favaloro and Kemp-Harper 2007). Thus the ability of IPA/NO to maintain its vasoprotective actions under disease conditions, together with its resistance to tolerance could lead to the use of HNO donors as an adjunct therapy in patients which are resistant to nitrovasodilators. Furthermore, the preservation of the vasoprotective actions of HNO may also be of use in the treatment of vascular dysfunction associated with angina, hypertension, atherosclerosis and of particular use in patients which display platelet NO• resistance or tolerance.

6.3 Endogenous HNO can serve as an EDRF in atherosclerosis

NO• was originally identified as the sole nitrogen oxide that could serve as an EDRF (Dierks and Burstyn 1996; Palmer et al. 1987), however there is growing evidence to suggest that HNO may also contribute to endothelium-dependent relaxation (Martin 2009). Indeed, it has been postulated that HNO is generated in the vasculature from both NOS-dependent (Donzelli et al. 2008; Hobbs et al. 1994; Pufahl et al. 1995; Rusche et al. 1998; Schmidt et al. 1996) and –independent sources (Irvine et al. 2008; Paolocci et al. 2007; Wong et al. 1998). Furthermore, exogenous HNO was previously

shown to elevate cGMP in the vasculature to induce vasorelaxation (Fukuto et al. 1992; Irvine et al. 2007) and we have confirmed that HNO can mediate vasorelaxation in cardiovascular disease states such as hypercholesterolemia (Chapter 2) and advanced atherosclerosis (Chapter 3). Thus, it is plausible that endogenous HNO has the capacity to induce relaxation in the vasculature. Furthermore, since HNO is resistant to inactivation by $^{\cdot}O_2^-$ (Leo et al. 2012; Miller 2013; Miranda et al. 2002) and its effect may be enhanced in the face of uncoupled eNOS and thiol-depletion (Park and Oh 2011; Rusche et al. 1998), this raises the possibility that the protective actions of endogenous HNO could be preserved in vascular disease. In accordance with previous studies (Andrews et al. 2009; Fukuto et al. 1992; Wanstall et al. 2001), we found that endothelium-dependent relaxation to ACh in WT mice was reduced in the presence of the $NO^{\bullet}$ scavenger, HXC and the HNO scavenger, L-cysteine (Chapter 3), indicating that both $NO^{\bullet}$ and HNO can serve as EDRFs under non-disease conditions. More importantly, this thesis has also demonstrated, for the first time, that the ability of HNO to serve as an EDRF was augmented in a mouse model of advanced atherosclerosis, as L-cysteine impaired ACh relaxation responses to a greater extent in $ApoE^{-/-}$ mice as compared to WT mice (Chapter 3). This was associated with a reduced contribution of $NO^{\bullet}$ to endothelium-dependent relaxation in $ApoE^{-/-}$ mice. Together, these findings raise the possibility that HNO may compensate for a loss of endogenous $NO^{\bullet}$ in hypercholesterolemic and atherosclerotic mice (Chapters 2 and 3), thereby maintaining endothelium-dependent relaxation responses *per se*. Whilst HNO appears to serve as an EDRF in animal models of disease, it will be important to elucidate its role in patients with vascular diseases. This latter point notwithstanding, this experimental evidence highlights a role for endogenous HNO as a vasoprotective signaling molecule and further emphasises its potential as a therapeutic target to improve vascular function in disease.

It is important to note however, that a role for endogenous HNO as an EDRF in health and disease can only be inferred from pharmacological studies. Definitive proof for its endogenous generation is at present, lacking due to the absence of methods to directly detect HNO, a concept which will be explored in more detail later in this discussion. Currently, HNO is assessed by utilising high concentrations of thiols as HNO scavengers (Ellis et al. 2000; Irvine et al. 2003; Irvine et al. 2007; Li et al. 1999; Wanstall et al. 2001) or through measuring by-products of HNO reactions (Fukuto et al. 1992; Keceli et al. 2013; Miranda et al. 2005). However, these methods have limitations due to the lack of specificity of thiols and the fact that the by-products measured do not account for all of HNO's

interactions. As such, more direct and sensitive methods need to be developed for the detection of HNO in mammalian cells. Nevertheless, with a potential to compensate for a loss in $NO^{\bullet}$ under disease conditions, endogenous HNO appears to be an attractive therapeutic target as a means to improve vascular function in disease.

6.4 Role of Nox2 oxidase in the pathogenesis of atherosclerosis

ROS have previously been demonstrated to be elevated in a number of cardiovascular diseases including atherosclerosis, hypertension, pulmonary hypertension and cardiac failure (Drummond et al. 2011; Konior et al. 2013) and are attributed primarily to increased expression and activity of the ROS generating enzymes, NAPDH oxidases (Drummond et al. 2011; Konior et al. 2013; Violi et al. 2009). Additionally, the Nox2 oxidase isoform represents a major source of $^{\cdot}O_2^-$ within the vasculature and its expression is upregulated in endothelial and inflammatory cells within human plaques (Guzik et al. 2006; Sorescu et al. 2002) and in an animal model of atherosclerosis ($ApoE^{-/-}$ mice) (Judkins et al. 2010). In atherosclerosis, enhanced $^{\cdot}O_2^-$ production can lead to endothelial dysfunction and impaired endogenous $NO^{\bullet}$ bioavailability (Landmesser et al. 2004; Vasquez et al. 2012). Similarly, overexpression of endothelial Nox2 oxidase led to increased vascular $^{\cdot}O_2^-$ production and macrophage recruitment in the aortic root when compared to $ApoE^{-/-}$ mice (Douglas et al. 2012). In the current thesis, we observed that genetic deletion of Nox2 oxidase significantly reduced vascular $^{\cdot}O_2^-$ production, yet did not improve endogenous $NO^{\bullet}$ bioavailability or decrease lesion size, per se, in a mouse model of advanced atherosclerosis. Given the absence of Nox2 oxidase in early atherosclerosis leads to improved endogenous $NO^{\bullet}$ bioavailability and a reduction in lesion size (Judkins et al. 2010; Miller et al. 2010), we postulate that the ability of Nox2 oxidase to modulate atherogenesis is dependent on the stage of lesional development.

Whilst limiting lesion development is an important therapeutic strategy, it is becoming increasingly apparent that the stabilisation of an atherosclerotic plaque is also critical to atherosclerosis, as many thrombotic complications arise from a physical disruption to a lesion (Silvestre-Roig et al. 2014). ROS can have previously been associated with endothelial cell activation, retention of inflammatory cells within the vessel wall, activation of MMPs and can oxidise LDL particles within the vessel wall (Libby et al. 2013; Tavakoli and Asmis 2012). As such, ROS may promote the development of a large

necrotic core, inflammation, thinning of the fibrous cap and reduced extracellular matrix deposition, resulting in unstable plaques that are prone to rupture (Libby et al. 2013; Silvestre-Roig et al. 2014; Tavakoli and Asmis 2012). However the role of Nox2 oxidase in plaque stability is currently unknown. In this thesis, we observed that genetic deletion of Nox2 oxidase led to improved plaque stability in advanced atherosclerotic lesions (Chapter 5). Specifically, in innominate arteries from $Nox2^{-/y}ApoE^{-/-}$ mice, α-SMC actin and collagen content was increased throughout the plaque and at the fibrotic cap (Chapter 5). Such changes stabilise the extracellular matrix within the lesion and lead to reinforcement of the fibrotic cap (Silvestre-Roig et al. 2014). Additionally, plaque lipid content was significantly reduced in the absence of Nox2 oxidase (Chapter 5), which may limit necrotic core formation and improve the structural integrity of the plaque.

Interestingly, we found Nox2 oxidase deletion had minimal impact on macrophage content, *per se*, within atherosclerotic plaques (Chapter 5). However, we observed that Nox2-derived ROS can influence macrophage polarisation in advanced atherosclerosis. Specifically, Nox2 deletion reduced M1 macrophage content, as reflected by decreased expression of the M1 macrophage marker TNFα and enhanced M2 macrophage content, as expression of the M2 macrophage marker Arg I was elevated in aorta from $Nox2^{-/y}ApoE^{-/-}$ mice (Chapter 5). Together these findings suggest that the absence of Nox2 oxidase was associated with M2 macrophage polarisation. Importantly, a previous study in $ApoE^{-/-}$ mice demonstrated that in M2 macrophages were more prevalent in early lesional development, but in advanced atherosclerosis there appeared to be a shift towards M1 polarised macrophages (Khallou-Laschet et al. 2010). Given macrophages maintained their plasticity in advanced atherosclerosis (Khallou-Laschet et al. 2010), this suggests that Nox2 oxidase may be facilitating a M2 < M1 macrophage switch in these lesions and may account for the increased expression of TNFα in advanced atherosclerosis. However, a limitation of the current study is that Nox2 deletion modified aortic mRNA expression of one M1 and one M2 macrophage marker. Thus, future experiments could examine the gene and protein expression of other macrophage polarisation markers in aortic and innominate arteries to confirm our findings. M1 macrophages are associated with the secretion of pro-inflammatory cytokines (TNFα, IL-1β, IL-6 and IL-12), are a major source of Nox2-derived ROS (Tavakoli and Asmis 2012) and can activate MMPs to promote plaque instability through VSMC and macrophage apoptosis, inflammation, thinning of the fibrous cap and reduced extracellular matrix deposition (Silvestre-Roig et al. 2014). In this study we observed that a loss of

Nox2 oxidase led to increased VSMC and collagen content within the lesion and at the fibrous cap, which further supports a role for Nox2 oxidase in plaque instability. Together, these findings suggest that target Nox2 oxidase could improve plaque stability and promote an atheroprotective M2 macrophage phenotype.

Both macrophages and the vascular wall appear to be important sources of Nox2 oxidase for atherosclerotic plaques, yet appear to have varying roles in the pathogenesis of atherosclerosis. A deficiency of Nox2 oxidase in the vessel wall was previously shown to limit endothelial activation and macrophage accumulation in early atherosclerosis (Vendrov et al. 2007) and an overexpression of endothelial Nox2 oxidase lead to increased macrophage recruitment (Douglas et al. 2012). Conversely, a loss of Nox2 oxidase from macrophages reduced plasma oxLDL levels and macrophage accumulation in early atherosclerosis (Vendrov et al. 2007). Thus both vascular and immune cells have been shown to play a role in lesion development. Whilst we did not examine the relative contributions of vascular and macrophage Nox2 in plaque stabilisation, we have clearly shown that global deletion of Nox2 promotes plaque stability and has atheroprotective actions. These findings, together with the observation that Nox2 oxidase expression was associated with more severe and unstable lesions in heart failure patients (Sorescu et al. 2002), suggests that Nox2 oxidase plays an important role in plaque instability. Consequently, developing therapeutic strategies that target Nox2 oxidase may be beneficial in preventing plaque rupture and reducing thrombotic complications associated with atherosclerosis. Given, HNO has been shown to have anti-inflammatory actions (Andrews *et al.*, unpublished) and can modulate the activity of Nox2 oxidase (Miller 2013) it is possible that HNO may also promote plaque stability. Having demonstrated that the acute vasoprotective actions of HNO are preserved in advanced atherosclerosis (Chapter 3), we anticipate that chronic treatment with HNO donors will lead to improved vascular function, reduced atherosclerotic lesion development and improved lesion stability.

6.5 Current limitations and future directions

Whilst HNO donors have the potential to display considerable advantages over traditional nitrovasodilators, a number of important issues must be addressed before their full therapeutic potential can be elucidated. Currently, it is not possible to directly detect HNO in intact mammalian cells and as a result indirect detection methods are utilised to discriminate between the effects of HNO and $NO^{\bullet}$. In this thesis we utilised the well-established HNO scavenger, L-cysteine and $NO^{\bullet}$ scavengers, carboxy-PTIO and HXC (Chapters 2, 3 and 4).

Furthermore, it appears that endogenous HNO can serve as a mediator of vascular function in health and disease, however direct detection methods are required to provide definitive proof of its endogenous generation. As discussed in the General Introduction, it is difficult to measure HNO directly as HNO undergoes rapid dimerisation and dehydration to yield N_2O (Miranda et al. 2005). Current techniques assaying final reaction products of HNO lack sensitivity and selectivity as many of these end products can be generated via other signaling pathways (Reisz et al. 2011). Importantly, there have been recent advancements in the development of HNO sensors that can bind directly to HNO. Cu^{2+}-based fluorescent probes (Apfel et al. 2013; Rosenthal and Lippard 2010; Wrobel et al. 2014) and triarylphosphine-based detectors (Kawai et al. 2013; Reisz et al. 2011) are both direct HNO sensors, are highly sensitive and can also measure the cellular-localisation of HNO in cultured cells (Kawai et al. 2013; Rosenthal and Lippard 2010; Wrobel et al. 2014). However, further development of these sensors is required to translate these findings from immortalised cell lines to the intact vasculature, *in vitro* and *in vivo* and thereby facilitate their use as a viable means to directly detect endogenous HNO generation.

A further drawback in the therapeutic use of HNO donors, is that currently used donors such as Angeli's salt and IPA/NO have short half-lives (~ 2.5 min; DuMond and King 2011; Hughes and Cammack 1999; Maragos et al. 1991). However, it is important to note that cGMP accumulation and relaxation responses to Angeli's salt were previously observed to be sustained over a 60 minute period (Irvine et al. 2007). Thus it appears that the effects of HNO itself and/or its downstream targets are maintained over an extended period of time despite the short half-life of its donor. The short half-life of Angeli's salt and IPA/NO are not amenable to chronic dosing in humans, nor can these donors be readily administered chronically in experimental models of disease. Maintaining donors at a basic

pH, prevents decomposition of the parent compound and a previous study demonstrated that Angeli's salt was stable for up to 4 days, therefore continuous infusion of this donor is possible over this time period (Irvine et al. 2011). However, we have observed that after 7-10 days there is a reduction in the efficacy of Angeli's salt and IPA/NO (Appendix I), precluding long-term administration of these donors. Additionally, IPA/NO generates a nitrosamine byproduct, which may exert non-specific effects (DuMond and King 2011; Miranda et al. 2005), which could also reduce the efficacy of IPA/NO. As a result, there is a need to develop pure HNO donors that possess longer half-lives, before we can fully assess the therapeutic potential of HNO donors.

Acyloxy nitroso compounds such as 1-NCA represent an important step in the development of novel HNO donors, as these donors have a longer half-life of ~13-15 hours at physiological pH levels (Sha et al. 2006). Additionally, 1-NCA primarily generates HNO and a corresponding ketone, with minimal amounts of $NO^{\bullet}$ and nitrite, potentially enabling it to serve as a more pure HNO donor than Angeli's salt and IPA/NO (Sha et al. 2006). However, further information is required with respect to the vasoprotective actions of 1-NCA, as 1-NCA has only been shown to induce vasorelaxation and serve as an anti-aggregatory agent (Donzelli et al. 2013; Sha et al. 2006). Additionally, the effectiveness of 1-NCA and any potential off target actions need to be fully explored before its therapeutic potential is realised.

Cardioxyl Pharmaceuticals (USA) are also in the process of developing a number of pure, longer lasting, small molecule HNO donors such as CXL-1020 for the treatment of cardiovascular diseases. In Phase I/IIA clinical trials CXL-1020 displayed positive inotropy and lusitropy actions in patients with congestive heart failure (Sabbah et al. 2013). Similarly, the haemodynamic effects of CXL-1427, a second generation HNO donor are currently being tested in Phase I clinical trials in patients with acute decompensated heart failure (Cardioxyl Pharmaceuticals 2014). Given the potential cardioprotective benefits of these molecules, it will be important to confirm if these molecules display similar vasoprotective actions to current HNO donors.

Whilst the cardioprotective and vasoprotective actions of HNO make donors of this nitrogen oxide an appealing therapeutic strategy, it is important to recognise any potential shortcomings of these donors. As discussed in the General Introduction, HNO has the potential to be cytotoxic (at high concentrations), exacerbate ischemia-reperfusion injury and impair neuronal function (Chlopicki et al.

2004; Choe et al. 2009; Ma et al. 1999; Vaananen et al. 2008). Similarly, the highly thiophilic nature of HNO may lead to potential non-specific effects, albeit its preference for thiolates over thiols would diminish these interactions (Kemp-Harper 2011). Although we did not examine the adverse effects of HNO donors in this thesis, others have previously reported that the anti-alcoholism drug, cyanamide, as well as the Cardioxyl compound, CXL-1020, displayed few side effects and were well tolerated (DuMond and King 2011; Sabbah et al. 2013). Additionally, to fully elucidate the therapeutic potential of HNO donors, the vasoprotective actions of HNO observed in experimental models of vascular disease, must be confirmed in patients with cardiovascular disease. Importantly, a recent report demonstrated that the anti-aggregatory actions of IPA/NO were preserved in patients with ischemic heart disease (Dautov et al. 2013) and we have shown that Angeli's salt mediates vasorelaxation in radial arteries isolated from patients undergoing coronary artery bypass surgery (Andrews *et al.*, unpublished; General Introduction). Together this data provides further evidence that the vasoprotective actions of HNO will be sustained in patients with cardiovascular disorders.

Given the importance of the immune system in cardiovascular diseases, particularly atherosclerosis (Libby 2012; Libby et al. 2013), there is also a need to explore the anti-inflammatory actions of HNO. Currently the role of HNO in immune cell modulation is unclear as HNO has previously been associated with increased neutrophil infiltration and ICAM-1 expression in rats (Takahira et al. 2001), is cytotoxic in human leukocytes at high concentrations (Augustyniak et al. 2013), but has also recently been shown to limit leukocyte adhesion in the vasculature (Andrews *et al.*, unpublished). Given the ability of HNO to supress Nox2 oxidase-derived $^{\cdot}O_2^-$ generation (Miller 2013), HNO may also influence macrophage function and limit their pro-inflammatory capacity, as oxidative stress is associated with M1 macrophage polarisation (Zhang et al. 2013).

Previously, Nox2 oxidase was linked with early atherosclerotic plaque formation and development (Barry-Lane et al. 2001; Judkins et al. 2010; Vendrov et al. 2007) and we have shown in this thesis, that Nox2 oxidase is also associated with plaque instability. Taken together, this raises the possibility that HNO could potentially modulate atherogenesis and plaque stability by limiting the activity of Nox2 oxidase in vascular and inflammatory cells. However, we await the development of longer acting HNO donors which will facilitate the chronic administration of these donors to determine if this nitrogen oxide can limit plaque formation and improve plaque stability in atherosclerosis.

Conclusions

In conclusion, this thesis has demonstrated that HNO plays a pivotal role as a vasoprotective signalling molecule in cardiovascular disease. Thus, the vasorelaxant, anti-aggregatory and $^{\cdot}O_2^-$ supressing actions of the HNO donor, IPA/NO were conserved in the setting of hypercholesterolemia and advanced atherosclerosis. Although the vasoprotective actions of the $NO^{\bullet}$ donor, DEA/NO were also maintained, the ability of the clinically used nitrovasodilator, GTN to limit $^{\cdot}O_2^-$ accumulation and inhibit platelet aggregation was compromised. Importantly, unlike GTN, IPA/NO and DEA/NO did not develop vascular tolerance, as they do not require biotransformation to generate their nitrogen oxide species. We also demonstrated that the contribution of endogenous HNO to endothelium-dependent relaxation was augmented in advanced atherosclerosis and may compensate for a disease-associated reduction in $NO^{\bullet}$ bioavailability. Thus HNO donors appear to be a superior alternative to traditional $NO^{\bullet}$ donors as a therapeutic treatment. Given HNO donors are resistant to cross-tolerance (Irvine et al. 2007; Irvine et al. 2010), this also raises the possibility that HNO donors could be used as an adjunct therapy in patients with acute coronary syndromes such as angina, which display nitrate tolerance or platelet $NO^{\bullet}$ resistance.

Moreover, combining the vasoprotective actions of HNO together with its cardioprotective actions, makes HNO donors an ideal therapy in patients with heart failure. Specifically, HNO donors have the ability to increase myocardial contractility, unload the heart, improve coronary blood flow and can limit vascular damage (Tocchetti et al. 2011). Indeed, Cardioxyl Pharmaceuticals have small molecule HNO donors in Phase I/IIA clinical trials for the treatment of congestive and acute decompensated heart failure. Lastly, HNO supresses Nox2 oxidase activity and limits vascular oxidative stress (Miller 2013). This property may be of particular relevance in the setting of atherosclerosis where we have shown, via this thesis, that limiting Nox2 oxidase significantly improves plaque stability and appears to promote an anti-inflammatory macrophage phenotype in lesions. As such, the vasoprotective and proposed atheroprotective actions of HNO donors would be beneficial in preventing the thrombotic complications associated with atherosclerosis.

Chapter 7: References

Anderson RA, Ellis GR, Chirkov YY, Holmes AS, Payne N, Blackman DJ, Jackson SK, Lewis MJ, Horowitz JD and Frenneaux MP (2004). Determinants of platelet responsiveness to nitric oxide in patients with chronic heart failure. *Eur J Heart Fail* 6(1): 47-54.

Anderson RA, Ellis GR, Evans LM, Morris K, Chirkov YY, Horowitz JD, Jackson SK, Rees A, Lewis MJ and Frenneaux MP (2005). Platelet nitrate responsiveness in fasting and postprandial type 2 diabetes. *Diab Vasc Dis Res* 2(2): 88-93.

Andrews KL, Irvine JC, Tare M, Apostolopoulos J, Favaloro JL, Triggle CR and Kemp-Harper BK (2009). A role for nitroxyl (HNO) as an endothelium-derived relaxing and hyperpolarizing factor in resistance arteries. *Br J Pharmacol* 157(4): 540-50.

Anfossi G, Mularoni EM, Burzacca S, Ponziani MC, Massucco P, Mattiello L, Cavalot F and Trovati M (1998). Platelet resistance to nitrates in obesity and obese NIDDM, and normal platelet sensitivity to both insulin and nitrates in lean NIDDM. *Diabetes Care* 21(1): 121-6.

Anfossi G, Russo I, Massucco P, Mattiello L, Cavalot F and Trovati M (2001). N-acetyl-L-cysteine exerts direct anti-aggregating effect on human platelets. *Eur J Clin Invest* 31(5): 452-61.

Anfossi G, Russo I, Massucco P, Mattiello L, Doronzo G, De Salve A and Trovati M (2004). Impaired synthesis and action of antiaggregating cyclic nucleotides in platelets from obese subjects: possible role in platelet hyperactivation in obesity. *Eur J Clin Invest* 34(7): 482-9.

Antoniades C, Shirodaria C, Leeson P, Antonopoulos A, Warrick N, Van-Assche T, Cunnington C, Tousoulis D, Pillai R, Ratnatunga C, Stefanadis C and Channon KM (2009). Association of plasma asymmetrical dimethylarginine (ADMA) with elevated vascular superoxide production and endothelial nitric oxide synthase uncoupling: implications for endothelial function in human atherosclerosis. *Eur Heart J* 30(9): 1142-50.

Apfel UP, Buccella D, Wilson JJ and Lippard SJ (2013). Detection of nitric oxide and nitroxyl with benzoresorufin-based fluorescent sensors. *Inorg Chem* 52(6): 3285-94.

Arnelle DR and Stamler JS (1995). NO^+, $NO^\cdot$, and NO^- donation of S-nitrosothiols: Implications for regulation of physiological functions by S-nitrosylation and aceleration of disulfide formation. *Arch Biochem Biophys* 318(2): 279-285.

Augustyniak A, Skolimowski J and Blaszczyk A (2013). Cytotoxicity of nitroxyl (HNO/NO-) against normal and cancer human cells. *Chem Biol Interact* 206(2): 262-71.

Balce DR, Li B, Allan ER, Rybicka JM, Krohn RM and Yates RM (2011). Alternative activation of macrophages by IL-4 enhances the proteolytic capacity of their phagosomes through synergistic mechanisms. *Blood* 118(15): 4199-208.

Barry-Lane PA, Patterson C, van der Merwe M, Hu Z, Holland SM, Yeh ET and Runge MS (2001). p47phox is required for atherosclerotic lesion progression in ApoE(-/-) mice. *J Clin Invest* 108(10): 1513-22.

Bartberger MD, Liu W, Ford E, Miranda K, Switzer CH and Fukuto JM (2002). The reduction potential of nitric oxide (NO) and it importance to NO biochemistry. *Proc Natl Acad Sci U S A* 99: 10958-10963.

Bea F, Blessing E, Bennett B, Levitz M, Wallace EP and Rosenfeld ME (2002). Simvastatin promotes atherosclerotic plaque stability in apoE-deficient mice independently of lipid lowering. *Arterioscler Thromb Vasc Biol* 22(11): 1832-7.

Bermejo E, Saenz DA, Alberto F, Rosenstein RE, Bari SE and Lazzari MA (2005). Effect of nitroxyl on human platelets function. *Thromb Haemost* 94(3): 578-84.

Bischoff E and Stasch JP (2004). Effects of the sGC stimulator BAY 41-2272 are not mediated by phosphodiesterase 5 inhibition. *Circulation* 110(12): e320-1; author reply e320-1.

Boerrigter G and Burnett JC, Jr. (2007). Nitric oxide-independent stimulation of soluble guanylate cyclase with BAY 41-2272 in cardiovascular disease. *Cardiovasc Drug Rev* 25(1): 30-45.

Bond AR and Jackson CL (2011). The fat-fed apolipoprotein E knockout mouse brachiocephalic artery in the study of atherosclerotic plaque rupture. *J Biomed Biotechnol* 2011: 379069.

Cardioxyl Pharmaceuticals (2014). Cardioxyl pharmaceuticals initiates first clinical trial of CXL-1427. Lacey-Moreira J. Chapel Hill, NC, PressComm PR, LLC.

Chan CT, Moore JP, Budzyn K, Guida E, Diep H, Vinh A, Jones ES, Widdop RE, Armitage JA, Sakkal S, Ricardo SD, Sobey CG and Drummond GR (2012). Reversal of vascular macrophage accumulation and hypertension by a CCR2 antagonist in deoxycorticosterone/salt-treated mice. *Hypertension* 60(5): 1207-12.

Chauhan S, Rahman A, Nilsson H, Clapp L, MacAllister R and Ahluwalia A (2003). NO contributes to EDHF-like responses in rat small arteries: a role for NO stores. *Cardiovasc Res* 57(1): 207-16.

Chen CA, Wang TY, Varadharaj S, Reyes LA, Hemann C, Talukder MA, Chen YR, Druhan LJ and Zweier JL (2010). S-glutathionylation uncouples eNOS and regulates its cellular and vascular function. *Nature* 468(7327): 1115-8.

Chen Z, Zhang J and Stamler JS (2002). Identification of the enzymatic mechanism of nitroglycerin bioactivation. *Proc Natl Acad Sci U S A* 99(12): 8306-11.

Cheong E, Tumbev V, Abramson J, Salama G and Stoyanovsky DA (2005). Nitroxyl triggers Ca^{2+} release from skeletal and cardiac sarcoplasmic reticulum by oxidizing ryanodine receptors. *Cell Calcium* 37: 87-96.

Chin KY, Qin C, Cao N, Kemp-Harper BK, Woodman OL and Ritchie RH (2014). The concomitant coronary vasodilator and positive inotropic actions of the nitroxyl donor Angeli's salt in the intact rat heart: contribution of soluble guanylyl cyclase-dependent and -independent mechanisms. *Br J Pharmacol* 171(7): 1722-34.

Chinetti-Gbaguidi G, Baron M, Bouhlel MA, Vanhoutte J, Copin C, Sebti Y, Derudas B, Mayi T, Bories G, Tailleux A, Haulon S, Zawadzki C, Jude B and Staels B (2011). Human atherosclerotic plaque alternative macrophages display low cholesterol handling but high phagocytosis because of distinct activities of the PPARgamma and LXRalpha pathways. *Circ Res* 108(8): 985-95.

Chirkov YY, Chirkova LP and Horowitz JD (1996). Suppressed anti-aggregating and cGMP-elevating effects of sodium nitroprusside in platelets from patients with stable angina pectoris. *Naunyn Schmiedebergs Arch Pharmacol* 354(4): 520-5.

Chirkov YY, De Sciscio M, Sverdlov AL, Leslie S, Sage PR and Horowitz JD (2010). Hydralazine does not ameliorate nitric oxide resistance in chronic heart failure. *Cardiovasc Drugs Ther* 24(2): 131-7.

Chirkov YY, Holmes AS, Chirkova LP and Horowitz JD (1999). Nitrate resistance in platelets from patients with stable angina pectoris. *Circulation* 100(2): 129-34.

Chirkov YY, Holmes AS, Martelli JD and Horowitz JD (2004). Effect of perindopril on platelet nitric oxide resistance in patients with chronic heart failure secondary to ischemic left ventricular dysfunction. *Am J Cardiol* 93(11): 1438-40, A10.

Chirkov YY, Holmes AS, Willoughby SR, Stewart S and Horowitz JD (2002). Association of aortic stenosis with platelet hyperaggregability and impaired responsiveness to nitric oxide. *Am J Cardiol* 90(5): 551-4.

Chirkov YY, Holmes AS, Willoughby SR, Stewart S, Wuttke RD, Sage PR and Horowitz JD (2001). Stable angina and acute coronary syndromes are associated with nitric oxide resistance in platelets. *J Am Coll Cardiol* 37(7): 1851-7.

Chlopicki S, Olszanecki R, Janiszewski M, Laurindo FR, Panz T and Miedzobrodzki J (2004). Functional role of NADPH oxidase in activation of platelets. *Antioxid Redox Signal* 6(4): 691-8.

Choe CU, Lewerenz J, Fischer G, Uliasz TF, Espey MG, Hummel FC, King SB, Schwedhelm E, Boger RH, Gerloff C, Hewett SJ, Magnus T and Donzelli S (2009). Nitroxyl exacerbates ischemic cerebral injury and oxidative neurotoxicity. *J Neurochem* 110(6): 1766-73.

Crane MS, Rossi AG and Megson IL (2005). A potential role for extracellular nitric oxide generation in cGMP-independent inhibition of human platelet aggregation: biochemical and pharmacological considerations. *Br J Pharmacol* 144(6): 849-59.

Crauwels HM, Van Hove CE, Holvoet P, Herman AG and Bult H (2003). Plaque-associated endothelial dysfunction in apolipoprotein E-deficient mice on a regular diet. Effect of human apolipoprotein AI. *Cardiovasc Res* 59(1): 189-99.

d'Uscio LV, Baker TA, Mantilla CB, Smith L, Weiler D, Sieck GC and Katusic ZS (2001). Mechanism of endothelial dysfunction in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol.* 21: 1017-1022.

d'Uscio LV, Smith LA and Katusic ZS (2001). Hypercholesterolemia impairs endothelium-dependent relaxations in common carotid arteries of apolipoprotein e-deficient mice. *Stroke* 32(11): 2658-64.

Dai T, Tian Y, Tocchetti CG, Katori T, Murphy AM, Kass DA, Paolocci N and Gao WD (2007). Nitroxyl increases force development in rat cardiac muscle. *Journal of Physiology* 580: 951-960.

Daiber A, Wenzel P, Oelze M and Munzel T (2008). New insights into bioactivation of organic nitrates, nitrate tolerance and cross-tolerance. *Clin Res Cardiol* 97(1): 12-20.

Daiber A, Wenzel P, Oelze M, Schuhmacher S, Jansen T and Munzel T (2009). Mitochondrial aldehyde dehydrogenase (ALDH-2)--maker of and marker for nitrate tolerance in response to nitroglycerin treatment. *Chem Biol Interact* 178(1-3): 40-7.

Dautov RF, Ngo DT, Licari G, Sverdlov AL, Ritchie RH, Kemp-Harper BK, Horowitz JD and Chirkov YY (2013). The nitric oxide redox sibling nitroxyl partially circumvents impairement of platelet nitric oxide responsiveness. *Nitric Oxide* 35: 72-78.

DeMaster EG, Redfern B and Nagasawa HT (1998). Mechanisms of inhibition of aldehyde dehydrogenase by nitroxyl, the active metabolite of the alcohol deterrent agent cyanamide. *Biochem Pharmacol* 55(12): 2007-15.

Demoncheaux EA, Foster PJ, Borland CD, Smith AP, Higenbottam TW and Davies MB (2003). Determination of trace concentrations of dissolved nitric oxide in a biological buffer. *Analyst* 128(10): 1281-5.

Dharmarajah J, Arthur JF, Sobey CG and Drummond GR (2010). The anti-platelet effects of apocynin in mice are not mediated by inhibition of NADPH oxidase activity. *Naunyn Schmiedebergs Arch Pharmacol* 382(4): 377-84.

Dierks EA and Burstyn JN (1996). Nitric oxide (NO), the only nitrogen monoxide redox form capable of activating soluble guanylyl cyclase. *Biochem Pharmacol* 51(12): 1593-600.

Donzelli S, Espey MG, Flores-Santana W, Switzer CH, Yeh GC, Huang J, Stuehr DJ, King SB, Miranda KM and Wink DA (2008). Generation of nitroxyl by heme protein-mediated peroxidation of hydroxylamine but not N-hydroxy-L-arginine. *Free Radic Biol Med* 45(5): 578-84.

Donzelli S, Espey MG, Thomas DD, Mancardi D, Tocchetti CG, Ridnour LA, Paolocci N, King SB, Miranda K, Lazzarino G, Fukuto JM and Wink DA (2006). Discriminating formation of HNO from other reactive nitrogen oxide species. *Free Radic Biol Med* 40(6): 1056-1066.

Donzelli S, Fischer G, King BS, Niemann C, Dumond JF, Heeren J, Wieboldt H, Baldus S, Gerloff C, Eschenhagen T, Carrier L, Boger RH and Espey MG (2013). Pharmacological characterization of 1-nitrosocyclohexyl acetate, a long acting nitroxyl donor that shows vasorelaxant and anti-aggregator effect. *J Pharmacol Exp Ther* 344(2): 339-47.

Douglas G, Bendall JK, Crabtree MJ, Tatham AL, Carter EE, Hale AB and Channon KM (2012). Endothelial-specific Nox2 overexpression increases vascular superoxide and macrophage recruitment in ApoE(-)/(-) mice. *Cardiovasc Res* 94(1): 20-9.

Drummond GR, Selemidis S, Griendling KK and Sobey CG (2011). Combating oxidative stress in vascular disease: NADPH oxidases as therapeutic targets. *Nat Rev Drug Discov* 10(6): 453-71.

DuMond JF and King SB (2011). The chemistry of nitroxyl-releasing compounds. *Antioxid Redox Signal* 14(9): 1637-48.

El-Armouche A, Wahab A, Wittkopper K, Schulze T, Bottcher F, Pohlmann L, King SB, DuMond JF, Gerloff C, Boger RH, Eschenhagen T, Carrier L and Donzelli S (2010). The new HNO donor, 1-nitrosocyclohexyl acetate, increases contractile force in normal and beta-adrenergically desensitized ventricular myocytes. *Biochem Biophys Res Commun* 402(2): 340-4.

Ellis A, Li CG and Rand MJ (2000). Differential actions of L-cysteine on responses to nitric oxide, nitroxyl anions and EDRF in the rat aorta. *British Journal of Pharmacology* 129: 315-322.

Fathi AR, Bakhtian KD and Pluta RM (2011). The role of nitric oxide donors in treating cerebral vasospasm after subarachnoid hemorrhage. *Acta Neurochir Suppl* 110(Pt 1): 93-7.

Favaloro JL and Kemp-Harper B (2009). Redox variants of NO (NO. and HNO) elict vasorelaxation of resistance arteries via distinct mechanisms. *Am J Physiol Heart Circ Physiol* 296: H1274-H1280.

Favaloro JL and Kemp-Harper BK (2007). The nitroxyl anion (HNO) is a potent dilator of rat coronary vasculature. *Cardiovasc Res* 73(3): 587-96.

Fenyo IM and Gafencu AV (2013). The involvement of the monocytes/macrophages in chronic inflammation associated with atherosclerosis. *Immunobiology* 218(11): 1376-84.

Feron O, Dessy C, Moniotte S, Desager JP and Balligand JL (1999). Hypercholesterolemia decreases nitric oxide production by promoting the interaction of caveolin and endothelial nitric oxide synthase. *J Clin Invest* 103(6): 897-905.

Flores-Santana W, Salmon DJ, Donzelli S, Switzer CH, Basudhar D, Ridnour L, Cheng R, Glynn SA, Paolocci N, Fukuto JM, Miranda KM and Wink DA (2011). The specificity of nitroxyl chemistry is unique among nitrogen oxides in biological systems. *Antioxid Redox Signal* 14(9): 1659-74.

Forstermann U and Sessa WC (2012). Nitric oxide synthases: regulation and function. *Eur Heart J* 33(7): 829-37, 837a-837d.

Froehlich JP, Mahaney JE, Keceli G, Pavlos CM, Goldstein R, Redwood AJ, Sumbilla C, Lee DI, Tocchetti CG, Kass DA, Paolocci N and Toscano JP (2008). Phospholamban thiols play a central role in activation of the cardiac muscle sarcoplasmic reticulum calcium pump by nitroxyl. *Biochemistry* 47(50): 13150-2.

Fujii M, Inoguchi T, Batchuluun B, Sugiyama N, Kobayashi K, Sonoda N and Takayanagi R (2013). CTLA-4Ig immunotherapy of obesity-induced insulin resistance by manipulation of macrophage polarization in adipose tissues. *Biochem Biophys Res Commun* 438(1): 103-9.

Fukuto JM, Bartberger MD, Dutton AS, Paolocci N, Wink DA and Houk KN (2005). The Physiological Chemisty and Biological Activity of Nitroxyl (HNO): The Neglected, Misunderstood, and Enigmatic Nitrogen Oxide. *Chemical Research in Toxicology* 18(5): 790-801.

Fukuto JM and Carrington SJ (2011). HNO signaling mechanisms. *Antioxid Redox Signal* 14(9): 1649-57.

Fukuto JM, Chiang K, Hszieh R, Wong P and Chaudhuri G (1992). The pharmacological activity of nitroxyl: a potent vasodilator with activity similar to nitric oxide and/or endothelium-derived relaxing factor. *J Pharmacol Exp Ther* 263(2): 546-51.

Fukuto JM, Cisneros CJ and Kinkade RL (2013). A comparison of the chemistry associated with the biological signaling and actions of nitroxyl (HNO) and nitric oxide (NO). *J Inorg Biochem* 118: 201-8.

Fukuto JM, Wallace GC, Hszieh R and Chaudhuri G (1992). Chemical oxidation of N-hydroxyguanidine compounds. Release of nitric oxide, nitroxyl and possible relationship to the mechanism of biological nitric oxide generation. *Biochem Pharmacol* 43(3): 607-13.

Gao WD, Murray CI, Tian Y, Zhong X, DuMond JF, Shen X, Stanley BA, Foster DB, Wink DA, King SB, Van Eyk JE and Paolocci N (2012). Nitroxyl-mediated disulfide bond formation between cardiac myofilament cysteines enhances contractile function. *Circ Res* 111(8): 1002-11.

Gaspari T, Welungoda I, Widdop RE, Simpson RW and Dear AE (2013). The GLP-1 receptor agonist liraglutide inhibits progression of vascular disease via effects on atherogenesis, plaque stability and endothelial function in an ApoE(-/-) mouse model. *Diab Vasc Dis Res* 10(4): 353-60.

Getz GS and Reardon CA (2012). Animal models of atherosclerosis. *Arterioscler Thromb Vasc Biol* 32(5): 1104-15.

Gkaliagkousi E, Ritter J and Ferro A (2007). Platelet-derived nitric oxide signaling and regulation. *Circ Res* 101(7): 654-62.

Gordon S (2003). Alternative activation of macrophages. *Nat Rev Immunol* 3(1): 23-35.

Gordon S and Martinez FO (2010). Alternative activation of macrophages: mechanism and functions. *Immunity* 32(5): 593-604.

Gouya G, Arrich J, Wolzt M, Huber K, Verheugt FW, Gurbel PA, Pirker-Kees A and Siller-Matula JM (2014). Antiplatelet treatment for prevention of cerebrovascular events in patients with vascular diseases: a systematic review and meta-analysis. *Stroke* 45(2): 492-503.

Grätzel M, Taniguchi S and Henglein A (1970). Pulsradiolytische Untersuchung kurzlebiger Zwischenprodukte der NO-Reduktion in wassriger Losung. *Ber-Bunsen-Ges. Phys. Chem.* 74(1003).

Gray SP, Di Marco E, Okabe J, Szyndralewiez C, Heitz F, Montezano AC, de Haan JB, Koulis C, El-Osta A, Andrews KL, Chin-Dusting JP, Touyz RM, Wingler K, Cooper ME, Schmidt HH and Jandeleit-Dahm KA (2013). NADPH oxidase 1 plays a key role in diabetes mellitus-accelerated atherosclerosis. *Circulation* 127(18): 1888-902.

Gudmundsdottir IJ, McRobbie SJ, Robinson SD, Newby DE and Megson IL (2005). Sildenafil potentiates nitric oxide mediated inhibition of human platelet aggregation. *Biochem Biophys Res Commun* 337(1): 382-5.

Guzik TJ, Chen W, Gongora MC, Guzik B, Lob HE, Mangalat D, Hoch N, Dikalov S, Rudzinski P, Kapelak B, Sadowski J and Harrison DG (2008). Calcium-dependent NOX5 nicotinamide adenine dinucleotide phosphate oxidase contributes to vascular oxidative stress in human coronary artery disease. *J Am Coll Cardiol* 52(22): 1803-9.

Guzik TJ, Sadowski J, Guzik B, Jopek A, Kapelak B, Przybylowski P, Wierzbicki K, Korbut R, Harrison DG and Channon KM (2006). Coronary artery superoxide production and nox isoform expression in human coronary artery disease. *Arterioscler Thromb Vasc Biol* 26(2): 333-9.

Hammond AH and Fry JR (1999). Effect of cyanamide on toxicity and glutathione depletion in rat hepatocyte cultures: differences between two dichloropropanol isomers. *Chem Biol Interact* 122(2): 107-15.

Haramaki N, Ikeda H, Takenaka K, Katoh A, Sugano R, Yamagishi S, Matsuoka H and Imaizumi T (2007). Fluvastatin alters platelet aggregability in patients with hypercholesterolemia: possible improvement of intraplatelet redox imbalance via HMG-CoA reductase. *Arterioscler Thromb Vasc Biol* 27(6): 1471-7.

Hirata Y, Tabata M, Kurobe H, Motoki T, Akaike M, Nishio C, Higashida M, Mikasa H, Nakaya Y, Takanashi S, Igarashi T, Kitagawa T and Sata M (2011). Coronary atherosclerosis is associated with macrophage polarization in epicardial adipose tissue. *J Am Coll Cardiol* 58(3): 248-55.

Hobbs AJ, Fukuto JM and Ignarro LJ (1994). Formation of free nitric oxide from L-arginine by nitric oxide synthase: Direct enhancement of generation by superoxide dismutase. *Proc Natl Acad Sci U S A* 91: 10992-10996.

Hobbs AJ and Moncada S (2003). Antiplatelet properties of a novel, non-NO-based soluble guanylate cyclase activator, BAY 41-2272. *Vascul Pharmacol* 40(3): 149-54.

Hoffman MD, Walsh GM, Rogalski JC and Kast J (2009). Identification of nitroxyl-induced modifications in human platelet proteins using a novel mass spectrometric detection method. *Mol Cell Proteomics* 8(5): 887-903.

Horowitz JD (2000). Nitrovasodilators. Nitric oxide and the cardiovascular system. Loscalzo J and Vita JA. Totowa, New Jersey, Humana Press**:** 397-400.

Hsich E, Segal BH, Pagano PJ, Rey FE, Paigen B, Deleonardis J, Hoyt RF, Holland SM and Finkel T (2000). Vascular effects following homozygous disruption of p47(phox) : An essential component of NADPH oxidase. *Circulation* 101(11): 1234-6.

Huang PL (2009). eNOS, metabolic syndrome and cardiovascular disease. *Trends Endocrinol Metab* 20(6): 295-302.

Hughes MN (1999). Relationships between nitric oxide, nitroxyl ion, nitrosonium cation and peroxynitrite. *Biochim Biophys Acta* 1411(2-3): 263-72.

Hughes MN and Cammack R (1999). Synthesis, chemistry, and applications of nitroxyl ion releasers sodium trioxodinitrate (Angeli's salt) and Piloty's acid. *Methods in Enzymology* 310: 279-287.

Hughes MN and Cammack R (1999). Synthesis, chemistry, and applications of nitroxyl ion releasers sodium trioxodinitrate or Angeli's salt and Piloty's acid. *Methods Enzymol* 301: 279-87.

Irvine J, Kemp-Harper BK and Widdop RE (2010). Chronic administration of the HNO donor, Angeli's salt does not lead to tolerance, cross-tolerance or endothelial dysfunction: comparison with GTN and DEA/NO. *Antioxid Redox Signal* 14(9): 1615-24.

Irvine J, Kemp-Harper BK and Widdop RE (2011). Chronic administration of the HNO donor, Angeli's salt does not lead to tolerance, cross-tolerance or endothelial dysfunction: comparison with GTN and DEA/NO. *Antioxid Redox Signal* 14(9): 1615-24.

Irvine JC, Favaloro JL and Kemp-Harper BK (2003). NO- activates soluble guanylate cyclase and Kv channels to vasodilate resistance arteries. *Hypertension* 41(6): 1301-7.

Irvine JC, Favaloro JL, Widdop RE and Kemp-Harper BK (2007). Nitroxyl anion donor, Angeli's salt, does not develop tolerance in rat isolated aortae. *Hypertension* 49(4): 885-92.

Irvine JC, Kemp-Harper BK and Widdop RE (2010). Chronic administration of the HNO donor Angeli's salt does not lead to tolerance, cross-tolerance, or endothelial dysfunction: comparison with GTN and DEA/NO. *Antioxid Redox Signal* 14(9): 1615-24.

Irvine JC, Ravi RM, Kemp-Harper BK and Widdop RE (2013). Nitroxyl donors retain their depressor effects in hypertension. *Am J Physiol Heart Circ Physiol* 305(6): H939-45.

Irvine JC, Ritchie RH, Favaloro JL, Andrews KL, Widdop RE and Kemp-Harper BK (2008). Nitroxyl (HNO): the Cinderella of the nitric oxide story. *Trends Pharmacol Sci* 29(12): 601-8.

Jang EK, Azzam JE, Dickinson NT, Davidson MM and Haslam RJ (2002). Roles for both cyclic GMP and cyclic AMP in the inhibition of collagen-induced platelet aggregation by nitroprusside. *Br J Haematol* 117(3): 664-75.

Judkins CP, Deip H, Broughton B, Mast AE, Hooker EU, Miller AA, Selemidis S, Dusting GJ, Sobey CG and Drummond GR (2010). Direct evidence of a role for Nox2 in superoxide production, reduced nitric oxide bioavailability and early atherosclerotic plaque formation in $ApoE^{-/-}$ mice. *Am J Physiol Heart Circ* 298(1): H24-32.

Kawai K, Ieda N, Aizawa K, Suzuki T, Miyata N and Nakagawa H (2013). A reductant-resistant and metal-free fluorescent probe for nitroxyl applicable to living cells. *J Am Chem Soc* 135(34): 12690-6.

Keceli G, Moore CD, Labonte JW and Toscano JP (2013). NMR detection and study of hydrolysis of HNO-derived sulfinamides. *Biochemistry* 52(42): 7387-96.

Keefer LK (2003). Progress toward clinical application of the nitric oxide-releasing diazeniumdiolates. *Annu Rev Pharmacol Toxicol* 43: 585-607.

Kemp-Harper B and Schmidt HH (2008). cGMP in the Vasculature. cGMP: Generators, Effectors and Therapeutic Applications. Schmidt HH. Berlin, Springer-Verlag. **191:** 447-467.

Kemp-Harper BK (2011). Nitroxyl (HNO): a novel redox signaling molecule. *Antioxid Redox Signal* 14(9): 1609-13.

Khallou-Laschet J, Varthaman A, Fornasa G, Compain C, Gaston AT, Clement M, Dussiot M, Levillain O, Graff-Dubois S, Nicoletti A and Caligiuri G (2010). Macrophage plasticity in experimental atherosclerosis. *PLoS One* 5(1): e8852.

Kim SY, Lee JG, Cho WS, Cho KH, Sakong J, Kim JR, Chin BR and Baek SH (2010). Role of NADPH oxidase-2 in lipopolysaccharide-induced matrix metalloproteinase expression and cell migration. *Immunol Cell Biol* 88(2): 197-204.

Kim WK, Choi YB, Rayudu PV, Das P, Asaad W, Arnelle DR, Stamler JS and Lipton SA (1999). Attenuation of NMDA receptor activity and neurotoxicity by nitroxyl anion, NO. *Neuron* 24(2): 461-9.

Kirk EA, Dinauer MC, Rosen H, Chait A, Heinecke JW and LeBoeuf RC (2000). Impaired superoxide production due to a deficiency in phagocyte NADPH oxidase fails to inhibit atherosclerosis in mice. *Arterioscler Thromb Vasc Biol* 20(6): 1529-35.

Klemenska E and Beresewicz A (2009). Bioactivation of organic nitrates and the mechanism of nitrate tolerance. *Cardiol J* 16(1): 11-9.

Kolovou G, Anagnostopoulou K, Mikhailidis DP and Cokkinos DV (2008). Apolipoprotein E knockout models. *Curr Pharm Des* 14(4): 338-51.

Konior A, Schramm A, Czesnikiewicz-Guzik M and Guzik TJ (2013). NADPH oxidases in vascular pathology. *Antioxid Redox Signal* 20(17): 2794-814.

Lale A, Herbert JM and Savi P (2003). The antiaggregating activity of clopidogrel is not affected by N-acetyl L-cysteine. *Thromb Haemost* 90(5): 839-43.

Lancel S, Zhang J, Evangelista A, Trucillo MP, Tong X, Siwik DA, Cohen RA and Colucci WS (2009). Nitroxyl activates SERCA in cardiac myocytes via glutathiolation of cysteine 674. *Circ Res* 104(6): 720-3.

Landmesser U, Hornig B and Drexler H (2004). Endothelial Dysfunction A Critical Determinant in Atherosclerosis? *Circulation* 109: II-27-II-33.

Laurindo FR, Araujo TL and Abrahao TB (2014). Nox NADPH oxidases and the endoplasmic reticulum. *Antioxid Redox Signal* 20(17): 2755-75.

Leitinger N and Schulman IG (2013). Phenotypic polarization of macrophages in atherosclerosis. *Arterioscler Thromb Vasc Biol* 33(6): 1120-6.

Leo CH, Joshi A, Hart JL and Woodman OL (2012). Endothelium-dependent nitroxyl-mediated relaxation is resistant to superoxide anion scavenging and preserved in diabetic rat aorta. *Pharmacol Res* 66(5): 383-91.

Li CG, Karagiannis J and Rand MJ (1999). Comparison of the redox forms of nitrogen monoxide with the nitrergic transmitter in the rat anococcygeus muscle. *Br J Pharmacol* 127(4): 826-34.

Li G, Scull C, Ozcan L and Tabas I (2010). NADPH oxidase links endoplasmic reticulum stress, oxidative stress, and PKR activation to induce apoptosis. *J Cell Biol* 191(6): 1113-25.

Li Z, Ajdic J, Eigenthaler M and Du X (2003). A predominant role for cAMP-dependent protein kinase in the cGMP-induced phosphorylation of vasodilator-stimulated phosphoprotein and platelet inhibition in humans. *Blood* 101(11): 4423-9.

Libby P (2008). The molecular mechanisms of the thrombotic complications of atherosclerosis. *J Intern Med* 263(5): 517-27.

Libby P (2012). Inflammation in atherosclerosis. *Arterioscler Thromb Vasc Biol* 32(9): 2045-51.

Libby P, Lichtman AH and Hansson GK (2013). Immune effector mechanisms implicated in atherosclerosis: from mice to humans. *Immunity* 38(6): 1092-104.

Libby P, Ridker PM and Hansson GK (2011). Progress and challenges in translating the biology of atherosclerosis. *Nature* 473(7347): 317-25.

Lies B, Groneberg D, Gambaryan S and Friebe A (2013). Lack of effect of ODQ does not exclude cGMP signalling via NO-sensitive guanylyl cyclase. *Br J Pharmacol* 170(2): 317-27.

Lin EQ, Irvine JC, Cao AH, Alexander AE, Love JE, Patel R, McMullen JR, Kaye DM, Kemp-Harper BK and Ritchie RH (2012). Nitroxyl (HNO) stimulates soluble guanylyl cyclase to suppress cardiomyocyte hypertrophy and superoxide generation. *PLoS One* 7(4): e34892.

Lopez BE, Rodriguez CE, Pribadi M, Cook NM, Shinyashiki M and Fukuto JM (2005). Inhibition of yeast glycolysis by nitroxyl (HNO): mechanism of HNO toxicity and implications to HNO biology. *Arch Biochem Biophys* 442(1): 140-8.

Lopez Farre A and Casado S (2001). Heart failure, redox alterations, and endothelial dysfunction. *Hypertension* 38(6): 1400-5.

Ma XL, Gao F, Liu GL, Lopez BL, Christopher TA, Fukuto JM, Wink DA and Feelisch M (1999). Opposite effects of nitric oxide and nitroxyl on postischemic myocardial injury. *Proc Natl Acad Sci U S A* 96(25): 14617-22.

Maragos CM, Morley D, Wink DA, Dunams TM, Saavedra JE, Hoffman A, Bove AA, Isaac L, Hrabie JA and Keefer LK (1991). Complexes of .NO with nucleophiles as agents for the controlled biological release of nitric oxide. Vasorelaxant effects. *J Med Chem* 34(11): 3242-7.

Marti MA, Bari SE, Estrin DA and Doctorovich F (2005). Discrimination of nitroxyl and nitric oxide by water-soluble Mn(III) porphyrins. *J Am Chem Soc* 127(13): 4680-4.

Martin E, Czarnecki K, Jayaraman V, Murad F and Kincaid J (2005). Resonance Raman and infrared spectroscopic studies of high-output forms of human soluble guanylyl cyclase. *J Am Chem Soc* 127(13): 4625-31.

Martin W (2009). Nitroxyl anion--the universal signalling partner of endogenously produced nitric oxide? *Br J Pharmacol* 157(4): 537-9.

Martinez FO, Gordon S, Locati M and Mantovani A (2006). Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J Immunol* 177(10): 7303-11.

Matsumoto T, D'Uscio L V, Eguchi D, Akiyama M, Smith LA and Katusic ZS (2003). Protective effect of chronic vitamin C treatment on endothelial function of apolipoprotein E-deficient mouse carotid artery. *J Pharmacol Exp Ther* 306(1): 103-8.

Maurice DH and Haslam RJ (1990). Molecular basis of the synergistic inhibition of platelet function by nitrovasodilators and activators of adenylate cyclase: inhibition of cyclic AMP breakdown by cyclic GMP. *Mol Pharmacol* 37(5): 671-81.

Melichar VO, Behr-Roussel D, Zabel U, Uttenthal LO, Rodrigo J, Rupin A, Verbeuren TJ, Kumar HSA and Schmidt HH (2004). Reduced cGMP signaling associated with neointimal proliferation and vascular dysfunction in late-stage atherosclerosis. *Proc Natl Acad Sci U S A* 101(47): 16671-6.

Meyrelles SS, Peotta VA, Pereira TM and Vasquez EC (2011). Endothelial dysfunction in the apolipoprotein E-deficient mouse: insights into the influence of diet, gender and aging. *Lipids Health Dis* 10: 211.

Mihaylova B, Emberson J, Blackwell L, Keech A, Simes J, Barnes EH, Voysey M, Gray A, Collins R and Baigent C (2012). The effects of lowering LDL cholesterol with statin therapy in people at low risk of vascular disease: meta-analysis of individual data from 27 randomised trials. *Lancet* 380(9841): 581-90.

Miller AA, De Silva TM, Judkins CP, Diep H, Drummond GR and Sobey CG (2010). Augmented superoxide production by Nox2-containing NADPH oxidase causes cerebral artery dysfunction during hypercholesterolemia. *Stroke* 41(4): 784-9.

Miller AA, Drummond GR, De Silva TM, Mast AE, Hickey H, Williams JP, Broughton BR and Sobey CG (2009). NADPH oxidase activity is higher in cerebral versus systemic arteries of four animal species: role of Nox2. *Am J Physiol Heart Circ Physiol* 296(1): H220-5.

Miller AA, Drummond GR, Schmidt HH and Sobey CG (2005). NADPH-oxidase activity and function are profoundly greater in cerebral versus systemic arteries. *Circ Res* 97: 1055-1062.

Miller AA, Maxwell, K.F., Bullen, M.L., Ku, J.M., De Silva, T.M., Selemidis, S., Hooker, E.U., Drummond, G.R., Sobey, C.G., Kemp-Harper, B.K., (2013). Nitroxyl (HNO) suppresses vascular Nox2 oxidase activity. *Free Radic Biol Med* 60: 264-71.

Miller TW, Cherney MM, Lee AJ, Francoleon NE, Farmer PJ, King SB, Hobbs AJ, Miranda KM, Burstyn JN and Fukuto JM (2009). The effects of nitroxyl (HNO) on soluble guanylate cyclase activity: interactions at ferrous heme and cysteine thiols. *J Biol Chem* 284(33): 21788-21796.

Miranda K (2005). The chemistry of nitroxyl (HNO) and implications in biology. *Coordination Chemistry Reviews* 249(3-4): 433-455.

Miranda K, Paolocci N, Katori T, Thomas DD, Ford E, Bartberger MD, Espey MG, Kass DA, Feelisch M, Fukuto JM and Wink DA (2003). A biochemical rationale for the discrete behavior of nitroxyl and nitric oxide in the cardiovascular system. *Proc Natl Acad Sci U S A* 100(16): 9196-9201.

Miranda KM, Katori T, Torres de Holding CL, Thomas L, Ridnour LA, McLendon WJ, Cologna SM, Dutton AS, Champion HC, Mancardi D, Tocchetti CG, Saavedra JE, Keefer LK, Houk KN, Fukuto JM, Kass DA, Paolocci N and Wink DA (2005). Comparison of the NO and HNO donating properties of diazeniumdiolates: primary amine adducts release HNO in Vivo. *J Med Chem* 48(26): 8220-8.

Miranda KM, Nagasawa HT and Toscano JP (2005). Donors of HNO. *Curr Top Med Chem* 5(7): 649-64.

Miranda KM, Nims RW, Thomas DD, Espey MG, Citrin D, Bartberger MD, Paolocci N, Fukuto JM, Feelisch M and Wink DA (2003). Comparison of the reactivity of nitric oxide and nitroxyl with heme proteins. A chemical discussion of the differential biological effects of these redox related products of NOS. *J Inorg Biochem* 93(1-2): 52-60.

Miranda KM, Yamada K, Espey MG, Thomas DD, DeGraff W, Mitchell JB, Krishna MC, Colton CA and Wink DA (2002). Further evidence for distinct reactive intermediates from nitroxyl and peroxynitrite: effects of buffer composition on the chemistry of Angeli's salt and synthetic peroxynitrite. *Arch Biochem Biophys* 401(2): 134-44.

Mitani H, Egashira K and Kimura M (2003). HMG-CoA reductase inhibitor, fluvastatin, has cholesterol-lowering independent "direct" effects on atherosclerotic vessels in high cholesterol diet-fed rabbits. *Pharmacol Res* 48(5): 417-27.

Moncada S and Higgs EA (2006). The discovery of nitric oxide and its role in vascular biology. *Br J Pharmacol* 147(Supplement 1): S193-S201.

Mondoro TH, Ryan BB, Hrinczenko BW, Schechter AN, Vostal JG and Alayash AI (2001). Biological action of nitric oxide donor compounds on platelets from patients with sickle cell disease. *Br J Haematol* 112(4): 1048-54.

Monteiro PF, Morganti RP, Delbin MA, Calixto MC, Lopes-Pires ME, Marcondes S, Zanesco A and Antunes E (2012). Platelet hyperaggregability in high-fat fed rats: a role for intraplatelet reactive-oxygen species production. *Cardiovasc Diabetol* 11: 5.

Moore JP, Sakkal S, Bullen ML, Kemp-Harper BK, Ricardo SD, Sobey CG and Drummond GR (2013). A flow cytometric method for the analysis of macrophages in the vascular wall. *J Immunol Methods* 396(1-2): 33-43.

Morganti RP, Cardoso MH, Pereira FG, Lorand-Metze I, De Nucci G, Marcondes S and Antunes E (2010). Mechanisms underlying the inhibitory effects of lipopolysaccharide on human platelet adhesion. *Platelets* 21(4): 260-9.

Mullershausen F, Russwurm M, Friebe A and Koesling D (2004). Inhibition of phosphodiesterase type 5 by the activator of nitric oxide-sensitive guanylyl cyclase BAY 41-2272. *Circulation* 109(14): 1711-3.

Naghavi M, Libby P, Falk E, Casscells SW, Litovsky S, Rumberger J, Badimon JJ, Stefanadis C, Moreno P, Pasterkamp G, Fayad Z, Stone PH, Waxman S, Raggi P, Madjid M, Zarrabi A, Burke A, Yuan C, Fitzgerald PJ, Siscovick DS, de Korte CL, Aikawa M, Juhani Airaksinen KE, Assmann G, Becker CR, Chesebro JH, Farb A, Galis ZS, Jackson C, Jang IK, Koenig W, Lodder RA, March K, Demirovic J, Navab M, Priori SG, Rekhter MD, Bahr R, Grundy SM, Mehran R, Colombo A, Boerwinkle E, Ballantyne C, Insull W, Jr., Schwartz RS, Vogel R, Serruys PW, Hansson GK, Faxon DP, Kaul S, Drexler H, Greenland P, Muller JE, Virmani R, Ridker PM, Zipes DP, Shah PK and Willerson JT (2003). From vulnerable plaque to vulnerable patient: a call for new definitions and risk assessment strategies: Part I. *Circulation* 108(14): 1664-72.

Nelli S, Hillen M, Buyukafsar K and Martin W (2000). Oxidation of nitroxyl anion to nitric oxide by copper ions. *Br J Pharmacol* 131(2): 356-62.

Norris AJ, Sartippour MR, Lu M, Park T, Rao JY, Jackson MI, Fukuto JM and Brooks MN (2008). Nitroxyl inhibits breast tumor growth and angiogenesis. *Int J Cancer* 122(8): 1905-10.

Palmer RM, Ferrige AG and Moncada S (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 327: 524-526.

Paolocci N, Jackson MI, Lopez BE, Miranda K, Tocchetti CG, Wink DA, Hobbs AJ and Fukuto JM (2007). The pharmacology of nitroxyl (HNO) and its therapeutic potential: not just the Janus face of NO. *Pharmacol Ther* 113(2): 442-58.

Paolocci N, Katori T, Champion HC, St. John ME, Miranda K, Fukuto JM, Wink DA and Kass DA (2003). Positive iontropic and lusitropic effects of HNO/NO- in failing hearts: Independence from β-adrenergic signalling. *Proc Natl Acad Sci U S A* 100: 5537-5542.

Paolocci N, Saavedra WF, Miranda KM, Martignani C, Isoda T, Hare JM, Espey MG, Fukuto JM, Feelisch M, Wink DA and Kass DA (2001). Nitroxyl anion exerts redox-sensitive positive cardiac inotropy in vivo by calcitonin gene-related peptide signaling. *Proc Natl Acad Sci U S A* 98(18): 10463-8.

Papaharalambus CA and Griendling KK (2007). Basic mechanisms of oxidative stress and reactive oxygen species in cardiovascular injury. *Trends Cardiovasc Med* 17(2): 48-54.

Park JG and Oh GT (2011). The role of peroxidases in the pathogenesis of atherosclerosis. *BMB Rep* 44(8): 497-505.

Pelegrin P and Surprenant A (2009). Dynamics of macrophage polarization reveal new mechanism to inhibit IL-1beta release through pyrophosphates. *EMBO J* 28(14): 2114-27.

Piedrahita JA, Zhang SH, Hagaman JR, Oliver PM and Maeda N (1992). Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cells. *Proc Natl Acad Sci U S A* 89(10): 4471-5.

Pignatelli P, Pulcinelli FM, Lenti L, Gazzaniga PP and Violi F (1998). Hydrogen peroxide is involved in collagen-induced platelet activation. *Blood* 91(2): 484-90.

Pino RZ and Feelisch M (1994). Bioassay discrimination between nitric oxide (NO.) and nitroxyl (NO-) using L-cysteine. *Biochem Biophys Res Commun* 201(1): 54-62.

Plump AS, Smith JD, Hayek T, Aalto-Setala K, Walsh A, Verstuyft JG, Rubin EM and Breslow JL (1992). Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell* 71(2): 343-53.

Pollock JD, Williams DA, Gifford MA, Li LL, Du X, Fisherman J, Orkin SH, Doerschuk CM and Dinauer MC (1995). Mouse model of X-linked chronic granulomatous disease, an inherited defect in phagocyte superoxide production. *Nat Genet* 9(2): 202-9.

Ponnuswamy P, Schrottle A, Ostermeier E, Gruner S, Huang PL, Ertl G, Hoffmann U, Nieswandt B and Kuhlencordt PJ (2012). eNOS protects from atherosclerosis despite relevant superoxide production by the enzyme in apoE mice. *PLoS One* 7(1): e30193.

Prendergast C, Quayle J, Burdyga T and Wray S (2014). Atherosclerosis affects calcium signalling in endothelial cells from apolipoprotein E knockout mice before plaque formation. *Cell Calcium* 55(3): 146-54.

Pufahl RA, Wishnok JS and Marletta MA (1995). Hydrogen peroxide-supported oxidation of NG-hydroxy-L-arginine by nitric oxide synthase. *Biochemistry* 34(6): 1930-1941.

Rajendran S and Chirkov YY (2008). Platelet hyperaggregability: imparied responsiveness to nitric oxide ("platelet NO resistance") as a therapeutic target. *Cardiovas Drugs Ther* 22: 193-203.

Reisz JA, Zink CN and King SB (2011). Rapid and selective nitroxyl (HNO) trapping by phosphines: kinetics and new aqueous ligations for HNO detection and quantitation. *J Am Chem Soc* 133(30): 11675-85.

Ritchie RH, Lin EQ, Cao AH, Patel R, Kaye DM and Kemp-Harper B (2007). The NO redox sibling, nitroxyl (HNO), blocks cardiomyocyte hypertrophy via suppression of NADPH-oxidase. *Circulation* 116(Supplement 2): S19-S20.

Roger S, Badier-Commander C, Paysant J, Cordi A, Verbeuren TJ and Feletou M (2010). The anti-aggregating effect of BAY 41-2272, a stimulator of soluble guanylyl cyclase, requires the presence of nitric oxide. *Br J Pharmacol* 161(5): 1044-58.

Rosenfeld ME, Averill MM, Bennett BJ and Schwartz SM (2008). Progression and disruption of advanced atherosclerotic plaques in murine models. *Curr Drug Targets* 9(3): 210-6.

Rosenthal J and Lippard SJ (2010). Direct detection of nitroxyl in aqueous solution using a tripodal copper(II) BODIPY complex. *J Am Chem Soc* 132(16): 5536-7.

Rusche KM, Spiering MM and Marletta MA (1998). Reactions catalyzed by tetrahydrobiopterin-free nitric oxide synthase. *Biochemistry* 37: 15503-15512.

Sabbah HN, Tocchetti CG, Wang M, Daya S, Gupta RC, Tunin RS, Mazhari R, Takimoto E, Paolocci N, Cowart D, Colucci WS and Kass DA (2013). Nitroxyl (HNO): A novel approach for the acute treatment of heart failure. *Circ Heart Fail* 6(6): 1250-8.

Schmidt HH, Hoffman MD, Schindler U, Shutenko ZS, Cunningham DD and Feelisch M (1996). No˙NO from NO Synthase. *Proc Natl Acad Sci U S A* 93: 14492-14497.

Schmittgen TD and Livak KJ (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 3(6): 1101-8.

Schrammel A, Behrends S, Schmidt K, Koesling D and Mayer B (1996). Characterization of 1-H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one as a heme-site inhibitor of nitric oxide-sensitive guanylate cyclase. *Molecular Pharmacology* 50: 1-5.

Selemidis S, Dusting GJ, Peshavariya H, Kemp-Harper B and Drummond GR (2007). Nitric Oxide supresses NADPH oxidase-dependent superoxide production by S-nitrosylation in human endothelial cells. *Cardiovasc Res* 75(2): 349-358.

Sha X, Isbell TS, Patel RP, Day CS and King SB (2006). Hydrolysis of acyloxy nitroso compounds yields nitroxyl (HNO). *J Am Chem Soc* 128(30): 9687-92.

Shafirovich V and Lymar SV (2002). Nitroxyl and its anion in aqueous solutions: Spin states, protic equilibria, and reactivities toward oxygen and nitric oxide. 99(11): 7340-7345.

Shaikh S, Brittenden J, Lahiri R, Brown PA, Thies F and Wilson HM (2012). Macrophage subtypes in symptomatic carotid artery and femoral artery plaques. *Eur J Vasc Endovasc Surg* 44(5): 491-7.

Sheehan AL, Carrell S, Johnson B, Stanic B, Banfi B and Miller FJ, Jr. (2011). Role for Nox1 NADPH oxidase in atherosclerosis. *Atherosclerosis* 216(2): 321-6.

Shen B and English AM (2005). Mass spectrometric analysis of nitroxyl-mediated protein modification: comparison of products formed with free and protein-based cysteines. *Biochemistry* 44(42): 14030-44.

Shiva S, Crawford JH, Ramachandran A, Ceaser EK, Hillson T, Brookes PS, Patel RP and Darley-Usmar VM (2004). Mechanisms of the interaction of nitroxyl with mitochondria. *Biochem J* 379(Pt 2): 359-66.

Shoeman DW, Shirota FN, DeMaster EG and Nagasawa HT (2000). Reaction of nitroxyl, an aldehyde dehydrogenase inhibitor, with N-acetyl-L-cysteine. *Alcohol* 20(1): 55-9.

Siegel-Axel D, Daub K, Seizer P, Lindemann S and Gawaz M (2008). Platelet lipoprotein interplay: trigger of foam cell formation and driver of atherosclerosis. *Cardiovasc Res* 78(1): 8-17.

Silvestre-Roig C, de Winther MP, Weber C, Daemen MJ, Lutgens E and Soehnlein O (2014). Atherosclerotic plaque destabilization: mechanisms, models, and therapeutic strategies. *Circ Res* 114(1): 214-26.

Sorescu D, Weiss D and Lasse`gue B (2002). Superoxide production and expression of nox family proteins in human atherosclerosis. *Circulation* 105(1429-1435).

Stasch JP, Becker EM, Alonso-Alija C, Apeler H, Dembowsky K, Feurer A, Gerzer R, Minuth T, Perzborn E, Pleiss U, Schroder H, Schroder W, Stahl E, Steinke W, Straub A and Schramm M (2001). NO-independent regulary site on soluble guanylate cyclase. *Nature* 410(212-215).

Stasch JP, Schmidt PM, Nedvetsky PI, Nedvetskaya TY, H SA, Meurer S, Deile M, Taye A, Knorr A, Lapp H, Muller H, Turgay Y, Rothkegel C, Tersteegen A, Kemp-Harper B, Muller-Esterl W and Schmidt HH (2006). Targeting the heme-oxidized nitric oxide receptor for selective vasodilatation of diseased blood vessels. *J Clin Invest* 116(9): 2552-61.

Stepien JM, Prideaux RM, Willoughby SR, Chirkov YY and Horowitz JD (2003). Pilot study examining the effect of cholesterol lowering on platelet nitric oxide responsiveness and arterial stiffness in subjects with isolated mild hypercholesterolaemia. *Clin Exp Pharmacol Physiol* 30(7): 507-12.

Stoger JL, Gijbels MJ, van der Velden S, Manca M, van der Loos CM, Biessen EA, Daemen MJ, Lutgens E and de Winther MP (2012). Distribution of macrophage polarization markers in human atherosclerosis. *Atherosclerosis* 225(2): 461-8.

Takahira R, Yonemura K, Fujise Y and Hishida A (2001). Dexamethasone attenuates neutrophil infiltration in the rat kidney in ischemia/reperfusion injury: the possible role of nitroxyl. *Free Radic Biol Med* 31(6): 809-15.

Tavakoli S and Asmis R (2012). Reactive oxygen species and thiol redox signaling in the macrophage biology of atherosclerosis. *Antioxid Redox Signal* 17(12): 1785-95.

Thomas SR, Witting PK and Drummond GR (2008). Redox control of endothelial function and dysfunction: molecular mechanisms and therapeutic opportunities. *Antioxid Redox Signal* 10(10): 1713-65.

Tocchetti CG, Stanley BA, Murray CI, Sivakumaran V, Donzelli S, Mancardi D, Pagliaro P, Gao WD, van Eyk J, Kass DA, Wink DA and Paolocci N (2011). Playing with cardiac "redox switches": the "HNO way" to modulate cardiac function. *Antioxid Redox Signal* 14(9): 1687-98.

Tocchetti CG, Wang W, Froehlich JP, Huke S, Aon MA, Wilson GM, Benedetto GD, O'Rourke B, Gao WD, Wink DA, Toscano JP, Zaccolo M, Bers DM, Valdivia HH, Cheng H, Kass DA and Paolocci N (2007). Nitroxyl improves cellular heart function by directly enhancing cardiac sarcoplasmic reticulum Ca^{2+} cycling. *Circ Res* 100: 96-104.

Tsihlis ND, Murar J, Kapadia MR, Ahanchi SS, Oustwani CS, Saavedra JE, Keefer LK and Kibbe MR (2010). Isopropylamine NONOate (IPA/NO) moderates neointimal hyperplasia following vascular injury. *J Vasc Surg* 51(5): 1248-1259.

Vaananen AJ, Kankuri E and Rauhala P (2005). Nitric oxide-related species-induced protein oxidation: reversible, irreversible, and protective effects on enzyme function of papain. *Free Radic Biol Med* 38(8): 1102-11.

Vaananen AJ, Salmenpera P, Hukkanen M, Miranda KM, Harjula A, Rauhala P and Kankuri E (2008). Persistent susceptibility of cathepsin B to irreversible inhibition by nitroxyl (HNO) in the presence of endogenous nitric oxide. *Free Radic Biol Med* 45(6): 749-55.

Vasquez EC, Peotta VA, Gava AL, Pereira TM and Meyrelles SS (2012). Cardiac and vascular phenotypes in the apolipoprotein E-deficient mouse. *J Biomed Sci* 19: 22.

Vendrov AE, Hakim ZS, Madamanchi NR, Rojas M, Madamanchi C and Runge MS (2007). Atherosclerosis is attenuated by limiting superoxide generation in both macrophages and vessel wall cells. *Arterioscler Thromb Vasc Biol* 27(12): 2714-21.

Verbeuren TJ, Coene MC, Jordaens FH, Van Hove CE, Zonnekeyn LL and Herman AG (1986). Effect of hypercholesterolemia on vascular reactivity in the rabbit. II. Influence of treatment with dipyridamole on endothelium-dependent and endothelium-independent responses in isolated aortas of control and hypercholesterolemic rabbits. *Circ Res* 59(5): 496-504.

Violi F, Basili S, Nigro C and Pignatelli P (2009). Role of NADPH oxidase in atherosclerosis. *Future Cardiol* 5(1): 83-92.

Violi F and Pignatelli P (2014). Platelet activation after radiofrequency ablation in atrial fibrillation: is there any clinical implication? *Circulation* 129(1): 5-7.

Wang J, Wang H, Hao P, Xue L, Wei S, Zhang Y and Chen Y (2011). Inhibition of aldehyde dehydrogenase 2 by oxidative stress is associated with cardiac dysfunction in diabetic rats. *Mol Med* 17(3-4): 172-9.

Wanstall JC, Jeffery TK, Gambino A, Lovren F and Triggle CR (2001). Vascular smooth muscle relaxation mediated by nitric oxide donors: a comparison with acetylcholine, nitric oxide and nitroxyl ion. *Br J Pharmacol* 134(3): 463-72.

Willoughby SR, Rajendran S, Chan WP, Procter N, Leslie S, Liberts EA, Heresztyn T, Chirkov YY and Horowitz JD (2012). Ramipril sensitizes platelets to nitric oxide: implications for therapy in high-risk patients. *J Am Coll Cardiol* 60(10): 887-94.

Wink DA, Feelisch M, Fukuto JM, Chistodoulou D, Jourd'heuil D, Grisham MB, Vodovotz Y, Cook JA, Krishna MC, DeGraff W, Kim S, Gamson J and Mitchell JB (1998). The Cytotoxicity of Nitroxyl: Possible Implications for the Pathophysiological Role of NO. *Arch Biochem Biophys* 351(1): 66-74.

Wink DA, Nims RW, Darbyshire JF, Christodoulou D, Hanbauer I, Cox GW, Laval F, Laval J, Cook JA, Krishna MC and et al. (1994). Reaction kinetics for nitrosation of cysteine and glutathione in aerobic nitric oxide solutions at neutral pH. Insights into the fate and physiological effects of intermediates generated in the NO/O2 reaction. *Chem Res Toxicol* 7(4): 519-25.

Wong P, Hyun J, Fukuto JM, Shirota FN, DeMaster EG, Shoeman DW and Nagasawa HT (1998). Reaction between S-nitrosothiols and thiols: generation of nitroxyl (HNO) and subsequent chemistry. *Biochemistry* 37(16): 5362-5371.

Worthley MI, Holmes AS, Willoughby SR, Kucia AM, Heresztyn T, Stewart S, Chirkov YY, Zeitz CJ and Horowitz JD (2007). The deleterious effects of hyperglycemia on platelet function in diabetic patients with acute coronary syndromes mediation by superoxide production, resolution with intensive insulin administration. *J Am Coll Cardiol* 49(3): 304-10.

Wrobel AT, Johnstone TC, Deliz Liang A, Lippard SJ and Rivera-Fuentes P (2014). A Fast and Selective Near-Infrared Fluorescent Sensor for Multicolor Imaging of Biological Nitroxyl (HNO). *J Am Chem Soc* [Epub ahead of print].

Wynne BM, Labazi H, Tostes RC and Webb RC (2012). Aorta from angiotensin II hypertensive mice exhibit preserved nitroxyl anion mediated relaxation responses. *Pharmacol Res* 65(1): 41-7.

Wynne BM, Labazi H, Tostes RC and Webb RC (2012). Aorta from angiotensin II hypertensive mice exhibit preserved nitroxyl anion mediated relaxation responses. *Pharmacol Res* 65(1): 41-47.

Yuill KH, Yarova P, Kemp XHBK, Garland CJ and Dora KA (2010). A Novel Role for HNO in Local and Spreading Vasodilatation in Rat Mesenteric Resistance Arteries. *Antioxid Redox Signal*: [Epub ahead of print].

Yuill KH, Yarova P, Kemp XHBK, Garland CJ and Dora KA (2011). A Novel Role for HNO in Local and Spreading Vasodilatation in Rat Mesenteric Resistance Arteries. *Antioxid Redox Signal* 14(9): 1625-35.

Zamora R, Grzesiok A, Weber H and Feelisch M (1995). Oxidative release of nitric oxide accounts for guanylyl cyclase stimulating, vasodilator and anti-platelet activity of Piloty's acid: a comparison with Angeli's salt. *Biochem J* 312 (Pt 2): 333-9.

Zarpelon AC, Souza GR, Cunha TM, Schivo IR, Marchesi M, Casagrande R, Pinge-Filho P, Cunha FQ, Ferreira SH, Miranda KM and Verri WA, Jr. (2013). The nitroxyl donor, Angeli's salt, inhibits inflammatory hyperalgesia in rats. *Neuropharmacology* 71: 1-9.

Zeller A, Wenzl MV, Beretta M, Stessel H, Russwurm M, Koesling D, Schmidt K and Mayer B (2009). Mechanisms underlying acivation of soluble guanylate cyclase by the HNO donor Angeli's Salt. *Molecular Pharmacology* 76(5): 1115-1122.

Zgheib C, Kurdi M, Zouein FA, Gunter BW, Stanley BA, Zgheib J, Romero DG, King SB, Paolocci N and Booz GW (2012). Acyloxy nitroso compounds inhibit LIF signaling in endothelial cells and cardiac myocytes: evidence that STAT3 signaling is redox-sensitive. *PLoS One* 7(8): e43313.

Zhang SH, Reddick RL, Piedrahita JA and Maeda N (1992). Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science* 258: 468-471.

Zhang SX, Khalyfa A, Wang Y, Carreras A, Hakim F, Neel BA, Brady MJ, Qiao Z, Hirotsu C and Gozal D (2013). Sleep fragmentation promotes NADPH oxidase 2-mediated adipose tissue inflammation leading to insulin resistance in mice. *Int J Obes (Lond)* Epub ahead of print.

Zhou RH and Frishman WH (2010). The antiplatelet effects of nitrates: is it of clinical significance in patients with cardiovascular disease? *Cardiol Rev* 18(4): 198-203.

Chapter 8: Appendix

Methods

Time-course studies

The time-course of vasorelaxation in response to the HNO donors, IPA/NO and Angeli's salt were assessed in order to elucidate their viability, as previously described (Irvine et al. 2011). IPA/NO and Angeli's salt stocks were freshly made at day 0 and were incubated for up to 10 days at 37˚C. On day 0, 1, 4, 7 and 10, cumulative concentration-response curves to the HNO donor IPA/NO (1 nmol/l - 30 µmol/l) and Angeli's salt (1 nmol/l - 30 µmol/l) were constructed in common carotid arteries from 8-12 week male, C57BL/6J (wild-type) mice. To ensure maximal relaxation, isoprenaline (1 µmol/l) and levcromakalim (10 µmol/l) were added at the end of each concentration-response curve. At each time point, vasorelaxation responses were compared to freshly made IPA/NO and Angeli's salt.

Data and statistical analysis

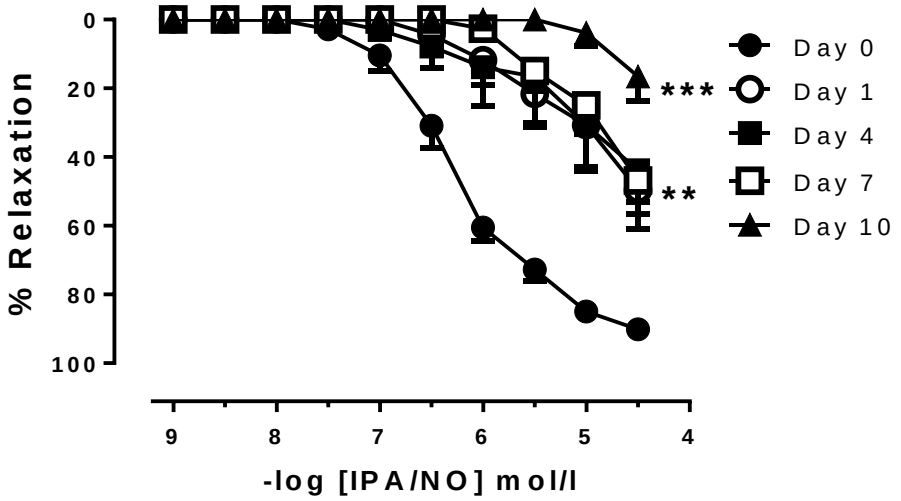
All results are presented as mean ± SE and $P<0.05$ was considered statistically significant. Vasorelaxation responses were expressed as a percentage reversal of U46619 precontraction. Individual relaxation curves were fitted to a sigmoidal logistical equation (GraphPad Prism, Version 5.0) to provide an estimate of the pEC_{50} value (concentration of agonist causing a 50% relaxation), expressed as –log mol/l and maximum relaxation (R_{max}). Differences between maximum relaxation values were tested using either a 1-way ANOVA with a Bonferroni post hoc test.

Drugs and their sources

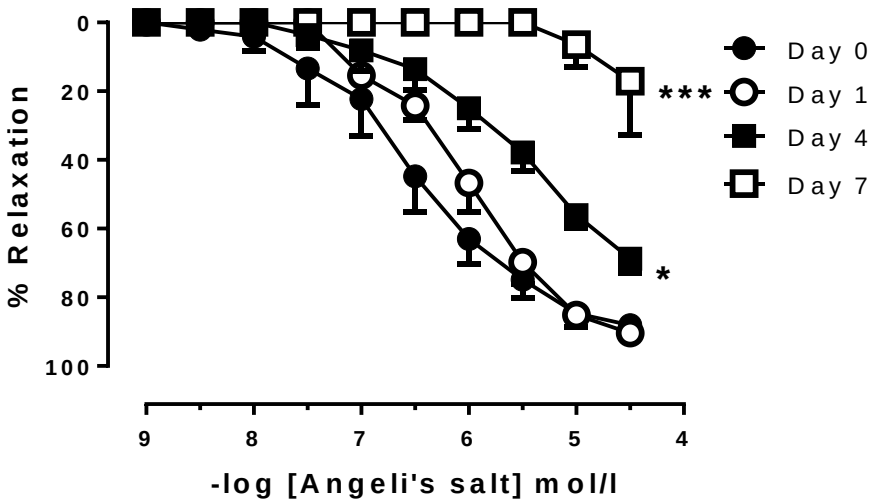
Drugs and there sources were: IPA/NO (kind gift from K. Miranda; University of Arizona, USA); levcromakalim (Tocris Bioscience, United Kingdom); U46619 (Sapphire Bioscience, Australia) and all other drugs (Sigma, Australia). IPA/NO and Angeli's salt were prepared at 10 mmol/l in 0.1 mol/l NaOH and all subsequent dilutions were in 0.1 mol/l NaOH. Levcromakalim was prepared at 10 mmol/l in 100% methanol and U46619 at 10 mmol/l in 100% ethanol. All subsequent dilutions were distilled water. All other drugs were dissolved and diluted in distilled water.

Appendix 1: Concentration-response curves to IPA/NO (A; n=4-6) and Angeli's salt (B; n=3-6) in common carotid arteries from WT mice. IPA/NO and Angeli's salt stocks were freshly made at day 0 and were incubated for up to 10 days at 37˚C and vasorelaxation responses were obtained at Day 0, 1, 4, 7 and 10. Values are expressed as the percent reversal of precontraction and given as mean ± SE, where n=number of animals. *****P<0.05, ******P<0.01 vs Day 0 (1-way ANOVA, Bonferroni post-hoc test).

A


% Relaxation
0
20
40
60
80
100
9
8
7
6
5
4
-log [IPA/NO] mol/l
***
**
Day 0
Day 1
Day 4
Day 7
Day 10

B


% Relaxation
0
20
40
60
80
100
9
8
7
6
5
4
-log [Angeli's salt] mol/l
***
*
Day 0
Day 1
Day 4
Day 7

Reference

Irvine J, Kemp-Harper BK and Widdop RE (2011). Chronic administration of the HNO donor, Angeli's salt does not lead to tolerance, cross-tolerance or endothelial dysfunction: comparison with GTN and DEA/NO. *Antioxid Redox Signal* 14(9): 1615-24.