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**REGULATION OF HAEM OXYGENASE-1 BY
NITROSATIVE STRESS IN CARDIAC CELLS**

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**THESIS SUBMITTED FOR THE DEGREE OF
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*FROM THE VASCULAR BIOLOGY UNIT, DEPARTMENT OF
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"The body possesses the high art of wrecking and also of restoring."

Paracelsus (1493-1541)

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ABSTRACT

The reactive nitrogen species (RNS) nitric oxide (NO), nitroxyl anion (NO⁻) and nitrosonium cation (NO⁺), modulate a myriad of biological processes. The microsomal haem oxygenases (HO-1, HO-2 and HO-3) oxidatively catabolise haem to bilirubin, carbon monoxide (CO) and ferrous iron (Fe²⁺). Sensitivity of the inducible isoform (HO-1) to a variety of inducers has identified HO-1 as an effective endogenous cytoprotectant against oxidative stress. Although nitrosative stimuli can enhance HO-1 expression, little is known about the biochemistry and mechanisms of this response. This Thesis examines a number of aspects related to HO-1 and nitrosative stimuli in cardiac cells, including: 1. induction by NO⁻; 2. the biochemistry of NO⁻/NO-mediated induction of HO-1; 3. identification of a possible mechanism for the activation of HO-1 by NO congeners; 4. the antinitrosative potential of bilirubin; and 5. the potential of glyceryl trinitrate (GTN), a clinically used NO donor, to activate the haem oxygenase pathway. These different aspects of HO-1 were addressed using biochemical, molecular biology and cell culture techniques. The results indicate that NO⁻, in analogy with other RNS, is a potent inducer of haem oxygenase activity and HO-1 mRNA and protein expression. A proposed mechanism for this response is modulation of thiol groups within redox-sensitive transcription factors. An antinitrosative and HO-1 inducing capacity was identified for bilirubin and GTN, respectively. Collectively, these findings suggest that the haem oxygenase pathway can act both as a sensor to, and target of, redox based mechanisms involving RNS, and extend our knowledge on the biological function of HO-1 in response to nitrosative stress.

LIST OF ABBREVIATIONS

μ l	microlitre
μ M	micromolar
AS	Angeli's salt
BR	bilirubin
BVR	biliverdin reductase
Ca^{2+}	calcium
CaM	calmodulin
C-PTIO	2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (potassium salt)
CO	carbon monoxide
cGMP	cyclic guanine monophosphate
DeaNO	diethylamine NONOate
DeaNO	diethylenetriamine NONOate
DMEM	Dulbecco's modified eagle's medium
DPBS	Dulbecco's phosphate buffered saline
Fe^{3+}	ferric iron
Fe^{2+}	ferrous iron
GTN	glyceryl trinitrate
g	gram(s)
h	hour(s)
HO	haem oxygenase
HO-1, -2 and -3	haem oxygenase-1, -2 and -3
l	litre(s)
min	minute(s)
ml	millilitre
mM	milimolar
MW	molecular weight
<i>n</i>	number of replicates per group
NADH	reduced nicotinamide adenine dinucleotide
NADP	oxidised nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
NOS	nitric oxide synthase
NO_3^-	nitrate
NO_2^-	nitrite
NO^+	nitrosonium cation
NO^-	nitroxyl anion
OD	optical density
<i>P</i>	probability
RNS	reactive nitrogen species
ROS	reactive oxygen species
Sec	seconds
S.E.M.	standard error of the mean
sGC	soluble guanylate cyclase
vs.	versus
$\times g$	times gravity

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1 INTRODUCTION

1.1 Historical perspective on nitric oxide

In 1847 the Italian scientist Ascanio Sobrero discovered nitroglycerin (NG). It wasn't until 1863 that Alfred Nobel, the person whose name is associated with the Nobel Prize, patented his detonator and harnessed the potential of using the stabilised form of NG as dynamite, the invention which gave him world wide success ¹. However, when in 1890 Nobel was recommended by his doctor to take NG to alleviate his symptoms of angina, he stated: "It is the irony of fate that I should be ordered by my doctor to take nitroglycerine internally" ². The reason for this medicinal usage of NG stemmed from work carried out during the preceding 50 years. In the mid to late 19th century a number of European scientists discovered that nitrite (NO_2^-) and nitrate (NO_3^-) containing compounds, e.g. amyl nitrite and glyceryl trinitrate (GTN), had a pharmacological potential as 'nitrovasodilators'. To this day nitrovasodilators are still used as therapeutic agents for cardiovascular disease ¹.

For most of the 20th century scientists tried in vain to elucidate the mechanism by which the medicinal effects of vasodilators are mediated. However, a breakthrough came in the 1970s when a series of experiments revealed that various vasodilators, including NG and nitric oxide (NO) gas, had the capacity to relax vascular smooth muscle ³. In each case relaxation coincided with an increase in the activity of cyclic guanine monophosphate (cGMP), the molecule whose synthesis is catalysed by soluble guanylate cyclase (sGC), which led to the suggestion that cGMP activation could occur via the formation

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of NO^{3,4}. With the discovery of endothelium-derived relaxing factor (EDRF) in the endothelial cells of arterial smooth muscle by Robert Furchgott and John Zawadzki in 1980, the mechanism of action of NO gradually began to emerge⁵. In the late 1980s two independent groups discovered that the effects mediated by EDRF was simulated by NO gas and that both were identical^{6,7}. It is now known that enzymatic metabolism of the vasodilators within the target tissue generates NO, which in turn mediates the drugs vasodilatory action⁸. Consequently, the perspective of NO changed in the late 20th century from a potentially toxic molecule into a signalling factor, thus generating a new era of scientific research.

The important status of NO is illustrated in a number of ways: 1. there has been an exponential growth in the number of publications related to NO since the 1980s; 2. a scientific journal, "Nitric Oxide", is devoted specifically towards NO research; 3. NO was declared "Molecule of the Year" by Science magazine in 1992⁹; and 4. in 1998 the Nobel Prize for physiology was awarded to Robert Furchgott, Louis Ignarro and Ferid Murad for their pioneering work into NO. However, the contribution of Salvador Moncada, another important figure involved in the discovery of NO, was overlooked much to the disappointment of others¹⁰. From a breakdown product of vasodilators to a versatile signalling molecule, NO has generated an explosion of research to examine its role in a myriad of physiological and pathological processes and highlighted its potential medical application and therapeutic manipulation.

1.2 Nomenclature and biochemistry of nitric oxide

The combination of one atom of molecular oxygen (O_2) with one atom of nitrogen (N_2) gives rise to the chemical nitrogen monoxide (NO), more commonly known as nitric oxide. The chemical symbol for the molecule is written with a superscript dot, (NO^\bullet), to indicate the presence of an unpaired electron, i.e. NO^\bullet is a radical ¹¹. Nitric oxide should not be confused with the anaesthetic nitrous oxide (N_2O), also known as 'laughing gas'. Although the abbreviations NO and NO^\bullet are used synonymously in the literature to refer to the same entity, the former takes precedence. This standardisation in the nomenclature of NO research was agreed upon by the International Union of Pharmacology (IUP) ¹². Similarly, in this Thesis, the abbreviation NO will be used unless otherwise indicated.

Nitric oxide has a number of physio-chemical properties which make it unique and set it apart from other molecules. Due to its radical nature, NO is generally perceived as highly reactive, but the number of target molecules that react with NO is limited to a few examples, e.g. O_2 , thiol- and metal-containing proteins and O_2^- ^{13,14}. Despite being a small simple molecule NO is produced by a group of complex enzymes, the nitric oxide synthases (NOSs, discussed further in Section 1.5) ¹⁵, at a concentration of 1-2 μM ^{16,17}. As it is a gas, NO can freely diffuse through cell membranes; this feature along with its radical nature gives the molecule a short half-life (between 6 and 30 seconds) ¹¹. However, when NO forms an adduct with cellular S-nitrosothiols (R-SNO) species, e.g. S-nitroso-serum albumin, the half-life can be extended to 60 minutes ¹⁸. Other fates for NO include: binding with haemoglobin (Hb) ¹⁹ or

myoglobin (Mb) ²⁰; activation of the enzyme sGC (discussed further in Section 1.7) ²¹; interaction with glutathione (GSH) to form S-nitrosoglutathione (GSNO) ²²; and slowly reacting with O₂ to form nitrogen dioxide (NO₂) ¹¹ or rapidly with superoxide anion (O₂⁻) to generate peroxynitrite (ONOO⁻) ²³. Although the quantity of NO targets is few their diverse range illustrates that the physiological effects of NO are controlled not just by its concentration but also its rates of interaction with other molecules and their respective concentrations ¹³.

Another interesting aspect of NO is its diverse chemistry which gives rise to different metabolites, known collectively as reactive nitrogen species (RNS, see Table 1.1 for examples), each having different oxidation states ¹⁷. The RNS have been implicated in a variety of environmental and occupational health effects, e.g. road traffic and air pollution, food and water contamination, as well as N₂O poisoning ²⁴. Among the more common biological species are the nitrosonium cation (NO⁺) and the nitroxyl anion (NO⁻), each of which has their own distinct properties and reactivities ^{13,25}. This raises the possibility that other NO redox subtypes (or NO congeners), i.e. NO⁺ and NO⁻, may exert biological effects akin to, or instead of, NO ¹¹.

Table 1.1 Examples of RNS and their oxidation states

Name	Symbol	Oxidation State
Nitroxyl anion	NO^-	+ 1
Nitrous oxide	N_2O	+ 1
Nitric oxide	NO	+ 2
Nitrite	NO_2^-	+ 3
Nitrosonium cation	NO^+	+ 3
Peroxynitrite	ONOO^-	+ 3
Nitrogen dioxide	NO_2^\cdot	+ 4
Nitrate	NO_3^-	+ 5

Formation of the oxidised form of NO, NO^+ , is feasible, but only on a transient basis, at pH 7 as its strong electrophilic nature limits its reactivity with other nucleophiles, the exception being water (H_2O) to generate nitrous acid (HNO_2)¹¹. At pH 7 a key role for NO^+ is in the process of nitrosation, i.e. transfer of NO^+ usually from a carrier compound (e.g. sodium nitroprusside (SNP, $\text{Na}_2[\text{Fe}(\text{CN})_5(\text{NO}^+)]$, S-nitroso-N-acetylpenicillamine (SNAP) and GSNO) to nucleophilic centres, e.g. thiol groups (R-SH), to generate R-SNO (where R can be one of a large range of chemical entities)²⁶. Endogenous formation of R-SNO, via reaction of NO with R-SH in the presence of an electron acceptor or as a nitrosyl complex formed by NO and ferric iron (Fe^{3+}) centres, is feasible^{25,27,28}. In addition, further transfer of NO^+ from a R-SNO to a second R-SH or other nucleophiles, a process known as transnitrosation, can occur until a critical nitrosation event takes place²⁵. Another possible reaction is S-thiolation, which involves addition of a thiolate anion (R-S^-) to the R-SNO group, giving rise to a disulfide and NO^- ^{29,30}.

The fact that R-SH groups are often found at receptor sites in control and signalling pathways has implicated nitrosation, transnitrosation and

S-thiolation as important mediators in the control of cell signalling pathways, gene expression and health and disease ^{26,31,32}. Despite recent confirmation of their existence *in vivo* R-SNO have long been suggested to exert a number of biological functions, e.g. vasodilation and activation of sGC ^{33,34}; act as a source of NO and its congeners ²⁹; inhibit platelet function ²⁶; and serve as neuromodulators ^{35,36}. Thus, a biological role for the NO⁺ species can be proposed, but this is exerted via NO⁺ carriers, i.e. R-SNO.

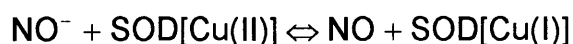
1.3 Nitroxyl anion: chemistry, sources and physiological aspects

Nitroxyl is the traditional name given to its protonated (HNO, nitroxyl ion or nitrosyl hydride) and unprotonated (NO⁻) species^{*}, both of which have their own distinct chemistry and are highly reactive molecules ³⁷. Unlike NO⁺, NO⁻, the one electron reduction product of NO, can exist freely in solution at pH 7 ³⁸. As NO⁻ is a short lived species (~5 milliseconds) identifying its presence can be difficult as it rapidly decomposes by dimerization and dehydration to yield N₂O ²⁵. Despite the previous observation NO⁻ is formed transiently when NO reacts with reductants, e.g. hydroxylamine ²⁹, and as an intermediate in NO type reactions ²⁵. The first direct observation of NO⁻ in aqueous solution suggested it was a weak acid (pKa = 4.7) ³⁷; however, a recent publication by Shafirovich and Lymar suggests a weaker acidity for NO⁻ (pKa ~11.4) ³⁹. In analogy with O₂, NO⁻ is isoelectronic, i.e. it can exist in singlet (¹NO⁻) or triplet

* The chemical notation for nitroxyl (NO⁻/HNO) is often used interchangeably, but in this Thesis the term NO⁻ will be used with the understanding that the molecule can exist as a protonated species as well.

($^3\text{NO}^-$) electronic spin states, with zero or two unpaired electrons, respectively. In aqueous solution the ground state for NO^- is $^1\text{NO}^-$, while the excited state is $^3\text{NO}^-$, with the former being the more reactive of the two ^{25,40}. However, $^1\text{NO}^-$ does not react with O_2 to produce ONOO^- , rather $^3\text{NO}^-$ does ²⁵, although ONOO^- formation was observed when NO^- was formed from the reduction of NO by ferrocyanochrome *c* ⁴¹, thus illustrating the complexity surrounding NO^- chemistry.

Chemical sources of NO^- include: 1. sodium trioxodinitrate (Angeli's salt; AS, $\text{Na}_2\text{N}_2\text{O}_3$); 2. benzenesulfohydroxamic acid (Piloty's acid; PA, $\text{C}_6\text{H}_5\text{SO}_2\text{NHOH}$); and 3. methanesulfohydroxamic acid (MSHA) ³⁸. Potential endogenous sources of NO^- include: 1. oxidation of L-arginine by NOS ^{42,43}, although this mechanism is still contentious ⁴⁴⁻⁴⁶; 2. decomposition of R-SNO in the presence of thiols ³⁰; 3. reduction of NO by ferrocyanochrome *c* ⁴¹; 4. metabolic oxidation of cyanamide (NH_2CN), an alcohol deterrent agent used to inhibit aldehyde dehydrogenase (A1DH, EC 1.2.1.3) ⁴⁷; and 5. oxidation of NO^- to NO by the ubiquitous electron acceptor superoxide dismutase (SOD), as illustrated in Equation 1.1 ⁴⁸.



Equation 1.1 Reversible conversion of nitroxyl anion to nitric oxide

A number of reports have shown that NO^- can elicit similar pharmacological effects to NO , and in some cases be more effective. Examples of beneficial effects include: vasodilation in aorta and arterial smooth muscle ^{42,49}; inhibition of platelet activation and stimulation of neutrophil migration ⁵⁰⁻⁵²; attenuation of

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post-ischaemic myocardial injury *in vivo*, in a manner analogous to NO⁵³; the ability to increase myocardial performance without altering heart rate in contrast to NO, thus suggesting a potential clinical application of NO⁻⁵⁴; and modulation of *N*-methyl-D-aspartate (NMDA) receptors in a neuroprotective manner unlike NO^{55,56}. Adverse effects mediated by NO⁻ include: cytotoxicity and DNA damage in the presence of strong oxidants, e.g. hydrogen peroxide (H₂O₂)⁵⁷; also, NO⁻ is more cytotoxic than NO, dinitrogen trioxide (N₂O₃) and ONOO⁻⁵⁸. The latter characteristics of NO⁻ may be harnessed for therapeutic applications, e.g. targeting cancer cells, but also indicates that the anion may play a role in the etiology of various pathological conditions. In analogy with NO^{59,60}, the aforementioned effects of NO⁻ can be mediated by cGMP dependent and independent (e.g. calcitonin gene-related peptide (CGRP))^{54,61} mechanisms. In the case of cGMP dependent reactions it is now accepted that intracellular oxidation of NO⁻ to NO occurs. In fact, a number of routes have been proposed, including oxidation by SOD^{48,62}, copper ions⁶³, quinones⁶⁴, O₂, flavins and methaemoglobin (Hb⁺)⁶².

Mechanisms for detecting the presence of NO⁻ include: trapping with thiols⁶⁵ and ferric (Fe³⁺) haem centres such as tetracyanonicklate anion [Ni(CN)₄]²⁻, metmyoglobin (Mb⁺) and Hb⁺⁶⁶; while electron paramagnetic resonance (EPR) can distinguish NO⁻ from NO as the former, in analogy with NO⁺, is diamagnetic, whereas NO is paramagnetic^{67,68}. The adverse effects of NO⁻ can be attenuated using cobalamins⁶⁹ and thiols (e.g. L-cysteine)⁶⁵. The latter reaction exploits the scavenging potential of thiols as well as the affinity of NO⁻ for sulphhydryl groups⁴⁷.

1.4 Nitric oxide biosynthesis

With the discovery that NO was the labile factor responsible for vascular smooth muscle relaxation ^{6,7}, interest in the way NO is produced intensified. Key discoveries related to this area included: 1. research into the basis for NO_2^- and NO_3^- synthesis *in vivo* ^{70,71}; 2. identification of L-arginine, but not D-arginine, as the principal source for mammalian NO synthesis ⁷²; 3. recognition that NO, L-citrulline and N^ω -hydroxy-L-arginine (NHA) are intermediates in the conversion of L-arginine to NO_2^- and NO_3^- , in macrophages ⁷³⁻⁷⁵ and endothelial cells ⁷⁶; and 4. discovery of the enzymes responsible for the synthesis of NO from L-arginine (i.e. the NOSs) in various mammalian tissues ⁷⁷. As a result, the enzymatic conversion of L-arginine to NO and L-citrulline by NOS in the presence of O_2 is known as the L-arginine-NO pathway (see Figure 1.1) ⁷⁸.

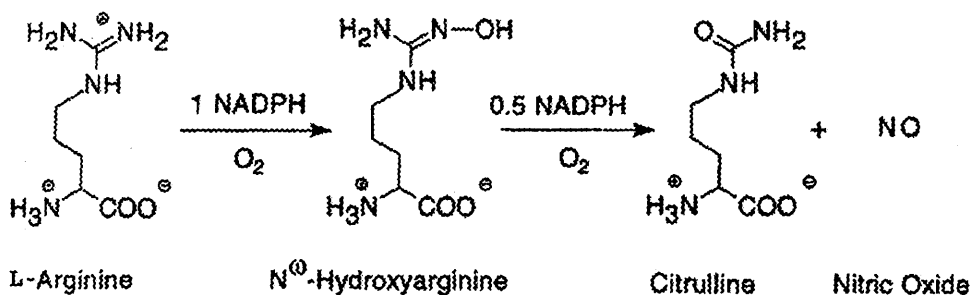


Figure 1.1 The L-arginine-NO pathway.

Adapted from ⁷⁹.

The semi-essential amino acid L-arginine is synthesised *in vivo* within the cytosolic and mitochondrial compartments of cells in the intestine, kidneys and liver from a number of sources, e.g. L-citrulline, L-glutamine and L-proline, via

a series of enzymatic reactions. The most important aspect of L-arginine metabolism is its catabolism, as this amino acid plays a central role in generating cell-signalling molecules, e.g. NO, glutamate, polyamines, agmatine and precursors for the synthesis of proteins, urea and creatine ⁸⁰. The principal regulators of L-arginine homeostasis are the NOSs and arginases, which form urea and L-ornithine ⁸¹. In the context of NO synthesis the importance of L-arginine is illustrated by the existence of a means to recycle L-citrulline to L-arginine via the citrulline/NO (or arginine/citrulline) cycle. The latter reaction is catalysed by the enzymes argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL), with L-argininosuccinate as an intermediate ⁸⁰.

1.5 The nitric oxide synthases

Having established the existence of the L-arginine-NO pathway, the next step was to identify the constituents of this pathway and its mechanism(s) of regulation. This began with the rapid characterisation, purification and cloning of the NOSs (E.C. 1.14.13.39), a group of highly regulated, but medium-sized (130-160 kDa) enzymes, which participate in a myriad of biological functions. The term 'synthases' is used instead of 'synthetases' as the NOSs do not employ ATP during their reactions ⁸². To date, four distinct isoforms of NOS have been identified in different cells types: 1. endothelial NOS (eNOS); 2. inducible NOS (iNOS); 3. neuronal NOS (nNOS); and 4. mitochondrial NOS

(mtNOS)^{*}. These isoforms are often classified according to their means of expression, either constitutive (eNOS, nNOS and mtNOS) or inducible (iNOS) and their calcium (Ca²⁺)-dependence (eNOS, nNOS and mtNOS) or -independence (iNOS), although such groupings are not clearcut⁸³.

1.5.1 Neuronal nitric oxide synthase

The first isoform to be purified and cloned was neuronal nitric oxide synthase (nNOS) from rat brain^{84,85}. This isoform has also been observed in non-neuronal tissues, e.g. skeletal muscle, and mapped to human chromosome 12⁸⁶. It is constitutively expressed in the cytosolic fraction (molecular weight (MW), 150-160 kDa) as a dimer in its native state and is highly conserved (93%) among species⁸⁷. Activation relies upon increased Ca²⁺ forming a complex with the Ca²⁺ binding protein calmodulin (CaM)⁸⁸.

1.5.2 Inducible nitric oxide synthase

The second isoform, inducible NOS (iNOS), was first discovered in murine macrophages following their exposure to an 'inducing' agent, e.g. lipopolysaccharides or cytokines⁸⁹. This dimeric enzyme (MW of 130 kDa) has been purified, cloned and ascribed to chromosome 17 in humans⁹⁰. Activity has been detected in the cytosolic fraction of immune and nonimmune cell types of various species, while interspecies amino acid sequence

^{*} The IUP recommends that a standardised terminology for the NOSs be based upon their cellular origin and location of their respective cDNAs¹², a similar nomenclature will apply in this Thesis.

similarity is $\geq 80\%$ ⁸⁶. The strong affinity between iNOS and CaM, even after boiling in SDS and PAGE, accounts for the Ca^{2+} -independence of this isoform ⁹¹.

1.5.3 Endothelial nitric oxide synthase

The second constitutive isoform, endothelial NOS (eNOS), has been purified and cloned in bovine endothelial cells ⁹². Analogous to the other isoforms eNOS is dimeric, but it has a MW of 135 kDa, is assigned to chromosome 7, and displays a very high ($> 94\%$) degree of interspecies amino acid sequence similarity ⁸⁶. This isoform is present in numerous cells types predominantly ($> 90\%$) in the particulate fraction, due to the presence of a N-myristoylation and palmitoylation sequence in the eNOS gene (see Figure 1.3), although cytosolic expression has been detected ⁷⁷. Akin to nNOS, eNOS requires binding of increased Ca^{2+} to CaM for its regulation and expression; however, similarity between these isoforms is only 57%, revealing that both are distinct gene products ⁸². As this isoform predominates in endothelial cells lining the lumen of blood vessels its expression can be regulated in a Ca^{2+} -independent manner as a result of haemodynamic forces, e.g. fluid shear stress (the dragging frictional force created by blood flow) and mechanical stretch (a cyclic strain stress created by blood pressure) ⁹³⁻⁹⁵. Of these stimuli shear stress induced activation of eNOS has assumed great importance as a means for sustained NO production independent of intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) ⁹⁶. Alterations in intracellular pH as well as activation of phosphatidylinositol 3-kinase (PI3K) and the subsequent phosphorylation of its downstream target Akt (also known as protein kinase B), which in turn

phosphorylates eNOS on Ser¹¹⁷⁷, have been proposed as mechanisms by which shear stress promotes an increase in eNOS activity⁹⁷⁻⁹⁹.

1.5.4 Mitochondrial nitric oxide synthase

Recently, a fourth dimeric NOS isoform, mitochondrial NOS (mtNOS), has been identified, purified and characterized from the inner mitochondrial membrane of rat liver^{100,101}. This enzyme shares similarities with other NOSs, e.g. it has a MW of 125-130 kDa, akin to iNOS, while its membranous location and Ca²⁺-dependence is similar to eNOS¹⁰¹. As mitochondria play a central role in a number of physiological processes, e.g. O₂ consumption, energy metabolism and Ca²⁺ buffering, it has been suggested that mtNOS may regulate such functions¹⁰².

1.6 Nitric oxide synthases: structure and mechanism of action

Although there are four isoforms of NOS details regarding the structure and mechanism of action of NOSs have been derived from eNOS, iNOS and nNOS. A number of common structural features exist suggesting the possibility of a common ancestral origin for the NOS gene⁸³. All NOSs contain a recognition site for their substrate, L-arginine, and possess a tightly bound haem (ferro-(Fe²⁺)protoporphyrin IX) prosthetic group which functions in arginine turnover⁷⁹. Their dimeric composition comprises two domains (see Figure 1.2 and Figure 1.3), a N-terminal oxygenase domain and a C-terminal reductase domain, which are linked by a CaM recognition site. The former domain contains the binding sites for L-arginine, haem and the cofactor (6R)-5,6,7,8-tetrahydrobiopterin (BH₄), while the latter domain contains binding

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sites for NADPH and other essential cofactors, i.e. flavoproteins (flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN))⁸². Also, different regulatory sites (see Figure 1.3) exist in certain NOS isoforms. For example, nNOS has a PDZ (PSD-95 Discs-large/ZO-1)¹⁰³ and a protein inhibitor of NOS (PIN)¹⁰⁴ domains which target this isoform to signalling regions and exert a negative influence upon its dimeric structure, respectively. In eNOS the N-myristoylation and palmitoylation sequences localise this isoform to the plasmalemmal caveolae⁸³.

Caveolae are dynamic non-clathrin-coated invaginations present on the cell surface membrane of many cell types, including endothelial cells and myocytes. They have been implicated in a variety of cellular functions, e.g. signal transduction, endocytosis, transcytosis, receptor-mediated uptake of small molecules (also known as potocytosis) and regulation of $[Ca^{2+}]_i$ ^{105,106}. A number of physiochemical features define caveolae, namely, an abundance of cholesterol, sphingomyelin and glycosphingolipids on their cytoplasmic surface and the presence of a Triton-insoluble protein coat called caveolin^{107,108}. So far four different caveolin isoforms have been identified (caveolin-1 α and -1 β , caveolin-2 and caveolin-3). Caveolin-1 and -2 are ubiquitously expressed whereas caveolin-3 predominates in muscle; however, caveolin-1 and -3 are the most studied¹⁰⁸. Given the variety of critical functions ascribed to caveolae a number of signalling molecules, e.g. G proteins, protein kinases, growth factor and hormonal receptors as well as eNOS and nNOS, are compartmentalized within them via the scaffolding protein caveolin^{108,109}.

The targeting of NOS to caveolin exhibits an isoform specificity,

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namely, eNOS and nNOS interact directly with caveolin via a 'caveolin scaffolding domain' in caveolin and a 'caveolin binding domain' in eNOS and nNOS ¹¹⁰⁻¹¹³, whereas iNOS, given its tight binding of calmodulin, is unlikely to be targeted to caveolae or regulated by caveolin ¹¹⁴. In endothelial cells eNOS is associated with caveolin-1, but with caveolin-3 in myocytes ¹¹⁵. As a result of these interactions caveolin exerts an inhibitory influence over eNOS release; however, this inhibition can be overcome by agonist stimulation, e.g. increased blood flow or binding of Ca²⁺-calmodulin to eNOS, leading to competitive displacement of caveolin and thus synthesis of NO by the active enzyme ^{111,112}. When basal levels of Ca²⁺ are reattained calmodulin detaches from eNOS allowing caveolin to rebind and deactivate the enzyme, thus illustrating the reciprocal regulatory cycle exerted by calmodulin (an activator) and caveolin (an inhibitor) ¹¹⁶. In addition, Flam *et al.* observed that NOS, and the enzymes involved in arginine regeneration, ASS and ASL, colocalise within plasmalemmal caveolae ¹¹⁷, thus illustrating the potential for rapid activation and deactivation of NO synthesis. Modulation of caveolin expression can play a role in a number of NO-related pathologies. For example, Feron *et al.* demonstrated that an abundance of caveolin-3 decreased NO production leading to an impairment of the muscarinic cholinergic negative chronotropic effect in isolated cardiac myocytes ¹¹⁸. Conversely, downregulation of caveolin could play a role in cardiac failure. Furthermore, exposure of endothelial cells to high levels of cholesterol increases caveolin mRNA leading to stabilisation of the caveolin/eNOS complex and thus decreased NO bioavailability ¹¹⁶.

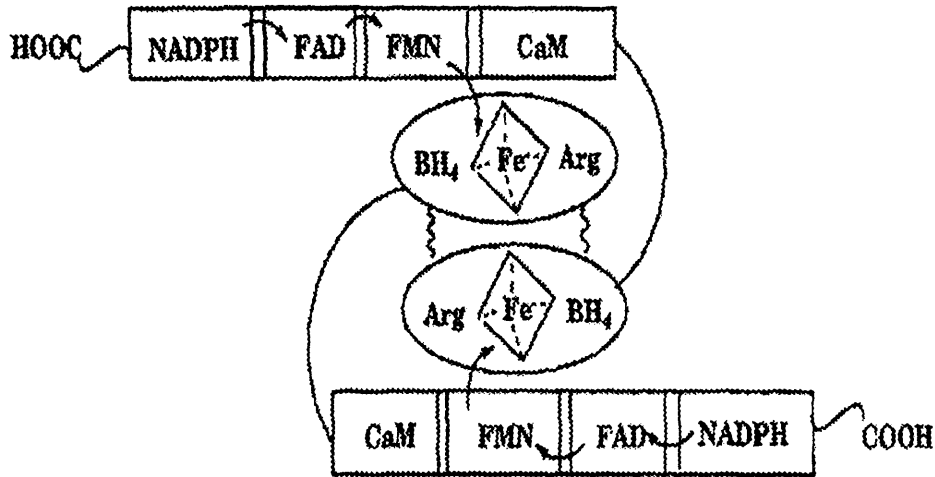


Figure 1.2 Structure of nitric oxide synthase dimer.

Adapted from ⁸¹.

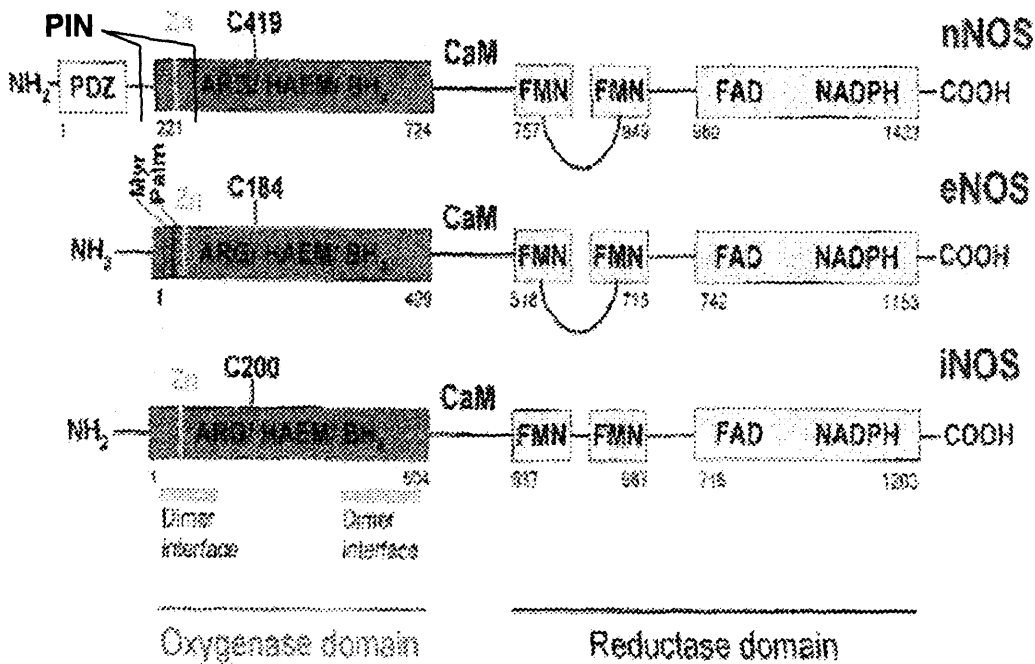


Figure 1.3 Regulatory sites within the nitric oxide synthase genes.

Myr = N-myristoylation; Palm = palmitoylation sequence; PDZ = PSD-95 Discs-large/ZO-1; PIN = protein inhibitor of nNOS (residues 161-245); and Zn = zinc. Adapted from ⁸³.

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Initially NOS was assumed to be a haemoprotein as its amino acid sequence bore a close homology with CYP-450 reductase (CPR)⁸⁵; however, they are distinct entities since NOS requires the cofactor BH₄ for dimerization, haem transition, efficient electron transfer and NO production⁵⁹. In addition, NOS is analogous to fatty acid monooxygenase (P-450_{BM3}) from *Bacillus megaterium*, as they are the only known self-sufficient P-450 type enzymes, i.e. they have their flavoprotein reductase and haem domains as part of the same polypeptide, thus illustrating the evolutionary conservation of the flavoprotein reductase in bacteria and mammals^{119,120}.

Despite their diverse locations, expression and regulation the mechanism by which NOSs catalyse the five-electron oxidation of L-arginine (the substrate) to NO and L-citrulline (the products), a reaction that is common to all isoforms, has assumed great importance given the key biological role played by NO⁵⁹. Although the exact mechanism is not fully understood the reaction, illustrated in Figure 1.4, is a highly regulated and complex process comprising two mono-oxygenation steps, with NHA as an intermediate, and shows an absolute requirement for reducing equivalents (NADPH), O₂, cofactors (FAD, FMN and BH₄), and in certain cases Ca²⁺ and CaM (i.e. eNOS and nNOS), for successful completion¹⁵.

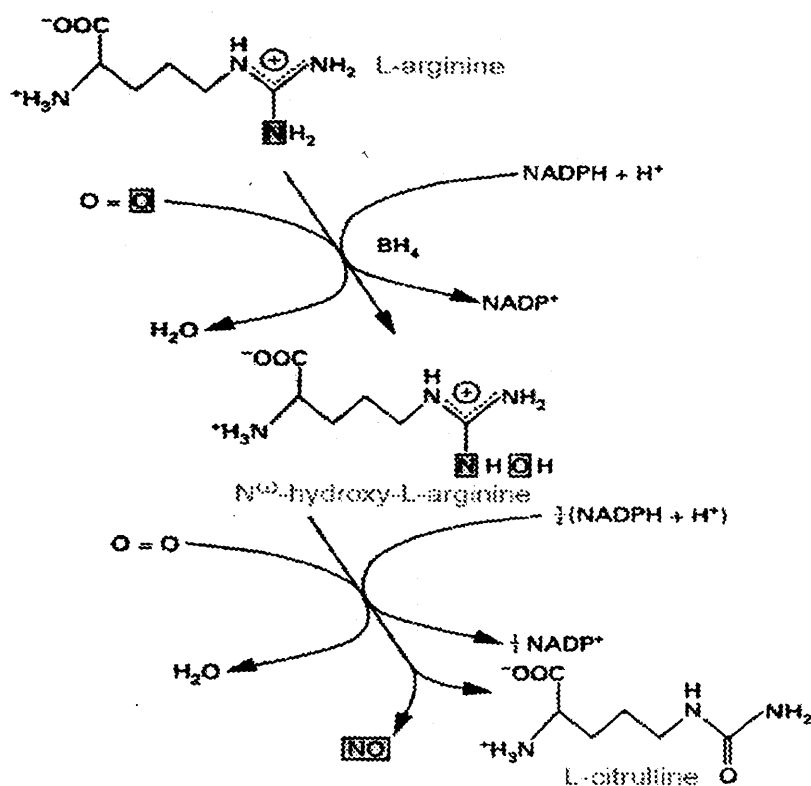


Figure 1.4 Mechanism of nitric oxide synthesis from L-arginine.

The shaded atoms show the origin of the constituent atoms of NO.
Adapted from ⁸².

In the first mono-oxygenation step one equivalent of NADPH and O_2 is used to hydroxylate one of the guanidino nitrogens of L-arginine, in the presence of BH_4 , to generate NHA ^{121,122}. In the absence of sufficient BH_4 the latter reaction proceeds but produces reactive oxygen species (ROS), e.g. $O_2^{\cdot-}$ and H_2O_2 , and less NO instead ⁸³. The second mono-oxygenation step oxidises NHA to N^G -oxo-L-arginine, followed by loss of H^+ to generate an unstable amino acid radical which fragments to yield NO and the amino acid carbodimine which reacts with H_2O to produce L-citrulline ¹²³.

In response to an increase in Ca^{2+} or immunogenic stimuli CaM binds to the enzyme, this has the dual effect of increasing the rate and efficiency with which electrons are transferred both within and between NOS domains (see Figure 1.2) ⁸⁸. Electrons, which are supplied by NADPH, are transferred through the reductase domain to sequentially reduce FAD and FMN and conveyed into the oxygenase domain. In the latter domain, the electrons interact with both haem iron and BH_4 at the enzymes active site to catalyse the reaction ⁸⁷. Binding of L-arginine promotes the reduction of NOS causing a conformational change of the prosthetic haem from a low spin six-coordinate (Fe^{3+}) to a high spin five-coordinate (Fe^{2+}) state, thus increasing the redox potential of the ligand ¹²⁴. Although BH_4 is present in all isoforms of NOS its function is somewhat unusual and controversial ¹²⁵. The suggested roles for BH_4 include: 1. a direct catalytic redox role in both monooxygenase cycles; 2. allosteric binding stabilises the enzyme; and 3. protection against inactivation and autoactivation ⁸⁷. Another important function ascribed to BH_4 in NOS catalysis is its critical role in the control of one- and two-electron mechanisms of NOS ¹²⁶. However, in the presence of low concentrations, or the absence of BH_4 or L-arginine, NOS catalyses the uncoupled oxidation of NADPH to liberate O_2^- and H_2O_2 ¹²⁷. This uncoupled reaction exhibits isoform specificity namely, eNOS coupling is controlled by BH_4 scavenging O_2^- ¹²⁸, whereas nNOS coupling is regulated via L-arginine decreasing the rate of NADPH oxidation ^{129,130}.

1.6.1 Regulation of nitric oxide production

Generally the intensity and rate of NO production varies according to need. For example, the activities of eNOS and nNOS are tightly regulated by fluctuations in Ca^{2+} /CaM to produce brief (seconds to minutes) but potent cell signalling effects, whereas iNOS, once expressed in response to an inducer (e.g. endotoxin and cytokines), is constitutively active for prolonged (hours to days) periods⁸¹. In disease states NO may be under (e.g. hypertension and atherosclerosis) or over (e.g. septic shock, inflammation and stroke) produced¹³¹. To effectively control imbalances in NO a number of strategies are available. Lack of NO can be overcome by pharmacological NO donors e.g. organic nitrates and nitrite esters such as GTN⁸. Control of excess NO has received the most attention, as a result there are a myriad of NOS inhibitors described and in use as pharmacological tools. Among the most widely used are the amino acid analogues of L-arginine (see Figure 1.5 for examples), which compete for the active site of NOS to impose a rapid, but reversible, inhibition of activity.

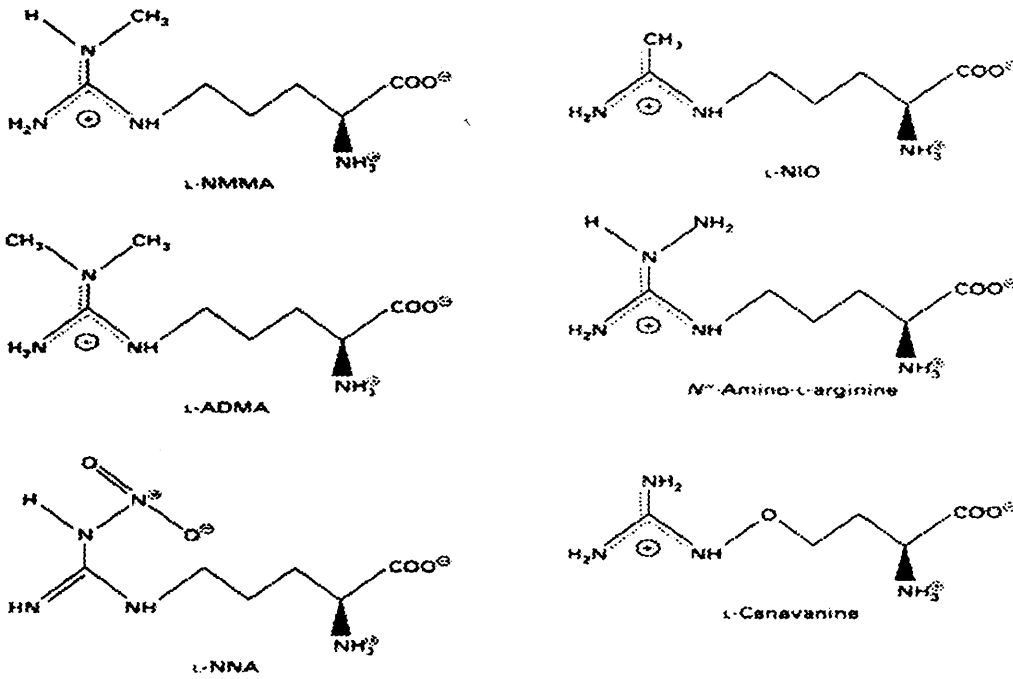


Figure 1.5 Examples of L-arginine analogues.

L-NMMA = NG-nitro-L-arginine methyl ester; L-NIO = N5-iminoethyl-L-ornithine; L-ADMA = NG-dimethyl-L-arginine; and L-NNA = N^ω-nitro-L-arginine. Adapted from ⁸².

Other inhibitors are based upon aromatic heterocyclics (e.g. 1-(2-trifluoromethylphenyl) imidazole (TRIM)) and nonamino acid (e.g. aminoguanidine) structures ⁸³. A major drawback with many of the aforementioned types of inhibitors include: their lack of isoform specificity; promotion of conformational changes in NOS; poor tissue uptake; and attachment to other essential enzymes ¹³². There are a number of post-translational mechanisms by which NO production can be regulated, these include: Ca²⁺ chelation with EDTA or EGTA; flavoprotein inhibitors, e.g. diphenyleneiodonium; CaM antagonists, e.g. trifluoperazine; and BH₄-binding site inhibitors (e.g. 4-amino-BH₄) or BH₄ synthesis inhibitors e.g. methotrexate

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Feedback inhibition by the product upon the enzyme is a common phenomenon, similarly with NO inhibiting the NOSs via binding to their haem irons ⁷⁹. Also, the intermediate NHA is a potent endogenous inhibitor of arginase (L-arginine-urea hydrolase), a binuclear manganese metalloenzyme which catalyses the hydrolysis of L-arginine to urea and L-ornithine. Therefore, interest in arginases has risen due to their ability to modulate the availability of substrate, i.e. L-arginine ^{80,81}. The asymmetric methylarginines (L-NMMA and asymmetric dimethylarginine (ADMA)) arise from the methylation of L-arginine residues in proteins by the enzyme protein arginine methyltransferase (PRMT) ¹³³. As endogenous guanidino substituted analogues of L-arginine both L-NMMA and ADMA inhibit NOSs and regulate the availability of L-arginine. The latter effect is achieved by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) metabolising L-NMMA and ADMA to citrulline, thus maintaining NO production ¹³³.

The ubiquitously expressed stress-activated gene heat shock protein 90 (HSP90) serves as a molecular chaperone involved in the folding, activation, stability, maturation, transport and targeting of important signalling proteins e.g. steroid hormone receptors, G protein $\beta\gamma$ subunits, protein kinases (c-Src and Raf-1) and NOSs ^{134,135}. The binding of HSP90 to eNOS and nNOS enhances the activation of both isoforms ^{136,137}. In response to stimuli (estrogen, fluid shear stress, histamine and vascular endothelial growth factor (VEFG)) HSP90 promotes an allosteric modulation of eNOS via recruitment of other proteins, e.g. Akt, to the caveolin-eNOS complex. This leads to phosphorylation of serine 1117 (human)/1179 (bovine) on eNOS by Akt and

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thus facilitating the CaM-induced dissociation of eNOS from the inhibitory influence of caveolin^{137,138}. In addition, HSP90 may act as a scaffold for the recruitment of other eNOS-associated proteins, e.g. kinases and phosphatases, as well as prevent eNOS uncoupling, i.e. its production of O₂⁻ instead of NO^{139,140}. The effects of HSP90 upon nNOS include increasing the isoforms affinity for CaM¹⁴¹ and facilitating the sustained opening of the haem binding cleft, and thus incorporation of haem into nNOS, especially so in *in vivo* situations where free haem is limited¹³⁶. The crucial regulatory role of HSP90 over the activation of eNOS and nNOS was confirmed using the specific inhibitor of HSP90, the ansamycin antibiotic geldanamycin (GA)¹³⁹.

Other modulators of NO production are the cytokines which can regulate NOS in a positive (interferon gamma (IFN- γ) and interleukin 1 beta (IL1- β)) and negative (e.g. transforming growth factor beta (TGF- β)) manner⁸¹. Hormones such as estrogen enhance eNOS and nNOS mRNA¹⁵, whereas glucocorticoids inhibit iNOS mRNA¹⁴². Interaction with proteins, e.g. caveolins for eNOS, proteins containing PDZ domains for nNOS and Rho family GTPases for iNOS, regulates the activity and cellular distribution of the isoforms¹⁴³. Collectively, the examples given illustrate a number of diverse, though sometimes ineffective, mechanisms to control NO production. Given the ubiquitous nature of NO and its association with a number of pathologies identifying specific and selective therapeutic modulators of NO is an area worthy of further investigation.

1.7 Cyclic guanosine monophosphate and guanylate cyclases

The cyclic nucleotides, cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP), have been shown to act as important intracellular second messengers ¹⁴⁴. Since its discovery in the 1960s, the biological roles of cGMP remained elusive due to cAMP superseding it in importance ¹⁴⁵. However, the identification of the enzymes responsible for its synthesis (guanylate cyclase; GC) and hydrolysis (cyclic nucleotide phosphodiesterase), along with the discovery of NO, led to a greater interest in the function of cGMP ³. The two mammalian GCs, particulate (pGC) and soluble (sGC), catalyse the conversion of guanosine triphosphate (GTP) to cGMP ¹⁴⁶, while enzymatic activity has been observed in a wide range of mammalian cells and tissues ¹⁴⁷.

Particulate GCs are membrane bound enzymes containing different subtypes (GC-A, GC-B and GC-C) that partition to the nuclear and microsomal fractions. They are found in mammals and bacteria and their principal functions are the regulation of fluid volume and movement ¹⁴⁶. Structurally, this group contains an extracellular ligand binding region, a single transmembrane domain, a protein kinase like domain and a catalytic domain ¹⁴⁶. A variety of agents, e.g. heat-stable endotoxin from *E. coli*, atriopeptins, atrial natriuretic peptide (ANP), intracellular Ca²⁺ binding proteins and hemin, have been shown to regulate this isoform ¹⁴⁵.

Soluble GC (sGC), the principal physiological target for NO, is found in the cytosolic fraction of all mammalian cells as a heterodimeric protein comprising

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two subunits, designated $\alpha 1$ - $\alpha 2$ and $\beta 1$ - $\beta 2$, which are derived from separate genes¹⁴⁸. The α and β subunits comprise three functional domains: 1. a haem with ferrous iron (Fe^{2+}) at its centre; 2. a catalytic domain; and 3. a dimerization domain (see structure in Figure 1.6). A catalytically active sGC heterodimer is formed when corresponding monomers, e.g. $\alpha 1$ and $\beta 1$, fuse via their respective dimerization domains^{148,149}. A second metal, copper, is also present in sGC, although its exact function remains elusive it may act as a cofactor for enzymatic activity¹⁵⁰. Activation of sGC can occur with protoporphyrin IX (the iron-free precursor of haem), NO, azide, CO and OH^{\cdot} , while methylene blue, Mb and Hb are potent inhibitors¹⁵¹.

Akin to other cell types, activation of cytosolic sGC by NO in cardiomyocytes promotes the formation of the second messenger cGMP from GTP^{3,4}. Therefore, cGMP can serve as a downstream effector of sGC and thereby one of the main routes through which NO mediates its signalling effects in the heart¹⁵². Like NO, cGMP can affect a variety of signalling pathways¹⁵³. Potential myocardial functions reported for cGMP include: modulation of sarcolemmal Ca^{2+} influx, reduction of myofilament Ca^{2+} sensitivity, altered sarcoplasmic reticulum (SR) function, changes in action potential, modulation of cell volume and reduction in O_2 consumption¹⁵⁴. The principal myocytic targets for cGMP include: cGMP-dependent protein kinases (cGKs type I (α and β) and II, also known as protein kinase G (PKG)); inhibitory or stimulatory phosphodiesterases (PDEs), e.g. iPDE or PDE type 3 and sPDE or PDE type 2, respectively; and cGMP-gated ion channels (also known as cyclic nucleotide-gated (CNG) cation channels)¹⁵⁵⁻¹⁵⁹.

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These mediate phosphorylation of proteins that regulate intracellular Ca^{2+} levels, cyclic nucleotide catabolism and cation influx across the membrane, respectively ^{158,160}. Despite the fact that cardiac cGMP levels change on the millisecond time scale to reflect the rapid changes characteristic of excitation-contraction (EC) coupling ¹⁶¹, the mechanisms by which sGC/cGMP regulates different cardiomyocyte functions, e.g. contraction (inotropy), relaxation (lusitropy) and heart rate (chronotropy), are becoming more distinct ¹⁶². The involvement of cGMP in inotropic function was first noted in 1977 and has since been confirmed in various mammalian myocytes using NO donors (e.g. SIN-1 and SNAP) and inhibitors of cGMP PDE (e.g. milrinone), sGC (e.g. LY 83583) or PKG (e.g. KT 5823) ¹⁵⁴.

The biphasic (positive or negative) nature of NO in cardiomyocytes is mediated by changes in the concentration of sGC/cGMP ^{163,164}. For example, low (nanomolar) concentrations of cGMP evoke a positive inotropic response via inhibition of PDE type 3 and a concomitant increase in the cAMP content of cardiomyocytes, which potentiates the stimulatory effect of cAMP upon the L-type Ca^{2+} channel (I_{Ca}) to promote Ca^{2+} influx and myocyte contraction ^{160,165}. The latter reaction occurs via phosphorylation by protein kinase A (PKA) of intracellular voltage-operated Ca^{2+} channels, e.g. phospholamban (PLN) and inositol trisphosphate (IP_3) receptors, in the SR of cardiomyocytes ^{162,166}. High (micromolar) cGMP concentrations have the opposite outcome, i.e. activation of PDE type 2, lower intracellular cAMP and Ca^{2+} concentrations, leading to a negative inotropic effect ^{167,168}. The latter effect, inhibition of the I_{Ca} , may also occur via cGMP activating PKG which promotes

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either direct phosphorylation of the channel, phosphorylation of an intermediate protein opposing the action of PKA or Ca^{2+} export ¹⁶⁹.

A positive lusitropic effect can be induced by PKG and PKA phosphorylation of troponin I (TnI) within cardiac myofilaments thus decreasing their sensitivity to Ca^{2+} ¹⁴⁰. In response to beta (β)-adrenergic stimulation eNOS-derived cGMP may affect myocyte chronotropy in a negative manner via inhibition of I_{Ca} through PDE type 2 degradation of cAMP ^{118,140,170}. A positive chronotropic effect has been observed in patients infused with the NO donor SNP after heart transplantation ¹⁶⁵. The importance of endogenous sGC/cGMP in the control of heart rate is illustrated by the fact that mice lacking the eNOS gene exhibit significant bradycardia (slow heart rate, < 60 beats per minute) ¹⁷¹. The cooperation of cGMP originating from pacemaker cells of the sinoatrial node should not be overlooked regards the chronotropic effects of sGC within target cardiomyocytes ¹⁷². Finally, it has been noted that the expression pattern and activity of sGC changes with different stages of cardiomyocyte development, i.e. higher expression in the neonatal period with a decline after birth followed by a reversion to higher levels with age or in septic hearts ¹⁷³⁻¹⁷⁵. The basis for these changes may be related to a switch in sGC expression to myocardial endothelial cells as well as the content of myocardial Mb, an endogenous scavenger of NO, as adult myocardium has a greater content of Mb compared with neonatal myocardium ^{173,174}.

Thus, in response to increased NO production sGC/cGMP can modulate a number of cardiomyocyte functions in a dynamic manner. One should also bear in mind that NO can exert its signalling effects independent of

sGC/cGMP production, e.g. post-translational modification of proteins via S-nitrosothiol bond formation, interaction with haem proteins, Ca^{2+} -dependent K^+ -channels and non-haem iron ^{13,17,60,176,177}. In addition, sGC can also be stimulated, but to a weaker extent, by carbon monoxide (CO; see Section 1.7.1 for more details), originating from the cytoprotective enzyme haem oxygenase ¹⁷⁸⁻¹⁸¹.

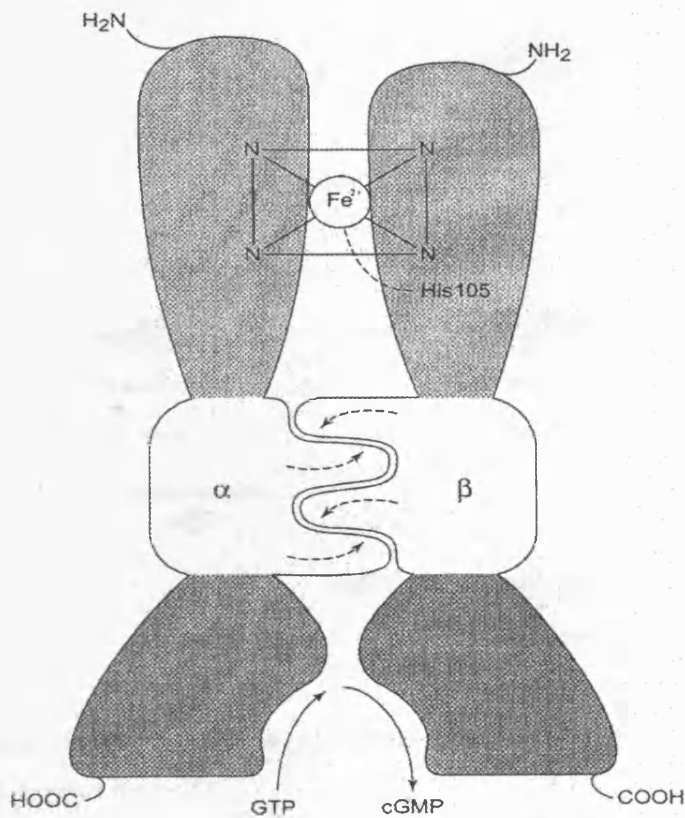


Figure 1.6 Structure of soluble guanylate cyclase.

Adapted from ¹⁴⁹.

1.7.1 Activation of soluble guanylate cyclase

Investigations of sGC regulation revealed that haem dependent (NO and related agents) and independent (protoporphyrin IX) mechanisms of activation proceeded via similar routes, i.e. formation of a Fe^{2+} complex (see examples in Figure 1.7) ¹⁸².

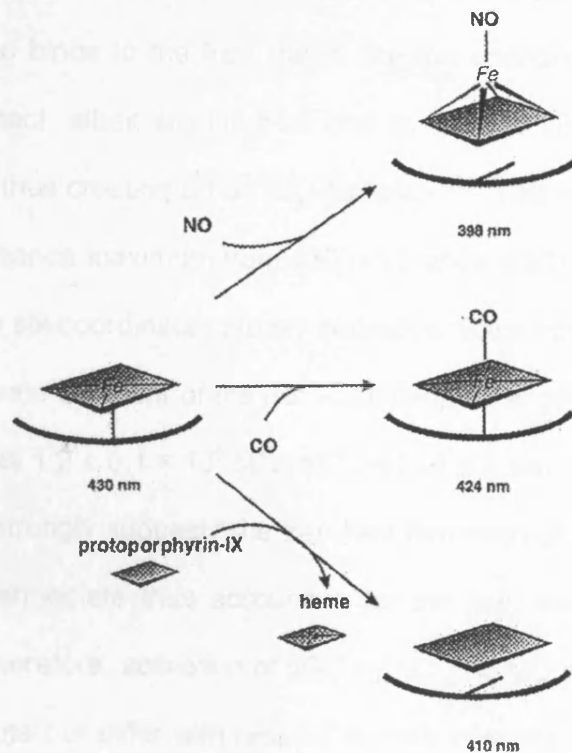


Figure 1.7 Activators of soluble guanylate cyclase.

Adapted from ¹⁵¹.

When NO binds to the six-coordinate position of the haem group it causes a number of physiochemical changes in sGC. Among these are disruption of the five-coordinate Fe^{2+} axial histidine (His 105) bond (absorption maximum 430 nm) and formation of a five-coordinate NO(nitrosyl)- Fe^{2+} complex (absorption maximum 398 nm) ²¹. Second, there is an increase in both enzyme activity

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(> 200-fold) and affinity for the substrate GTP brought about by the presence of the essential divalent cation magnesium (Mg^{2+})¹⁸³. Formation of the NO(nitrosyl)- Fe^{2+} complex is near diffusion controlled, while dissociation occurs quite rapidly, 5 sec at 37°C, thus enabling rapid deactivation of the enzyme in a biological scenario¹⁵¹. Another gaseous molecule, CO, has been shown to activate sGC, but activation occurs in a different manner to NO¹⁷⁸. Although CO also binds to the Fe^{2+} haem, the five-coordinate axial His 105 bond remains intact, albeit slightly bent due to the low vibration frequency arising from CO, thus creating a Fe^{2+} -CO complex¹⁸⁴. This complex causes a shift in the absorbance maximum from 430 nm (native sGC) to 424 nm which is suggestive of a six-coordinate carboxy derivative. Secondly, the association and dissociation rate constant of the Fe^{2+} -CO complex at pH 7.5 at 23°C has been calculated as $1.2 \pm 0.1 \times 10^5 M^{-1} sec^{-1}$ and $28 \pm 2 sec^{-1}$, respectively¹⁷⁸. The latter value strongly suggests the transient formation of a five-coordinate carboxyhaem intermediate thus accounting for the low, 4-fold, activation of sGC by CO²¹. Therefore, activation of sGC by NO and CO exhibit somewhat similar mechanisms but differ with respect to their intensity of increasing the catalytic capacity of the enzyme. Protoporphyrin IX (PPIX), the iron-free precursor of haem, strongly activates sGC in a manner similar to NO (absorbance maximum 410 nm)¹⁸⁵. In all these examples the prosthetic haem group is essential, as removal of the group abolishes activation, but can be restored by NO-haemoprotein complexes¹⁸⁶.

1.7.2 Modulation of soluble guanylate cyclase

Nitric oxide mediates a diverse range of biological effects via its interaction with a number of cellular targets, e.g. transition metal ions, non-haem Fe-proteins, ADP-ribosyltransferase, protein thiols, Fe-S enzymes, O_2 , O_2^- and transcription factors ^{13,31,176,187}. Given that sGC is the primary target of NO it would seem logical to focus on the former in order to modulate the beneficial and adverse aspects of NO. Currently there are a number of direct and indirect acting pharmacological modulators available. Examples of direct acting activators include NO donor compounds, e.g. GTN, nitroprusside, R-SNO and the NONOates (discussed further in Section 1.10), thus enabling precise modulation of sGC via metabolism to or release of NO. Isoliquiritigenin is another example of a direct acting sGC activator. Its mechanism of action is unclear but is thought to be multifactorial ¹⁴⁹.

The benzylindazole derivative 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole, YC-1, is a novel sGC stimulator which acts by a NO-independent mechanism ¹⁸⁸. This agent has the capacity to inhibit platelet aggregation, enhance vascular smooth muscle relaxation and potentiates the activation of sGC by NO, CO and PPIX by > 40, > 4000 and 360 percent, respectively ¹⁸⁹. Two possible mechanisms for YC-1 are: 1. allosteric modification of the sGC molecule; and 2. reduction of the dissociation rates for CO and NO ¹⁸⁹. The former mechanism requires the presence of an intact haem prosthetic group on the enzyme, even though YC-1 doesn't bind to haem, as evidenced by a lack of change in the Soret absorption when YC-1 was bound to native or

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haem-depleted sGC either on its own or in the presence of CO and NO ¹⁸⁹. The data obtained with YC-1, in particular allosteric modification, suggest that an endogenous 'YC-1 like' modulator may sensitise sGC to modulation by NO and even more so by CO ¹⁵¹. Although the identity of the endogenous 'YC-1 like' modulator is as yet unknown Colpaert *et al.* recently highlighted the ability of biliverdin and bilirubin to potentiate the CO-mediated relaxation of porcine gastric fundus by a sGC-dependent mechanism ¹⁹⁰. Even though there is still no categorical data identifying the endogenous 'YC-1 like' modulator, the results with the bile pigments (see Chapter 6) deserves further exploration with the ultimate aim of manipulating the potential of sGC endogenously.

A recent publication by Stasch *et al.* identified a regulatory site through Cys238 and Cys243, within the α 1 subunit of sGC that was sensitive to catalytic and haem ligand modulation via a new type of direct acting stimulator, BAY 41-2272 ¹⁹¹. BAY 41-2272 shares a number of similarities with YC-1, these include: 1. NO-independent, but haem-dependent, activation of sGC; 2. analogous structure; 3. lack of change in the Soret band in either non-stimulated or stimulated sGC; 4. antiplatelet and vasodilatory potentials; and 5. enhanced responsiveness of sGC is achieved without the need for biotransformation ¹⁹¹. These features of YC-1 and BAY 41-2272 represent a novel and potentially useful class of NO modulators as long term use of organic nitrates, e.g. GTN, can lead to tolerance as a result of impaired metabolism ¹⁹².

Examples of sGC inhibitors include methylene blue and 6-anilinoquinoline-5,8-quinone (LY83583). Their mechanism of action is not directed specifically

towards sGC, but proceeds via formation of O_2^- which inactivates NO, and direct inhibition of NOS activity¹⁴⁹. N-methylhydroxylamine is an example of a selective sGC inhibitor. A drawback with this agent is the necessity to administer millimolar quantities of the substance, thus questioning its selectivity¹⁴⁹. The compound, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), is an example of a highly selective inhibitor of sGC that has been used as a tool to distinguish between cGMP-dependent and cGMP-independent NO signalling in the brain and platelets^{193,194}. Although the mechanisms of ODQ are unclear, it is believed to compete with NO for the haem of sGC and once bound causes a reduction in the V_{max} as well as a shift in the absorbance maximum from 430 nm to 398 nm, thus irreversibly oxidising the haem from Fe^{2+} to Fe^{3+} , therefore limiting the use of ODQ to *in vitro* studies of sGC¹⁹⁵. From the examples given it is clear that further research in the field of sGC modulation is worthwhile in order to identify, refine and understand the mechanism of novel compounds which could be used as potential pharmacological modulators of NO.

1.8 Physiological and pathophysiological aspects of nitric oxide

For many decades NO was considered purely as yet another toxic molecule, possible carcinogen and an environmental pollutant; however, after the discovery of its production in the vasculature and other tissues, the importance of NO in mediating a variety of physiological functions started to gain credence¹⁹⁶. Nitric oxide participates in a wide range of biological functions in different systems, the most important being the nervous, vascular, immune and reproductive systems⁵⁹. In the subsequent sub-sections, the

beneficial and adverse consequences of NO will be discussed in relation to the aforementioned systems.

1.8.1 Nitric oxide in the nervous system

In 1982, Takeo Deguchi showed that L-arginine was the endogenous activator of sGC in the brain ¹⁹⁷. Later on, the identification of the nNOS isoform in neurons and nerve endings throughout the central and peripheral nervous system (CNS and PNS, respectively), firmly established that NO was produced within the nervous system ¹⁹⁸. Also, a link between NO and the excitatory amino acid glutamate was established as stimulation of *N*-methyl-D-aspartate (NMDA) receptors with glutamate results in an increase in Ca²⁺ which activates nNOS to produce NO, thus elevating cGMP levels, while NOS inhibitors attenuated cGMP production ¹⁹⁹. Excess NMDA receptor activation via glutamate can result in a variety of neurodegenerative disorders, e.g. cerebral ischaemia, epilepsy and Alzheimer's disease, which arise from enhanced NO production ²⁰⁰. The information derived from the work linking glutamate, NMDA and NO, has been used as a model for various neurological pathologies and also as a means to generate therapeutic treatments, in the form of NMDA antagonists ²⁰¹.

The principal site of nNOS production in the brain and peripheral tissues is in neurons ²⁰². It has been shown that nNOS neurons co-localise with a discrete set of neurons called diaphorase neurons, which incidentally make up 2% of the cerebral cortex ²⁰³. A unique feature of diaphorase neurons is their resistance to toxic insults in various models of neurotoxic disease, e.g.

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Alzheimer's and Huntington's disease ¹⁹⁶. The fact that neurons synthesising NO in response to increased glutamate activity could withstand the resulting neurotoxic effects was a paradox. A possible explanation for these observations was the suggestion that NOS/diaphorase neurons release a powerful burst of NO into the surrounding environment, thus killing adjacent neurons ²⁰⁴. One of the functions assigned to NO within the nervous system is that of a neurotransmitter ²⁰⁵. The evidence to support this hypothesis came from studies of neurotransmission in non-adrenergic non-cholinergic (NANC) nerves which serve as a means of innervation for the autonomic nervous system in the gastrointestinal tract, urogenital, respiratory and cardiovascular systems ²⁰⁶. These studies revealed that: 1. a 'NO-like' substance is released from stimulated NANC nerves; 2. in the presence of NANC inhibitors NO derived from NO donors mimicked NANC relaxation; 3. NO could be produced outside of the brain in the autonomic nervous system; and 4. inhibition of NOS activity in neurons innervating the anococcygeus muscle, the gut and blood vessels in the cerebral arteries and penis, led to a modulation of NANC nerve stimulation, thus supporting the neurotransmitter capacity of NO ²⁰⁵.

In the nervous system, NO fulfils the criteria for a neurotransmitter in a variety of ways: 1. the presence of the synthetic enzyme NOS within the relevant neurons; 2. release from the nerve terminals occurs following Ca^{2+} stimulation; 3. the effect induced by NO is similar to that invoked by physiologic nerve stimulation; and 4. inhibition of NO synthesis results in inactivation of the nerve stimulation ²⁰⁵. Nevertheless, NO does not have any characteristics that resemble the conventional chemical neuromodulators ²⁰⁷.

Firstly, it is a gas rather than a peptide and unlike other types of neurotransmitters it is not stored in synaptic vesicles but is produced on demand. Secondly, as a gas it diffuses freely out of cells rather than via exocytosis. In addition, NO has few receptors on postsynaptic cells, the best known 'receptor' being the iron in sGC, although it has also been shown to stimulate adenosine diphosphate (ADP) ribosylation of glyceraldehyde-3-phosphatedehydrogenase (GAPDH) ²⁰⁵. Despite such dissimilarities between the classical neurotransmitters and NO, the signalling capabilities of NO in the brain cannot be discounted, while the gaseous nature of NO creates a new class of neural messenger ²⁰⁷. Lastly, NO has been suggested to play a role in learning and memory by acting as a retrograde messenger in different types of neuronal plasticity such as long-term potentiation (LTP) and long-term depression (LTD) ^{202,208}, although this aspect remains to be proven.

1.8.2 Nitric oxide in the vascular system

Within the vascular system NO plays a key role in the maintenance of vascular tone, i.e. blood pressure, and preservation of an intact endothelium to prevent thrombus formation ²⁰⁹. Surrounding the inner walls of blood vessels, on top of the underlying smooth muscle layer, are endothelial cells which comprise the endothelium. The endothelial cells have receptors for a variety of neurotransmitters, e.g. acetylcholine (ACh), and in response to this stimulus they release a diffusible substance that acts on the underlying smooth muscle to stimulate relaxation and ultimately a lowering of blood pressure ²¹⁰. While working with rabbit thoracic aorta Robert Furchgott and John Zawadzki observed, by chance, that the vasodilatory response was

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absent from blood vessels denuded of their endothelium but was maintained in vessels with an intact endothelium⁵. The diffusible substance, which was very labile and nonprostanoid in nature, was first named EDRF²¹¹, but further experiments revealed that EDRF was in fact NO^{6,7}. *In vitro* and *in vivo* studies have confirmed that continuous release of NO from endothelial cells is essential to maintain vascular tone^{212,213}. As a result, NO has been suggested as an important pharmacological therapy for vascular disease in analogy with angiotensin and noradrenaline¹⁹⁶.

Within the vasculature the highest quantity of NO is derived from arteries, the purpose being to maintain a dilator tone between resistance (small diameter arteries) and the microvascular network²¹⁴. Continuous secretion of NO by the vascular endothelium helps to maintain basal levels (~1-2 μM) of NO⁵⁹; however, a number of physiological factors can augment this production. Examples include: platelet products, e.g. ADP and serotonin, thrombin, changes in oxygen tension, neuronal stimuli, pulsatile flow and shear stress. Of these stimuli shear stress and pulsatile flow are the main determinants of NO release⁵⁹. Maintenance of an intact endothelium for the release of NO is vital as numerous animal and human studies show that augmented NO production may be a causative factor in the pathogenesis of a number of cardiovascular related diseases, e.g. hypertension, arteriosclerosis, diabetes mellitus and septic shock²¹⁵.

Abnormalities in endothelium function can lead to impaired NO-mediated regulation of vascular tone, which is a causative factor in the development of essential hypertension in humans²¹⁶. Animal models of hypertension, e.g. the

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spontaneously hypertensive (SH) and deoxycorticosterone (DOCA)-salt hypertensive rats, have supported this theory, while induction of a prolonged hypertensive state in rabbits using L-NMMA could be attenuated by excess L-arginine ^{212,215}. Also, mice which are homozygous (-/-) for the eNOS gene exhibit hypertensive characteristics ¹⁷¹. A dysfunctional endothelium causes a disruption in NO release and function, which can lead to the development of a chronic inflammatory response within arteries as manifested by arteriosclerosis ²¹⁷. Animal and human models of arteriosclerosis show that certain risk factors, e.g. hypercholesterolemia and free radical mediated lipid peroxidation, promote fatty streaks and lesion development, the hallmarks of the disease, thus decreasing NO release and penetration to the lower smooth muscle ²¹⁵. Akin to arteriosclerosis, long term diabetes can lead to vascular complications at the endothelium and smooth muscle layers ²¹⁸, as a result NO release and function is altered.

Diabetes promotes these changes in NO via a number of mechanisms: 1. decreased availability or impaired transport or metabolism of the substrate L-arginine; 2. cellular stores of NADPH are consumed by the enzyme aldose reductase when it converts glucose to sorbitol (this reaction is known as the polyol pathway), as a result reducing equivalents for NOS are depleted; 3. alterations in the responsiveness of endothelial cells towards agonists, e.g. ACh, and reduced expression and structural modification of endothelial receptors, e.g. G proteins, via activation of diacylglycerol-protein kinase C; 4. increased production of vasoconstrictors, e.g. endothelin-1, attenuates the biological action of NO; and 5. enhanced nonenzymatic binding of glucose to

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lipids or to free amino acids on proteins generates advanced glycation end-products (AGEs) which inhibit the action of NO. The aforementioned examples can serve as sources of ROS, the causative factors of oxidative stress, which if left unchecked via endogenous antioxidants (e.g. GSH, SOD, catalase) can lead to endothelial dysfunction characterised by impaired release/inactivation of NO and vasoconstriction ^{219,220}. The latter changes predispose diabetic patients to an increased risk of vascular complications namely coronary heart disease (CHD) manifested by accelerated development of atherosclerosis, hypertension, dyslipidemia and a hypercoagulable state. Other cardiovascular pathologies include congestive heart failure and sudden cardiac death ²²¹.

When excess NO is released by the endothelium, e.g. septic shock arising from endotoxin exposure or underlying disease, there is a rapid fall in blood pressure leading to hypotension and myocardial depression ²⁰⁹. A link between NO and septic shock is illustrated by the fact that endotoxins cause a rapid and immediate increase in eNOS and iNOS expression, while NO_2^- and NO_3^- are particularly high in septic patients. Also, mice genetically deficient in iNOS have a greater resistance to endotoxin induced septic shock ^{222,223}. The ability of NO to modulate platelet function has been well documented and shown to be important to prevent thrombus formation and maintain endothelium integrity ²¹⁰. NO can inhibit ADP induced aggregation and promote disaggregation of preaggregated platelets via increased cAMP and cGMP concentrations in platelets ^{51,224,225}. The cAMP is derived from prostacyclin and cGMP originates from three sources: 1. NO itself; 2.

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synergism between NO and prostacyclin; and 3. NO produced within the platelets themselves, as human platelets can produce NO via activation of their own NOS upon stimulation ^{226,227}. Also, leukocyte aggregation and adhesion can be prevented by NO ²²⁸.

In addition to NO, the endothelium releases other vasoactive autacoids, e.g. prostaglandins such as prostacyclin (prostaglandin I₂, PGI₂), and endothelium-derived hyperpolarising factor (EDHF) ²²⁹. Initially discovered in 1976, PGI₂ exerts its principal effects, vasodilation and inhibition of platelet aggregation, via binding to specific plasma membrane receptors present on some, but not all, smooth muscle, blood elements and endothelial cells. This leads to activation of adenylate cyclase which in turn promotes an increase in the second messenger cAMP in target cells ²²⁹. A diverse range of stimuli (physical, chemical, hormonal and immunological) initiate the local *de novo* synthesis and release of PGI₂ from the endothelium ^{210,230}. Synthesis of PGI₂, which is swift (< 60 sec) in onset and transient (~5 min), occurs via the sequential activation of the enzymes cyclooxygenase (COX, also known as prostaglandin H (PGH) synthase) and PGI₂ synthase to metabolise their respective substrates, arachidonic acid (AA, which is released from membrane phospholipids by phospholipase A₂ (PLA₂)) and the endoperoxide prostaglandin H₂ (PGH₂) ^{210,230}. Next, PGI₂ is quickly hydrolysed non-enzymatically to 6-keto-PGF_{1α} followed by enzymatic conversion to the inactive metabolites 2,3-dinor-6-keto-PGF_{1α} and 6,15-diketo-2,3-dinor-PGF_{1α} ²³¹. The latter are excreted in urine and their measurement can serve as an index of PGI₂ generation ²¹⁰. The liberation of PGI₂ can be regulated at

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various points, e.g. glucocorticoids inhibit PLA₂, aspirin-like drugs and protein kinase C (PKC) respectively, inhibit and modulate COX and high concentrations of lipid peroxides impedes PGI₂ synthase^{210,229}. In addition to its vasodilator and haemostatic functions PGI₂ can prevent the development of atherosclerotic plaques via inhibiting the release of growth factors that cause abluminal occlusion, as well as enhancing and attenuating cholesterol ester metabolism and accumulation by smooth muscle cells and macrophages, respectively²¹⁰.

Along with NO and PGI₂ endothelial cells release a third diffusible vasodilator, endothelium-derived hyperpolarising factor (EDHF), that induces relaxation by hyperpolarising vascular smooth muscle cells. Although release of EDHF, NO and PGI₂ are mediated in response to increased [Ca²⁺]_i and haemodynamic changes²³² the identity and mechanism of action of EDHF are less clear-cut. However, a number of candidates have been forwarded, among these are potassium (K⁺) ions²³³, isoprostanes, which are COX-independent metabolites of AA,²³⁴ epoxyeicosatrienoic acids (EETs) generated by CYP-450 epoxygenase²³⁵ and endogenous cannabinoids²³⁶. EDHF mediates hyperpolarisation of the plasma membrane of vascular smooth muscle via opening of K⁺ channels, the net effect being to decrease [Ca²⁺]_i and impair activation of phospholipase C²³⁶. Akin to the ambiguous nature of EDHF, the aforementioned mechanism of action has since been challenged by more recent experimental data²³⁷. Among the proposed mechanisms are release of K⁺ from endothelial cells with hyperpolarisation transmitted to smooth muscle cells via gap junctions. Alternatively, an efflux of K⁺ ions from endothelial cells,

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via small and intermediate conductance Ca^{2+} -dependent K^+ (K_{Ca}) channels, could induce hyperpolarisation through activation of inwardly rectifying K^+ (K_{IR}) channels and/or the Na^+ - K^+ -ATPase on vascular smooth muscle cells ²³⁷.

The endothelium derived autacoids do not function in isolation, but interact together to regulate vascular function and one another. For example, NO and PGI_2 , which respectively activate the second messengers cGMP and cAMP, can synergise to inhibit platelet function, this effect can be enhanced by the inhibition of cGMP breakdown by cAMP in platelets, as well as in other target cells, e.g. smooth muscle and endothelial cells. Conversely, cGMP does not possess the ability to regulate cAMP in a similar manner ²³⁸. The association between NO and PGI_2 is further illustrated by the fact that absence or inhibition of one of these autacoids diminishes vasodilation by ~10%, whereas a combination of the two restored the vasodilatory response ²³⁸. Given that the precursor enzymes of NO and PGI_2 synthesis, NOS and COX, respectively, are regulated by Ca^{2+} Bolz and Pohl demonstrated that PGI_2 could regulate NO release. Using human umbilical vein endothelial cells (HUVEC) they observed that agonist stimulation of endothelial Ca^{2+} release using bradykinin led to enhanced NO synthesis and cGMP production in the presence of a PGI_2 inhibitor, i.e. indomethacin, but supplementation with forskolin, an adenylyl cyclase stimulator, or PGI_2 , attenuated $[\text{Ca}^{2+}]_i$ concentrations and consequently NO production. Thus, PGI_2 , by altering cellular cAMP, can exert a negative effect upon its own as well as NO synthesis via a Ca^{2+} -dependent feedback mechanism. Conversely, endogenous NO does not mediate a similar influence over $[\text{Ca}^{2+}]_i$ or PGI_2 production ²³⁹. Although NO may not be

able to modulate PGI₂ it has been shown to attenuate EDHF release in coronary arteries. Therefore, EDHF could compensate for diminished NO release in damaged arteries. However, this effect of EDHF may be suppressed by elevated levels of oxidised LDL²³⁸. Nevertheless, the fact that three different vasodilators, each with different mechanisms of synthesis and action, are released by the endothelium illustrates the potential for adaptation to different (patho)physiological scenarios and the possibility of manipulation via pharmacological means.

1.8.3 Nitric oxide synthases and nitric oxide in the heart

It is well recognised that NOS and its product NO play significant roles in the cardiovascular system^{240,241}. It has now been established that all three isoforms of NOS (eNOS, nNOS and iNOS) are expressed in the heart and exert different physiological roles depending upon their subcellular location and expression²⁴². Numerous studies at the molecular, cellular and organ level have verified that NO can enhance or impair a variety of cardiac parameters, namely: myocardial coronary vascular tone; myocyte chronotropy (heart rate), inotropy (contraction) and lusitropy (relaxation); and energy production and consumption^{154,243}. In addition, NO can regulate cardiac thrombogenicity, proliferation and antiinflammatory responses, as well as angiogenesis^{140,224}. The mechanism by which these different cardiac effects of NO occur are via activation of either cGMP-dependent (discussed further in Section 1.7) or -independent signal transduction pathways^{32,154,244}.

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In normal human myocardium eNOS and nNOS are constitutively expressed, whereas iNOS is synthesised under pathogenic conditions in response to inflammatory stimuli, resulting in high levels of myocardial NO production^{245,246}. In the case of eNOS, this isoform has been identified in a number of cardiac cells types, e.g. endothelium of the endocardium and coronary vasculature (both capillaries and venules), atrial and ventricular myocytes, as well as specialised cardiac conduction tissue, i.e. the sinoatrial and atrioventricular nodes (SAN and AVN, respectively)²⁴⁵. Subcellular locations within myocytes for eNOS include: plasmalemmal caveolae (via its association with muscle specific isoform caveolin-3); the sarcoplasmic reticulum (SR); T-tubule membranes; and mitochondria^{115,118,177}. As a result, eNOS can control Ca²⁺ flux and thereby participate in the regulation of myocardial force and rhythm contraction²⁴⁷, in addition to controlling energy (O₂) consumption²⁴⁸. Akin to other cell types myocytic eNOS can be regulated, either positively or negatively, by Ca²⁺-dependent or -independent mechanisms^{118,138,249}.

Although the number of studies relating to the role of eNOS in the heart and cardiomyocytes is constantly expanding the information derived is sometimes contradictory, i.e. NO exerts a bimodal (either stimulatory or inhibitory) response, due to the different models, sources (either endogenous or exogenous) and concentrations of NO used. Nevertheless, eNOS is known to regulate myocardial and cardiomyocyte functions under basal, stimulated and diseased scenarios¹⁴⁰. Under basal condition low concentrations of eNOS mediate paracrine, e.g. inhibition of platelet aggregation, promotion of

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vascular smooth muscle relaxation and inhibition of proliferation; and autocrine (within myocytes), e.g. increased diastolic relaxation (a positive lusitropic effect) and contraction (a positive inotropic effect) and decreased O₂ consumption and heart rate (a chronotropic effect), outcomes; whereas higher concentrations have the opposite outcome¹⁶⁹. With regards the chronotropic effects of NOS upon pacemaker cells within the SAN and AVN both eNOS and nNOS have been implicated as potential comodulators of these cells, i.e. nNOS acts presynaptically in cardiac ganglia, while eNOS acts postsynaptically in cardiomyocytes where it modulates the response to muscarinic cholinergic stimulation^{140,170,172,250}. An absence or reduced eNOS synthesis has been implicated as a causative factor in the development of atrial thrombosis (clots) and cardiac pathologies characterised by increased chronotropy, e.g. atrial fibrillation and stroke^{251,252}.

Under stimulated settings, such as during exercise, there is an increase in beating frequency (force-frequency relationship), cardiac muscle stretch and release of catecholamine signalling through β -adrenergic receptors (β 1 and β 2) all of which combine to increase cardiac inotropism¹⁴⁰. To accommodate these changes eNOS and nNOS promote increased O₂ consumption, coronary vasodilation, myocardial contractility (to protect against arrhythmia), inhibition of β -adrenergic-induced inotropy (the latter effect can be enhanced by β 3-adrenoceptors activation of eNOS) and sensitivity of the myocytes to Ca²⁺^{140,242,253}. In individuals with underlying cardiopathologies or vascular related diseases, e.g. diabetes and hypertension, the aforementioned

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responses to cardio-stressful exercises may be somewhat compromised.

In the diseased heart not only is there dysregulation in the type, quantity and expression of NOS and NO produced but there are also changes in the availability of substrates, cofactors, targets and activity of NO, and these parameters vary at different stages of disease development ¹⁴⁰. However, animal and human models of heart failure have noted spatial and temporal changes in the regulation of the expression of eNOS and nNOS towards enhanced iNOS within cardiomyocytes and other cardiac cell types ²⁵⁴. In the case of eNOS, its activity is reduced due to decreased release from peripheral and coronary vasculature sources, endothelial dysfunction ¹⁴⁰. As a result of which the β -adrenoceptors (β 1 and β 2), which are colocalised with eNOS (via caveolin-3) within caveolae, exert an enhanced inotropic response as the inhibitory effect of NO is attenuated ^{115,255}; in addition, control of contractility and relaxation is diminished. Despite these changes increased NO originating from nNOS within the SAN, infiltrating macrophages which produce iNOS, and β 3-adrenoceptors, which are coupled to eNOS production, may, during the initial stages of heart disease, exert a negative inotropic response ¹⁴⁰. However, sustained loss of eNOS, increased oxidative damage and cell loss (via apoptosis) can compound the situation in a defective myocardium.

Initial studies of nNOS suggested that, unlike skeletal muscle fibres, cardiomyocytes did not express significant amounts of this isoform ^{161,250,256}. Thus, eNOS was assumed to be the only constitutive isoform of NOS involved in autocrine regulation of Ca^{2+} homeostasis and myocardial contraction ¹⁵⁴. However, nNOS has since been identified in the atria, along epicardial

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coronary arteries and within cholinergic, nonadrenergic and noncholinergic nerve terminals of specialised cardiac conduction tissue, e.g. the SAN and AVN¹⁶⁹. The release of NO from nNOS within these different cell types can be down regulated through phosphorylation by protein kinases, e.g. Ca²⁺/CaM-dependent, cAMP-dependent, cGMP-dependent and PKC; while dephosphorylation by calcineurin and increasing the binding of CaM to nNOS, supported by the abundant cytosolic protein HSP 90, enhances catalytic activity to produce more NO^{136,257,258}.

Within myocytes, nNOS has been targeted to the SR²⁵⁹, where it reacts to changes in intracellular Ca²⁺ concentration by modulating sarcolemmal I_{Ca} involved in Ca²⁺ distribution, thus modulating excitation-contraction (EC) coupling within the heart^{260,261}. The targeting of nNOS to the SR is mediated by its N-terminal PDZ domain which can link with other muscle associated proteins, e.g. α 1-syntrophin and dystrophin, thereby integrating nNOS in synaptic signalling within cardiomyocytes¹⁰³. The latter integration is facilitated by binding of nNOS with the muscle specific variant of caveolin, i.e. caveolin-3, a component of specialized invaginations of the sarcolemma, called caveolae²⁶². Unlike brain nNOS cardiac SR nNOS is larger (155 and 160 kDa, respectively)²⁵⁹ and may be a new splice or posttranslationally modified variant of nNOS designated as the mu-isoform (nNOS _{μ})²⁶³. The SR of cardiomyocytes play an important role as intracellular sequesters and releasers of Ca²⁺ which is involved in the contraction/relaxation cycle of the beating heart²⁵⁹. Given its prominent role in Ca²⁺ homeostasis it is not surprising that a source of NO, i.e. nNOS, is found in close association with

the SR ²⁵⁹. Potential targets for NO within the SR include the I_{Ca} , the Ca^{2+} release channel (or ryanodine receptor (RyR)) and the Ca^{2+} -ATPase ^{259,260,264,265}. Proposed mechanisms by which NO can inhibit these proteins, and thus regulate cardiomyocyte contractility, include modification of their critical thiol groups or through GSNO ²⁶⁶.

Given its prominent neuronal distribution it is not surprising that nNOS may also regulate cardiac function both at the level of baroreceptor afferents within the NTS of the brainstem ^{267,268} and within the myocardium itself, as this isoform has been localised in cardiac ganglion cells and nerve fibres innervating the SAN ^{172,261}. In addition, nNOS colocalises with the enzyme choline acetyltransferase, which catalyses the synthesis of ACh, in intracardiac neurons ¹⁷⁰. Under normal conditions stimulation of the right vagus nerve leads to ACh release from nerve terminals into the synaptic cleft in response to depolarisation induced by Ca^{2+} influx. The liberated ACh diffuses across the synaptic cleft and binds to muscarinic M2 receptors, coupled to inhibitory heterotrimeric G proteins, on the SAN pacemaker cells. This causes a spontaneous depolarisation of these cells during diastole and the rhythmic generation of an action potential that regulates heart rate ¹⁷⁰. The latter comprises three parts: 1. the upstroke which is mediated by the I_{Ca} ; 2. the hyperpolarisation activated, or pacemaker, current (I_f), which comprises a mixture of sodium and potassium currents to maintain the pacemaker activity; and 3. repolarisation by potassium currents (I_K) ¹⁷⁰. Due to its central role in the regulation of heart rate modulation of the SAN by nNOS has been the subject of many investigations. Using low doses of NO donors *in vitro* and *in*

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vivo experiments have shown that nNOS can exert a positive chronotropic effect within the SAN via stimulation of the I_f as a result of a signal transduction cascade involving inhibition of PDE-3 by cGMP, which promotes increased cAMP/PKA-dependent enhancement of the I_f and intracellular Ca^{2+} mobilisation¹⁷². A dissimilar effect, prolongation of the sinus node recovery time, was observed when high doses of NO, NO and sGC inhibitors were included in the assay protocol, thus illustrating the biphasic ability of NO to directly regulate the SAN and thereby increase or decrease heart rate^{172,269}. Herring *et al.* have suggested that nNOS may also act presynaptically within right vagal neurons by promoting the release of ACh which subsequently stimulates M2 receptors on the SAN pacemaker cells¹⁷⁰.

In parallel with its role as an important physiological mediator of basal cardiac function NO also participates in cardiopathologies. For example, NO inhibits the positive inotropic response to β -adrenergic stimulation in humans with dilated cardiomyopathy more so compared with patients with normal ventricular function²⁴⁸. Cai *et al.* have shown a link between the most common cause of cardiac arrhythmia, i.e. atrial fibrillation, and reduced expression of eNOS and NO bioavailability²⁵². As a result of these changes expression of the prothrombotic stimulant plasminogen activator inhibitor-1 (PAI-1) is upregulated in parallel with an absence of NO, which is a potent antithrombotic agent, thus increasing the potential for clot formation within the left atrial endocardium²⁵². Myocardial expression of eNOS (diminished) and iNOS (enhanced) have been implicated as causative factors in heart failure²⁵⁴. As a result, contractility is reduced, O_2 consumption is increased and

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expression of genes involved in Ca^{2+} homeostasis are upregulated²⁷⁰. It has been suggested that a compensatory switch in NOS expression, i.e. from eNOS to nNOS, takes place in heart failure to maintain autocrine myocardial contractility¹⁵⁴. In fact, Damy *et al.* observed that nNOS and its regulatory protein HSP90 were significantly enhanced in an *in vivo* model of myocardial infarction (MI) in aging rats²⁷¹.

It is now known that the myocardium can express the inducible isoform of NOS, iNOS, in response to LPS and cytokine stimulation^{272,273}. Although the exact physiological role and pathophysiological consequences of this induction is unclear at present reports from studies of animal and human myocytes have observed increased iNOS expression following a cardiac allograft, as well as in different types of cardiac pathologies, e.g. heart failure, myocardial contractile dysfunction and ischaemic preconditioning^{245,274,275}. It has been noted that iNOS activity in cardiomyocytes is enhanced by increased intracellular cAMP or activation of PKC isoforms, which suggests that augmented intracardial or systemic levels of catecholamines and peptide autacoids, e.g. angiotensin II, all of which are an indication of heart failure, could promote and sustain iNOS expression in this pathology²⁴⁵. The generation of iNOS, and the subsequent RNS, can affect not only the heart via the induction of cardiomyocyte apoptosis, but can also give rise to increased systemic microvascular permeability²⁴⁵. Thus, therapeutic reduction of the aforementioned stimuli could lessen iNOS expression and the adverse effects associated with heart failure, i.e. cardiac hypertrophy, ventricular dilatation, increased apoptosis and interstitial fibrosis²⁷⁶⁻²⁷⁸. There

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is also the possibility of endogenous regulation of NO/iNOS as Godecke *et al.* observed that under normal circumstances myoglobin (Mb) can serve as an endogenous 'sink' for excess NO and thus protect the heart against nitrosative stress and subsequent heart failure ²⁷⁹. Given its role as an important O₂ carrier in heart and skeletal muscle, myoglobin can act as an effective trap for different types of RNS, e.g. NO and NO⁻, as oxygenated Mb (MbO₂) can scavenge and degrade NO leading to metmyoglobin (Mb⁺) which itself can react with NO⁻ ^{20,66,280,281}. The Mb⁺ is metabolised back to Mb, which can bind with more O₂, by the enzyme Mb⁺ reductase, thus continuing the cycle of O₂ delivery, RNS scavenging, and thereby protecting important myocytic targets, e.g. cytochromes, from the adverse effects of RNS and maintaining an ambient coronary blood flow and contractility ²⁸². The expression of iNOS is not always detrimental as it may be employed in the resolution of an intracardial inflammatory response, to attenuate low oxygen tension (i.e. hypoxia), protection against ischaemia-reperfusion (IR) injury, as well as maintaining cardiac homeostasis ²⁸³⁻²⁸⁶. Also, it is not restricted to just the cardiomyocytes as other cardiac cells, e.g. the endothelium and smooth muscle of the microvasculature, the endocardial endothelium and tissue macrophages, release this isoform ²⁴⁵. Therefore, the cellular constituents of the heart can act in unison to generate iNOS and RNS within focused areas as part of the normal innate immune response to pathogens ²⁸⁷, whereas widespread and uncontrolled iNOS activation can lead to more generalized myocardial dysfunction ²⁸³.

The above examples illustrate that all three isoforms of NOS and NO are

present within different spatial and temporal of the heart and myocytes. Production of NO at these locations serves to regulate and maintain normal homeostatic function, but excess enzymatic activity or diminished product can be indicative of an underlying cardiopathology or a causative factor of such. Despite the current knowledge on this subject there is still a great need for further research to understand the complex role of NOSs and NO in the heart and exploiting the therapeutic potential of this unique gas as the incidence of CHD is becoming more prevalent worldwide ²⁸⁸.

1.8.4 Nitric oxide in the immune system

A link between NO and the immune system was first made by Tannenbaum and colleagues when they demonstrated in animal and human models that NO_2^- and NO_3^- could be formed via endogenous metabolism and during inflammatory conditions ^{71,289-291}. Independent work by Michael A. Marletta and John B. Hibbs Jr. revealed that macrophages were the principal immunological source of NO_2^- and NO_3^- ^{70,72}. The identification of L-arginine as the source of NO_2^- and NO_3^- , along with the discovery of the iNOS gene in immunostimulated macrophages, strengthened the case for an immunological function for NO and confirmed earlier hypothesises suggesting that NO mediates the phagocytic and cytotoxic aspects of macrophages ^{72,74,292,293}.

Another source of RNS in macrophages, apart from NOS, is the peroxidase enzyme myeloperoxidase (MPO). This abundant haemoprotein (MW ~150 kDa) is stored in primary azurophilic granules and released during the respiratory burst by activated neutrophils, monocytes and macrophages

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^{294,295}. Parallel with MPO, secretion of O_2^- and its product H_2O_2 , respectively derived from the NADPH oxidase complex and nonenzymatic or enzymatic (via SOD) dismutation, enhance respiratory burst activity ²⁹⁶. A number of endogenous molecules, e.g. O_2^- , H_2O_2 , chloride (Cl^-), NO and NO_2^- (the oxidation product of NO), can serve as substrates, either individually or in combination, for MPO, thus generating a variety of reactive oxidants, e.g. hypochlorous acid (HOCl), and nitrating species such as nitryl chloride (NO_2Cl) and nitrogen dioxide (NO_2) ^{295,297}. As a result of these reactions the native ferric enzyme, MPO- Fe^{3+} , can generate multiple intermediates, e.g. Compound-I, -II and -III, to regenerate MPO- Fe^{3+} in a classical peroxidase cycle that varies in reaction rate depending upon the local pH ²⁹⁸. As a result of these varied reactions and products MPO has been implicated as a cause of, or contributor to, oxidative modification of lysine, tryptophan, tyrosine and cysteine targets within low density lipoprotein (LDL), proteins and antioxidants, all of which are causative factors in atherogenesis, both *in vitro* and *in vivo* ^{299,300}.

In the case of nitrating agents the powerful oxidising species NO_2 , derived from the MPO- H_2O_2 - NO_2^- system ³⁰¹, converts LDL into a high-uptake form (NO_2 -LDL) which is actively scavenged by macrophages via their CD36 receptor, thus generating lipid-laden or 'foam' cells characteristic of atherosclerotic lesions ^{298,302}. In the arterial wall NO and O_2^- , originating from endothelial cells and NADPH and xanthine oxidases, respectively, can readily combine to form $ONOO^-$, a RNS capable of modifying LDL ²⁹⁹. The endogenous antioxidants vitamin C (ascorbate) and E (α -tocopherol) have

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been proposed as a potential means to attenuate the adverse effects induced by MPO-mediated LDL modification originating from reactive oxygen, chlorine and nitrogen species ^{300,303,304}.

Production of, and sensitivity to, NO is not limited to macrophages as eNOS and nNOS are present in immune system (e.g. dendritic and mast cells) and immune responsive (e.g. fibroblasts and hepatocytes) cells ³⁰⁵. Immunological functions assigned to NO include: participation in the pathogenesis and control of infectious diseases; tumour development; and autoimmune and inflammatory reactions ³⁰⁶. These diverse effects are mediated primarily by iNOS-derived NO and RNS via: 1. modulating the development, proliferation and apoptosis of immune cells; 2. regulating the production and expression of cytokines, immune mediators; and 3. adhesion molecules along with the synthesis and deposition of extracellular matrix components ^{307,308}. Nitric oxide can promote the survival of an infectious agent through cytotoxic mechanisms and the provision of blood-borne nutrients concomitant with subversion of the hosts immune response ^{305,309}. Conversely, NO may subvert disease progression directly through mutation of DNA or indirectly through induction of iNOS, which depletes L-arginine, and promotion of neutrophil cell killing ^{17,80,305}. However, excessive activation of the latter mechanism can occur in chronic inflammatory diseases, e.g. rheumatoid arthritis (RA), as nitrotyrosine has been detected in the serum and synovial fluid of RA patients ³¹⁰.

The first role identified for NO in the immune system was modulation of tumour growth and cell death ⁷³. Since then opposing functions, both pro-

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(increasing tumour angiogenesis, immunosuppression and inhibiting leukocyte infiltration) and anti-tumour (cytotoxic, cytostatic and antimetastatic) actions have been purported for NO^{311,312}. The original perception of iNOS-derived NO in autoimmunity was a negative one as it activates macrophages which in turn causes tissue damage³⁰⁶. However, experimental models of autoimmune disease, e.g. experimental autoimmune arthritis (EAA) and experimental autoimmune nephritis (EAN), portrayed NO in a positive light with regards negative feedback regulation of T-helper (T_H1) cells, thus guarding against immunopathological consequences³⁰⁵. The antiinflammatory aspects of NO are manifested by inhibition of leukocyte and platelet function^{51,228}, while inhibition of iNOS expression can promote transplant accommodation and graft survival³⁰⁵.

The antiinflammatory mechanisms employed by RNS can be focused directly upon critical target such as DNA, proteins and lipids or indirectly via modulation of the immune response or other host cell functions³¹³. In the case of DNA, NO can promote deamination³¹⁴, ONOO⁻ induces DNA mutations³¹³, while NO₂⁻ can reversibly bind to the iron-sulphur (Fe-S) group of ribonucleotide reductase, the rate limiting enzyme in DNA replication, thus inhibiting DNA synthesis³¹⁵. A variety of proteins, e.g. reactive thiols, haem and Fe-S groups, tyrosyl radicals, phenolic and aromatic acid residues or amines, can be targeted by RNS. Nitric oxide can inactivate thiol and tyrosyl groups within nucleic adenoribonucleotidase, a key enzyme in translation processes¹³. In addition, formation of iron-nitrosyl complexes and inactivation of the Fe-S prosthetic group within aconitase (which converts citrate to

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isocitrate, in the tricarboxylic acid (or Krebs) cycle), as well as complex I (NADH dehydrogenase) and complex II (succinate dehydrogenase) of the electron transport chain, by ONOO^- and NO leads to metabolic inhibition and promotes depletion of iron, an essential element for microbial survival^{313,315,316}; the overall effect being cytostasis. Haemoproteins such as catalase, which mitigates the adverse effect of H_2O_2 , cytochrome *c* oxidase (complex IV), the terminal component of the respiratory chain, and cytochrome P450, are additional inhibitory targets of NO.

Thiol proteins are among the most prominent targets of NO and S-nitrosylation (transfer of NO^+) result in transnitrosation (reaction with other thiol groups) thus enhancing or inhibiting their activity³². An example of inhibition is the auto-ADP-ribosylation of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by NO, the outcome being loss of enzymatic activity and impairment of glycolysis³¹⁷. Nitration of tyrosine residues, not just by ONOO^- ³¹⁸ but also by the enzyme MPO in the presence of NO_2^- and H_2O_2 with NO_2 as the product²⁹⁹, can alter tyrosine phosphorylation thus disrupting protein function and turnover. Furthermore, ONOO^- and NO_2 can instigate lipid peroxidation which can lead to damage of the lipid-rich coat of microbes³¹³. Lastly, NO can: 1. promote vasodilation; 2. modulate the expression and function of endothelial cell adhesion molecules (ECAMs) via inhibition of the transcription factor $\text{NF-}\kappa\text{B}$ (possibly by stimulation of $\text{I}\kappa\text{B}$ an inhibitor of the latter) and the enzyme activity of PKC, as their expression affects recruitment of other inflammatory-related cells, e.g. neutrophils, macrophages and platelets; 3. regulate cytokine production, as

well as T-helper cell proliferation and differentiation; and 4. alter epithelial permeability ^{306,313,319}. Whether NO will exert a pro or antiinflammatory response will depend upon its quantity, the presence of ROS and other potential targets in the surrounding environment, in addition to the level of antioxidants which counteract toxic metabolites derived from RNS interactions ¹⁴.

1.8.5 Nitric oxide in the reproductive system

A role for NO in the male reproductive system stems from the detection of iNOS and eNOS in the neurons of the corpus cavernosum and the adventitial layer of penile arteries, respectively ³²⁰. Animal and human studies which employed NOS inhibitors, electrical field stimulation or NO donors, verified that NO mediates penile erection ^{320,321}. These reproductive aspects of NO have been harnessed for therapeutic purposes in the treatment of erectile dysfunction (ED) with the type V phosphodiesterase (PDE-5) inhibitor, sildenafil, more commonly known as Viagra® ³²². Development of ED is often associated with underlying cardiovascular disease, e.g. ischaemic heart disease, as both share similar risk factors, i.e. hypertension, smoking and diabetes ³²³. A comparison of the relaxant responsiveness of isolated strips of corpus cavernosum, from diabetic and non-diabetic men with impotence, towards nerve stimulation and ACh, noted a correlation between impaired NO responsiveness and underlying diabetes ³²⁴.

Preeclampsia, which has a prevalence of 6-8% of all pregnancies over 20 weeks, accounts for more than 75% of pregnancy induced-hypertensive

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disorders and is one of the main causes of foetal and maternal morbidity and mortality, but is resolved completely post partum^{325,326}. Although the exact cause of preeclampsia is unknown the trigger for the disease is believed to be placental in origin, as a result of which the mother responds with a strong systemic inflammatory response characterised by vascular dysfunction³²⁷. Risk factors for preeclampsia include: first pregnancy; asthma; a change in partner; a genetic disposition; and living at high altitude³²⁵. Normal pregnancy is associated with a number of vascular adaptations, i.e. a 50% increase in blood volume and cardiac output which leads to a fall in blood pressure due to peripheral vasodilation; the opposite occurs in preeclampsia³²⁸.

Preeclampsia is characterised by a triad of symptoms: 1. hypertension, 2. proteinuria and 3. oedema.³²⁵ Other characteristics of preeclampsia include: increased vascular resistance (the latter arises from increased foetalplacental hypoxia due to either decreased oxygen availability or transfer); poor perfusion; elevated circulating lipid peroxides because of oxidative stress; and an enhanced immune response which can result in a dysfunctional endothelium that promotes adhesion of platelets and leukocytes to the placental villi or intervillous spaces³²⁹. Nitric oxide is important during pregnancy as the activity of the NO system (eNOS, iNOS and L-arginine pathway) is shifted to a new higher equilibrium during normal pregnancy and maintained throughout gestation to inhibit uterine contractility and declines towards term to initiate labour³²⁹. Nitric oxide production, as measured by NO metabolites (NO_2^- and NO_3^-) and cGMP, are either significantly increased in preeclampsia and eclampsia, compared with normal pregnancy^{325,329,330}; or

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decreased ^{326,331}. When NO synthesis is increased it helps to promote vasodilation within the foetal capillaries, this results in better perfusion, reduced lipid peroxidation and prevention of platelet aggregation and leukocyte adhesion ³³⁰.

From the examples given above, it is clear that NO plays an important role in a diverse range of physiological functions. At the same time the effects mediated by NO are not always beneficial but can be detrimental and cytotoxic in the extreme, thus illustrating the two faced nature of NO ³³².

1.9 Oxidative and nitrosative stress: sources, biochemistry and relevance to cellular function and injury

Molecular oxygen (O_2) and nitrogen (N_2) constitute over 98% of the air we breath. Even though both are essential for plant and animal life reactions involving O_2 are more commonplace, due to its higher atmospheric quantity¹⁴. Although O_2 is an essential energy source, its redox derivatives can lead to alterations of cellular function, e.g. lipid peroxidation and modification of DNA and proteins, and have been implicated in the development of many diseases^{14,333-335}. These redox derivatives, which exist as radicals or non-radicals, are known collectively as ROS (see Table 1.2 for examples)¹⁴. Reactive oxygen species are constantly formed *in vivo* as 'accidents of chemistry' or deliberately. Examples of accidentally formed ROS include: O_2^- , H_2O_2 and the hydroxyl radical (OH^\bullet), while the generation of O_2^- by activated phagocytes during cellular killing is an example of deliberate formation³³³. In their 'free' form transition metal ions, e.g. iron (Fe^{2+}) and copper (Cu^{2+}), can act as powerful promoters of ROS formation³³⁶. When the levels of ROS are in excess of the capacity of endogenous cell protectants (see examples in Table 1.3) an increase in cellular damage and dysfunction can occur due to oxidative stress, while the causative agents are termed oxidants³³³.

Table 1.2 Examples of reactive oxygen and nitrogen species

Reactive oxygen species (ROS)	
Radicals	Non-radicals
Superoxide anion (O_2^-)	Hydrogen peroxide (H_2O_2)
Hydroxyl (OH^\cdot)	Hypochlorous acid (HOCL)
Peroxyl (LO_2^\cdot)	Ozone (O_3)
Alkoxyl (LO^\cdot)	Singlet oxygen (1O_2)
Hydroperoxyl (HO_2^\cdot)	Lipid peroxides
Reactive nitrogen species (RNS)	
Nitric oxide (NO)	Nitrous acid (HNO_2)
Nitrogen dioxide (NO_2)	Dinitrogen trioxide (N_2O_3)
	Dinitrogen tetroxide (N_2O_4)
	Peroxynitrite ($ONOO^-$)
	Alkyl peroxynitrites (LOONO)

Although the chemical and biological aspects of O_2 and ROS have been studied for almost 50 years, the same cannot be said of N_2 . Nevertheless, N_2 has a rich chemistry as this element has the ability to occupy a wide range of oxidation states from - 3 to + 5³⁷. Biologically important oxides of N_2 lie in the + 1 to + 5 range, examples include (NO^- , + 1; NO, + 2; NO^+ , + 3; and $ONOO^-$, + 3), other examples are shown in Table 1.2³³⁷. The discovery that the simplest N_2 and O_2 based molecule, NO, is produced *in vivo* and has many important physiological functions (see Section 1.8) and a diverse redox chemistry, opened up a new field of biological interest on a par with O_2 related biology^{17,25}.

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Maximal basal levels of NO, derived from NOS, are ~1-2 μM , while levels above 2 μM are associated with the formation of redox derivatives, which may be radical or non-radical in nature, and are known collectively as RNS¹⁷. The reactions mediated by NO can occur directly, e.g. with O_2 , O_2^- and the haem of sGC, or indirectly, e.g. nitration (addition of NO_2), oxidation, hydroxylation and nitros(yl)ation (addition of NO^+) of different molecular targets²⁹. The indirect effects of NO are mediated by RNS, which in excess can give rise to nitrosative stress, a common factor in many pathologies, while the causal species are referred to as nitrosants^{244,338}.

The reactions in which RNS and ROS participate are not confined to biomolecules or specific molecular targets as both types of reactants can interact with each other to generate even more reactive species. Among the best characterized of these examples is the rapid reaction ($6.7 (\pm 0.9) \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$) of NO with O_2^- to form ONOO^- **Reaction 1**³¹⁸. The concentration of ONOO^- is controlled by the activities of both NOS and SOD as a 10-fold increase in both NO and O_2^- leads to a 100-fold increase in ONOO^- production²³. Another potential *in vivo* source of ONOO^- is through reaction of NO with O_2 **Reaction 2**³³⁹. Under physiological conditions, ONOO^- is a strong oxidising species with a short half-life ($t_{1/2} = \sim 1 \text{ sec}$) and will decompose via peroxynitrous acid (ONOOH) to form NO_3^- through isomerisation **Reaction 3**

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$\text{NO} + \text{O}_2^{\bullet-} \rightarrow \text{ONOO}^-$	Reaction 1
$\text{NO}^- + \text{O}_2 \rightarrow \text{ONOO}^-$	Reaction 2
$\text{ONOO}^- + \text{H}^+ \rightleftharpoons \text{ONOOH} \rightarrow \text{NO}_3^- + \text{H}^+$	Reaction 3

Due to its very reactive nature ONOO^- can engage in oxidation, nitration and nitrosation type reactions, which are the causative factors for many pathologies, e.g. cardiovascular and neurological diseases as well as cancer^{17,341,342}. A positive aspect to ONOO^- formation is its role in mediating cell cytotoxicity towards pathogens within macrophages and neutrophils in response to increased iNOS activity¹⁷. As ONOO^- can cause nitration of aromatic amino acids, e.g. tyrosine, quantification of 3-nitrotyrosine could be used as a biomarker of RNS damage³¹⁰.

In order to combat the adverse effects of ROS and RNS the body has a repertoire of naturally occurring defence mechanisms in the form of: antioxidants; repair enzymes to remove damaged proteins, lipids and DNA; storage proteins; and scavengers³³⁴. These cellular protective systems can be distributed within (intracellular) or outside (extracellular) cells or occupy both compartments (see Table 1.3 for examples). A second line of cellular defence is the enzyme haem oxygenase, discussed further in Sections 1.12 and 1.13. Along with these natural defence mechanisms, 'synthetic' agents and a diet rich in fruit and vegetables have been suggested as an exogenous defence against radical based diseases³³⁴. In summary, ROS and RNS share a number of similarities: they both generate radical and non-radical derivatives from their respective precursors, O_2 and N_2 , on a continuous basis *in vivo*;

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their formation can be beneficial, i.e. killing pathogens, or adverse when excess quantities overwhelm endogenous attenuating mechanisms; they share similar molecular targets; and both have been shown to play a role in various pathologies either on their own or through interaction with one another.

Table 1.3 Types and location of naturally occurring cell protectants

Type	Location	
	Intracellular	Extracellular
<i>Antioxidants</i>		
Superoxide dismutase (SOD)	+	-
Catalase	+	-
Glutathione peroxidase	+	-
Reduced glutathione (GSH)*	+	+
<i>Storage Proteins</i>		
Ferritin	-	+
Transferrin	-	+
Lactoferrin	-	+
Caeruloplasmin	-	+
Haemopexin	-	+
Haptoglobin	-	+
Albumin	-	+
<i>Scavengers</i>		
α -tocopherol (TH)	+	+
Ascorbic acid (Vitamin C)	+	+
Urate	-	+
Ascorbate	-	+
Bilirubin (BR)	-	+
<i>Repair Enzymes</i>	+	-

* Reduced glutathione (GSH) is present mainly intracellularly at millimolar (0.1-10 mM) concentrations, but trace amounts exist extracellularly (≥ 2 $\mu\text{mol/L}$); however, fluids lining the lower respiratory tract have a much higher concentration (≥ 100 $\mu\text{mol/L}$)^{343,344}.

1.10 Nitric oxide donors

For over 130 years the organic nitrates and nitrites (known collectively as nitrovasodilators), e.g. amyl nitrite and GTN, have been used as pharmacological agents for the relief of cardiovascular complications¹. Despite their extensive usage it is only in the last 30 years that their mechanism of action has been unravelled, i.e. metabolic conversion to NO within the tissues³, although the factor(s) responsible for this effect are still unclear^{345,346}. In addition, prolonged usage of these agents can have a number of negative impacts, i.e. adverse haemodynamic effects, drug tolerance, lack of sensitivity and limited bioavailability⁸. The more conventional term 'NO donors' is used to describe compounds that release active NO and its congeners, i.e. NO⁺ and NO⁻, in a controlled manner through enzymatic, chemical, electrochemical or photochemical pathways³⁴⁷. Advantages of NO donors over nitrovasodilators include: 1. predictable amounts and release rates of the RNS; 2. instantaneous effect, i.e. they do not need to undergo biotransformation; and 3. lack of tolerance development³⁴⁷.

In order to study the wide range of physiological mechanisms mediated by NO, there are two choices open to the investigator: 1. inhibition of the NOS pathway to prevent endogenous NO production; or 2. administration of authentic NO gas, in solution or via inhalation, as an exogenous NO source. However, option 1 has the disadvantage of lack of specificity, while the second choice has further downsides, i.e. lack of solubility of NO gas in aqueous media and applicability to different experimental settings, plus the short half-life of NO adds to the difficulty of exogenous administration and

limits the use of the gas. These drawbacks have been overcome with the commercial availability of NO donors³⁴⁸. The NO donors are classified into six categories based upon the molecule to which the NO releasing moiety is attached, with further subclasses within each (see Table 1.4 for examples). Disadvantages associated with using these different types of NO donors is that they exhibit inter and intra class instability in the presence of light, thiols, oxygen and heat³⁴⁷. Nonetheless, research is ongoing towards the production of NO donors, from all categories, which are more stable, give greater control of NO release and are more tissue specific³⁴⁸.

Table 1.4 Classes and subclasses of commercial nitric oxide donors

Class of NO Donor	Subclasses
C-NO Donors	Nitro, nitroso, guanidine and oximes
N-NO Donors	N-nitrosamines, N-hydroxy-N-nitrosamines, N-nitrosoamides and diazeniumdiolates
D-NO Donors	Organic nitrates and organic nitrites
S-NO Donors	Thionitrates and thionitrites
Heterocyclic NO Donors	Sydonimines, oxadiazoles (furoxans) and oxatriazoles
Transition metal NO complexes	Nitrogen is bound to the metal instead of oxygen, e.g. sodium nitroprusside (SNP)

1.10.1 Diazeniumdiolates

The experiments detailed in this Thesis utilise diazeniumdiolates, which belong to the N-NO donor class (see Figure 1.8 for examples). Diazeniumdiolates are chemicals containing the structure $X-[N(O)NO]^-$, where X is a carrier molecule and $[N(O)NO]^-$ the anionic functional group³⁴⁹. Nucleophiles, e.g. primary amines, secondary amines, polyamines, oxygen (oxide) or sulphur (sulphite), can serve as carrier molecules, which allows for

compounds with specific NO releasing characteristics^{347,350}. Previously referred to as “NONOates”, “Drago complexes” and “NOC” compounds, these terms have been abandoned in favour of the term “diazoniumdiolates”, which is based on the structural and functional nomenclature of the functional group

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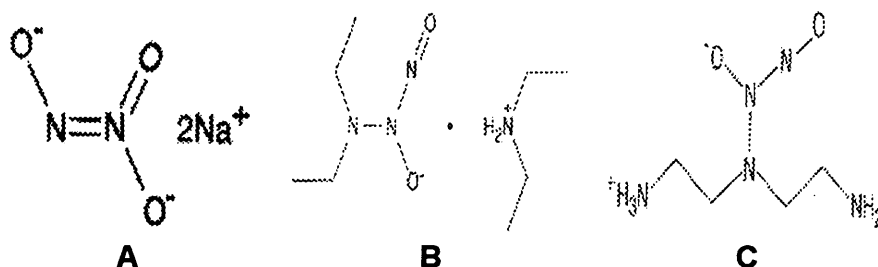
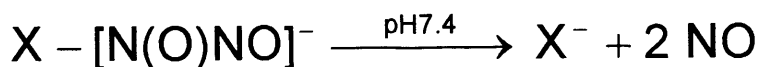


Figure 1.8 Structure of the diazeniumdiolates used in this Thesis.

A Sodium trioxodinitrate or Angeli's salt (AS); **B** diethylamine NO (DeaNO); and **C** diethylenetetraamine NO (DetaNO).

Although other classes of NO donors exist, the diazeniumdiolates have a number of physiochemical features to merit their use in NO research: 1. stability as solids at low temperatures; 2. known rates of NO generation, i.e. they decompose spontaneously, and dissociate according to first-order kinetics at constant pH to produce 2 moles of NO per mole of donor (Equation 1.2) thus, obviating the need for redox activation; 3. are insensitive to thiols or other redox-active agents; 4. half-lives range from 2 sec to 20 h at physiological pH and temperature (37°C); and 5. different types of RNS can be produced, e.g. NO⁻, NO⁺, NO, NO₂ or ONOO⁻³⁴⁹.



Equation 1.2 Decomposition pathway of diazeniumdiolates

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An interesting characteristic of diazeniumdiolates is that pH can affect their rate of decomposition, i.e. increased rate constants with decreasing pH and *vice versa*³⁵⁰. This feature enables the user to prepare stable alkaline stock solutions, e.g. 1-10 mM NaOH (pH 12), which become active to spontaneously generate a bioactive RNS at physiological pH or upon mixing with physiological buffer or media³⁴⁷. Examples of commonly used diazeniumdiolates, together with their respective acronyms and half-lives are shown in Table 1.5.

Table 1.5 Commonly used diazeniumdiolates

Chemical Name	Acronym	Half-life (t _{1/2}) at 37°C, pH 7.4
Sodium trioxodinitrate (Angeli's salt)	AS	2.3 Minutes
Diethylamine NONOate	DeaNO	2 Minutes
Diethylenetetraamine NONOate	DetaNO	20 Hours
Propylaminopropylamine NONOate	PAPANO	15 Minutes
Proline NO	PROLINO	1.8 Seconds
Methylamine hexamethylene methylamine NO	MAHMANO	1 Minute

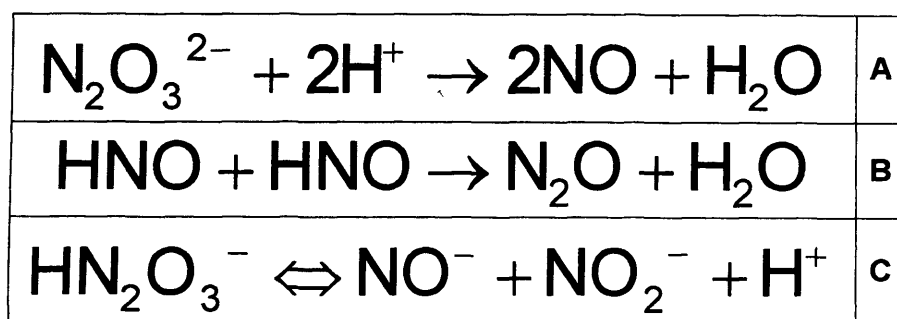
1.10.2 Chemistry and sources of sodium trioxodinitrate (Angeli's salt)

Sodium trioxodinitrate ($\text{Na}_2\text{N}_2\text{O}_3$, or Angeli's salt (AS)), see Figure 1.8 A for structure, spontaneously decomposes at neutral pH to liberate NO^- and NO_2^- ²⁵. Although AS is included in the category of diazeniumdiolates it differs from other members of this group as it releases NO^- instead of NO ³⁴⁹. Since its discovery by the Italian scientist A. Angeli in 1902 investigations into the chemistry of AS, in particular its decomposition reaction,^{37,38} and the physiological significance of its product NO^- ^{25,58,351}, have generated

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considerable interest as this agent is the most widely used synthetic source of NO^- to study the effect of the anion in biological systems. Angeli's salt can be synthesised through reacting hydroxylamine (NH_2OH) with an organic nitrate, e.g. ethyl, isopropyl and butyl nitrates, or purchased from commercial sources³⁸. Studies of AS decomposition and reactivity have revealed different answers depending upon the model (gas or aqueous phases), composition (presence of O_2 , thiols, metals and oxidants), pH and temperature of the surrounding environment^{25,37}.

Kinetic studies reveal that decomposition of AS is pH-dependent. In aqueous solution at pH below 4 the monoanion ($\text{N}_2\text{O}_3^{2-}$) decomposes to yield NO (Equation 1.3 **A**)³⁷. This reaction can be inhibited by NO_2^- . At pH above 4 two reactions take place: 1. the unstable conjugate acid of NO^- , nitroxyl ion (HNO), undergoes dimerization and dehydration via formation of hyponitrous acid ($\text{H}_2\text{N}_2\text{O}_2$) to give N_2O and water (H_2O) (Equation 1.3 **B**)⁴⁰; 2. spontaneous decomposition of hydrogen trioxodinitrate monoanion (HN_2O_3^-) liberates NO^- and NO_2^- (Equation 1.3 **C**). The latter reaction can be inhibited by NO_2^- , which illustrates the reversible nature of the reaction³³⁹. Thus, the decomposition of AS is independent of pH between pH 4.4-8.1 and proceeds via a first-order reaction involving dissociation of the monoanion, but different rate constants (k) and half-life ($t_{1/2}$) values apply depending upon the reaction temperature, i.e. $k = 6.8 \times 10^{-4} \text{ s}^{-1}$ at 25°C , $t_{1/2} = 17 \text{ min}$; $k = 4.1 \times 10^{-3} \text{ s}^{-1}$ at 37°C , $t_{1/2} = 2.3 \text{ min}$ ²⁵.



Equation 1.3 Decomposition sequence of Angeli's salt in aqueous solution

Although a N=N bond makes up the solid state of AS, Figure 1.8 A, cleavage occurs via heterolysis of a singly bonded N-N structure, i.e. tautomerisation between double and single bonded species occurs³⁸. Originally, NO⁻ was assumed to be a weak acid (pKa = 4.7)³⁷, however, recent reports indicated it has a weaker acidity (pKa ~7-11.4)^{39,351}. A number of suggestions have been put forward for the electronic spin state of the NO⁻ released from AS in different models (gas or aqueous phases). Similar to O₂, NO⁻ is isoelectronic, i.e. it can exist in singlet (¹NO⁻) and triplet (³NO⁻) states. Hughes suggests that thermal decomposition of AS in the presence of O₂ yields ¹NO⁻ (the ground state) as only ³NO⁻ (the excited state) reacts with O₂ to yield ONOO⁻²⁵. Recent articles by Bartberger *et al.* and Miranda and coworkers using NO⁻ in the gas phase and in aerobic conditions, respectively propose ³NO⁻ as the ground state^{58,351}.

Despite its short lived nature NO⁻ originating from AS is known to react with a number of endogenous targets resulting in different outcomes. For example, NO⁻ interacts with O₂ to generate potent oxidising species which can promote double strand DNA breaks and base mutations^{352,353}. Binding of NO⁻ with iron

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centres of haemoproteins such as hemin (the oxidised form of haem) yields nitrosyl complexes that act as potent stimuli for the induction of cytoprotective enzymes, e.g. haem oxygenase³⁵⁴. Akin to NO, critical thiol groups can serve as prominent targets for NO⁻ as illustrated by the latter's ability to downregulate the activity of the NMDA receptor via S-nitrosylation of a specific cysteine residue (Cys 399), as a result NO⁻ could provide neuroprotection from excitotoxic insults^{55,355}. Other important regulatory molecules that could be influenced by NO⁻ through S-nitrosylation include: the RyR³⁵⁶; I_{Ca} ²⁶⁰; Ca²⁺-dependent K⁺ channels (K⁺Ca)⁶⁰; caspase enzymes³⁵⁷; transcription factors, e.g. NF- κ B³¹; and indirect S-nitrosylation of haemoglobin possibly via the liberation of NO from the interaction of NO⁻ with R-SNO and thiols, the NO can then S-nitrosylate the Cys β 93 residue of haemoglobin^{30,358}. Lastly, NO⁻ has been shown to exert a beneficial effect in models of cardiac dysfunction⁵⁴ and IR injury⁴⁶.

Although other sources of NO⁻ exist, e.g. Nitroprusside (NP), discrepancies exist as to the product released, i.e. NO⁵⁰ or NO⁻³⁸, by this agent. The reason for these different observations stems from the fact that at pH 7 NP decomposes very slowly to give NO, while at pH 8 and above NO⁻ is the active product. Thus NP is more robust as a NO⁻ donor at physiological pH than NP, while the latter is a good NO⁻ generator at pH > 8³⁸. In summary, the chemistry of NP and its product NO⁻ is quite complex and diverse depending upon the environment and models used. This feature adds to the variety of beneficial and adverse effects of this agent, and at the same time merits further investigation to harness the potential of this unique compound.

1.11 The structure of haem

Haem (ferro-(Fe²⁺)protoporphyrin IX) is a nonpolypeptide unit which acts as the prosthetic group for a variety of important biological molecules, see Table 1.6 for examples ³⁵⁹. Haemoglobin (Hb), which is comprised of four globin molecules each with a central haem prosthetic group, acts as an O₂ carrier for red blood cells (RBC), thus representing the principal biological source of haem ³⁶⁰.

Table 1.6 Biologically important haemoproteins and their function

Molecule	Function
Myoglobin	Oxygen carrier in muscle
sGC	Endogenous receptor for NO
Cytochromes (b, c, c1, a and a3)	Cellular respiration and electron transfer
Cytochrome b5	Dehydrogenation of fatty acids in microsomes and reduction of methaemoglobin to haemoglobin in RBC
Cytochrome P-450 (CYP-450)	Drug metabolising enzymes
Catalase and peroxidase	Hydrogen peroxide scavengers
Tryptophan pyrrolase	Regulates L-tryptophan metabolism
Nitric oxide synthase (NOS)	Production of NO

Haem (Figure 1.9) consists of an organic protoporphyrin structure, four pyrroles each of which are linked by unsaturated methene (*meso*) bridges (α , β , γ and δ) to form a tetrapyrrole ring, surrounding a central iron (Fe) atom linked to four nitrogens (N₂). Attached to the tetrapyrrole ring are four methyl (CH₃), two vinyl (CH=CH₂) and two propionate (CH₂CH₂COOH) side chains. Fifteen different isomers can be formed by rearranging the location of these side chains; however, only one of these, protoporphyrin IX, is present in biological systems ³⁶⁰. In addition to its bonding with N₂, Fe is capable of forming two additional bonds either above or below the haem plane (also

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referred to as the fifth and sixth coordinate positions). As the Fe atom can exist in two different oxidation states (Fe^{2+} or Fe^{3+}) the corresponding forms of haem are ferro- (Fe^{2+}) protoporphyrin IX and ferri- (Fe^{3+}) protoporphyrin IX (or hemin, the oxidised form of haem). In RBC only ferro- (Fe^{2+}) haemoglobin can bind O_2 ³⁶⁰.

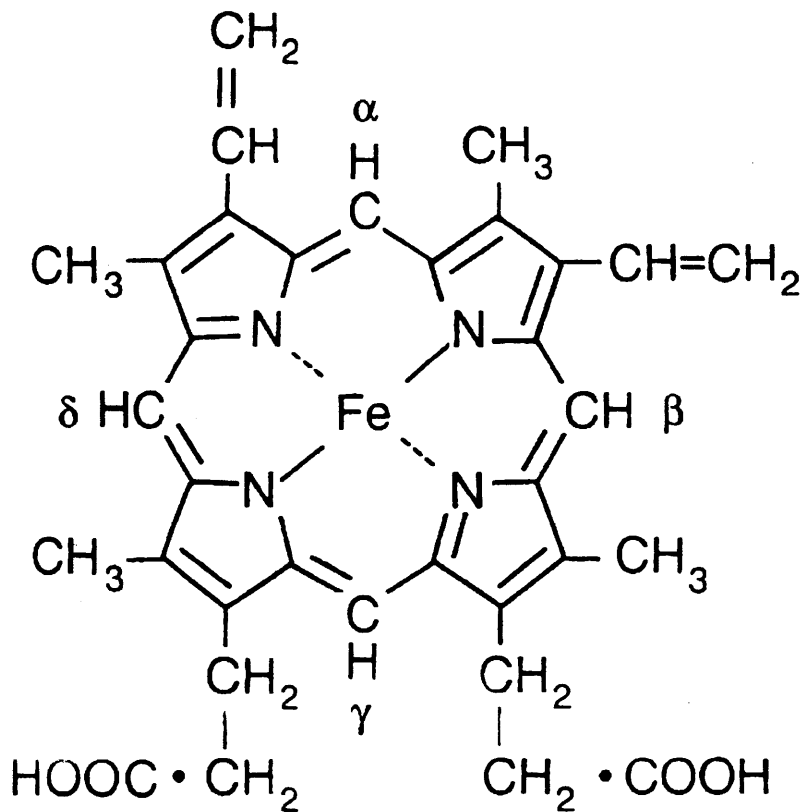


Figure 1.9 Structure of haem (ferro- (Fe^{2+}) protoporphyrin IX).

Adapted from ³⁵⁹.

1.11.1 The haem biosynthesis pathway

In the 1950s, Shemin and coworkers identified that glycine and succinyl coenzymes A (CoA) were the primary substrates in the biosynthesis of haem. Since then the pathway of haem synthesis, as well as the enzymes and their respective genes, have been characterized. The haem synthesis pathway (Figure 1.10) in mammals consists of eight enzymatic steps divided equally between the mitochondria and the cytosol³⁵⁹.

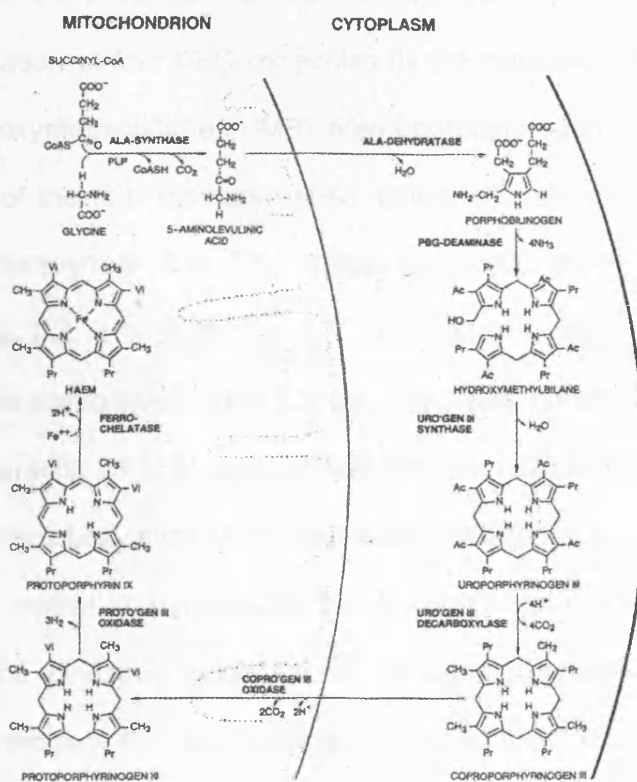


Figure 1.10 Pathway of haem synthesis.

Side chain abbreviations: Pr = Propionate (CH₂CH₂COOH); Vi = Vinyl (CH=CH₂); CH₃ = Methyl; and Ac = Acetic acid (CH₂COOH). Adapted from³⁵⁹.

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The first step of the pathway occurs in mitochondria and involves the condensation of glycine and succinyl CoA to form 5-aminolevulinic acid (ALA), concomitant with the release of CoA and carbon dioxide (CO₂). This reaction is catalysed by cytosolic ALA synthase (ALA-S) and requires pyridoxal-5'-phosphate (PLP) as a cofactor. The next four steps take place in the cytosol, beginning with the condensation of two molecules of ALA via the cytosolic enzyme ALA dehydratase (ALA-D). This results in the release of water (H₂O) and the production of the monopyrrole porphobilinogen (PBG). Next, polymerisation of four PBG molecules by the cytosolic PBG deaminase generates hydroxymethylbilane (HMB) preuroporphyrinogen concurrent with the elimination of the four methane (NH₃) molecules, one for each methene bridge of the tetrapyrrole ring. The structure of HMB resembles that of a tetrapyrrole ring, but is unstable as it is open on one side. Therefore, the cytosolic enzyme uroporphyrinogen (URO) III synthase catalyses the 'closure' of the ring, generating URO III, concomitant with the release of H₂O. The final cytosolic step, decarboxylation of the four acetic acid (CH₂COOH) side chains of URO III to methyl (CH₃) groups forms coproporphyrinogen III and is catalysed by the cytosolic enzyme URO III decarboxylase. This reaction requires four hydrogens (H⁺) and liberates four CO₂ molecules^{359,361}.

The remaining biosynthesis steps occur in the mitochondria. The first of these, oxidative decarboxylation of coproporphyrinogen III to protoporphyrinogen III, is catalysed by the intermembrane space enzyme coproporphyrinogen III oxidase. This reaction requires O₂ to convert two propionate (CH₂CH₂COOH) side chains to vinyl (CH=CH₂) groups, concomitant with the elimination of two

H⁺ and CO₂ molecules. In the penultimate reaction protoporphyrinogen III is oxidised to protoporphyrin IX by the mitochondrial enzyme protoporphyrinogen III in parallel with the loss of six H⁺. The final step, insertion of Fe²⁺ into protoporphyrin IX and release of two H⁺ ions to give haem, is catalysed by the mitochondrial enzyme ferrochelatase ^{359,361}.

1.11.2 Regulation of haem biosynthesis

Haem synthesis occurs predominantly in erythroid tissue, i.e. the spleen, but non-erythroid tissues, e.g. liver, muscle, adrenal glands, gonads and neuronal tissue, also produce haem ³⁵⁹. Identification and cloning of all the mammalian enzymes involved in the pathway of haem synthesis has enabled control points to be targeted as a mechanism to manipulate haem production ³⁶¹. The principal location for regulation of haem synthesis is the enzyme ALA-S; thus, it has been referred to as the rate-limiting step in the haem biosynthesis pathway ³⁵⁹. The enzymes PBG deaminase, coproporphyrinogen oxidase and ferrochelatase have also been proposed as possible targets for regulation, however, most of the evidence relating to regulation of haem biosynthesis is in favour of ALA-S ³⁶¹. The latter enzyme exists as two isoforms encoded by separate genes: the ubiquitous (ALA-S1 or ALAS-N) and the erythroid (ALA-S2 or ALAS-E) specific ³⁶¹.

Different mechanisms of ALA-S regulation prevail in liver and erythroid tissues. In non-erythroid tissues it is generally understood that the excess of haem suppresses ALA-S1 transcription and *vice versa* ³⁵⁹. However, recent evidence suggests that excess haem exerts feedback inhibition by decreasing

the half-life of ALA-S1 mRNA, thus blocking the movement of the ALA-S1 precursor proteins into mitochondria, whereas when haem is limited these mechanisms are subverted, leading to ALA-S1 activation. In erythroid cells, regulation of haem production is not necessarily via haem itself but rather iron, as the reaction is counter regulated by haem ³⁶¹.

1.12 The haem oxygenase pathway

Unlike the process of haem synthesis, which involves a series of complex enzymatic reactions, the process of haem degradation is far simpler. Haem catabolism, also referred to as the haem oxygenase pathway, see Figure 1.11, is carried out by the enzyme haem oxygenase (HO-1 and HO-2; EC 1.14.99.3) which is located within the endoplasmic reticulum ³⁶². This reaction produces equimolar quantities of carbon monoxide (CO), ferrous iron (Fe^{2+}) (which is sequestered by the iron storage protein ferritin) and the intermediate biliverdin, which is subsequently reduced to bilirubin via the cytosolic enzyme biliverdin reductase (BVR; E.C. 1.3.1.24) ³⁶³. As the process requires O_2 and a reducing agent, reduced nicotinamide adenine dinucleotide phosphate-cytochrome P-450 reductase (NADPH-CPR), it is known as coupled-oxidation. However, coupled-oxidation of haem can also proceed via non-enzymatic routes, but the haem oxygenase pathway is the most efficient and the only one to produce bile pigments and CO exclusively ³⁶⁴.

Depending upon the tissue, organ and cell type examined, as well as the underlying homeostatic state, i.e. normal or disease conditions, three major sources of haem exist: 1. intracellular haemoproteins (e.g. those listed in

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Table 1.6); 2. haem derived from haemoglobin in blood; and 3. *de novo* synthesis via the haem biosynthesis pathway described earlier. As haem is potentially toxic in the free (unbound) state and must be synthesised and degraded within the same cell, an exception being senescent erythrocytes phagocytosed by macrophages, the respective enzymes, ALA-S and haem oxygenase (constitutive HO-2 and inducible HO-1; discussed further in Section 1.13), act in tandem to regulate the essential iron containing prosthetic group haem as well as maintain the balance between basal and adverse physiological levels ³⁶⁵⁻³⁷⁰. Furthermore, the products of the haem oxygenases, bilirubin and CO, can serve as a constant (via HO-2) source of endogenous cytoprotective molecules, and more so in situations of cellular stress where HO-1 is activated by different stimuli ³⁷¹⁻³⁷³. In addition, haemoproteins, e.g. CYP-450, and the gaseous molecule NO can affect haem availability and turnover by HO-1. For example, CYP-450 and HO-1 are induced by similar electrophilic agents, e.g. drugs, chemicals, carcinogens and cellular toxins. As a result, induction of HO-1 is paralleled by a decrease in microsomal CYP-450 which can safeguard against subsequent toxicity ³⁶⁴. The combination of heme and NO can significantly augment haem oxygenase activity resulting in elevated bilirubin production and haem uptake ³⁷⁴; the mechanisms behind this observation will be explored further in this Thesis. Lastly, reversible inhibition of substrate binding of Bach1, a DNA-binding protein that senses intracellular haem content, to the HO-1 active site can lessen haem metabolism when haem is limited, but exposes the active site when it 'senses' that haem is plentiful ³⁷⁵; thus conserving haem for haemoproteins during periods of limited haem availability as well as

restricting the potential toxicity of excess bilirubin and CO release ^{376,377}.

The principal extracellular sources of haem for the haem oxygenase pathway include Hb and myoglobin, while those other molecules listed in Table 1.6 represent the main intracellular reservoir. In its unbound or "free" state, haem can be quite toxic to the body and therefore must be recycled, eliminated from the body or contained ^{361,365}. The vast majority of haem is recycled through oxidation via the haem oxygenase pathway ³⁶⁴. Other minor pathways for haem degradation exist in mammalian systems these include: 1. a NADPH-dependent system in heart mitochondria; 2. protein degradation by xanthine oxidase and H₂O₂ plus NADPH-CPR; and 3. degradation of haem by 2-allyl-2-isopropylacetamide. The latter pathway does not produce CO, while the two other pathways do not generate biliverdin or CO, rather pyrrolic complexes ^{372,378}. Although haem metabolism produces > 85% of endogenous CO the remaining fraction is derived from other metabolic pathways, including the CYP-450-dependent oxidation of particular xenobiotics (e.g. dihalomethanes, methylene chloride) and lipid substrates ³⁷⁹ and iron-dependent lipid peroxidation ³⁸⁰.

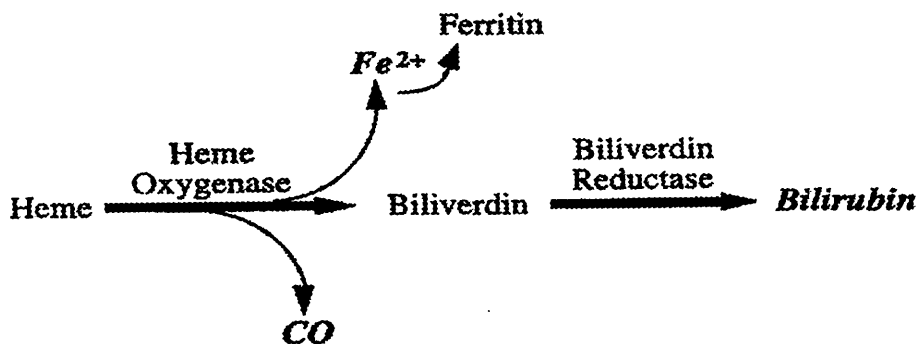


Figure 1.11 Pathway of haem degradation.

Adapted from ³⁸¹.

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Although the discovery of the components, products and mechanism of the haem oxygenase pathway has been elucidated during the past 30 years, the actual reaction has been manifested clinically for centuries in human skin during the process of cellular reconstitution following bruising. This is because the various steps in the haem oxygenase pathway are colour coded ³⁸². When skin becomes bruised, a characteristic black/purple colour, which represents haem from damaged RBC, appears. As the tissue heals in response to the damage, a green hue, which denotes the presence of biliverdin, develops. This is followed by a yellowing of the underlying cells, which represents bilirubin. While CO is represented by the increased flow of oxygenated (red) blood into the healed area ³⁸¹.

The reason(s) why humans should generate the final products bilirubin, CO and Fe^{2+} , all of which are potentially more toxic than the original substrate haem, puzzled scientists for some time ³⁸³. However, various research groups have identified a number of important physiological functions for the haem oxygenase pathway in the control of cellular homeostasis ³⁷³. For example: 1. iron can act as a regulator of gene expression and also be reutilised for the production of new haemoprotein molecules ³⁸⁴; 2. CO has similar characteristics to another gaseous molecule, NO, with respect to signal transduction and regulation of cellular function ^{180,377}; and 3. biliverdin and bilirubin have been proposed as major cytoprotectants ^{385,386}. In the subsequent sections the haem oxygenase pathway will be discussed further in terms of its biochemical mechanism, the haem oxygenase enzymes and the biological function of its products.

1.12.1 Biochemical mechanisms and regulation of the haem oxygenase pathway

Microsomal haem oxygenase catalyses the first and rate limiting step in the oxidative cleavage of the α -meso carbon bridge of the haemoprotein haem, which is in the ferric (Fe^{3+}) oxidation state, to yield equimolar quantities of biliverdin, CO and Fe^{2+} , in the presence of O_2 and the reductase NADPH-CPR³⁶⁴. The metabolism of biliverdin to bilirubin is discussed further in Section 1.14. Advancements in biochemistry procedures and detection equipment has enabled hypothetical mechanisms of haem catabolism to be postulated, which despite some ambiguities in their details, reflect common features³⁶⁷. The overall reaction (see Figure 1.12) involves a complex sequence of transformations over three stages with the formation of two intermediates, α -meso-hydroxyhaem and verdohaem, and requires three molecules of O_2 and at least seven electrons from NADPH-CPR³⁶⁸. Although NADH can also be used as a cofactor in the haem oxygenase pathway NADPH is a more effective electron donor³⁶⁹. Potential cellular sources of NADPH include: 1. the oxidative degradation of glucose 6-phosphate to ribose 5-phosphate by the dehydrogeases glucose 6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) via the pentose phosphate pathway (hexose monophosphate shunt); 2. oxidation of L-malate to pyruvate by the 'malic enzyme' NADP-linked malate dehydrogenase (NADP-MDH) during lipogenesis; 3. oxidative decarboxylation of isocitrate to 2-oxoglutarate by NADP-linked isocitrate dehydrogenase (NADP-ICDH) in the citric acid (Krebs) cycle; and 4. the enzyme H^+ -transhydrogenase (H^+ -Thase) catalyses

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the transfer of hydride ion between NADH and NADP⁺ coupled with the translocation of protons from the cytosol to mitochondria. Reactions 1-3 occur in the cytosol while reaction 4 occurs in the inner mitochondrial membrane ³⁹⁰.

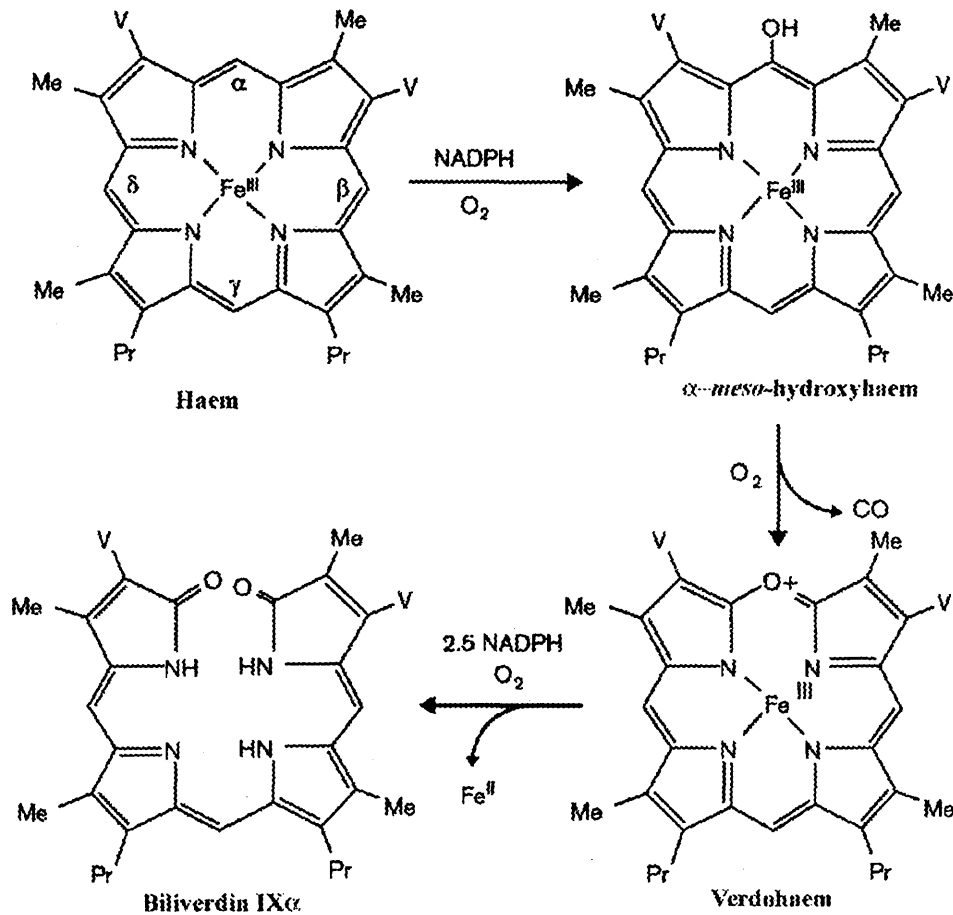


Figure 1.12 Structural pathway of haem catabolism.

Me = Methyl (CH₃); Pr = Propionate (CH₂CH₂COOH); and V = Vinyl (CH=CH₂). Adapted from ³⁸⁷.

In the first stage haem oxygenase forms a complex with its substrate haem to generate Fe³⁺ α-meso-hydroxyhaem. The Fe³⁺ is reduced to Fe²⁺ then O₂ binds to the latter to form a metastable oxy-form complex (HO-Fe²⁺-O₂). This

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is followed by formation of an iron-bound hydroperoxide intermediate which targets the α -*meso* carbon, thus converting HO-Fe²⁺-O₂ to a Fe³⁺ α -*meso*-hydroxyhaem-HO complex^{388,391}. The purpose of NADPH-CPR is to interact with the enzyme-substrate complex and transfer reducing equivalents to convert and maintain iron in the reduced (Fe²⁺) state and activate O₂ for attack at the α position, as only Fe²⁺ can bind O₂³⁶⁴. A unique feature of haem oxygenase is that it uses haem as both its substrate and prosthetic group, yet the enzyme is not a haemoprotein; however, when haem oxygenase binds haem in a 1:1 ratio as a HO-Fe²⁺-O₂ complex, a transitory 'haemoprotein' with its distinctive absorption maximum at 405 nm is formed³⁹².

The second stage transforms Fe³⁺ α -*meso*-hydroxyhaem to Fe²⁺-verdohaem and at the same time CO is released from the α -*meso* bridge. Two possible mechanisms have been proposed for this procedure: one mechanism requires O₂ and one electron, i.e. Fe³⁺ α -*meso*-hydroxyhaem is oxidised to Fe³⁺ verdohaem by O₂ followed by reduction to Fe²⁺-verdohaem after CO is released³⁹¹. The second mechanism, which has similar energy requirements, could proceed via two parallel routes: 1. reduction of the Fe³⁺ α -*meso*-hydroxyhaem-HO complex to its Fe²⁺ form, followed by oxygenation; or 2. oxygenation of the Fe³⁺ α -*meso*-hydroxyhaem-HO complex, followed by one-electron reduction³⁸⁸.

The final stage of haem degradation involves conversion of verdohaem to biliverdin and free Fe²⁺. Although the exact mechanism is unclear the fact that

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the O₂ incorporated into biliverdin originates from two different O₂ molecules rules out the possibility of a hydrolytic reaction and points towards the need for reducing equivalents and O₂³⁹³. A proposed sequence for this reaction is binding of O₂ to the Fe²⁺-verdohaem-HO complex, which is the rate limiting step in this phase, to generate an oxy-verdohaem-HO complex followed by conversion to unknown intermediates and lastly two electron reduction to produce the Fe³⁺-biliverdin-HO complex³⁸⁸. The latter product still remains attached to haem oxygenase, but is released as biliverdin and Fe²⁺ with the help of NADPH-CPR, thus completing the metabolism of haem and allowing the catabolic cycle to start again³⁹¹. However, in the absence of the reductase, iron is not reduced and the Fe³⁺-biliverdin complex is not a suitable substrate for BVR³⁹⁴.

Investigations into the mechanism of the aforementioned reactions reveals that steric orientation of the haem molecule within the enzyme haem oxygenase accounts for the regiospecific oxidation of the α -*meso* position, which is similar to the mechanism of other haemoproteins, e.g. myoglobin and haemoglobin, although oxidation can also occur at the other *meso*-positions, i.e. β , γ and δ ³⁹⁵⁻³⁹⁷. These observations are supported by the recent X-ray crystallographic structure of rat and human HO-1 with and without haem, respectively, as the protein sterically obstructs access to the β -, γ - and δ -*meso* carbons of haem in the closed conformation^{398,399}. Another factor suggested to control ligand and regiospecific metabolism is the presence of histidine (His) residues (His 25 and His 132) in the enzyme, as mutations in either leads to decreased catalytic activity⁴⁰⁰⁻⁴⁰². However, X-ray crystallography did

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not identify any His, or other polar residues, within the active site region of human HO-1, thus adding to the complexity surrounding the mechanism of haem metabolism ⁴⁰³.

The metabolism of haem can be regulated at the first committed step, i.e. α -meso-hydroxylation, to produce only biliverdin and no CO via α -meso substitutions ³⁹¹. A second site of regulation is at the Fe²⁺-verdohaem-HO complex, as binding of Fe²⁺ haem ligands, e.g. azide, cyanide and CO, can inhibit further progress of the reaction ³⁸⁸. Biliverdin can inhibit the activity of haem oxygenase through blocking access of the substrate to the catalytic site of the enzyme ⁴⁰⁴. Although NADPH-CPR has the capacity to oxidatively degrade haem, the products are propentdyopents rather than bile pigments, but simultaneous incubation with haem oxygenase is necessary to produce biliverdin-IX α ⁴⁰⁵.

Metabolism of haem to iron and bile pigments is not limited to mammals, as the pathogenic bacteria *C. diphtheriae* uses Hmu O, a protein homologous to haem oxygenase, to acquire iron from haem when the quantity of free extracellular iron is low ⁴⁰⁶⁻⁴⁰⁸. Also, cyanobacteria, algae and plants all have haem oxygenase enzymes which enables them to convert haem to biliverdin-IX α , the precursor of phytobilins (e.g. phycobilins and phytochromobilins) ^{409,410}. In summary, the X-ray crystal structure of human and rat HO-1 have provided a more detailed insight into the process of haem metabolism. Further investigations are required to fully clarify the identity of intermediaries in the pathway as well as make comparisons with other organisms that utilise the

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haem oxygenase pathway, so that a better understanding of the reaction mechanisms can be acquired ⁴⁰³.

1.13 Haem oxygenase and its isoforms

Due to the instability of biliverdin and bilirubin compared with haem, together with an inability to obtain pure samples of these bile pigments, haem oxygenase is the most studied component of haem metabolism⁴¹¹. The first documented existence of haem oxygenase was made by Wise and coworkers in 1964 with an *in vitro* assay showing haem was degraded to biliverdin⁴¹². Further work by Tenhunen and colleagues led to the identification, characterisation and localisation of haem oxygenase as the enzyme responsible for haem catabolism^{363,413}. The reaction catalysed by haem oxygenase was originally assumed to be a mixed function oxidase rather like the drug metabolising enzymes cytochrome P450 (CYP-450) as both required NADPH and O₂, but could be inhibited by CO³⁸⁹. However, closer inspection of both systems revealed sharp contrasts. Specifically, the wavelength at which monochromatic light could reverse the inhibition by CO is 450 nm and 464-468 nm for CYP-450 and haem oxygenase, respectively. Also, these two enzymes share different inducers, haem oxygenase activity is present in macrophages, but CYP-450 activity is absent in such cells³⁸⁹. These differences led to the haem oxygenase enzyme being classified as a distinct microsomal entity that does not require CYP-450 for its activity⁴¹⁴.

The haem oxygenase enzyme is found exclusively bound to membranes of the endoplasmic reticulum (ER), predominantly within the smooth endoplasmic reticulum (SER) fraction³⁶². Various mammalian tissues express haem oxygenase but the highest levels are found in spleen, brain and testes³⁸³. Interest in haem oxygenase increased following the observation that the

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inducible isoform, haem oxygenase-1 (HO-1) (or heat shock/stress protein 32; HSP32) was up-regulated by various forms of oxidative stress ⁴¹⁵⁻⁴¹⁷. Two other isoforms of haem oxygenase, HO-2 and HO-3, have since been discovered and like HO-1 they are rather diverse and highly conserved ^{415,418}. Both HO-1 and HO-2 have been fully characterized and shown to be phosphoproteins that utilise the same cofactors and substrate (haem) to produce bilirubin, CO and Fe²⁺; however, they differ with regards their genetic make up, chromosomal location, regulation, molecular and biochemical characteristics and cellular distribution ³⁷¹.

It is the sensitivity of HO-1 towards a variety of inducing agents, such as those listed in Table 1.7, which sets it apart not only from other haem oxygenase isoforms but also other biological enzymes ³⁸¹. HO-1 (MW 32 kDa) was first identified as a distinct enzyme in spleen and liver microsomes ^{392,419}, but it is also expressed in many other cells types in response to different inducers ³⁶⁴. Although HO-1 from rat is a HSP, human HO-1 is not inducible by heat shock, except in a hepatoma cell line Hep3B, despite the presence of a heat shock element (HSE) in its promoter region ⁴²⁰.

Table 1.7 Examples of haem oxygenase-1 inducers

Inducer	Reference Number
Haem	421
Heavy metals, e.g. arsenite	422
Cellular stress, e.g. hypoxia, hyperoxia	423,424
UV irradiation	425
GSH depletion	426
Nitric oxide	427
Heat shock	416
Bacterial toxins, e.g. endotoxin	428
Oxidative/nitrosative stress	373,429
Ischaemia-reperfusion (IR) injury	430
Plant-derived phenolics, e.g. curcumin, caffeic acid phenethyl ester (CAPE)	431,432
Disease states, e.g. anaemia, cancer	433

The second isoform, HO-2 (MW 36 kDa), was first identified in rat liver microsomes ⁴¹⁵. In contrast to HO-1 this isoform is constitutively expressed and has few, if any inducers, although developmental factors, adrenal glucocorticoids, opiates and possibly NO, have been suggested as possible candidates ³⁷¹. Secondly, HO-2 is not a HSP ⁴³⁴ and despite its continuous expression the brain ⁴³⁵ and testes ⁴³⁶ represent the major sites of activity. The most recently discovered isoform of haem oxygenase is HO-3 (MW 33 kDa) ⁴¹⁸. This isoform is similar to HO-2 in that it is also constitutively

expressed, but differs compared to HO-1 with respect to structure. Also, HO-3 is a weak metaboliser of haem and so far its physiological role has not been fully clarified³⁸³. Therefore, this isoform will not be discussed any further.

1.13.1 Structure and regulation of the haem oxygenase gene

Advances in molecular genetic techniques have revealed that the amino acid and nucleotide sequences of HO-1 and HO-2 are translation products of two gene loci, i.e. they are isoforms, and not derived from one another by posttranslational modifications^{437,438}. Polymerase chain reaction (PCR) and fluorescence *in situ* hybridisation (FISH) techniques have assigned human HO-1 and HO-2 to chromosome regions 22q12 and 16p13.3, respectively⁴³⁹. Structurally, the isoforms of haem oxygenase have five exons, four introns and numerous binding sites for transcription factors within their N-terminal coding regions (see Figure 1.13), while a hydrophobic carboxy terminus anchors them to the ER³⁶⁴. Also, the haem oxygenase protein is posttranslationally integrated into the SER as a functional protein, thus obviating the need for prior processing by a precursor molecule⁴⁴⁰.

The isoforms vary in their content of amino acids and nucleotides (HO-2 316 amino acids and 12563 nucleotides vs. HO-1 289 amino acids and 6830 nucleotides)^{438,441}. The HO-1 gene is a product of a single transcript of ~1.8 kb, whereas HO-2 is the product of two or more transcripts, ~1.3 and ~2.1 kb⁴³⁵. The coding sequence for HO-1 begins in exon 1, whereas exon 1 of HO-2 is a 5'-untranslated region (UTR) with exon 2 containing the start codon. Analysis of the amino acid sequences of both isoforms showed that HO-2

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contains three cysteine residues, while HO-1 has none ⁴³⁷. There is a high degree of similarity in the primary amino acid and nucleotide sequences of HO-1 (85-95%) and HO-2 (> 90%) among different mammalian species suggesting a common ancestral gene; however, homology between the different haem oxygenase isoforms is limited to about 40% ⁴⁴².

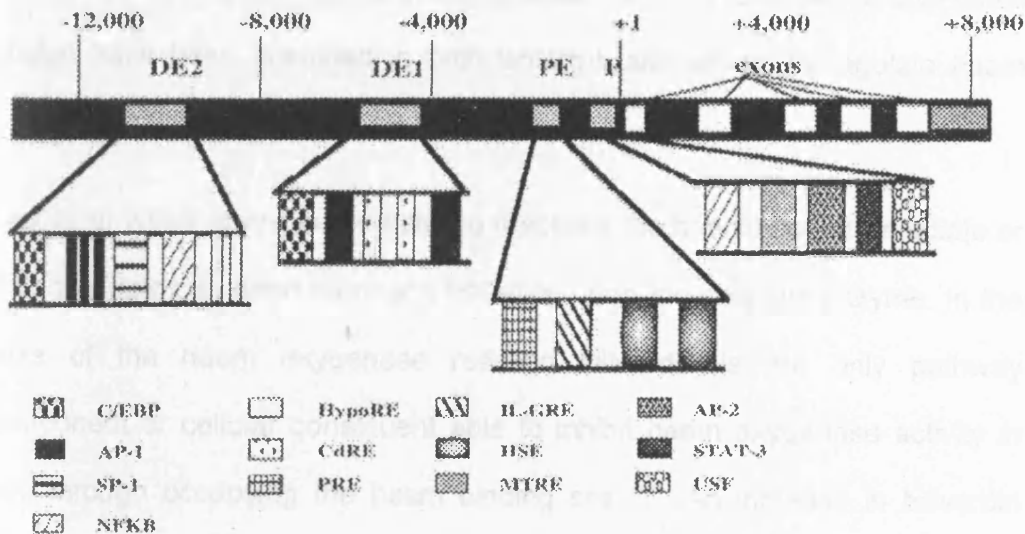


Figure 1.13 Structure of the haem oxygenase-1 gene.

The white boxes represent exons the black boxes between the exons are introns. The promoter (P), proximal enhancer (PE) and two distal enhancers (DE1 and DE2) regions contain the known binding site for transcription factors (patterned boxes). C/EBP CCAT enhancer binding protein; AP-1 activator protein-1; SP-1 stress protein-1; CdRE cadmium response element; IL-GRE glucocorticoid response element; HSE heat shock element; MTRE metal response element; PRE proximal response element; NF-κB nuclear factor-kappa B; AP-2 activator protein-2; STAT-3 signal transduction and activator of transcription; and USF upstream stimulatory factor. Adapted from ⁴⁴³.

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Despite the lack of homology between the two isoforms of haem oxygenase, a highly conserved (96%) sequence of 24 amino acids is present within exon 3 of HO-1 and exon 4 of HO-2 ⁴⁴⁴. This hydrophobic region, known as the haem pocket, contains the consensus sequence Leu-Leu-Val-Ala-His-Ala-Tyr-Th-Arg or haem signature, and has been implicated in orientating the haem molecule so that the α -methane bridge is broken ^{437,438}. Conserved His residues (His 25 and 132 of HO-1 and His 151 of HO-2) within the haem pocket have been identified in both isoforms and shown to regulate haem catalysis ^{400,401,445}.

Like other types of enzyme catalysed reactions the quantity of intermediate or final products can exert feedback inhibition upon the catalytic enzyme. In the case of the haem oxygenase reaction biliverdin is the only pathway component or cellular constituent able to inhibit haem oxygenase activity *in vivo* through occupying the haem binding site ⁴⁰⁴. An increase in biliverdin occurs via feedback inhibition of the serine/threonine kinase, BVR, by its product bilirubin ⁴⁴⁶. Availability of the substrate haem can influence the activity of haem oxygenase ⁴⁴⁷, as well as other metabolic pathways ⁴⁴⁸. In situations where there is excess haem oxygenase activity synthetic derivatives of haem, i.e. metalloporphyrins, can act as competitive inhibitors of the enzyme ⁴⁴⁹. This form of haem oxygenase inhibition is frequently used in the suppression of jaundice ⁴⁵⁰. However, certain metalloporphyrins, e.g. tin proto- and meso-porphyrin, are photosensitisers, which can generate cytotoxic oxygen species, such as singlet oxygen ($^1\text{O}_2$). Other drawbacks include: 1. inhibition and stimulation of sGC and NOS activity, respectively; 2.

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lack of discrimination between the HO-1 and HO-2 isoforms; and 3. long term treatment with zinc or tin protoporphyrin (ZnPP or SnPP, respectively) can induce HO-1 ⁴⁵¹.

As HO-1 has many more inducers compared with HO-2, studies of HO-1 transcription and translation have provided most of the information pertaining to the regulation of haem oxygenase gene expression. Nevertheless, recent evidence suggests a role for HO-2 with regards gene regulation in the cell ⁴⁵². Metals were among the first type of HO-1 inducers ⁴²², since then the list has expanded to cover a wide range of agents (see Table 1.7 for examples). Cloning and sequencing of the HO-1 and HO-2 gene has identified a number of regulatory transcription factor-binding sites, or regulatory elements (Figure 1.13), some of which are repeated within the promoter region of both isoforms ^{438,441,453}. Although HO-1 and HO-2 may share similar regulatory elements (see **Table 1.8** for examples), those within the HO-2 isoform are refractory towards stimulation. Consequently, HO-1 is more responsive to a variety of inducers ³⁶⁴. However, glucocorticoids can promote HO-2 induction but suppress HO-1 expression ^{454,455}.

Presently, the exact molecular mechanism(s) by which an inducer elicits an increase in HO-1 gene expression is not fully understood; however, studies of the HO-1 gene in various species has revealed that the stimuli, which bind to cell surface receptors, act through a signal transduction process known as the mitogen-activated protein kinases (MAPKs) pathway, thus initiating a cascade of events leading to the release of transcription factors, e.g. AP-1 and -2, NF- κ B, hypoxia-inducible factor 1 (HIF-1) and nuclear factor-erythroid 2 related

factor (Nrf2) ^{412,456}. The transcription factors propagate the signal to the nucleus where they bind to their respective binding elements in the HO-1 gene, which ultimately leads to upregulation of HO-1 mRNA to counteract the negative stimuli ⁴⁵⁷.

Table 1.8 Examples of haem oxygenase regulatory elements

Regulatory Elements	Reference Number
Heat shock element (HSE)	416
Haem-binding regulatory motif (HRM)	474
Glucocorticoid response element (GRE)	475
Stress response element (StRE)	412
Hypoxia responsive element (HRE)	476
GCN4 binding site	416
Activator protein-1 (AP-1) binding element	457
Metal responsive element (MRE)	416
Nuclear factor-kappa B (NF- κ B) response element	477
Sp1 binding site	416
TATA-like box sequence	416
Antioxidant response element (ARE)	465

The MAPKs signal transduction pathway is part of a family of evolutionary conserved serine-threonine (Ser-Thr) protein kinases that play a prominent role in coupling an array of extracellular signals, e.g. growth factors, cytokines, irradiation, osmolarity and fluid shear stress, to intracellular targets such as nuclear transcription factors, cytosolic kinases, PLA₂, membrane-bound tyrosine kinase receptors (e.g. EGF receptor) and cytoskeletal components.

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As a result, a variety of biological responses including: gene expression, cell proliferation, differentiation and cell death, can be elicited ⁴⁵⁶⁻⁴⁵⁹. So far four different subfamilies of MAPKs have been identified: 1. the extracellular signal-regulated kinases 1 and 2 (ERK1/2); 2. c-Jun amino(NH₂)-terminal kinases 1, 2 and 3 (JNK 1/2/3); 3. the p38 (a homologue of the yeast HOG1 kinase) enzymes (p38 α , p38 β , p38 γ and p38 δ); and 4. ERK5 ⁴⁵⁸. In response to upstream interactions with small GTP-binding proteins (GTPases) of the Ras or Rho family, or phosphorylation (addition of a phosphate group) by MAP kinase kinase kinase kinases (MAP(4)K), a sequential phosphorylation cascade, beginning with MAP(3)K followed by the dual-specific kinase MAP(2)K terminates with activation of one of the four MAPK subfamilies. The latter response is achieved via phosphorylation of a threonine and tyrosine (ThrXTyr) tripeptide motif, where X can be different amino acids, within the activation loop of the MAPKs. Once activated the MAPKs initiate proline-directed phosphorylation of the Ser-Thr-proline (Ser-Thr-Pro) sequence within their downstream target substrates ⁴⁶⁰. The activity of MAPKs are governed by the balance between upstream kinase phosphorylation (to activate) and protein phosphatases dephosphorylation (to inactivate) of the threonine and tyrosine residues. Examples of inactivators include, dual specificity (threonine/tyrosine) phosphatases (DSPs, also called MAPK phosphatases (MKPs)), tyrosine-specific phosphatases and serine/threonine-specific phosphatases ⁴⁶¹.

The most studied MAPKs, i.e. ERK, JNK and p38, have been classified according to their method of regulation, i.e. ERK expression is enhanced by

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signals associated with growth and proliferation, while JNK and p38 are regulated by cellular stress signals and as such are known collectively as the stress-activated protein kinases (SAPKs) ⁴⁶⁰. The fact that HO-1 and MAPKs are activated by stressful stimuli, via tyrosine phosphorylation, suggests a link between these two pathways ⁴¹². For example, Kietzmann and colleagues showed that arsenite, a potent inducer of MAPKs and HO-1, promoted HO-1 protein and mRNA induction in primary cultures of rat hepatocytes via the JNK pathway ⁴⁶², whereas Elbirt *et al.*, using a chick hepatoma cell line, suggested that ERK and p38, but not JNK, MAPK pathways were responsible for arsenite-dependent upregulation of HO-1 ⁴⁵⁷; while Alam and coworkers identified p38, but not ERK or JNK, as the route through which cadmium activates Nrf2 thereby inducing HO-1 gene expression in mammary epithelial cells ⁴⁶³. In contrast, Yu and collaborators observed that the MAPKs could differentially regulate Nrf2, i.e. JNK activates but p38 deactivates Nrf2 ⁴⁶⁴, which in turn can bind to the antioxidant response element (ARE) leading to induction of several cytoprotective genes among them HO-1 ^{465,466}. In primary rat cardiomyocytes, p38, but not ERK, was shown to induce HO-1 during hypoxia ⁴⁶⁷; however, Ryter *et al.* noted that both ERK and p38 MAPK pathways negatively regulated HO-1 in vascular endothelial and smooth muscle cells exposed to hypoxia ⁴⁶⁸. A number of reports have documented an association between CO and the MAPK pathways. In particular, CO may exert its cytoprotective influences, i.e. as an antiinflammatory, antiproliferative and antiapoptotic agent, via a sGC-independent mechanism using p38 and ERK as mediators of these effects ⁴⁶⁹⁻⁴⁷². Recently, Taillé *et al.* showed that *in vitro* and *in vivo* induction of HO-1 could exert an antiproliferative effect

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upon airway smooth muscle (ASM) through a bilirubin-mediated redox modulation of phosphorylation of ERK1/2 ⁴⁷³. Collectively, these examples illustrate the ability of the MAPKs to serve as an effective link between extracellular chemical signals and intracellular protective responses mediated by HO-1. In addition, the type of MAPK that is activated, as well as the eventual outcome, will vary with the stimulus provided, cell type and species examined.

1.13.2 Physiological role of the haem oxygenase pathway

The degradation of haem by haem oxygenase is the principal mechanism for *in vivo* production of bile pigments (biliverdin and bilirubin), CO and Fe²⁺ ³⁶⁴. Why a catabolic pathway for the elimination of a toxic agent, haem, should produce even more lethal products: CO a poisonous gas; bile pigments can cause jaundice and possible brain damage at excessive levels; and iron may exacerbate oxidative stress and haematological pathologies, puzzled biologists for many decades ^{382,478,479}. In this subsection these enigmatic features of the haem oxygenase enzyme, in particular HO-1, will be discussed with respect to cellular stress, the immune, vascular and nervous systems.

Since its discovery, the negative perception of the haem oxygenase pathway has been challenged by strong evidence to support a role for endogenously generated bile pigments and CO as important physiological mediators of cellular and tissue homeostasis ⁴⁸⁰. In response to alterations in cellular homeostasis due to oxidative (e.g. UV irradiation, H₂O₂, metals) ^{422,425} and nitrosative (e.g. NO, ONOO⁻) ^{481,482} stress two events take place. Firstly, there

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is a decline in the quantity of the most abundant cellular antioxidant, GSH, as a result of increased ROS and RNS generation¹⁴; and 2. specific stress proteins, in particular HO-1, are expressed^{373,417}. Combating oxidative stress is not limited to the HO-1 isoform as HO-2 can protect neurons against oxidative stress through the production of bilirubin⁴⁸³. The mechanism(s) by which HO-1 mediates cytoprotection is not clear; however, bilirubin, CO and ferritin are seen as potential candidates³⁸¹.

Although 'free' iron derived from the haem oxygenase pathway can be deleterious, the parallel induction of the iron sequestering protein, ferritin, scavengers iron, thus preventing its adverse effects⁴⁸⁴. The importance of HO-1 in the regulation of cellular iron has been illustrated in both animal and human models. Mice with a targeted deletion of the HO-1 gene, HO-1^{-/-} null mice, exhibit alterations in iron metabolism characterized by severe iron deficiency, pathological iron-loading, as well as a reduced responsiveness towards oxidative challenge, both *in vitro* and *in vivo*^{369,429}. More recently, Yachie *et al.* reported the first case of HO-1 deficiency in a six year old boy³⁷⁰. Like the animal model, the patient exhibited similar biochemical and pathological features, e.g. growth retardation, anaemia and increased sensitivity to oxidative injury. A comparison of apoptosis levels in HO-1^{-/-} mice and those expressing HO-1 revealed that HO-1 was cytoprotective via modulation of intracellular iron levels⁴⁸⁵. In some circumstances, e.g. UV irradiation or enhanced HO-1 activity, iron released via the haem oxygenase pathway can exert a negative influence due to overloading the capacity of other iron homeostasis proteins, e.g. ferritin and transferrin (an iron binding

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protein), which can lead to deleterious effects through redox-cycling of the iron⁴⁸⁶. These examples emphasise the key role played by HO-1 with regards to *in vivo* and *in vitro* iron homeostasis.

The importance of HO-1 within the immune system is illustrated in the following examples. In rats, prior enhancement of haem oxygenase activity and HO-1 protein expression, using haem, resulted in greater protection towards a subsequent acute inflammatory challenge, compared with animals given the haem oxygenase inhibitor tin-protoporphyrin (SnPP), prior to and at the time of administration of the immunological activator⁴⁸⁷. Preservation of organs for transplants as well as manipulation of the hosts immunological response are important determinants to prevent rejection and enable successful accommodation by the recipient. Two recent publications have highlighted the possibility of HO-1 attenuating organ rejection. In a model of xenografting, mouse to rat transplants which were accommodated by immunosuppressive drugs, it was noted that the degree of HO-1 expression correlated with the extent of survival and accommodation, as mice which had altered HO-1 gene expression rejected the transplants sooner⁴⁸⁸. A common feature of heart transplant rejection is accelerated arteriosclerosis due to a chronic immune response⁴⁷⁸. An *in vitro* and *in vivo* study of accelerated arteriosclerosis by Hancock and colleagues revealed that prior expression of a range of antioxidant and apoptotic genes, including HO-1, was associated with an absence of arteriosclerosis induced by alloantibodies, thus protecting allografts against chronic rejection⁴⁸⁹. The mechanism(s) by which HO-1 mediates its immunomodulatory function has not been fully elucidated.

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However, prevention of oxidative stress mediated expression of adhesion molecules on endothelial cells, thus inhibiting the production of proinflammatory mediators (e.g. complement components, IgM and TNF- α), together with the antioxidative and anticomplement effects of bilirubin and the vasodilatory and antiinflammatory effects of CO, represents a powerful stratagem in the prevention of an adverse inflammatory response^{470,487,490}.

Vascular diseases, e.g. arteriosclerosis, hypertension, hypoxia and reperfusion injury, are a common cause of morbidity and mortality world wide. A characteristic feature of such diseases are oxidative and nitrosative stress induced injury¹⁴. Even though antioxidants have been proposed as a possible therapy, they are not always effective⁴⁹¹. However, HO-1 and HO-2 genes have been described as possible endogenous protectors against a variety of vascular diseases⁴⁹². Carbon monoxide originating from HO-1 within vascular smooth muscle and HO-2 expressed by endothelial cells has been suggested as the mechanism by which haem oxygenase regulates vascular tone both physiologically and pathophysiologically⁴⁹³⁻⁴⁹⁵. Recently, the application of gene transfer of HO-1 has demonstrated its usefulness in promoting angiogenesis, long-term myocardial protection and safeguarding against hyperoxia-induced lung injury^{424,496,497}. Sammut *et al.* observed that aortas expressing high levels of HO-1 were more resistant to the vasoconstrictor effect of phenylephrine (PE), possibly due to elevation of cGMP by CO⁴⁹⁸. Using an *in vivo* model of acute hypertension Motterlini *et al.* reported that CO derived from enhanced HO-1 expression was effective towards suppression of the hypertensive response⁴⁹⁹. Reperfusion injury is a common cause of organ

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transplant failure and post operative injury, but a number of publications have highlighted the potential of HO-1 and its products, bilirubin and CO, as possible protectants ^{430,500,501}.

The nervous system is another area that shows a high level of haem oxygenase activity, with HO-2 being the more prominent isoform ⁵⁰². A unique characteristic of haem oxygenase expression in the nervous system is that it is found colocalised with NOS enzymes or in areas lacking the latter enzymes ^{503,504}. Bilirubin and CO derived from both isoforms have been highlighted as protective agents in models of oxidative stress injury in neurons, glial and astrocytes ^{483,505-507}. Neuronal expression of HO-2 has been shown to account for neurotransmission in the peripheral and autonomic nervous system as well as the developmental and growth response of neurons to adrenal steroids ^{371,454,507}. Within the baroreceptor region of the brain, the nucleus tractus solitarius (NTS), both endogenous and exogenous CO can lower blood pressure, while inhibition of haem oxygenase leads to vasoconstriction ³⁷⁷.

The aforementioned physiological effects of haem oxygenase illustrate the significance of its metabolites and widespread distribution of both isoforms in different organs and systems. Further mechanistic studies of HO-1 should help to elucidate the mechanism(s) of cytoprotection, while new scientific procedures, e.g. gene regulation and transfer as well as selective induction of the HO-1 gene by pharmacological means, could both be used to harness the therapeutic potential of the haem oxygenase pathway ⁴⁵⁷.

1.14 Bile pigments

The bile pigments, biliverdin and bilirubin, are continuously formed *in vivo* by their respective ubiquitous enzymes haem oxygenase and BVR, as a result of oxidative cleavage of the α -meso bridge of ferric (Fe^{3+}) haem (see Figure 1.14) ³⁶⁴. Both prokaryotic and eukaryotic species have the ability to oxidise haem and generate bile pigments. The overall reaction is manifested in nature and biology as a specific colour sequence, red \rightarrow green \rightarrow yellow. Ferrous (Fe^{2+}) haem, which has a crimson colour, gives rise to the blue-green colour range of the biliverdins which are used by birds, to camouflage their eggs, and as precursors for essential photosynthetic bilins by algae, bacteria and plants, while the yellow-golden colouring of jaundice is due to excess bilirubin ⁴¹¹. Disruption of the α -meso bridge in haem by microsomal haem oxygenase generates the corresponding isomer of biliverdin, i.e. $\text{IX}\alpha$, although other isomers, i.e. $\text{IX}\beta$, $\text{IX}\gamma$ and $\text{IX}\delta$, are possible (see Figure 1.15). The IX denotes the order of ring substituents and Greek letters identify the meso bridge that is cleaved ⁴¹¹. The biliverdin-IX isomers are rapidly converted by cytosolic BVR (discussed further in Section 1.14.1) to the corresponding isomer of bilirubin. In adult bile, bilirubin- $\text{IX}\alpha$ (or simply bilirubin) is the most common (95%) isomer, while 5% are non- α isomers ⁵⁰⁸. Bilirubin is often represented as a linear two-dimensional tetrapyrrole structure (Figure 1.16, upper diagram), although a more accurate representation is that of a three-dimensional ridge-tile shape (Figure 1.16, lower diagram) which is more stable and has the ability to flex, bend and invert to a mirror image ridge-tile without loss of its intramolecular hydrogen bonds ⁵⁰⁹. The presence of propionic acid

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(CH₂CH₂CO₂H) groups at positions 8 and 12 accounts for the high hydrophobicity and lipophilic nature of bilirubin and explains why it cannot be excreted intact, but must undergo metabolism in the liver before elimination via the bile ^{509,510}. This is in stark contrast to the other isomers, i.e. IX β , IX γ and IX δ , which are more polar and less lipophilic, as they do not engage in intramolecular hydrogen bonding, but are excreted intact by the liver ⁴¹¹.

Although the pathways and mechanism of haem biosynthesis and catabolism have been well studied, due to the stability of haem and the importance of haem homeostasis, the same cannot be said about bile pigments as bilirubin, biliverdin and BVR are unstable, difficult to work with and obtain in a pure form ⁴¹¹. Despite these drawbacks a number of physiochemical and physiological properties have been identified for the enzyme BVR and mammalian bile pigments.

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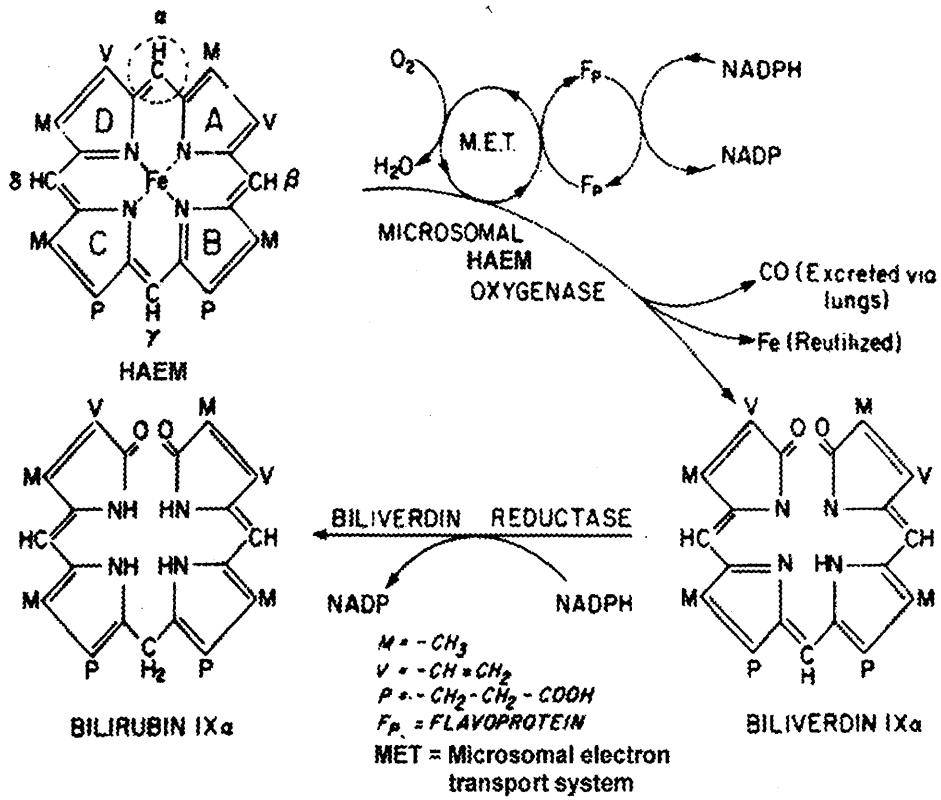


Figure 1.14 Pathway for formation of biliverdin and bilirubin.

Adapted from ⁵¹¹.

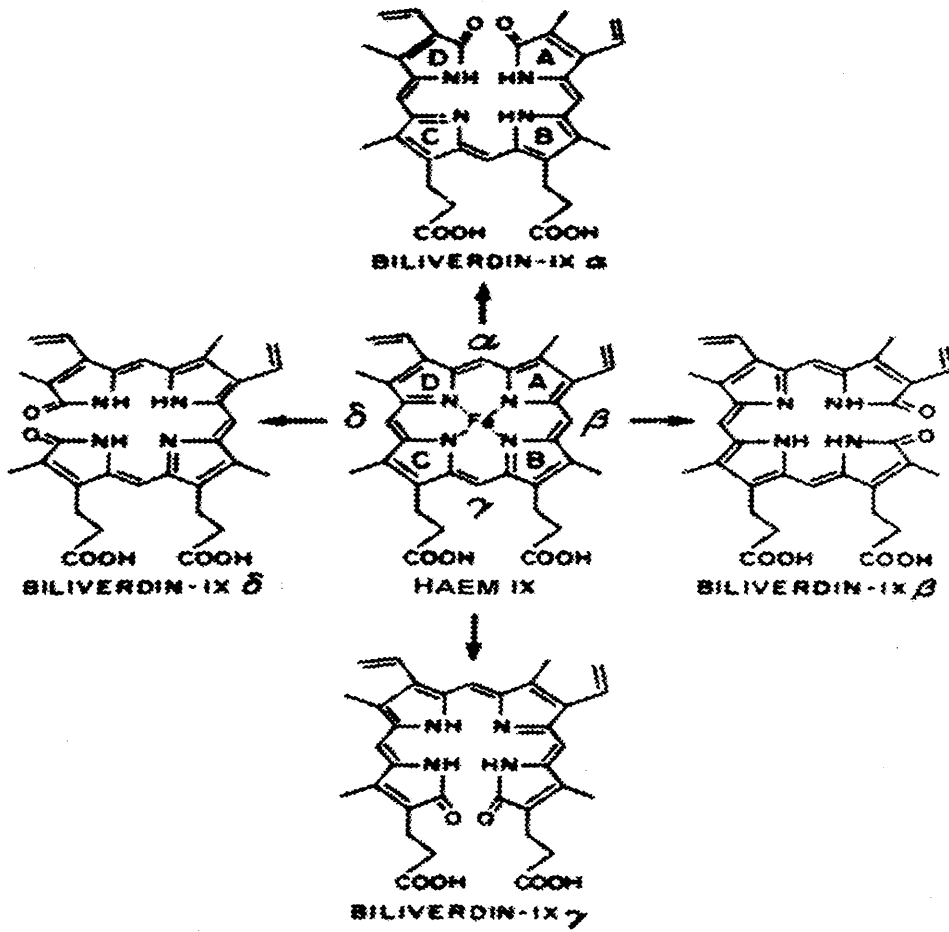


Figure 1.15 Generation of biliverdin isomers from haem.

Adapted from ⁵¹².

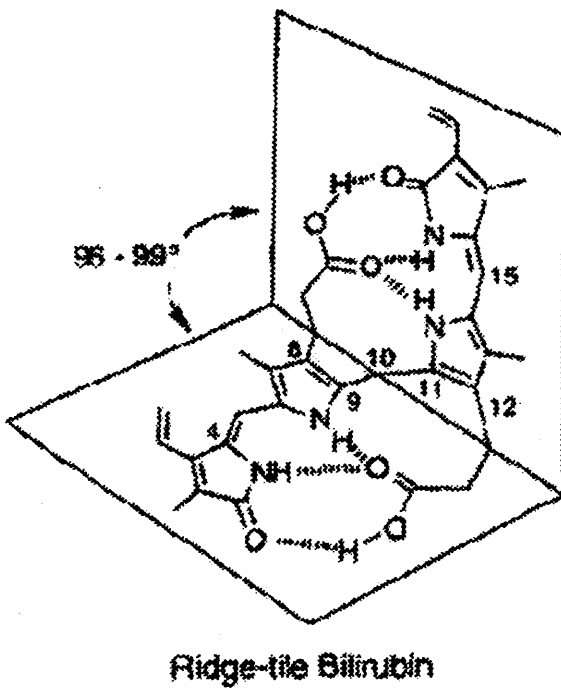
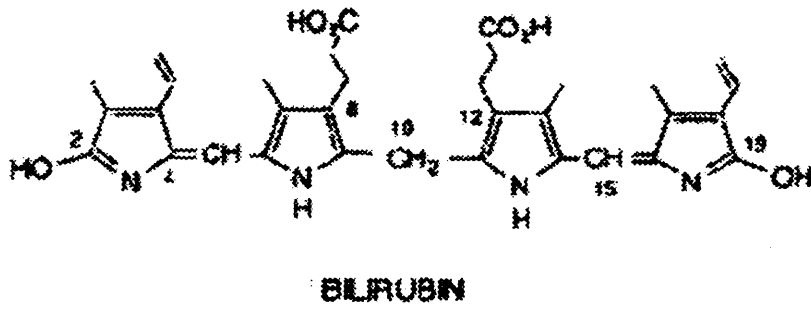


Figure 1.16 Structural representations of bilirubin.

Upper diagram of two-dimensional structure, lower diagram illustrating the more stable three-dimensional ridge-tile shape and intramolecular hydrogen bonds. Adapted from ⁵⁰⁹.

1.14.1 Biliverdin reductase

As shown in Figure 1.15 haem oxygenase can open up the haem molecule at either of its four *meso* bridge carbons to generate the respective isomers of biliverdin. These isomers are subsequently metabolised to the corresponding isomer of bilirubin by the cytosolic enzyme BVR³⁸⁵. Purification and characterisation of a highly stable form of rat (MW ~34 kDa) and human (MW ~41-42 kDa) BVR show that this enzyme has quite unique properties^{513,514}. For example, the enzyme is encoded by a single copy gene and is postrationally modified; it is thiol (SH)-dependent; BVR is a kinase enzyme that can be autophosphorylated and reversibly phosphorylated on its serine/threonine residues, which is essential for enzymatic activity; it is the only enzyme to have a dual pH (pH 6.6-6.9 and pH 8.5) and cofactor (NADH or NADPH) requirement, with pH 8.5 and NADPH being the optimal conditions for activity^{446,513}.

The complete amino acid sequences of four different isomers, two BVR-IX β (BVR-B) reductases (isoforms I and II, MW ~21 kDa) and two BVR-IX α (BRV-A) reductases (isoforms III and IV, MW ~34 kDa), have been cloned and characterized in human liver cytosolic fractions and mapped to chromosomes 19 and 7, respectively⁵¹⁵⁻⁵¹⁹. The BVR-IX α reductases are limited to the regiospecific reduction of the α -*meso* (C10) hydrogen on biliverdin-IX α to produce bilirubin-IX α , whereas BVR-IX β reductases catalyse the reduction of the non- α isomers of biliverdin to the corresponding bilirubin isomers, as well as a variety of flavins and ferric ions^{516,520}.

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Recently the crystal structure of BVR-IX α and BVR-IX β have been characterized in rats and humans, respectively, and revealed a number of structural and mechanistic details about these two isoforms^{521,522}. Firstly, the rat BVR-IX α comprises two tightly packed domains, whereas human BRV-IX β is a single monomer^{521,522}. Second, the substrate binding site is large enough to accommodate substrate and cofactor (NADH or NADPH) simultaneously, but separate mechanisms are employed for each cofactor⁵²¹. Although the mechanism of catalysis used by BVR is unclear, the discovery of a 'conserved EYP motif (Glu 96-Tyr 97-Pro 98), which corresponds to other critically important structural motifs, at the proposed active site of BVR-IX α is important⁵²¹. Also, electrostatic interactions aid enzyme-substrate binding and recognition, e.g. the presence of two negatively charged residues enables BVR-IX β to have a wider repertoire of substrates, but prevents metabolism of biliverdin IX α as it has propionate side chains. Therefore, the latter is the only substrate for BVR-IX α ^{520,522}.

1.14.2 Physiological and pathophysiological aspects of biliverdin and bilirubin

The reason why energy should be expended to metabolise biliverdin to bilirubin has been an enigma for many years⁵²³. In fact, biliverdin is non-toxic and easily excretable whereas bilirubin can be neurotoxic and is unexcretable unless it is metabolised by a third enzyme system, uridine diphosphate glucuronyl transferase (UDPGT)⁵²⁴. However, it has been suggested that biliverdin is the only *in vivo* agent capable of regulating the activity of haem

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oxygenase⁴⁰⁴ and it also has potential antioxidant³⁸⁵ and immunoprotective⁵²⁵ capacities. Although the exact physiological role of biliverdin may be unclear, what is apparent is that the synthesis of its product, bilirubin, is essential. For example, the percentage of biliverdin isomers varies among adult and foetal liver: in adults 95-97% is IX α , 3-5% is IX β , with little or none of IX δ and IX γ ; in the foetus 87% is IX β , 6% is IX α and IX δ , while 0.5% is IX γ ⁵⁰⁸. The physiological relevance for the higher quantity of biliverdin-IX β in the foetus and biliverdin-IX α in adults is not clear entirely, but may be due to: 1. immaturity of the conjugating enzymes in the foetus required to dispose of bilirubin-IX α , as the other isomers of bilirubin are hydrophilic and excretable without the need for conjugation; 2. the switch from foetal to adult haemoglobin; also 3. bilirubin-IX α may be more effective than the IX β isomer at combating oxidative stress due to the higher oxygen pressure of pulmonary respiration after birth compared with placental respiration^{508,515}.

The oxidative metabolism of haemoproteins by the enzyme haem oxygenase generates approximately 250 mg/day of bilirubin in healthy humans⁵⁰⁹. Due to its poor water solubility bilirubin is bound to the transport protein albumin in the systemic circulation. Normal plasma levels range from ~5 to 17 μ M, while levels greater than 300 μ M are associated with disease states, especially of a neurological nature, due to the lipophilicity of unbound 'free' bilirubin⁵²⁶. Even before the discovery that bilirubin was derived solely from the haem oxygenase pathway its toxic aspects were well known, e.g. it can cause neurotoxicity, uncouple oxidative phosphorylation, inhibit NADH oxidase and interfere with mitochondrial structure and function^{527,528}. This negative

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perception of bilirubin remained until a series of publications during the late 1980s and early 1990s hypothesised a beneficial role for bilirubin. In an article by Antony F. McDonagh, 'Is bilirubin good for you?'⁵²³, the author puts forward two answers to this question, saying bilirubin is "definitely not" good due to the pathologies arising from disorders of bilirubin metabolism, but "may be" good on the basis of evidence showing its protective function as a naturally occurring antioxidant against radical induced injury.

The beneficial aspects of bilirubin were first proposed by Roland Stocker and colleagues following the observation that free and albumin-bound bilirubin were effective scavengers of peroxy radicals, a cause of lipid peroxidation⁵²⁹. Furthermore, bilirubin is more effective than another endogenous antioxidant, i.e. α -tocopherol or vitamin E, at attenuating lipid peroxidation⁵²⁶. The fact that bilirubin is one of the most powerful *in vitro* and *in vivo* antioxidants has led to its utilisation as a defence against oxidative injury of lipids and lipoproteins, which are early event in atherosclerosis and subsequent development of coronary artery disease (CAD)^{428,530,531}. Also the presence of bilirubin on albumin protects the latter from damage inflicted by OH[•] radicals

532

The novel concept that bilirubin is an endogenous antioxidant has gained widespread acceptance, but other beneficial aspects as a cytoprotectant, antimutagen and anticomplement agent, have been muted⁴⁸⁰. The cytoprotective nature of bilirubin has been documented in rat liver and hepatocytes, bovine vascular smooth muscle cells, human erythrocytes, human monocytes and human blood plasma following exposure to

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oxidants⁵³³⁻⁵³⁷. In premature infants, their antioxidant capacity is often reduced due to immaturity of the necessary enzymes; as a result, susceptibility to oxygen free radical based disease is increased⁵³⁸. Similarly, such infants often have elevated levels of bilirubin (hyperbilirubinemia), therefore bilirubin may exert a protective function against free radical injury, thus compensating for the lack of antioxidant enzymes^{539,540}.

Although hyperbilirubinemia can be detrimental a number of publications have queried whether such a state could be a marker for, or possibly attenuate, the development of CAD^{541,542}. Evidence to support this theory has shown both positive and inverse correlations between serum bilirubin levels and the development of CAD in humans and animals⁵⁴³⁻⁵⁴⁷. Furthermore, risk factors associated with the development of CAD, e.g. smoking, high LDL-cholesterol and obesity, lead to lower plasma levels of bilirubin, thus attenuating the protective effect of bilirubin^{548,549}. Prospective cohort studies in adult and young males and females revealed that higher serum bilirubin levels, particularly in men, were associated with a lower incidence of cardiovascular and cancer mortality⁵⁴⁹⁻⁵⁵². In a study of IR injury Clark *et al.* noted that upregulation of cardiac HO-1 using hemin (75 µM/kg i.p.) 24 h beforehand, significantly ameliorated myocardial dysfunction due to an increased rate of bilirubin formation, while exogenously administered bilirubin (100 nM) mediated a similar cardioprotective effect⁴³⁰. Using a rat model of IR injury of the liver, Yamaguchi and colleagues reported that bilirubin was protective as an *in vivo* antioxidant in response to oxidative stress⁵⁵³.

Due to its high lipophilicity the neurotoxic nature of free bilirubin has been

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known for almost a century³⁷⁶. Despite this adverse image experiments have concluded that bilirubin derived from haem oxygenase exerts a neuroprotective function and also compensates for the poor antioxidant capacity of the brain^{483,554}. In Alzheimer's, a disease characterized by chronic oxidative stress, increased bilirubin formation as a consequence of HO-1 induction could modulate the severity of the condition⁵⁵⁵. A number of recent reports have indicated that the brain may be able to protect itself from bilirubin toxicity through the oxidation of bilirubin^{556,557}. Although the enzyme responsible for bilirubin oxidation has not been fully characterized a number of details are known, e.g. enzyme activity is cytochrome c dependent; activity increases with age; the expression is subject to genetic variability; and is lowest among neurons compared with glial cells^{557,558}.

Due to its lipophilic nature free bilirubin can traverse cell membranes and cross the blood brain barrier, where it can induce toxic effects⁵⁵⁹. To prevent such reactions occurring, bilirubin is bound to albumin in the systemic circulation. The albumin-bilirubin complex is transported to the liver to undergo further metabolism by a series of intricate and poorly understood reactions so that bilirubin is excreted in bile. The process of bilirubin metabolism begins with the passage of the albumin-bilirubin complex through fenestrae of the endothelial cells lining the hepatic sinusoids into the space of Disse⁵¹⁰. At this location bilirubin dissociates from albumin at the surface of the hepatocyte and is taken up by protein mediated transporters located in the sinusoidal membrane⁵²⁴. Within the hepatocyte bilirubin is bound to ligandin, a glutathione-S-transferase (GST) isomer that has a high affinity for bilirubin,

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and carried to the ER where it is translocated into the ER lumen via a protein mediated transport pathway⁵¹⁰.

In the ER, a family of microsomal membrane bound enzymes called uridine diphosphate (UDP)-glucuronosyltransferases (UGTs) catalyse the conjugation of bilirubin to UDP-glucuronic acid (UDPGA)⁵⁶⁰. The latter process, which is also known as glucuronidation, esterifies bilirubin's two $\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$ groups to generate bilirubin mono- and di-glucuronides, thus making the molecule more water soluble. The water soluble glucuronides are returned to the cytosol via transporters located in the ER membrane and excreted from the hepatocyte into the bile via an ATP-dependent anion transporter, termed canalicular multispecific organic anion transporter (cMOAT) in rats and multidrug resistance protein 2 (MRP2) in humans, which is located in the bile canalicular membrane^{524,560}. The bile then passes from the liver to the gastrointestinal tract (GIT) and within the colon the glucuronides are enzymatically converted to urobilinogens, and other products, and ultimately excreted in the faeces⁵⁶¹.

A number of pathological conditions or hereditary disorders of hepatic bilirubin metabolism can disrupt the above pathway leading to hyperbilirubinemia. Hyperbilirubinemia can be classified as 'unconjugated' or 'conjugated' to indicate its respective causes, free (unconjugated) bilirubin, defective canalicular excretion of glucuronides or obstruction of bile flow^{560,562}. Physiological hyperbilirubinemia is a common non-life threatening feature in jaundiced neonates and those with Gilbert's syndrome. However, most cases are transitory or can be alleviated using phototherapy and metalloporphyrins,

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e.g. tin (Sn) and zinc (Zn) metalloporphyrin, which act as competitive inhibitors of haem oxygenase ^{450,563,564}. In severe cases of hyperbilirubinemia, kernicterus and bilirubin encephalopathies can develop and ultimately lead to irreversible neurotoxicity, developmental abnormalities and death ⁵²⁷.

The toxic effects of bilirubin can also be manifested when: 1. the buffering capacity of albumin is exceeded; 2. when drugs compete with bilirubin for, or displace it from, albumin binding sites; and 3. due to the transitory and reversible nature of the binding of bilirubin with albumin at body temperature ^{563,565,566}. In addition, the characteristic yellow/orange hue of jaundiced babies and Gilbert's syndrome subjects, as well as the yellow staining of the basal ganglia in post-mortem victims of kernicterus, are due primarily to the formation of colloid (micropolymer crystals of bilirubin) in extravascular sites, although flocculants (macroaggregates of colloids) can also develop ⁵⁶⁷. Despite having a partial deficiency of liver UGTs which results in chronic, but non-fatal, hyperbilirubinemia those with Gilbert's syndrome have a reduced risk of ischaemic heart disease (IHD). The basis for this observation was suggested to be due more so to elevated concentrations of free bilirubin, which protects LDL cholesterol (a common target in the process of atherogenesis) against the deleterious effects of oxidative stress, than increased high density lipoprotein (HDL) cholesterol levels ⁵⁴⁶. These observations are comparable to those in other human and animal studies where hyperbilirubinemia was correlated with protection against postischemic myocardial dysfunction ⁴³⁰ and reduced oxidative stress, thereby mitigating the development of IHD ^{544,545,547}.

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The availability of animal models for hyperbilirubinemia has enabled the elucidation of the molecular mechanisms responsible for this disease as well as providing a means to test new therapeutic agents⁵⁶⁰. Crigler-Najjar (CN) syndrome, which is an example of unconjugated hyperbilirubinemia, can be of three types: Type 1 or severe CN syndrome is characterized by very high (> 340 μM) serum bilirubin; Type 2 or moderate CN syndrome has serum bilirubin levels of 85-340 μM ; and Type 3 or mild CN syndrome, also known as Gilbert's syndrome, has serum bilirubin of < 84 μM ⁵²⁴. CN syndrome is caused by mutations in the UGTs, but the Gunn rat, which is a mutant strain of Wistar rats, serves as a model for Type 1 CN syndrome⁵⁶⁸.

Dubin-Johnson (DJ) and Rotor's syndromes are examples of conjugated hyperbilirubinemias⁵⁶². Of the two, DJ syndrome has been the most studied as it is caused by mutations in the MRP2 gene leading to mild or chronic conjugated hyperbilirubinemia as a result of the secretion of conjugated bilirubin, and organic anions, into the bile canaliculus, even though bile acid excretion into bile is normal⁵⁶⁹. Two animal models of DJ syndrome, both of which exhibit autosomal recessive defects in hepatobiliary excretion of non-bile acids and organic anions, exist. They are the GY/TR- rat, which is a homozygous mutant of Wistar rats, and the Eisai (or spontaneous) hyperbilirubinemic rat (E(S)HBR), which is a mutant strain of inbred Sprague Dawley rats⁵⁶⁹.

In summary, the specific cleavage of the haem molecule at the α -meso bridge by haem oxygenase and formation of the bile pigments biliverdin and bilirubin is not without significance or energetically wasteful. These bile

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pigments, particularly bilirubin, have since been shown to possess quite unique and previously unrecognised beneficial attributes, i.e. as antioxidants and cytoprotectants. Further research into the mechanism(s) of these effects along with the development of pharmacological BVR active site inhibitors is needed to gain a better understanding of the properties of bile pigments and modulate their formation for therapeutic purposes.

1.15 Carbon monoxide: history, metabolism and physiological applications

Another product of the haem oxygenase reaction is the gaseous molecule carbon monoxide (CO) ³⁷². The toxic, "silent killer", nature of CO has been recognised for centuries. The basis for this conception stems from a number of factors: 1. CO is a colourless, odourless, tasteless, non-corrosive gas which is insoluble in water and has a similar density to air; 2. it is produced by the incomplete combustion of fossil fuels and is widely studied due to its presence in cigarette smoke and as a means of suicide; and 3. global atmospheric levels are estimated to be 0.19 parts per million (ppm), 90% of which is derived from natural sources e.g. the oceans and microorganisms, the other 10% from human activity. Thus, avoiding daily exposure to CO is an impossible task ⁵⁷⁰. The first demonstration that human blood carries a small but measurable amount of CO occurred over 50 years ago ⁵⁷¹. Subsequent studies by Sjöstrand and Coburn revealed that CO is constantly formed *in vivo* from the haem in haemoglobin and that certain pathological conditions, e.g. anaemia and polycythaemia, could augment CO formation ⁵⁷²⁻⁵⁷⁶. The discovery by Tenhunen *et al.* that the microsomal enzyme haem oxygenase was responsible for catalysing the aforementioned reaction stimulated a deeper interest in the function of endogenous CO production ^{363,413}.

Under normal circumstances, endogenous CO originating from haemoprotein metabolism is transported to the lungs via binding to RBC, producing carboxyhaemoglobin (COHb). Within the lungs COHb equilibrates with the inhaled oxygen and is eliminated from the body via the breath, although a

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small proportion (< 1%) is retained⁵⁷⁷. In a pathological situation, excess CO produces a myriad of toxic effects, the earliest symptom being an increase in the level of COHb (10-20%) which interferes with oxygen delivery and use by the RBC, as the binding affinity of Hb for CO is ~240 times greater than for O₂⁵⁷⁸, while levels of 40-60% are associated with death due to CO binding with other haemoproteins, e.g. CYP-450 and myoglobin³⁷⁷. As CO is excreted primarily via the lungs, and is derived from haem oxygenase in a 1:1 ratio with bilirubin, measurement of CO in the blood (as COHb) and breath can be used as an indicator of haem degradation and bilirubin formation⁵⁷⁹. Akin to the outward manifestations of bilirubin toxicity, i.e. yellowing of the skin, excess COHb in the blood gives a rosy hue to the complexion of the victim⁵⁷¹. In adults, CO is formed at a rate of 0.4 ml/h (16.4 µmol/h)⁵⁷⁷. Normally the rate of CO production (V_{CO}) is relatively constant in males over time; however, newborns exhibit a higher V_{CO} compared to adults, while in females the V_{CO} varies due to the menstrual cycle. Under pathological conditions the V_{CO} can increase in an adult male from 18 to 160 µmol/h⁵⁸⁰. The majority (> 86%) of endogenous CO is derived from the enzymatic metabolism of haem via the haem oxygenase pathway, the remaining (< 14%) is acquired from non-enzymatic sources which include: 1. lipid peroxidation; 2. photooxidation of natural or synthetic photosensitisers, e.g. riboflavin, bilirubin and some metalloporphyrins; and 3. intestinal bacteria^{571,581}.

A great deal of information is known about the mechanisms and pathological effects of CO poisoning^{377,582,583}. Similar to the other products of haem metabolism, CO was long considered a mere waste product until 1991, when

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Marks *et al.* published a paper entitled: "Does carbon monoxide have a physiological function?"⁵⁸⁴. In their article the authors suggested that CO, derived from endogenous haem catabolism, may play a similar role to NO in the regulation of cell function and communication, as both gases have similar chemical and biological properties, i.e. both diatomic molecules with a complex biochemistry. The basis for the latter suggestion was influenced by evidence showing that the other derivatives of haem metabolism, i.e. biliverdin and bilirubin, were beneficial³⁸⁵. The idea that CO, regarded primarily as a toxic agent, could have a widespread physiological function was dismissed initially. However, the evidence in support of this hypothesis has grown strongly, but questions still remain⁵⁸⁵. Since 1991 the number of publications confirming a physiological role for CO continues to rise³⁸³. No longer viewed as a superfluous by-product of haem catabolism, CO is now considered to play a central role in many aspects of human health and disease, e.g. in the vasculature, CNS, reproductive and immune systems⁵⁷⁸.

Exogenous CO has been shown to maintain vascular tone in large and small arteries and directly relax vascular smooth muscle in a reversible manner^{494,495,586,587}. A threshold concentration of 1 μM CO was sufficient to induce these effects and was not dependent upon the presence of an intact endothelium, unlike the situation of vasorelaxation induced by NO^{5,588}. In the liver, where all the haem oxygenase isoforms are expressed, inhibition of their activity (but not NOS activity) increased hepatic vascular resistance, which was reversed by 1 μM exogenous CO⁵⁸⁹. Moreover, the constitutive expression of HO-2 or the induction of HO-1 in endothelial cells points to the

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possibility of CO regulating blood pressure *in vivo*, during normal and stressful conditions ^{493,499}. The vasorelaxation capability of CO is not common to all vascular tissues as cerebral arteries seem to be refractive to CO, but responsive to only NO; the differential response to these two gases in cerebral arteries is not entirely clear at present ⁵⁷⁸. Situations that lead to increased HO-1 expression, e.g. IR injury and hypoxia, are associated with increased CO production, which would ultimately increase blood flow and oxygenation of the affected tissues ^{495,554,590-592}. The effects of CO on blood pressure may be directly through the promotion of vasodilation or indirectly via acting on the nucleus tractus solitarius in the brain, which leads to an alteration of glutamatergic transmission and thus a lowering of blood pressure ⁵⁹³. It has been suggested that CO acts in conjunction with bilirubin, or on its own through inhibition of platelet aggregation via cGMP, to prevent the development of atherosclerosis, as the expression of HO-1 is induced by many proatherogenic stimuli, e.g. oxidised LDL, hypoxia and proinflammatory cytokines ^{428,594}.

The discovery of a neuronal role for NO led to the suggestion that CO may function as a neurotransmitter, intercellular messenger and modulator of neuroendocrine function ^{207,505,506,595}. The discovery of haem oxygenase isoforms, particularly HO-2, throughout the CNS and PNS has added weight to the hypothesis that CO is an important gaseous neural messenger, while in certain cases CO formation may substitute for decreased or absent NOS activity ^{502-504,596}. In the digestive system, mice lacking the HO-2 gene have an impaired NANC relaxation response in their mesenteric plexus, a phenotype

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that was also observed in mice lacking NOS⁵⁰⁷. In another study, it was reported that CO and NO act as coneurotransmitters in the modulation of neuronal activity in the enteric nervous system⁵⁹⁷. These examples illustrate the neurotransmitter capacity of CO and support the possibility that CO and NO act as coneurotransmitters in the same neurons. Certain neurodegenerative disorders, e.g. Alzheimer's and Parkinson's disease (AD and PD, respectively), show increased expression of HO-1 protein in regions with senile plaques and neurofibrillary tangles, while amyloid precursor protein, which is postulated to be a major cause in the neurotoxicity of AD, leads to inhibition of haem oxygenase⁵⁹⁸. The precise function and mechanism of action of haem oxygenase, and therefore CO, in these and other neurodegenerative disorders is unclear at present. Another possible neurological function for CO is in the area of learning and memory as there is evidence to support a role for the molecule in long-term potentiation^{599,600}.

Carbon monoxide also seems to play a role in different aspects of reproduction. During pregnancy, CO maintains normal vascular tone to protect against preeclampsia and relaxes smooth muscle in the uterus⁵⁷⁸. In the placenta, CO has been suggested to control functional and developmental maturity as haem oxygenase isoforms are widely distributed in the placenta at different periods of gestation^{578,601}. The reproductive effects of CO are conveyed through the regulation of the endocrine system. For example, CO inhibits the release of corticotrophin-releasing hormone (CRH), arginine vasopressin and oxytocin, but promotes the release of luteinising hormone-releasing hormone, all of which are involved in the hypothalamus-pituitary-

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adrenal axis control of pregnancy⁶⁰². Within the male reproductive track, CO originating from specific neuronal populations within the vas deferens may act as a neurotransmitter in the regulation of ejaculation as mice with a targeted deletion of HO-2 exhibit ejaculatory abnormalities⁶⁰³. Secondly, cholinergic nerves which innervate the corpus cavernosum (CC) and spongiosum (CS) contain haem oxygenase isoforms, which suggests a role for CO in penile erection⁶⁰⁴.

Another important role for CO is modulation of immune responsive cells, e.g. inhibition of platelet activation and neutrophil migration via increasing the concentration of cGMP^{594,605}. These characteristics have been suggested as a mechanism by which CO promotes survival of transplanted organs as expression of HO-1 has been shown to protect against chronic rejection⁴⁸⁹. Furthermore, a direct link between CO formation and suppression of xenograft rejection in mouse-to-rat cardiac and liver transplant models was demonstrated by Soares and coworkers, as inhibition of HO-1 induction with SnPP promoted an acute rejection response, whereas exogenous CO restored long-term xenograft survival^{488,490}. A number of publications have highlighted the antiinflammatory ability of HO-1 derived CO to suppress endothelial cell apoptosis via activation of the p38 MAPK signal transduction pathway^{469,470}.

The discovery that CO can bind to the iron within the haem moiety of sGC, thus activating the enzymatic conversion of GTP to cGMP, suggests that CO mediates its biological effects via a cGMP pathway⁶⁰⁶. This mechanism of activating sGC is similar to that elicited by NO but differs with respect to the

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bonds formed and intensity of upregulation of the enzyme ^{21,178,184}. Furthermore, the benzylindazole derivative YC-1 can enhance the activation of sGC by CO > 4000% ^{188,189}, suggesting an endogenous 'YC-1 like' molecule may enable a higher degree of activation of sGC by CO akin to NO ¹⁵¹. Akin to NO, the gaseous molecule CO can exert its effects through a variety of sGC-independent mechanisms. For example, CO can enhance vasorelaxation by directly activating Ca²⁺-dependent K⁺ (K_{Ca}) channels, possibly via a histidine residue on the extracellular side of the channel, but without elevating intracellular Ca²⁺ concentration ^{588,607,608}. The K_{Ca} channel complexes comprise noncovalently linked α and β subunits which affect the electrophysiological and pharmacological properties of the pore. More detailed studies have revealed that the α subunit is the preferred target for CO, but supplementation with NO, which preferentially activates the β subunit, significantly attenuated the CO-mediated excitatory effect upon the α subunit, possibly via modulation of a histidine residue within the latter subunit. However, CO was unable to exert a similar influence on the responsiveness of the β subunit towards NO ⁶⁰⁹. Selective modulation of the p38 MAPK signal transduction pathway, which in turn leads to downregulation of proinflammatory cytokines (e.g. TNF α and IL-1 β), may represent an important target of CO action ^{469-471,610}. In contrast to NO which has an affinity for both Fe²⁺ and Fe³⁺ haems, CO only binds with reduced (Fe²⁺) haemoproteins ¹⁸⁰. Although CO may activate sGC and cyclooxygenase (COX), a haemoprotein that is involved in prostaglandin synthesis, it has an inhibitory effect upon CYP 450, catalase, iNOS, Hb, Mb, cytochrome c oxidase and NADPH:oxidase ⁶¹¹⁻

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⁶¹⁴. Given the variety of potential targets for CO it is no surprise this gaseous molecule, in analogy with NO, can exert an influence upon a number of important homeostatic functions namely, vasoregulation, inflammation, apoptosis, cellular respiration, oxygen transport and drug detoxification.

Although many studies of CO function have employed metalloporphyrins (e.g. SnPP) as inhibitors of haem oxygenase the use of such agents has been questioned due to possible inhibition of sGC and modulation of NOS activity ⁴⁵¹. Nevertheless, the physiological effects of CO can no longer be viewed from a purely toxic perspective as the molecule plays a vital role in many important aspects of human health and disease. The weight of evidence in favour of a physiological role for the haem oxygenase/CO pathway is strong and increasing, while at the same time there is a growing understanding of the mechanism(s) by which CO exerts its effects. The possibility of harnessing the beneficial aspects of CO for therapeutic purposes was brought a step closer with the discovery that carbon monoxide-releasing molecules (CO-RMs) liberate CO to elicit direct biological activities ⁶¹⁵. Much knowledge about CO has still to be attained and as Barinaga commented in Science in 1993, "... this gas is likely to provide fuel to run plenty of labs" ⁶¹⁶.

1.16 Iron: metabolism, mechanism of regulation and physiological function

Haemoglobin (Hb) from RBC represents the principal endogenous source of iron, one of the most abundant transition metals ⁴⁷⁹. When RBC die, after about 120 days in the circulation, their Hb is liberated and catabolised further to release its four globin chains each of which contains a haem (Fe-protoporphyrin IX) molecule with ferric iron (Fe^{3+}) at its centre. Within splenic macrophages the globin molecules are metabolised to amino acids, while haem is degraded via the haem oxygenase pathway to Fe^{2+} , CO and biliverdin ⁴⁷⁹. Both free (unbound to protein) iron and haem can be deleterious due to their ability to cause oxidative injury and stimulate inflammation, but NO and haem oxygenase can counteract these adverse effects ^{365,368,617}. In addition, specific binding proteins, e.g. haemopexin (Hx) for haem and transferrin (Tf) for iron, can form complexes to make iron inert and deliver it to other cells for further metabolism and recycling, thus ensuring there is very little iron lost through excretion ^{365,479,618,619}.

Due to the toxic nature of iron, its metabolism is finely regulated in terms of its transport, processing and storage via transcriptional and post-transcriptional control of the proteins involved at each stage. Among the major players in this act are: the plasma carrier protein Tf; transferrin receptors (TfRs); the cellular storage protein ferritin; and lastly, iron-regulatory proteins (IRPs) of which there are two types (IRP1 and IRP2) ⁶²⁰. When iron levels are low, IRPs bind to the iron response elements (IREs), which are mRNA stem-loops, present in the 3' or 5' untranslated region (UTR) of ferritin (which has 1 IRE in the 5'

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UTR) and TfRs (which has 5 IREs in the 3' UTR). This has the effect of decreasing ferritin mRNA translation, but enhancing the stability of the TfRs mRNA and increasing the availability of intracellular iron through uptake by the TfRs. The opposite occurs when iron is plentiful⁶²¹. The human iron-binding protein (IBP) Tf (MW 80 KDa), or apotransferrin (apoTf) in its unsaturated state, is a monomer glycoprotein that has a low capacity, it becomes maximally active with Fe³⁺ iron atoms (this confirmation is called diferric transferrin), but high affinity (dissociation constant (K_d) = 10⁻²³ M) for iron⁶²⁰. The importance of Tf in iron homeostasis is illustrated by the fact that absence of the gene, which is located on human chromosome 3, causes severe hypochromic microcytic anaemias (hereditary atransferrinemia) associated with iron overloading³⁶¹.

The acquisition of iron from Tf by cells, especially erythroid cells, is a finely regulated procedure (see Figure 1.17). The process begins with binding of diferric Tf to TfRs on the cell surface to form a Tf-TfR complex. Internalisation of the complex, via receptor-mediated endocytosis within clathrin coated pits, forms an intracellular endosome within which an acidic environment promotes the dissociation of iron from Tf to form an apoTf-TfR complex. Exocytosis of the latter leads to reattachment of the TfR to the cell surface and liberates apotransferrin to collect more Fe³⁺, thus maintaining the cycle. The natural-associated macrophage protein 2 (Nramp2; also known as divalent cation transporter 1 (DCT1)) assists in the transport of iron, which must be in the Fe²⁺ form as Nramp2 can not bind Fe³⁺, from the endosome^{361,620}. This iron enters an enigmatic compartment known as the intracellular labile iron

pool. From here it can be: 1. incorporated into low or high molecular weight iron complexes; 2. modulate the activity of the IRPs; or 3. taken up by the intracellular iron storage protein ferritin³⁶¹.

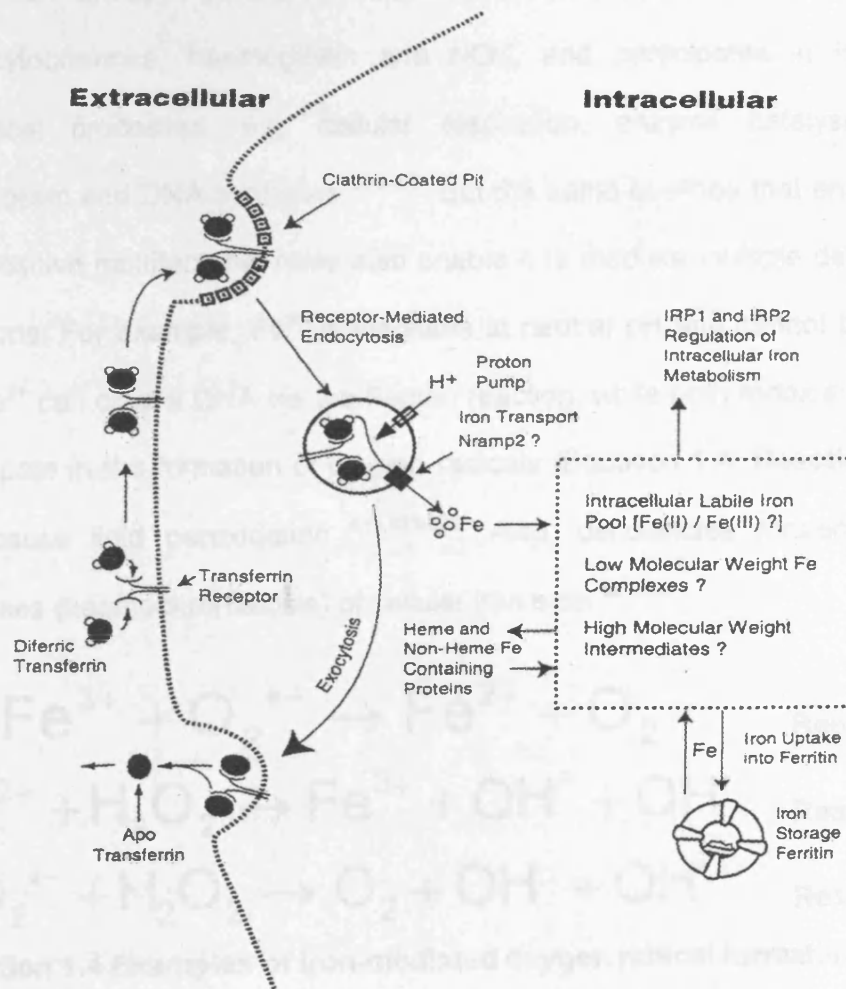
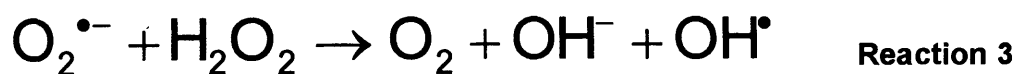
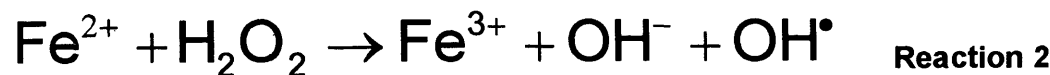
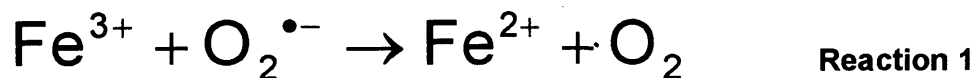


Figure 1.17 Cellular iron uptake by receptor-mediated endocytosis.

Adapted from³⁶¹

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The versatility and reactivity of iron stems from its ability to reversibly change its oxidation state from reduced (Fe^{2+}) to oxidised (Fe^{3+}) and vary the oxidation potential and electron spin configuration of different ligands ³⁸⁴. Thus, iron functions as a protein cofactor of many essential haemoproteins, e.g. cytochromes, haemoglobin and NOS, and participates in important biological processes, e.g. cellular respiration, enzyme catalysis, drug metabolism and DNA synthesis ^{384,622}. But the same qualities that endow iron with positive multifactorial roles also enable it to mediate multiple deleterious reactions. For example, Fe^{3+} is insoluble at neutral pH and cannot bind with O_2 , Fe^{2+} can cleave DNA via the Fenton reaction, while both redox states can participate in the formation of oxygen radicals (Equation 1.4, **Reactions 1-3**) and cause lipid peroxidation ^{618,623-625}. Also, deficiencies (anaemia) and excesses (haemochromatosis) of cellular iron exist ^{621,626}.



Equation 1.4 Examples of iron-mediated oxygen radical formation

The Fenton reaction (Reaction 2), which produces the hydroxyl radical (OH^{\bullet}), can be combined with Reaction 1 in the Haber-Weiss reaction (Reaction 3).

The cytotoxic potential of free iron is kept to a minimal by the actions of haem oxygenase and ferritin ^{484,627,628}. This cytoprotective feature can be modulated by nitric oxide, haem and oxidative stress ^{617,629,630}. Akin to the other haem

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oxygenase pathway products, bilirubin and CO, the physiological role of iron has also generated considerable interest. The principal function of Fe^{2+} is its reuse in new haemoproteins. Animal studies of iron metabolism reveal that a high proportion of mice which are genetically deficient in HO-1 protein (HO-1^{-/-}) die *in utero*, those that survive exhibit a number of pathologies, e.g. severe anaemia, due to low serum iron, and increased sensitivity to oxidative stress^{369,429}. The significance of these animal studies was reinforced with the first report of human HO-1 deficiency in a boy aged six³⁷⁰. The boy exhibited similar features to the mice, i.e. anaemia, growth retardation, tissue iron deposition, and increased sensitivity to oxidative stress, and died prematurely.

Exposure of fibroblasts to stress inducers, e.g. staurosporine, etoposide and serum deprivation, was tolerable by wild type cells, but caused apoptotic death in HO-1^{-/-} cells. However, transfection of the HO-1 gene or iron chelation therapy protected cells, this led the authors to conclude that regulation of intracellular iron efflux was the mechanism by which HO-1 protects cells⁴⁸⁵. Although haem oxygenase cannot pump iron from cells the coexpression of a specific iron ATPase pump that is inducible in tissues with high levels of HO-1 protein activity suggests that both factors cooperate to exert cytoprotection⁵⁰⁴. These examples illustrate that production and regulation of cellular iron is another important cytoprotective aspect of haem oxygenase.

1.17 Haem oxygenase and nitric oxide in cardiomyocytes

The oxidative enzymes NOS (eNOS, iNOS and nNOS) and haem oxygenase (HO-1 and HO-2) share a number of similarities as well as differences^{180,504}. For instance, they both utilise NADPH as an electron donor to generate bioactive gaseous end products, NO (from NOS) and CO (from haem oxygenase), which can modulate similar cellular targets to various degrees^{32,372,609}. Both constitutive and inducible biosynthetic enzymes exist in each case^{83,364}, however, the effects of their inducible forms are dissimilar, i.e. iNOS generates cytotoxic NO to kill other cells⁶³¹, whereas HO-1 exerts a cytoprotective influence via its products bilirubin and CO⁴²⁸ and its ability to eliminate iron from cells⁴⁸⁵. Their cellular and subcellular locations often coincide, e.g. in the nervous, reproductive, digestive and vascular systems^{372,595,597,604,632,633}, or serve as a substitute for the other in locations where one is absent or weakly expressed^{503,634,635}. In addition, recent publications have highlighted an intimate link between HO-1 and NO in the context of cellular homeostasis and regulation of each others expression^{373,636}, while Otterbein *et al.* have suggested that HO-1 may serve as a therapeutic funnel through which the beneficial aspects of NO/NOS are mediated⁶³⁷.

The haem oxygenase enzymes, along with their products bilirubin and CO, have been detected in different cardiac cell types, including cardiac muscle cells (cardiomyocytes), where they exert positive effects^{536,634,638}. Specifically, HO-1 is targeted to the intercalated disc area and sarcoplasmic reticulum of the cardiomyocytes⁶³⁹, while HO-2 is present in the sarcolemma region where it serves as a constant source of bilirubin and CO⁶³⁵. The physiological

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importance of the haem oxygenase system, in particular HO-1, has been documented in recent reports of cardiac pathologies, e.g. hypoxia, I/R injury and myocardial infarction^{181,590,640}. Studies of HO-1 in cardiomyocytes have used similar models to illustrate the protective influence of HO-1 and its products bilirubin and CO. For example, Lakkisto *et al.* suggested that induction of HO-1 shortly after a myocardial infarction may be an important adaptive mechanism⁶³⁹. Loss or damage to cardiomyocytes in response to ROS and RNS can, if left unchecked, be the catalyst for a variety of cardiac pathologies⁶⁴¹⁻⁶⁴³. In addition to its cytoprotective influence in other cell types HO-1 has been shown to be beneficial in cardiomyocytes. In response to metalloporphyrins, e.g. cobalt and iron tetrakis-(4-benzoic acid) porphyrin (CoTBAP and FeTBAP, respectively), HO-1 inhibited doxorubicin (DOX)-induced apoptosis and promoted ROS scavenging in cardiomyocytes⁶⁴⁴. Using cultured neonatal rat cardiomyocytes Lüss and coworkers demonstrated that inhibition of proteasomes, intracellular multisubunit complexes that regulate the degradation of regulatory as well as damaged proteins, with the proteasome inhibitor carbobenzoxy-leuciny-leuciny-leucinal (MG132) led to activation of p38 MARK concomitant with HO-1 induction, and thereby protection against hyperthermic and oxidative injury⁶⁴⁵. Enhanced expression of HO-1 *in vivo* was correlated with protection of cardiomyocytes against I/R injury and improved recovery of cardiac function⁶⁴⁶. Exposure of cardiomyocytes to acute and chronic episodes of hypoxia leads to increased expression of HO-1 mRNA, protein and augmented haem oxygenase activity and bilirubin levels, all of which are associated with a greater degree of cardioprotection and responsiveness towards

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hypoxia-induced stress^{467,647}. Conversely, mice lacking the HO-1 gene exhibited a greater degree of cardiomyocyte apoptosis and developed right ventricular dilatation, compared with their wild type counterparts, following exposure to chronic hypoxia *in vivo*⁶⁴⁸.

Given their ubiquitous nature all three isoforms of NOSs (eNOS, iNOS and nNOS) have been identified in cardiomyocytes where they serve as a constant source of NO^{242,649}. Normally NO functions as a paracrine molecule, i.e. it is made in one cell and diffuses outwards towards its cellular targets in other neighbouring cells⁶⁵⁰; however, cardiomyocytes are unique in that the source (NOS) and key targets (e.g. sGC, K⁺ channels, iron centres and thiols) of NO reside within the same cell. Therefore, NO can function as a paracrine, autocrine and intracrine molecule in the regulation of different cardiomyocyte functions¹⁵⁴. For example, eNOS has been identified in sarcolemma and T-tubule membranes^{115,118} where it exerts direct effects over cardiomyocyte contraction, relaxation and heart rate¹⁴⁰. Neuronal NOS, which is localised to the SR, can assist in the regulation of these physiological aspects of myocytes as it participates in Ca²⁺ handling via inhibition of the RyR within the SR membrane^{259,261,264,651}. The high output and Ca²⁺-independent NOS (iNOS) which is expressed by myocytes during inflammatory responses (e.g. cytokines, sepsis, heart failure) serves as a continuous source of NO that negatively modulates myocyte function²⁷⁴.

Under pathological conditions (e.g. I/R injury, heart failure and heart transplantation) NO/NOS may exert beneficial and adverse effects in myocytes¹⁵⁴. An increase in the expression and activity of eNOS and iNOS

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has been observed in the early (up to 30 min) stage of ischaemia (or hypoxia)^{272,652}. The purpose of this upregulation is to maintain coronary blood flow and myocyte function, lessen the concentration of deleterious ROS, as well as inhibit the build up of and infiltration (via increased vascular permeability) by inflammatory cells^{154,284,653}. Upregulation of HO-1 expression and CO release, in response to increased NO, can serve as another adaptive response in myocytes^{286,590,654}. However, reperfusion can rapidly reverse the above responses due to the build up of ROS, which inactivates NO, and deficiency of substrate and cofactor availability¹⁵⁴. Haem oxygenase may also serve as a positive regulatory of myocyte NO production^{452,636,655,656} and *vice versa*⁶⁵⁷. In addition, expression of HO-1 and its products may compensate for lack of NO production⁴⁹⁹.

The above examples illustrate that both NOS/NO and the haem oxygenase system exert important roles within cardiomyocytes. It is well known that oxidants can be potent catalyst for this enhancement of HO-1⁴¹⁷, in addition, the importance of RNS as mediators of HO-1 is gaining credence³⁷³. Nevertheless, little is known at present about the interaction between these two important regulatory systems within cardiomyocytes, therefore, this Thesis will endeavour to examine this association.

1.17.1 Hypothesis and aims

The ability of HO-1 to counteract oxidative stress is well established³⁸¹, while a number of recent reports have documented a link between RNS and activation of the haem oxygenase pathway^{373,636}. This Thesis will utilise cell culture, molecular biology and biochemical techniques to elucidate the biochemistry and mechanisms responsible for HO-1 induction by nitrosative stimuli. The hypothesis for this work is:

Increased haem oxygenase activity and induction of HO-1 protein and gene expression in response to reactive nitrogen species, particularly NO[•], attenuates nitrosative stress in cardiac cells.

To verify this theory a number of objectives were set:

- (i) Establish that Angeli's salt (AS), a NO[•] generator, activates the haem oxygenase pathway.
- (ii) Dissect the mechanism of AS-mediated HO-1 induction.
- (iii) Investigate the mechanism and identify possible molecular targets which lead to increased haem oxygenase activity and HO-1 expression in response to cellular stress.
- (iv) Utilise bilirubin, an end product of the haem oxygenase pathway, as an effective means to counteract nitrosative stress.
- (v) Examine the potential of a clinically used NO donor, GTN, to promote activation of the haem oxygenase pathway.

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2.1 Materials and equipment

Hemin (ferriprotoporphyrin IX chloride), bilirubin and biliverdin were purchased from Porphyrin Products Inc. (Logan, Utah, USA). The NO donors DeaNONOate (DeaNO) and DetaNONOate (DetaNO) as well as the NO⁻ liberator, Angeli's salt (AS), along with the NO trapping agent carboxy phenyl-tetramethyl imidazolineoxyl-oxide (potassium salt) (C-PTIO), were all obtained from Alexis Biochemicals (Nottingham, UK). All other reagents were acquired from Sigma (Dorset, UK) unless otherwise stated.

Solutions were pHed using a pH meter (Fisher Scientific UK, Loughborough, UK) and a Russell combination pH electrode (Thermal Russell Ltd., Fife, Scotland). Samples were weighed on electronic balances (Sartorius AG, Goettingen, Germany). A NO meter (ISO-NO Mark II), sensor (ISO-NOP) and NO chamber (NOCHM) were purchased from World Precision Instruments (Sarasota, FL, USA). Consumables were acquired from Fisher Scientific UK.

2.2 Cell culture

2.2.1 Preparation and maintenance of the cardiomyocytes

Rat H9c2 cardiomyocytes were obtained from American Type Culture Collection (ATCC, Manassas, Virginia, USA). The H9c2s are a subclone of the original cell line derived from embryonic BD1X rat myocardium tissue and exhibit many of the properties of skeletal muscle. The morphology is of a myoblastic variety in which cells fuse to form multinucleated myotubes and

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respond to ACh stimulation. Cardiomyocytes were handled aseptically at all times and cultured in fresh complete Dulbecco's modified Eagle's media (DMEM) which was supplemented with 10% foetal bovine serum (FBS), 4 mM L-glutamine, all from Sigma, and 100 U/ml of penicillin-streptomycin (Invitrogen Life Technologies Ltd., Paisley, Scotland). Upon receipt of a 1 ml vial of H9c2 cardiomyocytes, cells were quickly thawed in a water bath (37°C) by gentle agitation. The contents were transferred carefully to a 75 cm² cell culture flask (Sarstedt Ltd., Leicester, UK) containing 9 ml of complete media, followed by gentle swirling to disperse the cells. The flask was incubated at 37°C in an incubator in a 95% O₂/5% CO₂ environment. The following day, the media containing the cytoprotective agent was removed and replaced with fresh complete media. Subsequent media changes took place every two days.

In this Thesis an established cell line (H9c2) was used instead of a primary cell line because of its convenience and unlike the latter the H9c2s were more stable and less labour intensive to maintain. Although the product description highlights that H9c2s are similar to skeletal muscle the cells maintain a myoblastic morphology akin to myocytes. To prevent any possibility of a switch in phenotype, i.e. to skeletal muscle, cells were utilised in experiments up to passage 26, as a greater passage number may lead to inconsistency of results. Furthermore, repeated use of this cell line within the above passage limit provided consistent and repeatable results. In addition, the mechanisms examined in this Thesis were replicated in other cell lines, e.g. endothelial cells, smooth muscle, keratinocytes and macrophages, by our group suggesting a generalised response towards the agents administered. Thus,

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the H9c2 cells were utilised to ascertain the effect, and potential mechanism, of RNS upon the haem oxygenase system in cardiac cells.

2.2.2 Subculturing the cardiomyocytes

In order to maintain the cell line and prevent loss of the myoblastic cells the cardiomyocytes were subcultured every 3-4 days when they reached about 70%-80% confluency. The old media was discarded and replaced with 10 ml of warm sterile phosphate buffered saline (PBS) (10X), without Ca^{2+} and Mg^{2+} (Invitrogen LifeTechnologies Ltd.). After a brief wash the latter was removed and replaced with 4 ml of warm 0.25% (w/v) trypsin-0.02% (w/v) ethylenediaminetetraacetic acid (EDTA) solution (Sigma). The flasks were placed in the incubator for a few minutes to allow the trypsin to detach the cardiomyocytes from the base of the flask and each other. The contents of the flasks were transferred carefully to individual 15 ml tubes containing 6 ml of warm (37°C) fresh complete DMEM. After centrifuging (1000 x g) for 5 min at room temperature in a bench-top centrifuge (MSE Harrier 15/80, Sanyo Gallenkamp Plc., Leicestershire, UK) the supernatant was discarded and the pellets resuspended gently in sufficient DMEM to prepare a new set of flasks, final volume 10 ml, in a 1 to 4 ratio. The flasks were returned to the incubator to allow the cells to grow. Cells were subcultured until passage number 26, after which time a new vial was subcultured. In some cases cardiomyocytes were seeded into 24 well plates (Sarstedt Ltd.) in a ratio of three plates per flask. The procedure is similar to that just described with minor variations. The pellets were gently resuspended in fresh complete DMEM (6 ml). The cell suspension (4 ml) was dissolved in a sufficient volume of DMEM so that the

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final volume in each well was 1 ml. Cells were ready in 2-3 days, thus media changes were not necessary.

2.2.3 Preparation of agents and treatment of cardiomyocytes

Stock solutions (50 mM) of AS (a source of NO⁻), DeaNO and DetaNO (sources of NO) were prepared in 0.01 M NaOH according to the manufacturer's instructions. A 100 mM stock solution of *N*-acetylcysteine (NAC) was prepared with 5% (v/v) 2 M NaOH. Stock solutions (50 mM) of the NO scavengers, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (potassium salt) (C-PTIO) and hydroxocobalamin (HCB) Vitamin B_{12a} acetate salt, were dissolved in ethanol and dH₂O, respectively. A stock solution of copper sulphate (CuSO₄) (10 mM) was prepared with sterile PBS (10X). All of the agents were prepared fresh in opaque Eppendorfs, or a 15 ml tube wrapped in foil for CuSO₄, and placed on ice until required. Hemin (ferri-(Fe³⁺)protoporphyrin IX) (Frontier Scientific Europe, Carnforth, Lancashire, UK) was prepared as a 2 mM stock using 0.5% (v/v) NaOH (2 M), phosphate buffer (2 mM MgCl₂ and 100 mM KH₂PO₄, pH 7.4) and dH₂O.

The agents were incubated with confluent cardiomyocytes in 75 cm² flasks or 24 well plates, final volumes 10 ml and 1 ml, respectively, within different vehicles. The vehicles used were fresh complete DMEM and Dulbecco's PBS (DPBS), modified with Ca²⁺ and Mg²⁺ (Sigma). Decomposed AS was prepared by placing the agent at 37°C for 1 h in one or both vehicles. Where DMEM was the vehicle, both freshly prepared and decomposed AS were applied to the cells for the required duration. When DPBS was used as the

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vehicle, fresh and decomposed AS were preincubated with the cells for 15 min, removed and replaced with fresh complete media for the remaining duration. A variety of concentrations and durations (0-24 h) were used with each experiment. Negative controls, which consisted of vehicle alone, were included in each experiment.

2.2.4 Cell metabolism assay

In order to examine the metabolic effect of the agents applied to the cardiomyocytes a cell metabolism assay using the reduction-oxidation (REDOX) indicator Alamar blue (Serotec, Oxford, UK) was performed. This reagent contains various stabilising agents along with the non-fluorescent tetrazolium blue-based dye resazurin, which upon oxidative metabolism by metabolically active cells yields the reduced product resorufin. The latter has a pink/red colour and the extent of colour change in the growth media can be measured fluorometrically or colourimetrically, the former method being more sensitive^{658,659}. Alamar blue has been used to assess cytotoxicity, viability and antimicrobial efficacy in both animal, bacterial and plant cells^{658,660-663}. Previous studies in eukaryotic cells have revealed that Alamar blue can undergo reduction within living cells via cytosolic, microsomal and mitochondrial enzymes, e.g. alcohol and aldehyde oxidoreductases, NAD(P)H:quinine oxidoreductase, flavin reductase and NADH dehydrogenase and cytochromes, as a result the quantifiable reduction product resorufin is released into the medium^{658,664}. Given that Alamar blue has a positive redox potential (+ 0.38 V) it can be reduced by viable cells without the need for energy expenditure, therefore, in mitochondria the reagent acts between the

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final reduction of O₂ and cytochrome *c* oxidase by substituting for O₂ as an electron acceptor without affecting the activity of the respiratory chain as the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay does⁶⁶¹. In addition, the indicator is not affected by antioxidant or free radical scavenging enzymes in the media and is stable in reduced media under CO₂ incubation⁶⁶⁰. It has been suggested that FBS might interfere with the Alamar blue readings when it is coincubated with a treatment or during prolonged (> 24 h) incubation in the medium. However, this can be overcome by applying the Alamar blue after the compound incubation period in fresh media and for no longer than 6 h⁶⁵⁸. In addition, Rasmussen *et al.* have observed no interference with Alamar blue reduction in the presence of DMEM⁶⁶⁵. As a decrease in Alamar blue fluorescence or absorbance is an indication of diminished metabolic activity, and *vice versa*, the assay can serve as a sensitive means to monitor intracellular cytoplasmic, mitochondrial and nuclear changes, all of which are sensitive to imbalances of the intracellular redox state.

Cardiomyocytes, which were seeded into 24 well plates according to Section 2.2.2, were incubated with the required agent(s) in fresh complete DMEM (total volume 1 ml per well). After mixing by gentle swirling, the plates were placed in the incubator for the duration required. At the end of the incubation period the media was removed and replaced with 1 ml of fresh complete media containing 10% (v/v) Alamar blue. The plates were then returned to the incubator for 4-5 h until a colour change appeared. Addition and removal of media were performed carefully so as not to dislodge any cells from the base

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of the wells. After 4-5 h, 200 μ l of each sample was transferred to its respective well of a microtitre plate and the absorbance was read at 570 nm and 600 nm against a blank set of wells containing DMEM plus 10% Alamar blue, in a microplate reader (Versamax, Molecular Devices Ltd., Wokingham, UK). The difference between the absorbance at 600 nm and 570 nm was expressed as a percentage of the control group.

2.3 Biochemical procedures

2.3.1 Collection of cardiomyocytes

For this procedure, all reagents and collecting tubes (15 ml) were kept on ice. At the end of an experiment the media was discarded and cells were washed with 10 ml of ice-cold non-sterile PBS (10X). After a brief wash the latter was discarded and replaced with 5 ml of ice-cold non-sterile PBS (1X) (non-sterile PBS 10 X diluted 1:10 with dH₂O). Cells were gently scraped from the base of the flasks with a plastic scraper (policeman) (Thomas Scientific, Swedesboro, New Jersey, USA), followed by careful transfer to pre-labelled collecting tubes. A further 5 ml of PBS (1X) was added to each flask to collect any residual cells and then transferred to its respective tube. The tubes were centrifuged (1000 \times *g*) for 5 min at room temperature to pellet the cells. The supernatants were discarded and the tubes inverted on tissue paper to remove any excess of liquid. The cell pellets requiring haem oxygenase activity analysis (Section 2.3.4) were resuspended in 550 μ l of phosphate buffer (2 mM MgCl₂ and 100 mM KH₂PO₄, pH 7.4). Samples for HO-1 protein analysis (Section 2.4.2) were resuspended in 150 μ l of PBS (pH 7.4), containing 1% (v/v) Triton X-100, to lyse the cells. In both cases, samples

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were dissolved fully using a bench-top vortexor and stored at -70°C until required for analysis.

2.3.2 Protein determination in cells and tissues

The DC Protein Assay kit (Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, UK) was used to determine protein concentration. A standard curve was prepared using serial dilutions (0 to 2.8 mg/ml) of a bovine serum albumin (BSA) standard (Bio-Rad Laboratories Ltd.), prepared in triplicate, in phosphate buffer (pH 7.4). The absorbance readings at an optical density of 750 nm (OD_{750}) were plotted against their respective protein standard concentrations (see Figure 2.1 for a representative plot). The procedure for protein determination in cell and tissue samples was performed as follows. A 100 μl aliquot of the cell suspension and a blank of phosphate buffer or PBS Triton X-100 was added to 10 ml polystyrene tubes (Sarstedt Ltd.). When tissue samples were used, the aliquot was diluted 1:10 in phosphate buffer (pH 7.4). To each tube, 500 μl of A' (20 μl of Reagent S per ml of Reagent A) was added, then vortexed briefly without frothing. Next, 4 ml of Reagent B was added to each tube, mixed well then left at room temperature for 20 min before reading the OD_{750} against the blank using a UVikon 810P spectrophotometer (Tegimenta AG, Switzerland). A numerical value (mg/ml) for the protein concentration in the unknowns was derived by multiplying the respective OD_{750} by the slope of the standard curve (Equation 2.1).

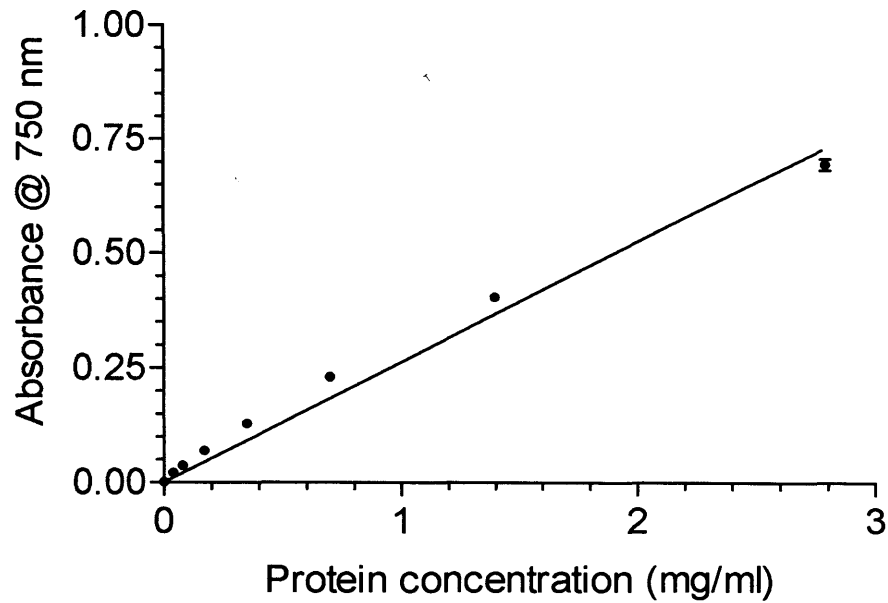


Figure 2.1 Representative standard curve for protein determination.

Serial dilutions of bovine serum albumin (BSA) (0-2.8 mg/ml) were prepared according to Section 2.3.2. The OD_{750} was plotted against its respective protein concentration.

$$OD_{750} \times \text{Slope of standard curve}$$

Equation 2.1 Calculation of protein concentration

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2.3.3 Preparation of liver cytosol and liver microsomes

A male Sprague Dawley rat (250-300 g) was used as a source of liver cytosol. Following sacrifice by anaesthetization with phenobarbital Lethobarb® sodium BP (500 µl, i.p.) and cervical dislocation, a 50 ml syringe was used to perfuse cold (4°C) buffer (1.15% (w/v) KCl) through the liver lobes to remove any residual blood, while clotted tissue was discarded. The organ was weighed, finely chopped with a scissors and homogenised in 2-3 volumes of homogenising buffer (20 mM Tris-HCl, pH 7.4, containing 1.15% (w/v) KCl), followed by partition among a set of polyallomer centrifuge tubes (Beckman Coulter Ltd., High Wycombe, UK). After centrifuging (5,000 x g) for 20 min at 4°C in a refrigerated ultracentrifuge (Optima LE 80, Beckman Instruments, Palo Alto, California, USA) the supernatant was retained and recentrifuged (105,000 x g) for 90 min at 4°C. Following each centrifugation, the turbid lipid layer was removed with a Pasteur pipette. The supernatant was collected, and protein and haem oxygenase activity were measured as described in Sections 2.3.2 and 2.3.4, respectively, then aliquoted and stored at -70°C.

The preparation of liver microsomes is similar to that for liver cytosol with the following variations. The rat was pre-treated with hemin (50 mg/kg, i.p.) 24 h beforehand to induce HO. The lobes were perfused with cold (4°C) saline (0.9% (w/v) NaCl) and homogenised in 5 volumes of sucrose solution (0.25 M sucrose, 0.05 M Tris-HCl, pH 7.4) then centrifuged. After the last centrifugation step, the resulting microsomal pellet was resuspended gently in 1 ml of phosphate buffer (pH 7.4) and protein and haem oxygenase activity were determined according to Sections 2.3.2 and 2.3.4, respectively, followed

by storage at -70°C .

2.3.4 Determination of haem oxygenase activity

Stock solutions of the assay reagents, i.e. glucose-6-phosphate (G6P) (20 mM), glucose-6-phosphate dehydrogenase (G6PDH) (50 U/ml) and NADPH (40 mM), were prepared with phosphate buffer (pH 7.4) and stored at -70°C until required. The NADPH was wrapped in foil as it is light sensitive. Hemin (2 mM) was prepared freshly for each assay as described in Section 2.2.3. Rat liver cytosol and liver microsomes were prepared according to Section 2.3.3.

The samples, collected according to Section 2.3.1, were disrupted by three rounds of freeze-thawing (-70° to 37°C), followed by sonication for 15 sec, then placed on ice. A 100 μl aliquot was retained for protein determination according to Section 2.3.2. To a set of glass tubes, which were maintained on ice, the cell suspension (400 μl) was added to the reaction mixture (final volume, 900 μl) containing: phosphate buffer (pH 7.4), 20 μM hemin, 2 mM G6P, 0.5 U/ml G6PDH, 3 mg of rat liver cytosol, as a source of biliverdin reductase (BVR), and 0.8 mM NADPH. The negative and positive controls (rat liver microsomes) contained proportionate volumes of phosphate buffer (pH 7.4) instead of the cell suspension. The tubes were vortexed well and incubated in the dark for 1 h at 37°C . The activity of the cytosolic and microsomal fractions was verified by replacing hemin with biliverdin (1 mM), while the samples were incubated in the dark for 30 min at 37°C . The reaction was terminated by addition of chloroform (1 ml), followed by thorough vortexing and centrifuging ($200 \times g$) 2-3 times for 5 min at room temperature

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using a bench-top centrifuge until three distinct layers were formed. Using a quartz cuvette and the UVikon 810P spectrophotometer the absorbance (at 464 nm and 530 nm) of the lower organic layer, which contains bilirubin (extinction coefficient (ϵ) for bilirubin in chloroform, $40 \text{ mM}^{-1}\text{cm}^{-1}$), was read against a blank of chloroform. Haem oxygenase activity was expressed as picomoles of bilirubin formed/mg protein/h using Equation 2.2.

$$\frac{\text{pmoles bilirubin}}{\text{mg protein in 60 min}} = \left(\frac{\Delta\text{OD} (\text{OD}_{464} - \text{OD}_{530})}{40} / \text{mg protein} \right) \times 10^6$$

Equation 2.2 Calculation of haem oxygenase activity

2.4 Molecular biology procedures

2.4.1 Preparation of nuclear extracts[‡]

In the cytosol the redox-sensitive transcription factor Nrf2 lies dormant as a result of its association with Keap1⁶⁶⁶. In response to electrophiles, or agents that modify thiol groups, e.g. nitrosants, the repression of Nrf2 by Keap1 is lost and the former migrates to the nucleus where it activates the transcription of defensive genes, e.g. HO-1⁴⁶⁵. In order to determine the responsiveness of Nrf2 towards nitrosants nuclear extracts were prepared as follows. Cells were washed twice with PBS (1X) then harvested in 1 ml of PBS (1X) followed by centrifugation ($800 \times g$) for 3 min at 4°C . The resulting pellet was resuspended carefully in 200 μl of cold Buffer A (10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, final volume 950 μl , followed by 10 μl of 0.1 M

[‡] All nuclear extracts were kindly prepared by Martha Hoque, Dept. of Surgical Research, NPIMR.

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dithiothreitol (DTT) and 40 μ l of complete protease inhibitor cocktail (PIC) (Roche, Mannheim, Germany)). Next, the pellet was placed on ice for 15 min to allow the cells to swell then 15 μ l of 10% Nonidet P-40 was added followed by a 10 sec vortex-mix and centrifuging ($800 \times g$) of the resulting homogenate for 3 min at 4°C. The supernatant was discarded and the nuclear pellet was resuspended in 30 μ l of cold Buffer B (20 mM Hepes (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, final volume 950 μ l, followed by 10 μ l of 0.1 M DTT and 40 μ l of complete PIC). The pellet was placed on ice for 15 min and vortex-mixed for 10-15 sec every 2 min. After a final centrifugation ($10000 \times g$) for 5 min at 4°C the supernatant, which contains the nuclear proteins, was retained and stored at -70°C until further analysis of the Nrf2 protein by Western blot (Section 2.4.2) was performed.

2.4.2 Western blot protein analysis

Total protein of each sample was calculated as per Section 2.3.2. An appropriate volume of each unknown sample was combined with loading buffer (Laemmli buffer (Bio-Rad Laboratories Ltd.) containing 5% (v/v) β -mercaptoethanol), total volume 30 μ l, so that the final concentration loaded onto each lane was equal to 30-50 μ g. The HO-1 positive control (Bioquote Ltd., York, UK) was diluted to 1 μ g/ml in loading buffer. Samples were pulsed for 20-30 sec on a bench-top microcentrifuge (MSE Microcentaur, Sanyo Gallenkamp Plc., Leicestershire, UK) followed by denaturing of the proteins for 10 min at 100°C on a heating block (Techne DB2A, Techne GmbH, Germany). The molecular weight marker (MWM) (Invitrogen LifeTechnologies Ltd.) was not heated but placed on ice until required. After heating, the

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samples were re-pulsed and 15-30 μ l of samples, MWM and positive control was loaded carefully using gel loading tips (Fischer, UK) into the wells of a 12% Tris-glycine Ready gel (Bio-Rad Laboratories Ltd.). Electrophoresis was carried out at room temperature in a tank containing running buffer (0.025 M Tris, 0.192 M glycine, 0.1% (v/v) SDS) (Fischer, UK) using the Mini-PROTEAN® II system and Power-Pac 300 power supply (Bio-Rad Laboratories Ltd.) at a constant voltage of 110 V until the loading buffer had migrated to, but not beyond, the base of the gel.

The subsequent procedures were carried out using the Mini Trans-Blot® electrophoretic transfer cell (Bio-Rad Laboratories Ltd.) and gloved hands to avoid contamination of membranes. While the gel was running appropriately sized sheets of nitrocellulose membrane 45 μ m pore size (Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, UK) and 3MM Whatman™ blotting paper were cut and presoaked in dH₂O and cold (4°C) transfer buffer (0.025 M Tris, 0.192 M glycine, 0.1% (v/v) SDS, 20% (v/v) methanol) for 10 and 5 min, respectively. After electrophoresis was completed a 'gel sandwich' (see Figure 2.2 for assembly) was prepared upon the clear (positive electrode) side of a gel cassette within a shallow vessel flooded with transfer buffer. After removing any air bubbles the cassette was closed securely via the latch and placed into the electrode module with the black (negative electrode) side facing the black (cathode) panel of the module. This orientation ensures the proteins migrate from the gel onto the nitrocellulose membrane and not into the transfer buffer while transferring. Extra transfer buffer was added and overnight transfer was carried out at 4°C with a

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constant voltage of 30 V using a 1000/500 transfer unit from Bio-Rad Laboratories Ltd.

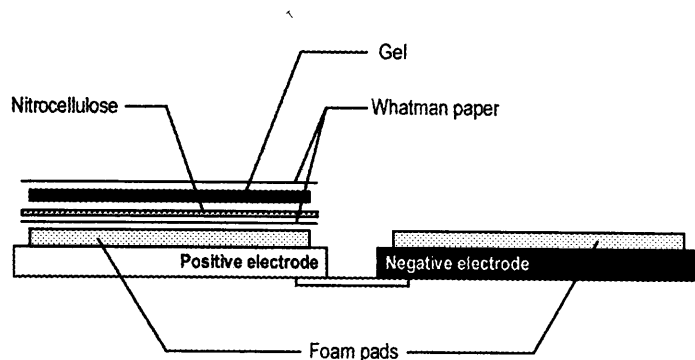


Figure 2.2 Arrangement of gel sandwich assembly.

Next, equal protein transfer in each lane of the nitrocellulose membrane was verified by reversible protein staining with Ponceau S (3% (v/v) acetic acid, 0.2% (w/v) Ponceau S), then washed off thoroughly with dH₂O. All subsequent procedures were performed at room temperature on an orbital shaker (Denley Instruments Ltd., Sussex, UK) at low speed. Non-specific binding (NSB) of the antibodies was prevented by incubating the membrane in blocking solution (5% (w/v) non-fat dried milk in PBS (0.01 M phosphate buffer, pH 7.4, containing 0.2% Tween-20) for 2 h at room temperature. This was followed by a single 5 min wash with 10 ml of PBS (pH 7.4). All subsequent washes were for 5 min with 10 ml of the named solution. The membrane was incubated for 2 h with anti-HO-1 (Bioquote Ltd., York, UK) or anti-Nrf2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibodies, diluted 1:1000 or 1:500, respectively, in Tris buffered saline (TBS) (0.05 M Tris-HCl, 0.0037 M KCl, 0.7137 M NaOH, pH 7.4). Next, the

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membrane was washed three times, once with PBS-T (PBS 0.01 M, pH 7.4, containing 0.05% (v/v) Tween 20), then twice with TBS (pH 7.4).

Proteins were visualised using an ExtrAvidin® alkaline phosphatase staining kit (Sigma). The biotinylated anti-rabbit IgG antibody, diluted 1:1000 in TBS (pH 7.4), was incubated with the membrane for 1 h. The membrane was washed three times with TBS (pH 7.4) and the ExtrAvidin® alkaline phosphatase conjugate, diluted 1:1000 in TBS (pH 7.4), applied for 1 h. The three washes with TBS (pH 7.4) were repeated. After the last wash a freshly prepared substrate solution (9.8 ml of 0.1 M Tris buffer, pH 8.2, containing 200 µl of 1% (w/v) Naphthol-AS-BI phosphate in N-N-N-dimethyl formamide and a 10 mg Fast Red DT salt tablet) was incubated with the membrane until red/pink bands, which indicates the location of the antigen-antibody complexes of the protein on the nitrocellulose membrane, was observed. In all Western blot experiments only one blot (replicate) was prepared. After maximal colour change was attained, any excess substrate solution was washed off with dH₂O and the membrane stored in foil, until scanned into a PC.

2.4.3 Isolation and determination of total haem oxygenase-1 mRNA

In order to maintain the integrity of the ribonucleic acid (RNA) and prevent contamination or degradation by RNases (enzymes that degrade RNA) a number of precautions were taken: gloves were worn at all times; all glassware was sterilised by baking for 6 h at 200°C beforehand; and the equipment and solutions were washed and prepared with dH₂O which was

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pre-treated with diethyl pyrocarbonate (DEPC). The latter was prepared by adding DEPC (0.5 ml/litre) to dH₂O, within pre-baked glass bottles, shaking vigorously to mix, followed by baking at 40°C for 24 h. The water was purged of any remaining DEPC by autoclaving at 121°C for 25 min.

The method for HO-1 mRNA isolation was based upon that described by Chomczynski and Sacchi⁶⁶⁷. Samples were collected by washing the flasks twice with sterile PBS (10X) followed by the addition of 1 ml of guanidine thiocyanate (GT) lysis buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% sarkosyl (pH 7.0), containing 7.2 µl (v/v) of 0.1 M 2-mercaptoethanol). The flasks were gently swirled to cover all the cells and placed at an angle for 5 min to pool the viscous lysate, followed by transfer to 15 ml tubes. The following extraction agents were added to each tube sequentially: 0.2 ml of 2 M sodium acetate (pH 7.4); 2.5 ml of phenol (pH 4.3); and 0.4 ml of chloroform:isoamylalcohol (in a ratio of 49:1 v/v). This was followed by thorough vortexing, 15 min on ice, then centrifuging (10,000 x g) for 20 min at 4°C in a bench-top centrifuge (Avanti 30 Centrifuge, Beckman Instruments). The upper aqueous layer was transferred to a new 15 ml tube and the addition of GT lysis buffer, extraction agents, vortexing and standing on ice were repeated.

The tube contents were divided equally among 1.5 ml Eppendorfs then recentrifuged. The upper aqueous layer was retained and divided equally among 1.5 ml Eppendorfs. An equal volume (~600-700 µl) of cold 2-propanol was added to each. After inverting three times the samples were placed at -20°C for at least 1 h or overnight. Next, the samples were centrifuged (10,000

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x g) for 30 min at 4°C. The supernatants were discarded and GT lysis buffer (300 µl) was used to resuspend and pool the pellets from each sample into a single Eppendorf. Cold 2-propanol (300 µl) was added to each Eppendorf, then mixed by inversion three times and left at -20°C for 1 h. The samples were centrifuged (10,000 x g) for 30 min at 4°C. The supernatants were discarded and the pellet was washed with 75% ethanol (500 µl). After a brief vortexing to dislodge the pellet samples were centrifuged (10,000 x g) for 15 min at 4°C. The supernatant was discarded and the Eppendorfs inverted to air dry the pellet on the bench top for 30 min. The pellets, containing the RNA, were dissolved in DEPC water (20 µl).

The RNA concentration was determined spectrophotometrically with 3 µl of each sample diluted in 97 µl of DEPC water. The absorbance was read at 260 nm and 280 nm against a blank of DEPC water using a UV-visual spectrophotometer (Helios α, Unicam Ltd., Cambridge, UK). The total RNA concentration of the entire sample was derived from Equation 2.3. The quality of the extracted RNA was based on the ratio of the readings at OD₂₆₀/OD₂₈₀. A ratio of 1.6-1.8 indicates good quality while anything less than 1.6 signifies that the sample contains DNA instead of RNA.

$$OD_{260} \times 4 = \text{RNA}(\mu\text{g} / \mu\text{l})$$

Equation 2.3 Calculation of total RNA concentration

2.4.4 Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of HO-1 mRNA[§]

Having isolated and calculated the total RNA concentration as described above sufficient DPEC water was added to each sample so that a final concentration of 50 ng RNA was obtained. The reverse transcriptase-polymerase chain reaction (RT-PCR) consists of two parts, 1. denaturing of the enzyme RT, and 2. amplification of the cDNA products. The RT reaction was carried out in a 50 µl reaction volume using Ready-To-Go™ RT-PCR Beads (Amersham Biosciences UK Ltd., UK) in 0.2 ml thin-walled tubes. Each bead contains all the necessary reagents, i.e. Moloney murine leukaemia virus (M-MuLV) RT in 1.5 mM MgCl₂, *Taq* DNA polymerase and RNase inhibitor, except primers and template, to generate PCR amplification products from an RNA template. To each reaction tube 35 µl of DPEC water was added and left for 5-10 min to dissolve the beads. Next, 5 µl of first strand primer (pd(N)₆ random hexamer primers) and 2 µl of sample was added to each tube and mixed by gentle pipetting. A 50 µl aliquot of mineral oil was added onto the latter mixture, then the tubes were placed in a UNO II thermal cycler (Biometra Ltd., Maidstone, Kent, UK) and underwent RT denaturation for a duration of 20 min (15 min at 42°C and 5 min at 95°C).

The RT reaction was amplified by PCR, for this 8 µl of primer mixture (2 µl of the following primers: HO-1 sense, HO-1 antisense, β-actin sense and β-actin

[§] All RT-PCR assays were kindly performed by Martha Hoque, Dept. of Surgical Research, NPIMR.

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antisense (see Table 2.1 for sequences) in 152 µl of DPEC water) was added under the mineral oil. The second cycle parameters consisted of a 30 sec denaturing step at 94°C, a 30 sec annealing step at 60°C and a 1 h 30 min extension step at 68°C. A total of 26 cycles were used per amplification and the annealing temperature for HO-1 and β-actin was 65°C. In the mean time a 1.5% agarose gel (1.5 g of agarose in 100 ml of 0.5% TBE buffer (10 mM Tris-HCl, 1 mM EDTA), containing 0.5 µg/ml of ethidium bromide (EB), was prepared. The gel was poured into a gel casting mould and pre-run for 10 min at 120 V in a tank containing 0.5% TBE buffer.

After completion of the PCR the samples were placed on ice while prepared for running on the gel. To a fresh set of Eppendorfs 3 µl of loading buffer and 10 µl of sample (taken from below the mineral oil layer) was added and mixed by gentle pipetting. A molecular weight marker (MWM) (5 µl of 100 base pair (bp) PCR ladder, 5 µl of DPEC water and 3 µl of loading buffer) was prepared in parallel. Aliquots (13 µl) of samples and MWM were carefully loaded into their respective wells, their loading order recorded, and the gel run at 120 V for about 1-1.5 h. The PCR products were visualised using a UV dual-intensity transilluminator TM 20 (UVP, Genetic Research Instrumentation Ltd., Dunmow, Essex, UK) and photographed using a Polaroid DS 34 camera with a DS H-8 hood (Polaroid UK Ltd., St Albans, Hertfordshire, UK) and Polaroid film (Type 667) with a No. 15, deep yellow filter. In all PCR experiments only one assay (replicate) was performed.

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Table 2.1 RT-PCR primer sequences for HO-1 and β -actin

Primer	Sequence 5' – 3'	Product size (bp)	Primer length
HO-1			
Sense	CTT TCA GAA GGG TCA GGT GTC CA	246	23
Antisense	CTG AGA GGT CAC CCA GGT AGC GG		23
β-actin			
Sense	C GTG GGC CGC CCT AGG CAC CA	309	21
Antisense	CG GTT GGC CTT AGG CTT CAG AGG GG		25

2.4.5 Northern blot analysis of haem oxygenase-1 mRNA

A 1.3% (w/v) denaturing agarose gel was prepared using 1.4 g of agarose-LE (Ambion Inc., Austin, Texas, USA) per 100 ml, 10X MOPS buffer (3-(N-morpholino) propane sulfonic acid) and DPEC water. The 10X MOPS buffer (pH 5.5-7.0) was made as a stock solution using, 0.2 M MOPS, pH 6.0; 50 mM sodium acetate; and 10 mM EDTA. The gel mixture was heated for 6 min, or until all the agents were fully dissolved, on low-medium power in a microwave. After a short cooling period at room temperature 2.2 M formaldehyde was added and mixed by gentle swirling. The gel was poured into a casting tray on a flat surface and left to set. The set gel was placed into an electrophoresis apparatus and pre-run at 120 V for 10 min in 1X MOPS buffer (0.02 M MOPS, pH 6.0; 5 mM sodium acetate; and 1 mM EDTA) containing 5% (v/v) EB.

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A 10-20 µg concentration of extracted total RNA was added to 10 µl of denaturing buffer (1X MOPS, 50% (v/v) deionised formamide, 2.2 M formaldehyde), vortexed briefly, then heated in a water bath at 60°C for 10 min to denature the RNA, followed by rapid cooling on ice. To each sample, 2 µl of loading dye (5% glycerol, 10% bromophenol blue, saturated solution) and 1 µl of EB (diluted 1:2 with DPEC water) was added, then mixed by pulsing (30 sec) in a Microcentaur centrifuge (Sanyo Gallenkamp Plc.). Samples (10-20 µg RNA/lane) were loaded onto the 1.3% gel and run at 120 V in 1X MOPS buffer until the loading dye had migrated a distance of 5 cm from the wells, i.e. a duration of 2-2.5 h. A positive control, derived from cells exposed to hemin (50 µM) in DMEM for 3 h followed by fresh media for a further 3 h, was run in parallel with the treated samples. The migration pattern was observed using a UV dual-intensity transilluminator TM 20 and recorded with a Polaroid DS 34 camera with a DS H-8 hood and Type 667 film (Polaroid UK Ltd.). After rinsing with DPEC water, the gel was washed once with 1 X rinse solution (75 mM NaOH, 100 mM NaCl) for 45 min followed by a second wash second with Tris-HCl (100 mM, pH 7.5) for 1 h, on the orbital shaker. The gel was blotted overnight by upward capillary action onto a positively charged nylon membrane (GeneScreen™, NEN® Research Products, Du Pont de Nemours & Co. Inc., Boston, MA, USA) in a tank containing 1 X phosphate buffer (25 mM Na₂HPO₄, 25 mM NaH₂PO₄, pH 6.5), using the assembly shown in Figure 2.3.

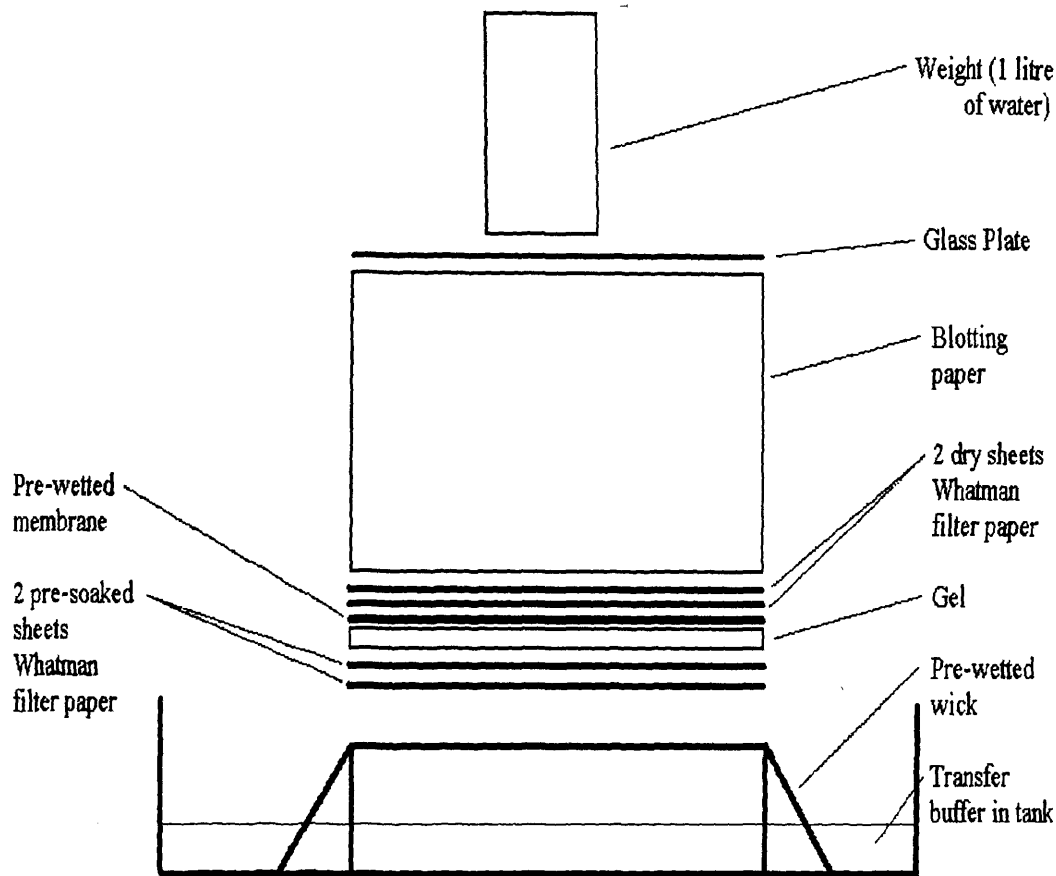


Figure 2.3 Assembly of Northern blot for blotting process.

Following overnight blotting, the membrane was baked in an oven for 2 h at 80°C. The membrane was incubated for 1 h at 65°C in 15-20 ml of Rapid-Hyb buffer (Amersham Biosciences UK Ltd., UK), while rotating in a rotisserie (SI 20H Hybridisation Oven/Shaker, Stuart Scientific Co. Ltd., Redhill, Surrey, UK). All subsequent procedures were carried out at 65°C using the rotisserie, unless stated otherwise. A rat HO-1 cDNA probe (kindly provided by Professor Shibahara, Tohoku University, Japan) was labelled with Redivue™ [$\alpha^{32}\text{P}$]-dCTP (Amersham Biosciences UK Ltd., UK) using a

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commercial Nick Translation kit (Boehringer Ingelheim Ltd., UK., Bracknell, Berkshire, UK). Unincorporated nucleotides were removed using a second commercial kit QIAquick™ Nucleotide Removal Kit (Qiagen Ltd., Dorking, Surrey, UK). After denaturing by boiling for 10 min, followed by standing on ice for 10 min, the labelled probe was incubated with the membrane in the Rapid-Hyb buffer for 24 h.

Next, the membrane was washed in 2X SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0)/0.1% (w/v) SDS) for 20 min at room temperature. This was followed by washing with 1X SSC/0.1% (w/v) SDS for 15 min at 65°C. A third wash using 0.1% SSC/0.1% (w/v) SDS was carried out at 65°C for 15 min. The purpose of these post-hybridisation washes is to increase the stringency conditions in order to remove non-specific binding from the membrane, yet allowing the stronger affinity of the probe to the gene of interest to be unaffected. The membrane was placed in a radiographic cassette on top of a sheet of radiographic film (BioMax MR, Eastman Kodak Co., Rochester, New York, USA) and exposed for 3-4 days at -70°C. The film was developed and the image scanned using a scanner and cropped to size using Adobe Photoshop 6.0 (Adobe Systems UK, Uxbridge, UK). Staining of the 18S ribosomal RNA (rRNA) band was used to confirm integrity and equal loading of the RNA. In all Northern blot experiments only one blot (replicate) was prepared.

2.5 Spectrophotometric and electrochemical studies of bile pigments and NO congeners

2.5.1 Spectrophotometric analysis of bile pigments and NO congeners

Stock solutions (10 mM) of the bile pigments, bilirubin and biliverdin, and NO congeners, AS (a NO⁻ liberator) and DeaNO (a NO donor), were dissolved in 0.1 M (bile pigments) or 0.01 M (NO congeners) NaOH and diluted down to 0.5 or 1 mM working solutions, respectively. The agents were prepared freshly on the day of use in opaque Eppendorfs and placed on ice until required. All spectra were recorded using a Helios α UV-visual spectrophotometer (Unicam Ltd., Cambridge, UK). The absorbance readings, between 300 and 750 nm, were conducted at 37°C against a blank of DPBS (pH 7.4) containing 5 μ M vehicle (0.1 M NaOH). Initial spectra of the bile pigments (final concentration 5 μ M in DPBS) alone were compared with absorption spectra in the presence of freshly prepared AS or DeaNO over time (0-30 min) and across a concentration range (0-300 μ M). Changes in spectra were also recorded after exposure of bilirubin to 50 μ M of decomposed AS or DeaNO, i.e. the agents were preincubated in DPBS at 37°C for 1 h before the addition of bilirubin (5 μ M). The data was plotted using the accompanying Convert application together with Microsoft Excel and GraphPad Prism 3.0 software.

2.5.2 Detection of NO release from NO congeners**

Release of NO from AS, DeaNO and DetaNO was measured amperometrically using a NO-sensitive electrode (ISO-NO Mark II) and sensor (ISO-NOP, detection limit 1 nM) both from World Precision Instruments (Sarasota, FL, USA). Calibration of the electrode was performed daily by measuring known amounts of NO in solution according to the manufacturer's instructions. This consisted of generating NO by cumulative additions (25-100 μM) of potassium nitrite (KNO_2) to an acidic solution (0.1 M H_2SO_4 , sulphuric acid) containing 0.1 M potassium iodide (KI). The product of plateau value minus peak height was plotted against the respective NO concentrations as a calibration curve, a representative plot is shown in Figure 2.4. Experiments were performed in a nitric oxide chamber (NOCHM, World Precision Instruments) maintained at 37°C. The electrode was immersed in either 1 ml of complete DMEM, DPBS or rat plasma, followed by addition of the agents being analysed, and NO concentration was recorded continuously under constant stirring. The amount of NO released over time was expressed as the area under the curve (AUC) calculated after 15 min of readings ($\mu\text{M} \times \text{sec}$).

** All NO electrode assays were kindly performed by Sandip K. Bains, Dept. of Surgical Research, NPIMR.

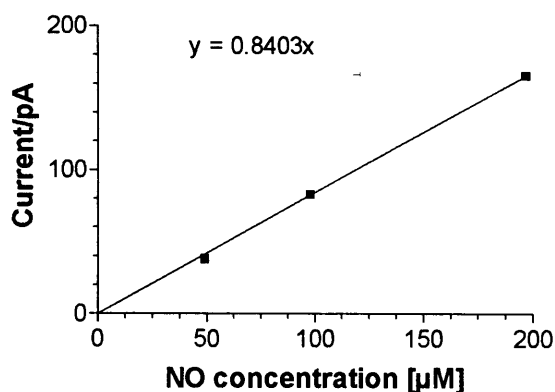


Figure 2.4 Representative calibration curve for nitric oxide release

2.6 Statistics Analysis

The statistical analyses employed in this Thesis included a one-way analysis of variance (ANOVA) combined with the Bonferroni post test to compare all treated values to one another. The one way ANOVA compares the means from three or more groups, assuming that data are sampled from Gaussian populations. The aforementioned tests were applied to time course, multiple concentrations and different treatments, data. Linear regression analysis was used to plot standard curves while non-linear regression (one phase exponential decay) was employed for the bilirubin reaction rate graphs in Chapter 6. The values were expressed as the means \pm standard error of the mean (S.E.M.) and differences between the groups were considered to be statistically significant at probability (P) $<$ 0.05. All analyses were performed using GraphPad Prism 3.00 for Windows (GraphPad Software, San Diego, California, USA, www.graphpad.com).

3 INDUCTION OF HAEM OXYGENASE-1 BY THE NITROXYL ANION RELEASER, ANGELI'S SALT

3.1 Introduction

First identified in the late 1960s the enzyme system HO represents the primary biological route of haem catabolism^{363,413}. So far, inducible (HO-1, also referred to as HSP 32) and constitutive (HO-2 and HO-3) isoforms of the enzyme have been discovered. Two isoforms (HO-1 and HO-2) degrade haem to the final products CO, bilirubin and ferrous iron (Fe^{2+})³⁶⁴. However, HO-1 has generated most interest due to its greater sensitivity to induction by a wide range of inducers (e.g. haem, UV irradiation, heavy metals and pathological states such as atherosclerosis and inflammation) and its ability to mitigate vascular and cellular dysfunction⁴³³. The basis for the cytoprotective actions attributed to HO-1 stem from compelling evidence showing that CO and bilirubin act as a potent vasodilator and antioxidant, respectively⁴²⁸. A common factor leading to the induction and overexpression of the HO-1 gene, and thus CO and bilirubin formation, is an increased generation of ROS leading to oxidative stress⁴²⁹. More recently, a number of publications have highlighted the ability of the gaseous molecule NO and its different redox derivatives, known collectively as RNS, to induce the HO-1/bilirubin/CO pathway^{372,373}.

Due to the complex chemistry of NO and its ability to exist in a variety of interrelated redox-activated forms¹³, investigations are now required to

identify the NO congeners responsible for stimulating HO-1 induction. The NO^+ and NO^- , respectively the one electron oxidation and reduction products of NO, are possible candidates as both of these redox forms can invoke a variety of biological responses depending upon their concentration, location, the presence of thiols and the composition of the cellular milieu ²⁹. In this chapter, experiments conducted in cardiac H9c2 cells to examine the effect of the NO^- generator AS on HO-1 protein and mRNA expression as well as haem oxygenase activity will be described. A comparison of the extent of HO-1 induction will be made with known NO donors, i.e. DeaNO and DetaNO, to determine the cellular response to stress, i.e. nitrosative stress, inflicted by NO and its congeners.

3.2 Objective

The aim of this chapter is to demonstrate that haem oxygenase activity and HO-1 protein and mRNA expression are increased in the cardiac cell line H9c2 in response to different forms of nitrosative stress, specifically from the NO^- liberator AS.

3.3 Materials and Methods

3.3.1 Preparation of reagents

Stock solutions (50 mM) of the NO^- generator AS and the NO donors (DeaNO and DetaNO) were prepared in 0.01 M NaOH. A 2 mg/ml stock solution of actinomycin D was prepared in ethanol. All reagents were prepared in opaque Eppendorfs shortly before each experiment and placed on ice until use.

3.3.2 Maintenance and treatment of cells

Rat H9c2 cardiomyocytes were grown to confluence in flasks and 24 well plates as described in Section 2.2.2. The reagents (AS, DeaNO and DetaNO) were applied to cells directly or following their decomposition (see Section 2.2.3 for methodology) in a specific vehicle (fresh complete DMEM or DPBS, modified with Ca^{2+} and Mg^{2+}) for the durations and concentrations specified in each graph. In the case of actinomycin D it was applied to cells in DMEM 30 min before the addition of AS to the same media. After incubation, cells were collected for analysis of either haem oxygenase activity, Western blot of HO-1 protein, RT-PCR or Northern blot of HO-1 mRNA expression, or a combination of such assays, as described in Sections 2.3.4, 2.4.2, 2.4.4 and 2.4.5, respectively. To determine the possible cytotoxic effects of AS, DeaNO and DetaNO and correlate this with their respective effect upon haem oxygenase activity and HO-1 protein expression a concentration range (0-2 mM) of these compounds was applied to cells cultured in 24 well plates. Cytotoxicity was assessed spectrophotometrically via the cell metabolism assay (Section 2.2.4). Negative controls, which consisted of vehicle alone, were included in all experiments.

3.4 Results

3.4.1 Concentration-dependent effect of Angeli's salt (AS) on haem oxygenase activity, HO-1 protein and mRNA expression in H9c2 cardiomyocytes

In order to establish the effect of AS on haem oxygenase activity and HO-1 protein and gene expression, cardiomyocytes were treated with a concentration range (0 to 2 mM) of the NO⁻ donor for 6 h in complete DMEM. As shown in Figure 3.1 **A**, AS (0-1 mM) elicited a concentration-dependent increase in haem oxygenase activity (254.8 ± 16.2 to 1062 ± 43.6 pmoles bilirubin/mg protein/h; $P < 0.05$) with a slight decline in activity at the higher concentrations (1.5-2 mM). Western blot analysis (Figure 3.1 **B**; $n = 1$) demonstrated a change in the expression of a 32 kDa protein band, which corresponds to HO-1 protein, over the same concentration range. After 6 h exposure to various concentrations of AS, the intensity of HO-1 mRNA gene expression levels also increased gradually with maximal levels visible at 2 mM AS (Figure 3.1 **C**; $n = 1$). This correlated with the results depicted in Figure 3.1 **B**. The 18S rRNA band is also shown to confirm integrity and equal loading of the RNA of the samples on the gel, while hemin (50 μ M) was used as a positive control. We hypothesised that the mechanism by which AS mediates an increase in haem oxygenase activity occurs at the transcriptional level. In fact, the use of the transcription inhibitor actinomycin D completely abolished any increase in haem oxygenase activity and mRNA expression when it was combined with AS in the media (see Figure 3.3).

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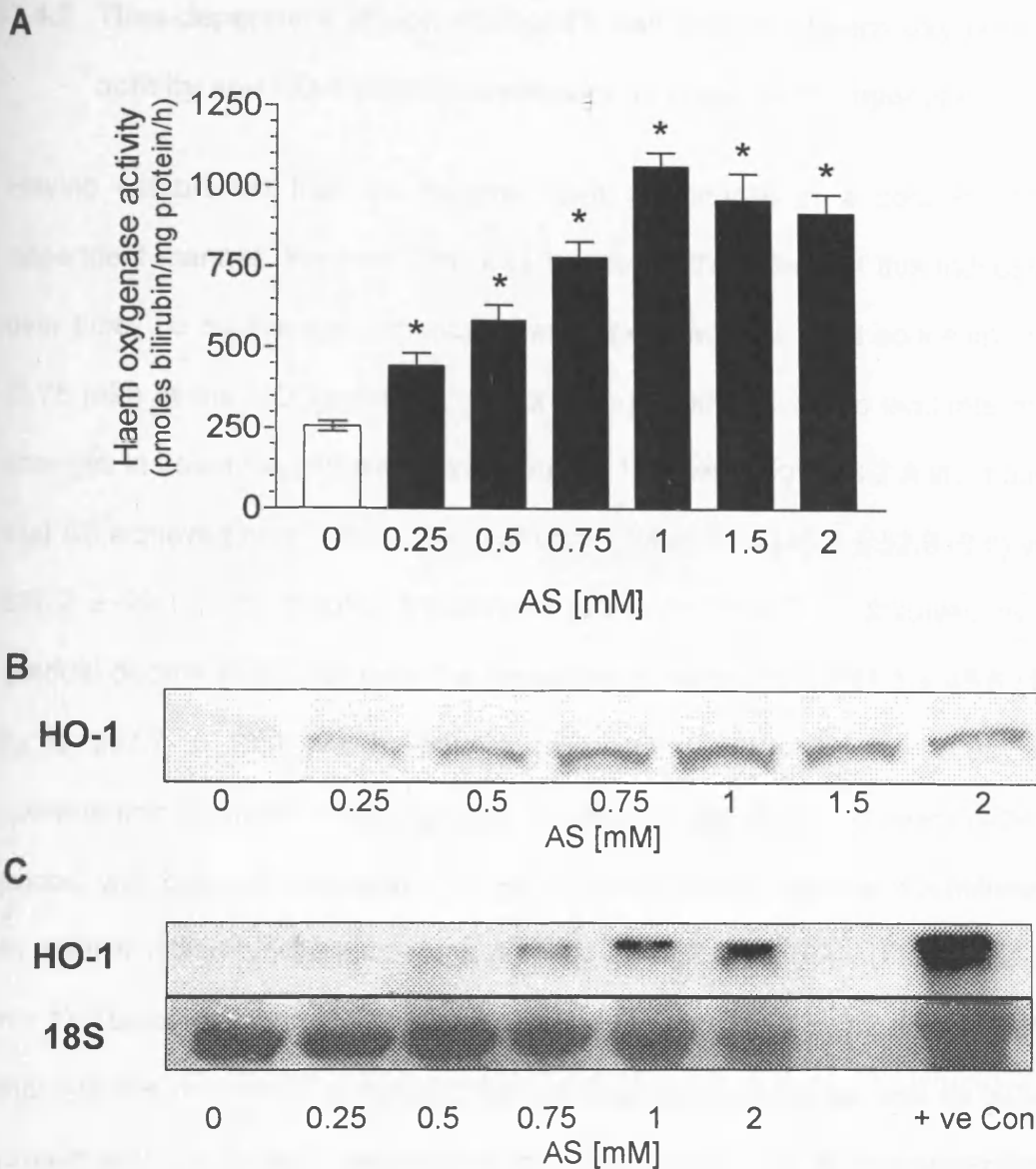


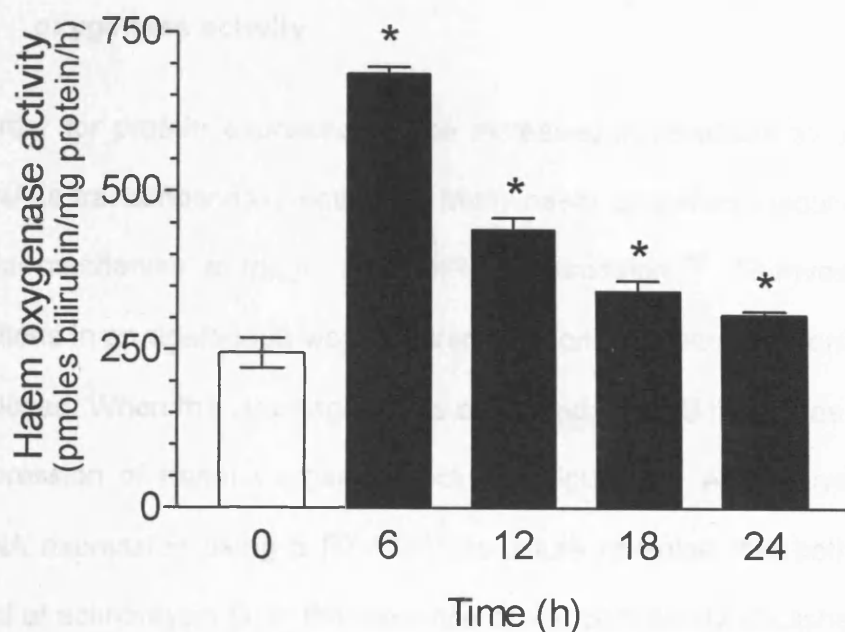
Figure 3.1 Concentration-dependent effect of Angeli's salt on haem oxygenase activity, HO-1 protein and mRNA expression in H9c2 cardiomyocytes.

Cells were incubated with Angeli's salt (AS, 0-2 mM) in fresh complete DMEM. After 6 h, cells were collected and analysed for: **(A)** haem oxygenase activity; **(B)** HO-1 protein; and **(C)** HO-1 mRNA expression, as described in Sections 2.3.4, 2.4.2 and 2.4.5, respectively. Control (0 mM AS) represents cells exposed to media alone. The +ve Con in panel C was generated using hemin (50 μ M). The 18S rRNA band is shown to confirm integrity and equal loading of RNA. Blots **(B)** and **(C)** are representative of one replicate. Bars represent the mean \pm S.E.M. of $n = 6$ experiments. * $P < 0.05$ vs. 0 mM AS.

3.4.2 Time-dependent effect of Angeli's salt (AS) on haem oxygenase activity and HO-1 protein expression in H9c2 cardiomyocytes

Having established that AS induces haem oxygenase in a concentration dependent manner, the next step was to assess the extent of this induction over time. To do this cardiomyocytes were exposed to a fixed concentration (0.75 mM) of the NO⁻ generator over a 24 h period in order to evaluate any changes in haem oxygenase activity and HO-1 protein. Figure 3.2 A illustrates that AS achieved maximal induction of activity after 6 h (245.5 ± 52.9 (0 h) vs. 685.2 ± 29.1 (6 h) pmoles bilirubin/mg protein/h; $P < 0.05$) followed by a gradual decline in activity over the remaining duration (from 441.1 ± 46.6 (12 h) to 307.7 ± 13.8 (24 h) pmoles bilirubin/mg protein/h; $P < 0.05$). A corresponding pattern of HO-1 protein expression was observed over the 24 h period, with maximal intensity of the HO-1 protein band visible at 6 h followed by weaker intensity with increasing duration of exposure to AS (Figure 3.2 B; $n = 1$). Taken together, the data presented in Figures 3.1 and 3.2 demonstrate that AS has the ability to induce haem oxygenase activity, as well as HO-1 protein and mRNA gene expression, in a concentration- and time-dependent manner.

A



B

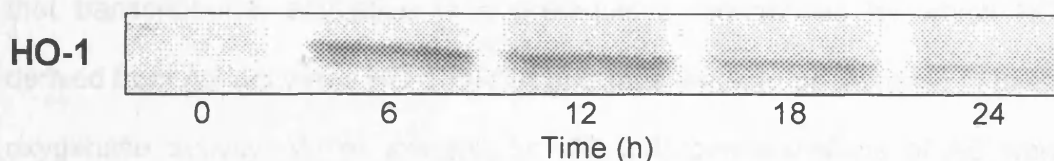
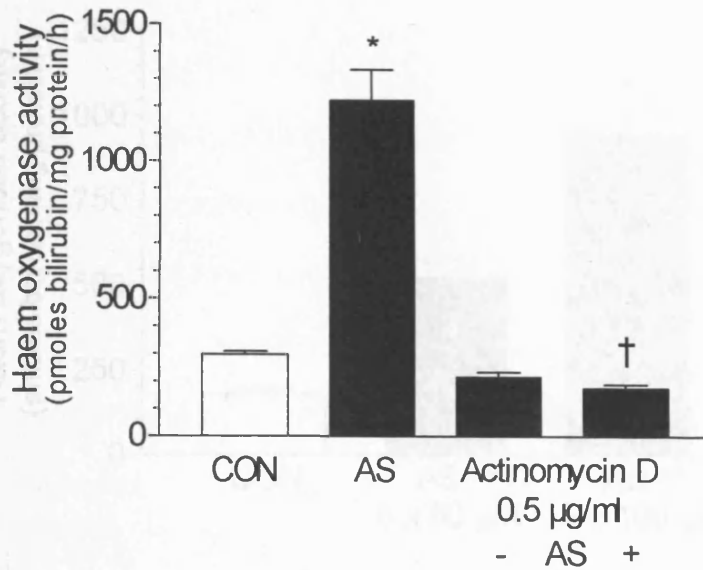


Figure 3.2 Time-dependent effect of Angeli's salt on haem oxygenase activity and HO-1 protein expression in H9c2 cardiomyocytes.

Following exposure to a fixed concentration (0.75 mM) of Angeli's salt (AS) for various times (0-24 h), haem oxygenase activity (**A**) and Western blot assay (**B**) for HO-1 protein were assessed as described in Sections 2.3.4 and 2.4.2, respectively. Control (0 h) represents cells exposed to media alone. The Western blot panel is representative of one experiment. Bars represent the mean \pm S.E.M. of $n = 6$ experiments. * $P < 0.05$ vs. 0 h.

A



B

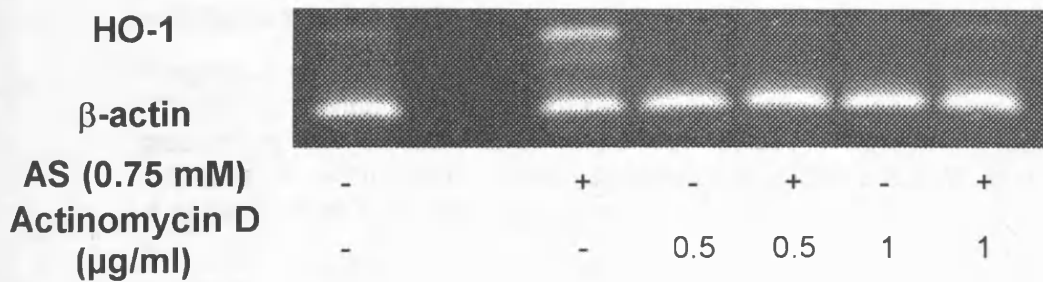


Figure 3.3 Effect of Angeli's salt and actinomycin D on haem oxygenase activity and HO-1 mRNA expression in rat H9c2 cardiomyocytes.

Cells were exposed to Angeli's salt (AS, 0.75 mM) in the absence (-) or presence (+) of actinomycin D (0.5 or 1 µg/ml) in fresh complete DMEM. In the latter case actinomycin D was preincubated with cells for 30 min and AS was added for the remaining duration. After 6 h haem oxygenase activity (**A**) and HO-1 mRNA expression (**B**) were determined according to Sections 2.3.4 and 2.4.4, respectively. Control (CON) represents cells exposed to media alone. The PCR image (panel **B**) is representative of a single experiment with β -actin serving as a loading control. Bars represent the mean \pm S.E.M. of $n = 4$ experiments. * $P < 0.001$ vs. CON. † $P < 0.001$ vs. AS alone.

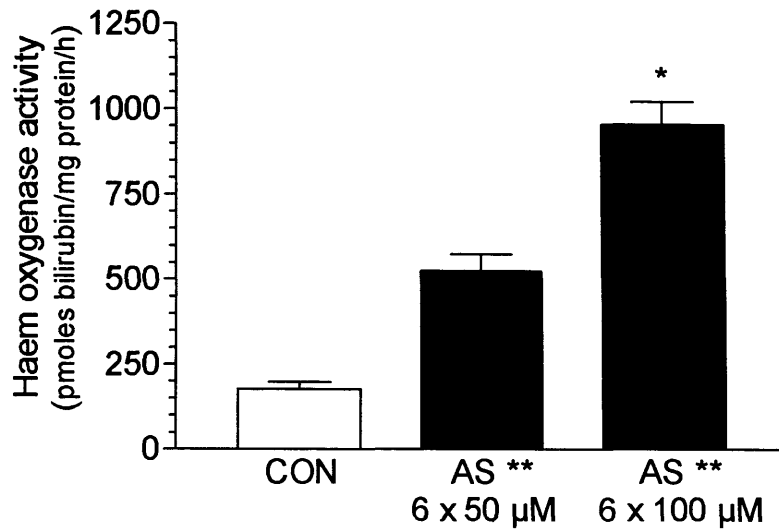


Figure 3.4 Multiple additions of Angeli's salt stimulates haem oxygenase activity in H9c2 cardiomyocytes.

Angeli's salt (AS, 50 or 100 µM) was added to cells at 1 h intervals (**) over a period of 6 h. Haem oxygenase activity was determined according to Section 2.3.4. Control (CON) represents cells exposed to media alone. Bars represent the mean \pm S.E.M. of $n = 4$ experiments. * $P < 0.01$ vs. control (CON).

3.4.4 Effect of different vehicles on the haem oxygenase inducing potential of Angeli's salt

Given that AS has quite a short half-life ($t_{1/2} = 2.3$ min), it was decided to investigate whether the agent had any inducing potential upon haem oxygenase after its capacity to liberate NO^- had expired. To do this AS (0.75 mM) was allowed to 'decompose' for 1 h at 37°C (see Section 2.2.3) in two different vehicles, complete DMEM or DPBS. A comparison was also made with freshly prepared AS in both vehicles. Following a 6 h exposure to AS, cells were collected for haem oxygenase activity determination. Figure 3.5 shows that freshly prepared AS had a greater ability to induce haem oxygenase activity in both vehicles compared with the decomposed form. Fresh AS had the greatest influence on haem oxygenase activity when cells were cultured in DMEM compared with DPBS (1132 ± 328.2 vs. 705.8 ± 133.5 pmoles bilirubin/mg protein/h, respectively; $P < 0.05$). When decomposed AS was applied to cells the level of haem oxygenase activity was significantly less ($P > 0.05$) in both vehicles. A possible reason for the higher level of haem oxygenase activity observed when freshly prepared AS was added to cells in media compared with DPBS could be due to the influence of constituents within the media enhancing the inducing potential of AS. The fact that freshly prepared AS elicited a stronger induction suggests that the NO^- , derived from AS, is likely to promote the signalling cascade. This point is reinforced by the lower level of haem oxygenase activity observed when decomposed AS was incubated with both vehicles.

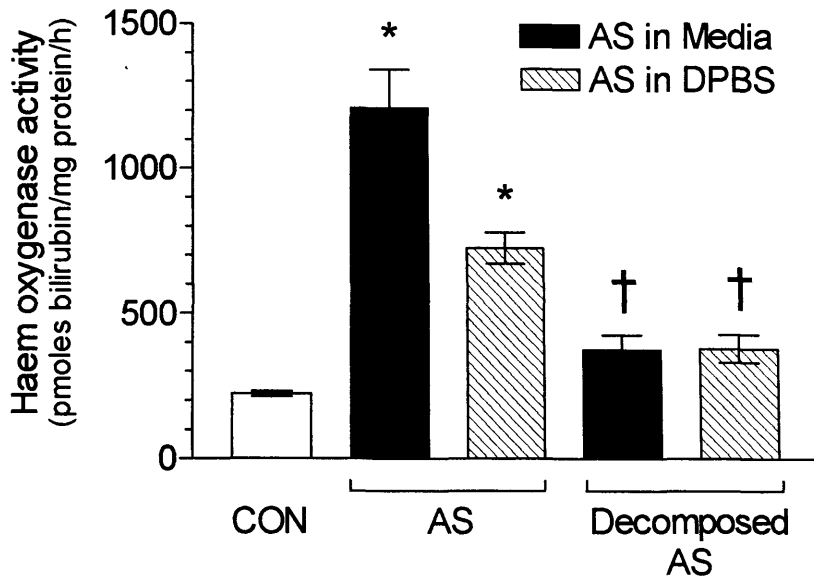


Figure 3.5 Effect of Angeli's salt decomposition on haem oxygenase activity elicited in different culture vehicles.

Angeli's salt (AS, 0.75 mM) was applied to cardiomyocytes either directly or following preincubation (see Section 2.2.3 for preparation procedures) in fresh complete DMEM (filled bars) or DPBS (hatched bars). Haem oxygenase activity was determined after 6 h treatment as described in Section 2.3.4. Control (CON) represents cells exposed to media alone. Bars represent the mean \pm S.E.M. of $n = 4$ experiments. * $P < 0.05$ vs. CON. † $P < 0.05$ vs. directly applied AS.

3.4.5 Potency of nitric oxide donors on HO-1 induction and activity in H9c2 cardiomyocytes

It has been shown that NO can induce haem oxygenase activity in endothelial cells^{373,423}. To complement the work carried out so far two different types of NO donors were used: diethylamine NONOate (DeaNO), which has a short half-life ($t_{1/2} = 2$ min); and diethylenetetraamine NONOate (DetaNO), which has a long half-life ($t_{1/2} = 20$ h). Stock solutions of both NO donors were prepared according to Section 2.2.3. Cardiomyocytes were treated with a concentration range (0-2 mM) of DetaNO for 6 and 18 h in DMEM to examine the change, if any, in haem oxygenase activity and HO-1 protein expression. The same parameters were assessed using DeaNO (0-2 mM), but this agent was applied to cells for just 6 h.

Following a 6 h exposure to DetaNO (0-2 mM) (Figure 3.6 A) there was a concentration-dependent increase in haem oxygenase activity, with maximal activity attained at 1.5 mM ($P < 0.05$). At 2 mM there was a slight decline in activity, possibly due to cell toxicity at this concentration (see data in Figure 3.9). Western blot analysis (Figure 3.6 B; $n = 1$) identified a protein band corresponding to HO-1 at 0.25 to 2 mM DetaNO, the intensity of which increased with increasing concentration of the drug. An 18 h incubation (Figure 3.7 A) with DetaNO caused a more significant ($P < 0.05$) increase in haem oxygenase activity compared with a 6 h exposure. As DetaNO has a long half-life it is often used to mimic physiological NO release. The fact that activity levelled off from 1 mM onwards indicates that high concentrations of the drug are tolerable over the duration of exposure. After 18 h a

corresponding change in HO-1 protein expression was observed, with similar intensity of staining from 0.75 to 2 mM (Figure 3.7 B; $n = 1$).

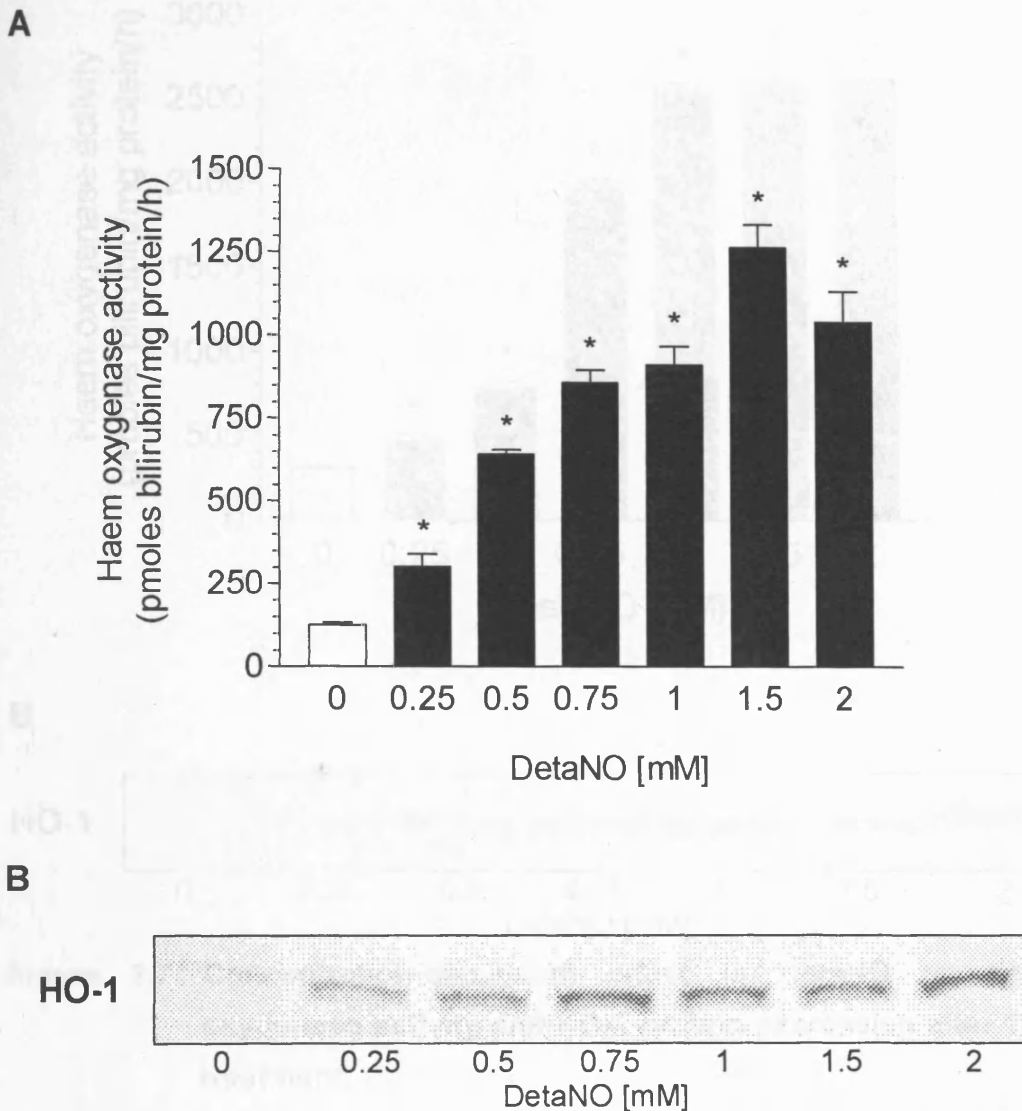
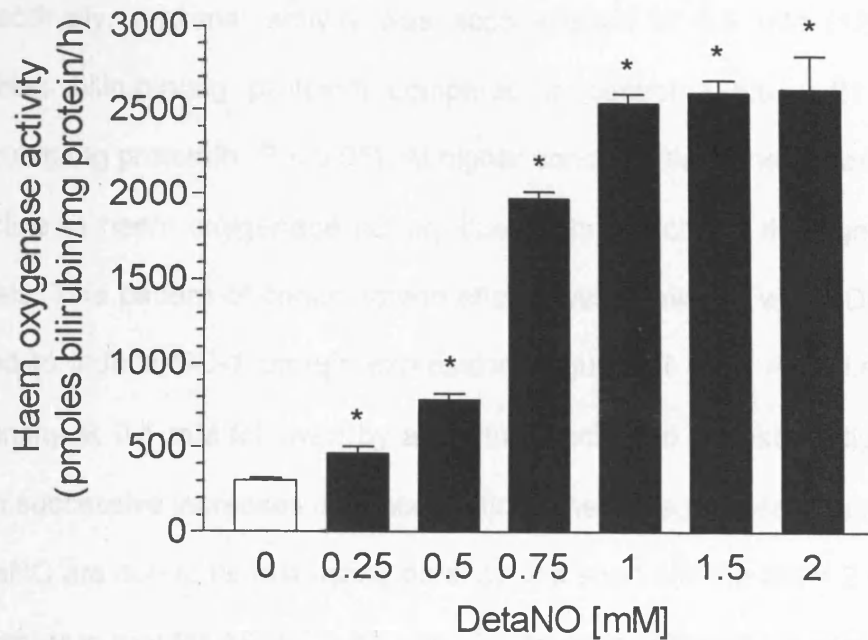


Figure 3.6 Concentration-dependent effect of DetaNO on haem oxygenase activity and HO-1 protein expression after 6 h treatment.

A concentration range of DetaNO (0-2 mM) was applied to cells in fresh complete DMEM for 6 h. Haem oxygenase activity (**A**) and Western blot (**B**) assays were performed as described in Sections 2.3.4 and 2.4.2, respectively. Control (0 mM DetaNO) represents cells treated with fresh DMEM alone. The Western blot panel is representative of one experiment. Bars represent the mean \pm S.E.M. of $n = 3-4$ experiments. * $P < 0.05$ vs. 0 mM DetaNO.

A



B

HO-1

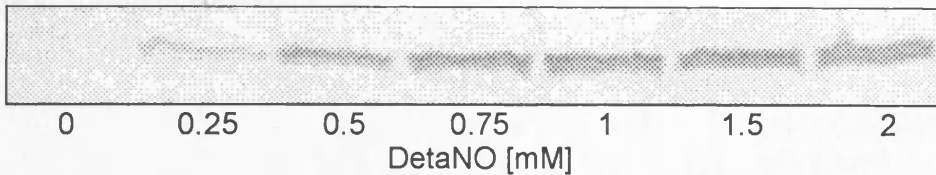


Figure 3.7 Concentration-dependent effect of DetaNO on haem oxygenase activity and HO-1 protein expression after 18 h treatment.

A concentration range of DetaNO (0-2 mM) was applied to cells in fresh complete DMEM for 18 h. Haem oxygenase activity (**A**) and Western blot (**B**) assays were performed as described in Sections 2.3.4 and 2.4.2, respectively. Control (0 mM DetaNO) represents cells treated with fresh DMEM alone. The Western blot panel is representative of one experiment. Bars represent the mean \pm S.E.M. of $n = 3-4$ experiments. * $P < 0.05$ vs. 0 mM DetaNO.

INDUCTION OF HO-1

When DeaNO (0-2 mM) was applied to cardiomyocytes for 6 h a different pattern of haem oxygenase activity was observed (see Figure 3.8 **A**). Specifically, maximal activity was accomplished at 0.5 mM (1265 ± 622 pmoles bilirubin/mg protein/h compared to control 125.6 ± 21.5 pmoles bilirubin/mg protein/h; $P < 0.05$). At higher concentrations there was a gradual decline in haem oxygenase activity due to the toxicity of the agent at such levels. This pattern of concentration effects was mimicked when DeaNO was used to induce HO-1 protein expression (Figure 3.8 **B**; $n = 1$), i.e. maximal intensity at 0.5 mM followed by a gradual decline to almost negligible levels with successive increases in concentration. The pattern of results produced by DeaNO are due to its fast acting potency and short half-life ($t_{1/2} = 2$ min) which is similar to that for AS ($t_{1/2} = 2.3$ min). In the case of DeaNO, it can be used to simulate short but intense bursts of NO release rather like that seen under pathological conditions involving NO.

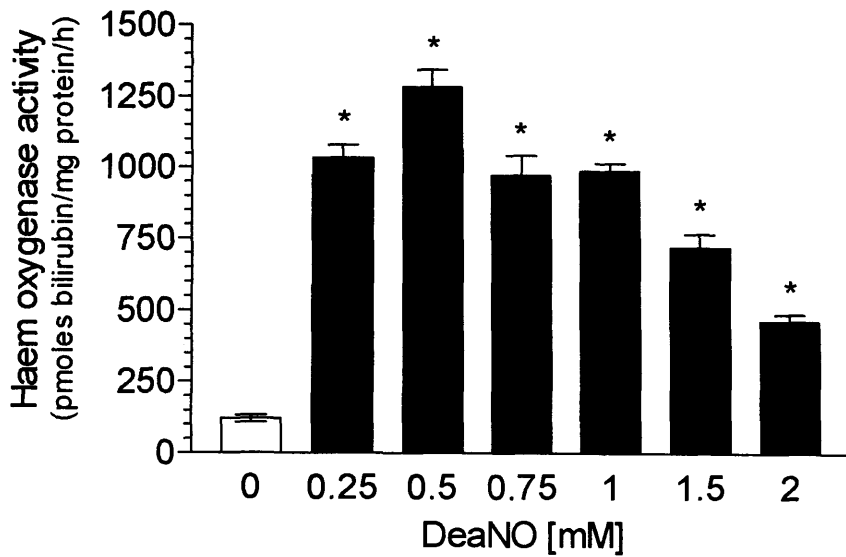
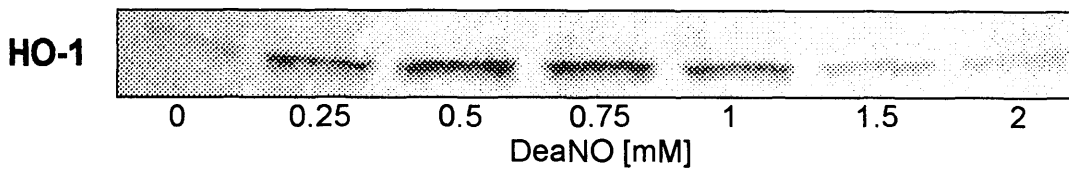
A**B**

Figure 3.8 Concentration-dependent effect of DeaNO on haem oxygenase activity and HO-1 protein expression.

A concentration range of DeaNO (0-2 mM) was applied to cells in fresh complete DMEM for 6 h. Haem oxygenase activity (**A**) and Western blot (**B**) assays were performed as described in Sections 2.3.4 and 2.4.2, respectively. Control (0 mM DeaNO) represents cells treated with fresh DMEM alone. The Western blot panel is representative of one experiment. Bars represent the mean \pm S.E.M. of $n = 3-4$ experiments. * $P < 0.05$ vs. 0 mM DeaNO.

3.4.6 Effects of nitric oxide (NO) and nitroxyl anion (NO⁻) on cell metabolism

The procedures detailed so far have focused on the modulation of haem oxygenase activity and HO-1 protein expression in H9c2 cardiomyocytes by NO⁻ (AS) and NO (DeaNO and DetaNO). The potential cytotoxic effect of these compounds was tested in a concentration range (0-2 mM) using the Alamar blue cell metabolism assay. Following a 6 h exposure to agents that liberate NO⁻ or donate NO and a subsequent 5 h incubation with the Alamar blue reagent, each agent had a different effect upon cell metabolism (Figure 3.9). When AS was applied to cells, cell metabolism was maintained at approximately 100% across the concentration range. After DetaNO was applied, there was an overall reduction in cell metabolism by 20%, while DeaNO promoted a significant (40%; $P < 0.05$) concentration-dependent reduction in cell metabolism. These results suggest that the H9c2s show different levels of tolerance to the agents chosen. Of the three, AS is the most acceptable followed by DetaNO with DeaNO being the least tolerable.

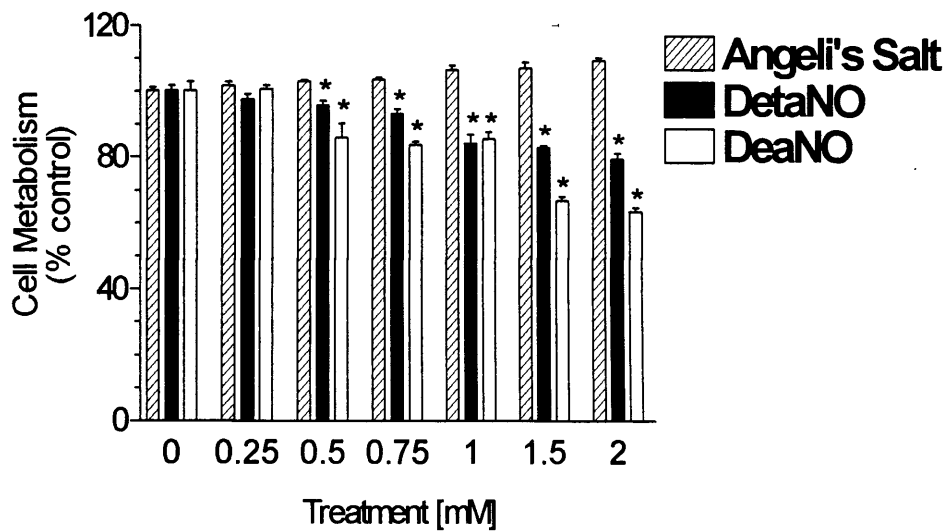


Figure 3.9 Effect of NO and NO⁻ on cell metabolism.

Cardiomyocytes were treated with similar concentrations (0-2 mM) of AS (hatched bars), DetaNO (filled bars) and DeaNO (empty bars) for 6 h in fresh complete media. Cell metabolism, which was determined according to Section 2.2.4, was expressed as a percentage of the control (assigned 100%). Control (0 mM agent) represents cells treated with fresh DMEM alone. Bars represent the mean \pm S.E.M. of $n = 6$ experiments. * $P < 0.05$ vs. 0 mM agent.

3.5 Discussion

For the first time this study demonstrates that a commercially available agent that liberates NO⁻, AS, can induce haem oxygenase activity, HO-1 protein and HO-1 mRNA expression in cardiac cells in a time- and concentration-dependent manner. Although the exact mechanism of haem oxygenase induction by NO⁻ is unknown, it appears to involve transcriptional activation of the HO-1 gene because increases in both HO-1 mRNA expression and haem oxygenase activity were completely suppressed by the transcriptional inhibitor actinomycin D. It is also plausible that other NO intermediates, e.g. ONOO⁻, may activate the transcription of the HO-1 gene as this powerful oxidant can be generated from NO⁻ at physiological pH^{41,58} and has been shown to increase endothelial HO-1 protein and haem oxygenase activity *in vitro*^{482,668}. At this stage it is not known what transcription factor(s) are responsible for upregulating the expression of the HO-1 gene and protein in response to nitrosative reactions; however, Chapter 5 will explore this topic in more detail.

It is interesting to note that multiple additions of low (50 or 100 μM) concentrations of AS over a 6 h period was sufficient to induce a high level of haem oxygenase activity. This suggests that the short half-life of AS ($t_{1/2} = 2.3$ min) is a limiting factor in HO-1 protein induction and by simulating sustained NO⁻ production, as would be the case under physiological/pathophysiological conditions, it is possible to preserve the signalling mechanisms involved in AS-mediated haem oxygenase activation. The latter observation is further supported by the experiments carried out in different vehicles, as the level of

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haem oxygenase activity is higher when freshly prepared AS was applied directly to cells in DMEM and DPBS, compared with decomposed AS in the same vehicles. The difference in the activity levels between DMEM, which contains a variety of nutrients, and DPBS, which comprises mainly water and Mg^{2+} and Ca^{2+} ions, also suggests that NO^- can directly stimulate the enzyme system, but the possibility that constituents of the media may oxidise NO^- to NO, and eventually other RNS, thus stimulating haem oxygenase activity, cannot be excluded.

It is known that various cell types respond to nitrosative stress induced by NO via the activation of haem oxygenase ^{427,481,669}. Using specific NO releasing compounds, which release NO at different rates, a comparison was made with NO^- in terms of the degree and duration of haem oxygenase induction and toxicological effects exhibited by the cardiomyocytes in response to different forms of nitrosative stress. The data reveal that DetaNO, which releases NO at a slow rate ($t_{1/2} = 20$ h), was less effective than DeaNO, which releases NO at a faster rate ($t_{1/2} = 2$ min), at stimulating an early increase in HO-1 protein expression and haem oxygenase activity. When using DeaNO maximal haem oxygenase activity was obtained with a concentration of 0.5 mM after 6 h which is rather similar to that observed with AS (0.75 mM). Chronic exposure of the cardiomyocytes to low fluxes of NO from DetaNO for a prolonged period of time was manifested by the preservation of HO-1 induction and haem oxygenase activity at maximal levels over the duration of exposure. In contrast, exposure to a high concentration of DeaNO (2 mM) for 6 h did not result in a marked increase in HO-1 protein and haem oxygenase activity, but

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an increase of cytotoxicity which was manifested by a decline in the level of cell metabolism. The data from the NO releasers is in line with previous findings which showed that compared to treatment with slow NO releasers, a burst of NO considerably extends the half-life of HO-1 mRNA, suggesting that translation-independent mRNA stability could be an important mechanism by which cells sense NO challenge ⁶⁷⁰.

Notably, the present data would indicate that NO^- is less harmful than NO since exposure of cells to AS, which has a similar half-life to DeaNO, did not result in any detectable cytotoxicity over a range of concentrations (0-2 mM). Although the current findings contrast with previous studies showing significant cytotoxic activity for NO^- ³⁵², it must be pointed out that the latter studies employed much higher concentrations of AS (2-5 mM) and cellular damage was only aggravated when AS was combined with hydrogen peroxide ⁵⁷. In agreement with previous data using NO donors, the NO^- -mediated induction of HO-1 expression is transient and gradually disappears once the spontaneous generation of NO^- ceases. These observations about NO^- and its interaction with the haem oxygenase pathway may have important implications in the design of agents that are capable of amplifying the induction of antinitrosative systems without causing a major threat to the cellular components.

3.6 Conclusion

This chapter demonstrates that NO^- , the one electron reduction product of NO, stimulates cardiac HO-1 protein and mRNA expression as well as haem oxygenase activity. Decomposition leads to a loss of potency towards inducing haem oxygenase activity suggesting that NO^- liberated by freshly prepared AS promotes HO-1 protein induction. This upregulation occurs via transcriptional activation as actinomycin D completely abolished the responsiveness of the HO-1 gene towards AS. Although it is not possible to say whether the concentrations of AS employed in these current experiments are biologically relevant because sensitive methodologies for the measurement of NO^- generated *in vivo* still need to be developed, Chapter 4 provides information from *in vitro* experiments to support the notion that NO^- is the chemical entity responsible for the induction of HO-1.

4 DISSECTING THE CONTRIBUTION OF NITROXYL ANION AND NITRIC OXIDE TO ANGELI'S SALT-MEDIATED INDUCTION OF HAEM OXYGENASE ACTIVITY

4.1 Introduction

Following the discovery that NO mediates vasodilatation a number of important biological functions have been purported for this gaseous molecule in the immune, nervous and reproductive systems⁵⁹. In response to increased Ca^{2+} or immunogenic stimuli a group of enzymes known as NOSs catalyse the formation of NO from the amino acid L-arginine⁷⁸. The NO molecule can bind with sGC to promote the conversion of GTP to cGMP, the latter mediates the biological effects of NO¹⁴⁹. Recently a number of reports have suggested that NO⁻ is the active product of NOS metabolism^{43,671}, although this issue remains controversial⁴⁵. At the same time interest in the biochemistry of NO⁻ and its role in modulating specific biological reactions has expanded^{25,46}. Due to its unstable and short lived nature the fate of NO⁻ within the cell remains elusive. However, reaction with thiols, molecular oxygen and oxidants, can generate other types of RNS and thus modulation of specific cellular targets^{30,58,672}. Although the mechanism(s) behind these effects is still under scrutiny we can say that, based upon the data in Chapter 3 NO⁻ activates HO-1. The biochemical mechanisms leading to this induction will be explored further in this chapter.

DISSECTING THE MECHANISM OF HO-1 INDUCTION

4.2 Objective

This chapter aims to dissect the contribution of NO^- and NO in increasing haem oxygenase activity and HO-1 protein expression in rat H9c2 cardiomyocytes following their exposure to AS and DeaNO. In addition, the quantity of NO released by the latter agents, as well as DetaNO, will be assessed in vehicle (DMEM or DPBS) using a sensitive NO electrode.

4.3 Materials and Methods

4.3.1 Preparation of reagents

Fifty millimolar (50 mM) stock solutions of the NO^- liberator AS, the NO donors (DeaNO and DetaNO) and the glutathione synthesis inhibitor buthionine-sulfoximine (BSO) were prepared in 0.01 M NaOH. Nitrite (NO_2^-) was prepared as a 50 mM stock solution in dH_2O . A 100 mM stock solution of *N*-acetylcysteine (NAC) was prepared with 5% (v/v) 2 M NaOH. Stock solutions (50 mM) of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (potassium salt) (C-PTIO) and hydroxocobalamin (HCB) were dissolved in ethanol and dH_2O , respectively. A 10 mM stock solution of copper sulphate (CuSO_4) was prepared with sterile PBS (10X). All of the agents were prepared fresh at the time of each experiment in opaque Eppendorfs, or a 15 ml tube wrapped in foil for CuSO_4 , and placed on ice until required.

4.3.2 Maintenance and treatment of cells

Cardiomyocytes were grown to confluence in flasks as described in Section 2.2.2. The reagents (AS, DeaNO or DetaNO) were applied to cells directly or

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following their preincubation (see Section 2.2.3 for methodology) in a specific vehicle (fresh complete DMEM or DPBS, modified with Ca^{2+} and Mg^{2+}) for the times and concentrations specified in each graph. When AS was coincubated with CuSO_4 or NAC the agents were preincubated with cells for 15 min in vehicle (DPBS or DMEM) followed by DMEM for the remaining duration. In the case of BSO, the agent was applied to cells for 6 h in DMEM followed by AS for a further 6 h in fresh complete media. At the end of each experiment cells were collected for analysis of haem oxygenase activity or HO-1 protein expression as described in Sections 2.3.4 and 2.4.2, respectively. Negative controls, which consisted of vehicle alone, were included in all experiments.

4.3.3 NO electrode experiments

The quantity of NO released by AS and the NO donors, DeaNO and DetaNO, was examined in fresh complete DMEM or DPBS, final volume 1 ml, using a NO-sensitive electrode under constant stirring at 37°C as described in Section 2.5.2. Controls consisted of vehicle alone.

4.4 Results

4.4.1 Modulation of the haem oxygenase-inducing potential of Angeli's salt (AS) by copper sulphate and a nitric oxide and nitroxyl anion scavenger

It has been shown that copper ions (Cu^{2+}) can oxidise NO^- derived from AS to NO ^{62,63}. Copper ions can also lead to the induction of haem oxygenase ⁶⁷³. Cardiomyocytes were exposed to various concentrations of copper sulfate (CuSO_4 , 10, 50, 100 or 200 μM) in DMEM to establish its effect on haem oxygenase activity and to choose an optimal concentration for further experiments. Figure 4.1 illustrates that CuSO_4 produced a concentration-dependent increase in haem oxygenase activity, with maximal levels being observed at 100 μM CuSO_4 (296.1 ± 36.1 (control) vs. 1627 ± 123.2 (100 μM) pmoles bilirubin/mg protein/h; $P < 0.001$). There was no significant increase in haem oxygenase activity if cells were incubated with 10 μM CuSO_4 (486.1 ± 38.58 pmoles bilirubin/mg protein/h; $P > 0.05$). Therefore, 10 μM CuSO_4 was chosen to examine its effect on haem oxygenase activity in the presence or absence of AS.

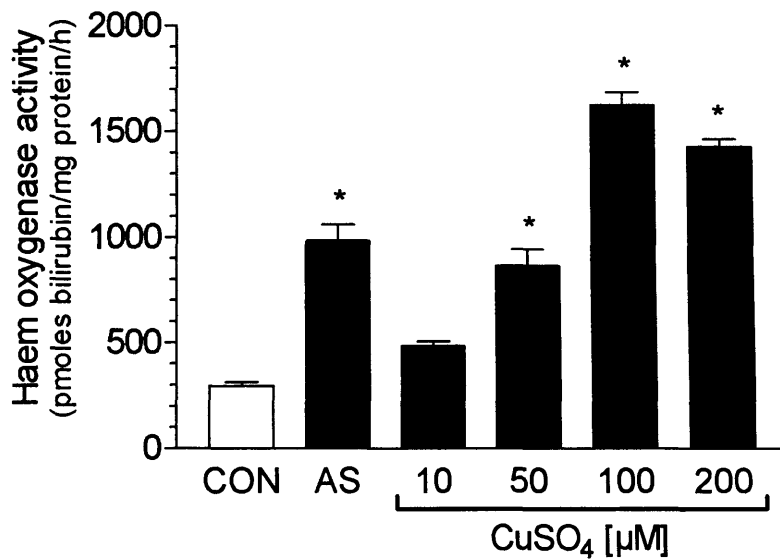


Figure 4.1 Concentration-dependent effect of copper sulfate on haem oxygenase activity.

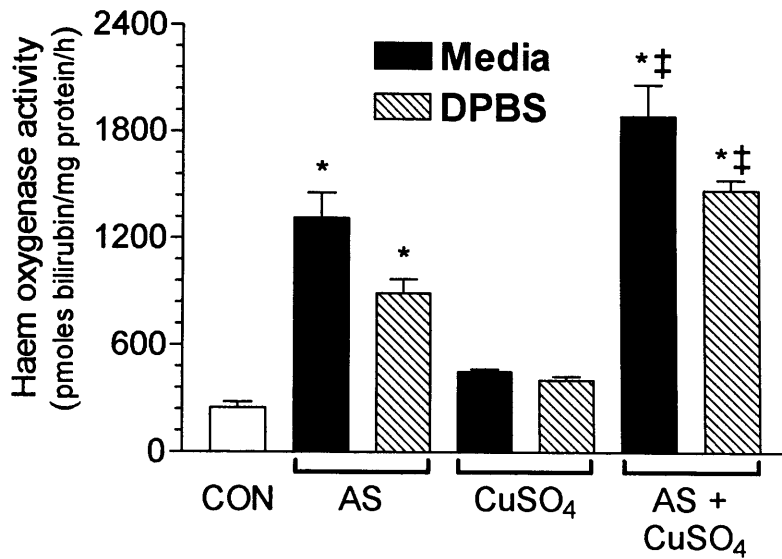
Cells were treated with copper sulfate (CuSO₄, 10, 50, 100 or 200 μM) in fresh complete DMEM for 6 h. Experiments with Angeli's salt (AS, 0.75 mM) are shown for comparison. Haem oxygenase activity was determined as described in Section 2.3.4. Control (CON) represents cells treated with fresh DMEM alone. Bars represent the mean ± S.E.M. of $n = 4$ experiments. * $P < 0.05$ vs. CON.

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In a second set of experiments (Figure 4.2 A) AS (0.75 mM) was incubated with or without CuSO₄ (10 µM) in DMEM or DPBS for 15 min followed by 6 h in fresh DMEM. As in the previous experiments, incubation of AS in DMEM gave rise to a greater level of haem oxygenase induction compared with DPBS (1260 ± 142.2 vs. 907.1 ± 78.46 pmoles bilirubin/mg protein/h, respectively). Once again, this particular experiment shows that AS on its own can promote haem oxygenase induction in both vehicles. When CuSO₄ was added in combination with AS there was a potentiation in haem oxygenase activity in both vehicles, the effect being more pronounced in DMEM (1802 ± 179.2 pmoles bilirubin/mg protein/h; *P* < 0.05). Western blot analysis (Figure 4.2 B; *n* = 1) of cells treated with a range of concentrations of CuSO₄ (10, 50, 100 or 200 µM) in the absence or presence of AS (0.75 mM) revealed that individually both agents were capable of eliciting an increase in HO-1 protein expression at the different concentrations examined. In combination a synergistic interaction between the two agents brought about an augmentation in the intensity of HO-1 protein expression at all concentrations of CuSO₄ used. These observations indicate that AS and CuSO₄ have the ability to act synergistically to promote an induction of haem oxygenase activity and HO-1 protein expression in cardiomyocytes.

DISSECTING THE MECHANISM OF HO-1 INDUCTION

A



B

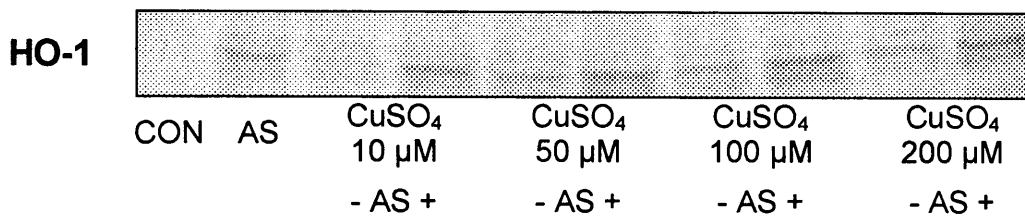


Figure 4.2 Synergistic influence of copper sulphate and Angeli's salt on haem oxygenase activity and HO-1 protein expression.

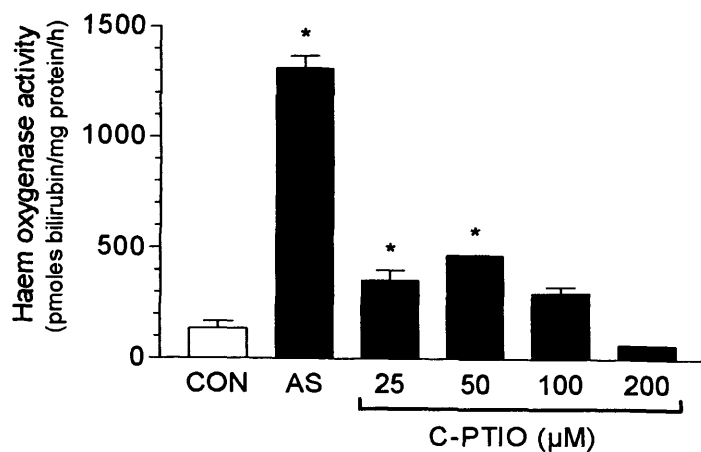
Cardiomyocytes were treated with Angeli's salt (AS, 0.75 mM) in the absence (-) or presence (+) of 10 μM copper sulphate (CuSO₄) in fresh complete DMEM (filled bars) or DPBS (hatched). Haem oxygenase activity (**A**) was determined after 6 h incubation according to Section 2.3.4. Cells were treated with CuSO₄ (10, 50, 100 or 200 μM) in the absence (-) or presence (+) of AS (0.75 mM) in fresh DMEM. After 6 h a Western blot assay for HO-1 protein (**B**) was performed as described in Section 2.4.2. Control (CON) represents cells exposed to fresh DMEM alone. The Western blot panel is representative of one experiment. Bars represent the mean ± S.E.M. of *n* = 4 experiments. * *P* < 0.05 vs. CON. ‡ *P* < 0.05 vs. AS.

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To support the hypothesis that NO^- induces haem oxygenase activity and HO-1 protein expression, a set of experiments were performed to test the possibility of scavenging NO, which could be derived from the redox conversion of NO^- , using the NO scavengers C-PTIO and HCB. Cardiomyocytes were challenged with AS (0.75 mM) in the absence or presence of C-PTIO (25 μM) in fresh complete media. The latter concentration was chosen as a result of a preliminary set of experiments (Figure 4.3 A) showing this concentration was the most suitable in terms of haem oxygenase induction and potential toxicity. Exposure to AS promoted a 5.5-fold increase in haem oxygenase activity compared with the control group (100%) (Figure 4.3 B), whereas C-PTIO had an insignificant ($P > 0.05$) effect. Combining both agents caused a significant (6-fold; $P < 0.05$) increase in activity similar to that produced by AS alone. Although C-PTIO is a known scavenger of NO⁶⁹ it had no significant attenuating effect upon AS-induced haem oxygenase induction. This suggests that C-PTIO is unable to attenuate the effects of NO^- and that any NO eventually formed does not contribute to AS-mediated haem oxygenase activation. The reason for the significant stimulation of haem oxygenase activity by CPTIO (25 and 50 μM) observed in Figure 4.3 A could be explained by the high sensitivity of the HO-1 enzyme to any external stimuli that creates an imbalance in the cellular redox state. However, it has been noted that C-PTIO itself promotes a weak but measurable increase in HO-1 mRNA expression⁶⁷⁰ supporting the observations in Figure 4.3 A.

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A



B

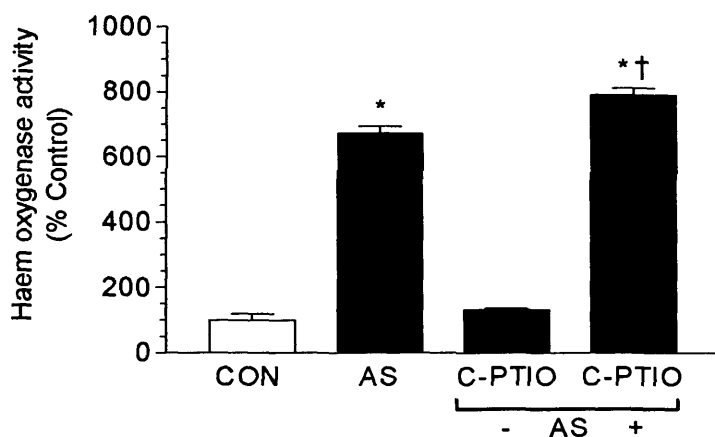


Figure 4.3 Effect of carboxy-PTIO and Angeli's salt on haem oxygenase activity.

Cells were treated with carboxy-PTIO (C-PTIO, 25, 50, 100 or 200 μM) in fresh complete DMEM for 6 h. The effect of Angeli's salt (AS, 0.75 mM) is shown for comparison (**A**). A 0.75 mM concentration of AS was applied to cells in the absence (-) or presence (+) of C-PTIO (25 μM) (**B**) in fresh DMEM for 6 h. Haem oxygenase activity (assayed as described in Section 2.3.4) was expressed as a percentage of the control (CON), i.e. cells treated with fresh DMEM alone. Bars represent the mean ± S.E.M. of n = 3-4 experiments. * $P < 0.05$ vs. CON. † $P < 0.05$ vs. AS.

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When HCB (0.75 mM), a scavenger of both NO and NO⁻ ⁶⁹, was incubated in the absence or presence of a corresponding concentration of AS, striking changes in the level of haem oxygenase were observed (Figure 4.4). On its own AS promoted a significant increase in activity compared with control (1358 ± 50.79 vs. 243.2 ± 36.4 pmoles bilirubin/mg protein/h, respectively; $P < 0.05$) in contrast to HCB which was similar to baseline. Coincubation of HCB with AS caused a significant decrease in haem oxygenase activity levels (597.4 ± 45.83 pmoles bilirubin/mg protein/h; $P < 0.05$). Possible reasons for this observation include the ability of HCB to scavenge both NO and NO⁻, also HCB may enhance the redox conversion of NO⁻ to NO thus enabling scavenging to occur more effectively ⁶⁹. Taken together these two sets of data, using CuSO₄ and HCB, add further weight towards the hypothesis that NO⁻ liberated from AS induces haem oxygenase in cardiomyocytes by a mechanism that possibly involves redox conversion of NO⁻ to NO.

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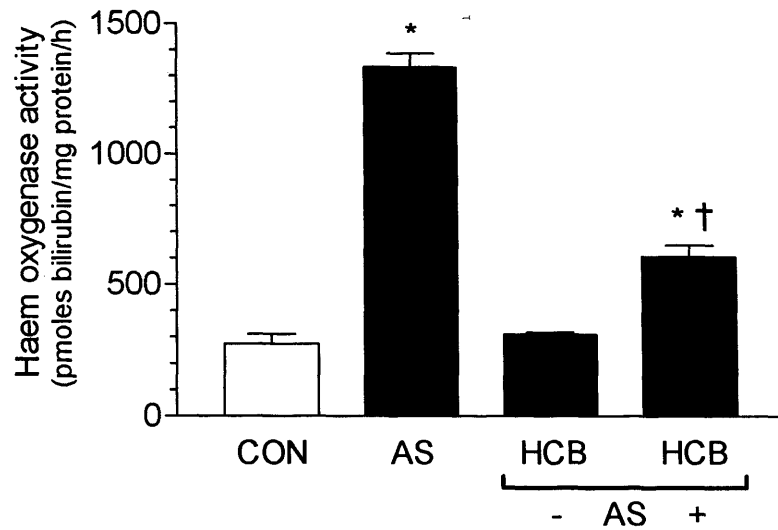


Figure 4.4 Attenuation of Angeli's salt-mediated induction of haem oxygenase activity by hydroxocobalamin.

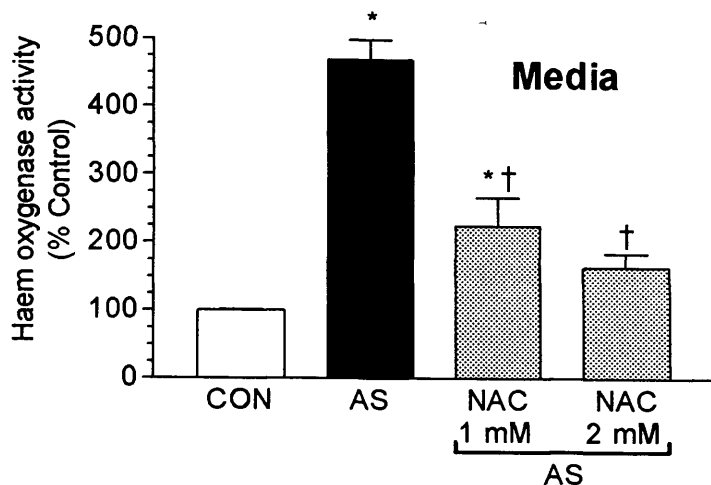
Angeli's salt (AS, 0.75 mM) was applied to cells in the absence (-) or presence (+) of hydroxocobalamin (HCB, 0.75 mM) in fresh complete DMEM for 6 h. Haem oxygenase activity was assayed as described in Section 2.3.4. Control (CON) represents cells treated with fresh DMEM alone. Bars represent the mean \pm S.E.M. of $n = 3-4$ experiments. * $P < 0.05$ vs. CON. † $P < 0.05$ vs. AS.

4.4.2 Interaction of Angeli's salt (AS) with thiols: effect on haem oxygenase activity and HO-1 protein expression

It has been shown that thiols, in particular the glutathione precursor *N*-acetylcysteine (NAC), have the potential to modulate haem oxygenase activity in different cell types under 'stressful' conditions^{423,668}. With this in mind, cardiomyocytes were treated with AS (0.75 mM) in the absence or presence of NAC (1 or 2 mM) in either fresh complete media or DPBS. After 6 h cells were collected and analysed for haem oxygenase activity and HO-1 protein expression. The data shows that in DMEM AS alone increased haem oxygenase activity by ~4-fold with respect to the control group (100%); however, the addition of NAC (1 or 2 mM) significantly ($P < 0.05$) attenuates the upregulating potential of AS 3- and 3.5-fold, respectively (Figure 4.5 A). A similar pattern of results was obtained for AS with and without NAC when DPBS was the vehicle (Figure 4.5 B). Of the two vehicles DMEM promoted a stronger (2-fold) enhancement of haem oxygenase activity in response to AS compared with a similar treatment in DPBS. In addition, NAC (2 mM) attenuated AS-mediated activity more effectively than 1 mM NAC in media compared with a similar, but still significant ($P < 0.05$), scenario in DPBS.

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A



B

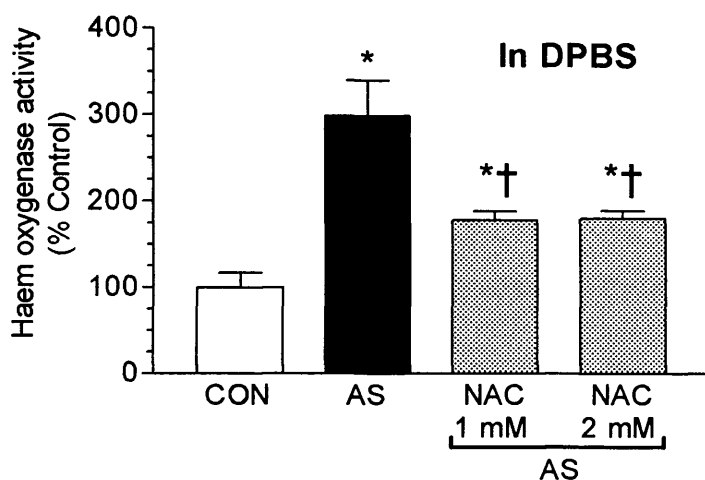


Figure 4.5 Attenuation of haem oxygenase activity following interaction of Angeli's salt with thiols.

Cells were exposed to Angeli's salt (AS, 0.75 mM) in the absence (black bars) or presence (grey bars) of *N*-acetylcysteine (NAC, 1 or 2 mM) either directly in DMEM (**A**) or after its decomposition (see Section 2.2.3 for preparation) in DPBS, followed by DMEM (**B**). After 6 h haem oxygenase activity was assessed according to Section 2.3.4. The activity was expressed as a percentage of control (CON) (assigned 100%), where control represents cells treated with fresh DMEM or DPBS alone. Bars represent the mean \pm S.E.M. of $n = 4$ experiments. * $P < 0.05$ vs. CON. † $P < 0.05$ vs. AS.

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The ability of NAC to prevent the induction of HO-1 protein by AS is illustrated in Figure 4.6 ($n = 1$). When cardiomyocytes were incubated with AS (0.75, 1 or 2 mM) in DMEM a strong HO-1 protein signal was observed at each concentration used. However, when equal concentrations of NAC and AS were coincubated the intensity of the HO-1 bands were substantially decreased. These effects upon HO-1 protein were due to the interaction of NAC with AS as NAC on its own was unable to induce HO-1. These three sets of data (Figure 4.5 and Figure 4.6) demonstrate that in this model exogenous thiols have the capacity to subvert the haem oxygenase inducing potential of NO^- released from AS.

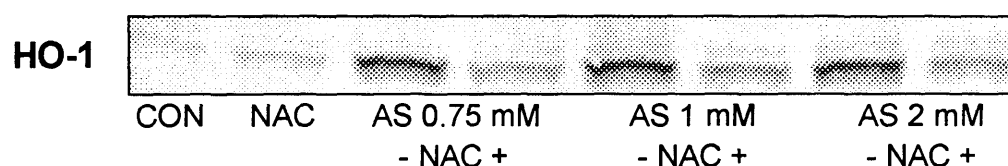


Figure 4.6 Attenuation of HO-1 protein expression following interaction of Angeli's salt with thiols.

Cells were treated with Angeli's salt (AS; 0.75, 1 or 2 mM) in the absence (-) or presence (+) of an equal concentration of *N*-acetylcysteine (NAC) for 6 h in fresh complete DMEM. Western blot detection of HO-1 protein was performed as described in Section 2.4.2. Control (CON) and NAC represent cells exposed to media or media containing 2 mM NAC, respectively. The Western blot panel is representative of one experiment.

The latter set of results illustrate the crucial role thiols play in attenuating the activation of haem oxygenase by AS. To further illustrate the importance of thiols in preserving the bioactivity and signal transducing properties of NO^- , an

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experiment using the glutathione synthesis inhibitor buthionine-sulfoximine (BSO) was performed. Following a 6 h preincubation with BSO (0.5 or 1 mM), which was a sufficient time to deplete cellular glutathione levels, AS (0.75 mM) was applied for a further 6 h in fresh DMEM. Measurement of haem oxygenase activity (Figure 4.7) revealed that BSO on its own did not affect haem oxygenase activity, unlike AS alone, whereas coincubation with AS significantly ($P < 0.05$) decreased the activation of haem oxygenase.

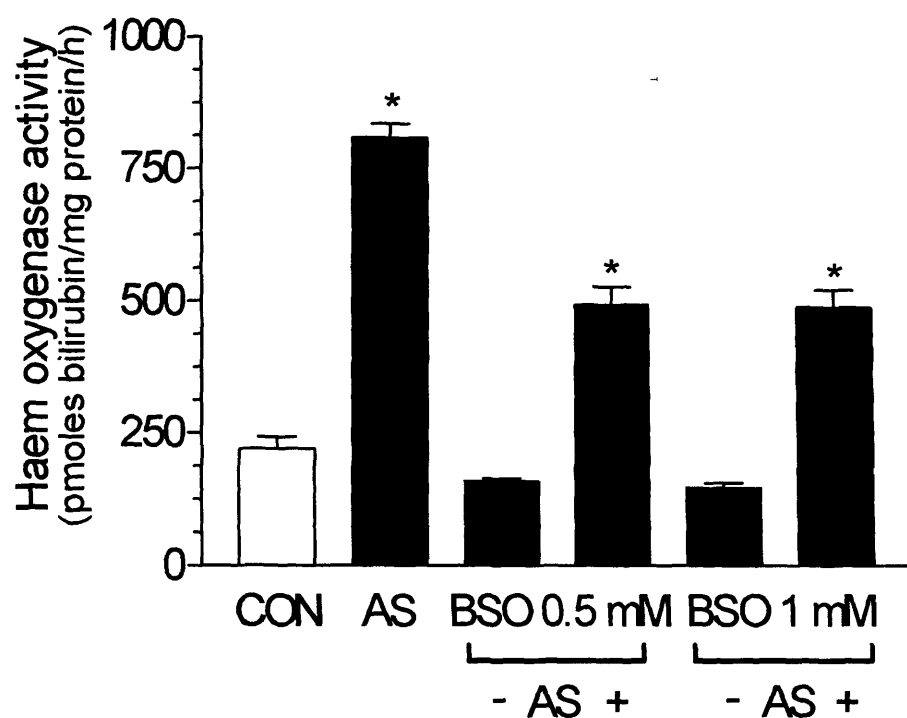


Figure 4.7 Attenuation of Angeli's salt-mediated induction of haem oxygenase activity by the glutathione synthesis inhibitor buthionine-sulfoximine.

Cells were exposed to the glutathione synthesis inhibitor buthionine-sulfoximine (BSO, 0.5 or 1 mM) in fresh complete DMEM for 6 h. Next, Angeli's salt (AS; 0.75 mM) was added to the BSO treated cells, and on its own, for a further 6 h in fresh DMEM. After 6 h haem oxygenase activity was assessed according to Section 2.3.4. Control (CON) represents cells exposed to media alone, $n = 4$ experiments, * $P < 0.05$ vs. CON.

4.4.3 Dissecting the NO redox forms responsible for Angeli's salt (AS)-mediated induction of haem oxygenase-1

It is known that NO can readily interconvert between its different redox-activated forms within biological systems¹³. In subsection 3.4.4 it was noted that the haem oxygenase inducing capacity of AS differs according to its biological state, i.e. freshly prepared or decomposed agent, and surrounding environment (fresh complete DMEM or DPBS). So far the data reveal that combining AS with HCB and thiols leads to an attenuation of AS-mediated induction of haem oxygenase activity, whereas C-PTIO and CuSO₄ promotes an increase. To further probe the biochemistry of these reactions measurements of NO release from equal concentrations (0.5 mM or 20 μM) of AS and two commonly used NO releasing agents, i.e. DeaNO and DetaNO, were made in fresh complete DMEM or DPBS using a Clark-type NO electrode.

The table in Figure 4.8 reveals a number of biochemical details about AS. Firstly, 0.5 mM of AS in DMEM generates much less NO ($0.31 \pm 0.01 \mu\text{M} \times \text{s}$) compared with DeaNO ($10.1 \pm 0.78 \mu\text{M} \times \text{s}$, $P < 0.05$) but significantly more than DetaNO ($0.03 \pm 0.01 \mu\text{M} \times \text{s}$, $P < 0.05$). Secondly, the amount of NO generated from AS in culture media ($0.31 \pm 0.01 \mu\text{M} \times \text{s}$) is significantly ($P < 0.05$) higher than the quantity measured in DPBS ($0.078 \pm 0.01 \mu\text{M} \times \text{s}$), thus indicating that conversion of NO⁻ to NO is indeed a feasible process within the cell culture environment. Lastly, a concentration of 20 μM DeaNO releases the same amount of NO over time as 0.5 mM AS in the cell culture medium

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($0.32 \pm 0.01 \mu\text{M} \times \text{s}$ and $0.31 \pm 0.01 \mu\text{M} \times \text{s}$, respectively). When these two concentrations, 0.5 mM AS and 20 μM DeaNO, were applied directly to cells in culture for 6 h the level of haem oxygenase activity for DeaNO was comparable to control levels (300.7 ± 29.02 vs. 324.7 ± 49.7 pmoles bilirubin/mg protein/h, respectively), whereas AS caused a significant ($P < 0.05$) increase in activity (see Figure 4.8). Taken together, this set of data confirms that NO^- liberated from AS can directly stimulate the haem oxygenase pathway and suggest that the extent and rate of NO^- oxidation to NO augments the contribution of NO to stimulating the haem oxygenase pathway.

Treatment	NO release (AUC, $\mu\text{M} \times \text{sec}$)	
	DPBS	DMEM
AS (0.5 mM)	0.078 ± 0.1	$0.31 \pm 0.01^*$
DeaNO (0.5 mM)	21.7 ± 0.65	$10.1 \pm 0.78^{\dagger}$
DetaNO (0.5 mM)	0.079 ± 0.02	$0.03 \pm 0.01^{\dagger}$
DeaNO (20 μM)	0.60 ± 0.02	$0.32 \pm 0.01^*$

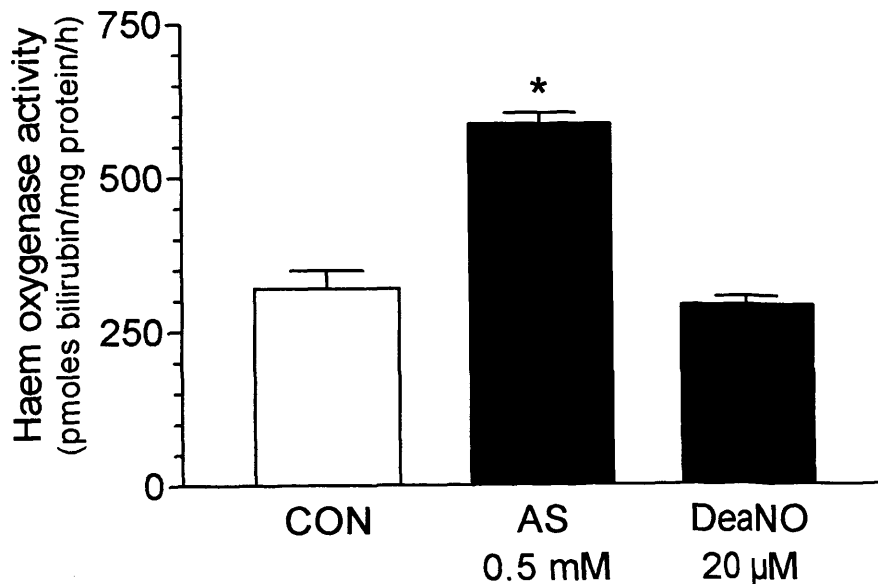


Figure 4.8 Direct involvement of Angeli's salt-derived NO⁻ in increasing haem oxygenase activity.

The Table inserted in this figure reports data on the amount of NO released over time from various agents measured amperometrically using a NO-sensitive electrode (see Section 2.5.2). Each value represents the mean (\pm S.E.M.) of 4 experiments performed independently. * $P < 0.05$ vs. DPBS; $\dagger P < 0.05$ vs. AS. The figure reports the effect of Angeli's salt (AS, 0.5 mM) or DeaNO (20 μM) on H9c2 haem oxygenase activity (see Section 2.3.4 for method) after 6 h in DMEM. Control (CON) represents cells treated with complete DMEM alone. Each bar represents the mean (\pm S.E.M.) of 5-6 experiments performed independently. * $P < 0.05$ vs. control (CON).

4.4.4 Nitrite (NO_2^-) liberated from the metabolism of Angeli's salt (AS) does not affect haem oxygenase activity

Ever since Angeli's salt (AS, $\text{Na}_2\text{N}_2\text{O}_3$) was first identified in 1903 there have been numerous studies into its chemistry, reactivity and decomposition in various solutions and under different conditions^{38,281,672}. Under neutral or mildly alkaline conditions AS rapidly breaks down according to first-order kinetics to liberate the intermediates nitrosyl hydride (HNO , commonly referred to as NO^-) and NO_2^- . The former can undergo dimerization and dehydration to form N_2O as the final product⁶⁷⁴. To eliminate the possibility of NO_2^- having an effect on the reactions involving AS similar concentrations (0.75 mM) of these agents were added to cells in DMEM for 6 h. Figure 4.9 reveals that AS promotes a significant ($P < 0.05$) enhancement of haem oxygenase activity (1413 ± 60.41 (AS) vs. 372.7 ± 49.22 (control) pmoles bilirubin/mg protein/h), whereas NO_2^- did not have any effect upon this parameter ($P > 0.05$). Therefore, this data suggests that NO^- liberated from AS is the principal factor responsible for the results observed.

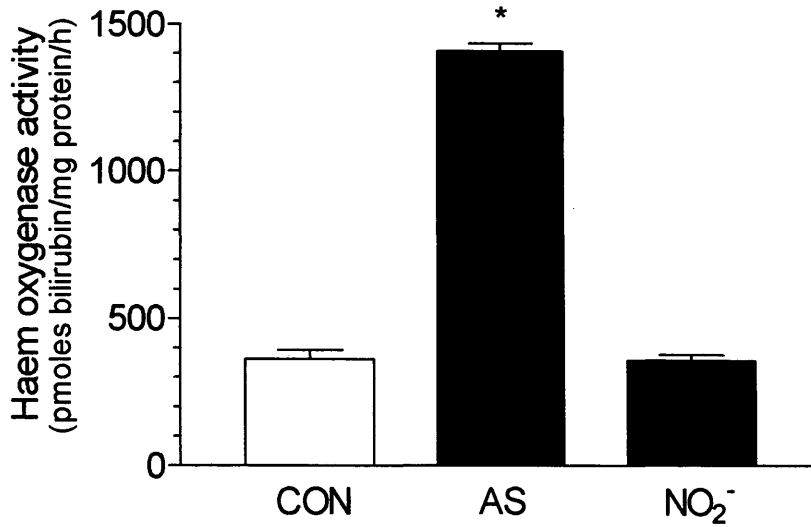


Figure 4.9 Nitrite does not influence haem oxygenase activity.

Cells were treated with equal concentrations (0.75 mM) of Angeli's salt (AS) and nitrite (NO₂⁻) in fresh complete DMEM. After 6 h haem oxygenase activity was assessed according to Section 2.3.4. Control (CON) represents cells exposed to media alone, $n = 4$ experiments, * $P < 0.05$ vs. CON.

4.5 Discussion

Having established in Chapter 3 that NO^- liberated from AS induces haem oxygenase activity, HO-1 protein and gene expression, this chapter further examined the chemistry of these responses. It is known that the composition of the cellular milieu and the surrounding environment can influence the type and rate of NO congener formation¹³. Also, oxidants, e.g. superoxide dismutase and copper ions, can bring about the oxidation of NO^- to NO^{48,62,63}. With this in mind, a series of experiments were conducted to alter the microenvironment towards the conversion of NO^- to NO and also to examine the influence of NO^- on haem oxygenase activity and the HO-1 protein. Incubation of a low (10 μM) concentration of CuSO_4 in the presence of AS in different vehicles, DMEM and DPBS, elicited a synergistic increase in haem oxygenase activity in both vehicles, particularly so in DMEM (1802 ± 179.2 pmoles bilirubin/mg protein/h; $P < 0.05$). The possibility that CuSO_4 was exerting an influence *per se* was excluded as the activity levels remained similar to control in both vehicles using CuSO_4 alone. The synergistic capacity of both agents was repeated when increasing concentrations of CuSO_4 were combined with a fixed concentration of AS, thus augmenting the intensity of HO-1 protein expression in cardiomyocytes. However, at high concentrations (50-100 μM) CuSO_4 *per se* was able to stimulate haem oxygenase activity. This set of data suggests that the effects of NO^- appear to be independent of NO but can be amplified by accelerating the rate of NO^- oxidation to NO. In parallel with the previous results showing a greater degree of haem oxygenase activity in DMEM compared with DPBS, one cannot discount the

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possibility of media components exerting an influence.

The results from the latter experiment suggest that the oxidation of NO^- to NO leads to an elevation in haem oxygenase activity. To fully verify the hypothesis that AS-derived NO^- induces haem oxygenase activity and HO-1 gene expression a set of experiments were performed to test the feasibility of scavenging the NO eventually generated from AS using the NO scavenger C-PTIO and HCB, a scavenger of both NO and NO^- ^{69,675}. Thus, in the presence of a NO scavenger NO^- should represent the principal mediator of haem oxygenase induction. When the cardiomyocytes were treated with AS and C-PTIO there was no significant increase in haem oxygenase activity compared with that generated by AS alone. Possible reasons for this result could be: (a) the concentration of the agents applied, AS [mM] and C-PTIO [μM], thus AS would subdue the scavenging capabilities of C-PTIO *[Note: we could not use concentrations of C-PTIO greater than 25 μM because toxicity would prevail]; (b) the C-PTIO is unable to attenuate the NO^- derived from AS ⁶⁷⁶; and (c) any NO that is formed does not contribute to AS-mediated haem oxygenase activation. When HCB was used as a NO/ NO^- scavenger in the presence of AS there was a significant reduction in haem oxygenase activity compared with AS alone (1358 ± 50.79 vs. 597.4 ± 45.83 pmoles bilirubin/mg protein/h; $P < 0.05$). Recent publications suggest that HCB may mediate the oxidation of NO^- to NO, by an unknown mechanism, thus facilitating more effective scavenging, a similar reaction could account for the results observed with the cardiomyocytes ^{69,677,678}. Taken together, the data obtained with C-PTIO and HCB substantiate the hypothesis that NO^- induces haem oxygenase

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in cardiomyocytes, at the same time the data suggests a possible mechanism for this induction, i.e. redox conversion of NO^- to NO.

In the context of HO-1 modulation by NO and NO-related species a number of reports have highlighted the protective capacity of the glutathione precursor *N*-acetylcysteine (NAC) ^{423,668}. The experiments using the thiol donor NAC support the notion that NO^- originating from AS metabolism, rather than the agent itself, is the chemical entity responsible for the induction of HO-1 protein and increased haem oxygenase activity. When NAC (1 or 2 mM) was combined with a fixed concentration of AS (0.75 mM) in different vehicles, DMEM and DPBS, a considerable (3-3.5-fold) reduction in haem oxygenase activity was observed using both vehicles, while coincubation of equal concentrations of the two agents, in DMEM, significantly attenuated HO-1 protein expression. These results indicate that NO^- can directly influence haem oxygenase activity and transcriptionally activate the HO-1 gene in addition to the notion that thiols exert a protective influence, possibly through the formation of endogenous R-SNO, in different cell types under 'stressful' conditions through their ability to scavenge harmful molecules ^{26,679}. This idea is substantiated by the observation that BSO, a glutathione synthesis inhibitor, reduced the ability of AS to induce haem oxygenase activity. Thus, endogenous glutathione participates in the preservation of NO^- bioactivity and signalling properties.

To dissect the contribution of NO^- and NO towards the upregulation of haem oxygenase activity experiments were conducted using a sensitive NO electrode and the agents AS, DeaNO and DetaNO. A comparison between

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AS and DeaNO, both of which have similar half-lives ($t_{1/2} = 2.3$ min for AS and $t_{1/2} = 2$ min for DeaNO), but release either NO^- or NO, respectively, revealed that in DMEM, 0.5 mM AS generated the same quantity of NO as 20 μM DeaNO. However, only AS was capable of promoting a significant increase in haem oxygenase activity at these concentrations. The low concentration of NO release from AS in DPBS ($0.078 \pm 0.1 \mu\text{M} \times \text{s}$), which is 4-fold less than that in DMEM, correlates with the results shown in Figure 3.5. The very low concentration of NO released by DeaNO in DMEM ($\sim 0.03 \mu\text{M} \times \text{s}$) can be explained by the long half-life of this agent ($t_{1/2} = 20$ h). To complement the latter experiments the data in Figure 4.9 support the notion that NO^- , rather than other intermediates, e.g. NO_2^- , is the principal factor responsible for activating the haem oxygenase pathway in this model.

4.6 Conclusion

The aim of this chapter was to explore the chemistry of NO^- -mediated induction of haem oxygenase activity and HO-1 protein expression. This study demonstrates that: 1. NO^- can directly influence haem oxygenase activity and HO-1 protein levels; 2. this effect can be enhanced by increasing the rate of NO^- oxidation to NO (or other RNS) or by media components; and 3. thiols are essential to convey the signalling properties of NO^- . Collectively, these data reveal that the haem oxygenase pathway is not only a highly sensitive and effective stratagem for cellular defence against oxidative challenge, but can also be finely modulated by redox reactions involving nitrosative chemistry.

5 INTERACTION BETWEEN NITROXYL ANION OR NITRIC OXIDE AND HEMIN AMPLIFIES HAEM OXYGENASE-1 INDUCTION: A ROLE FOR THE TRANSCRIPTION FACTOR Nrf2

5.1 Introduction

Ferro-(Fe²⁺)protoporphyrin IX (or haem) plays a crucial role in many mammalian functions and tissues ⁴⁴⁸. This highly lipophilic and hydrophobic tetrapyrrole participates as a cofactor in complex biological reactions and acts as a ligand for a wide range of haemoproteins, e.g. haemoglobin, cytochromes, NOS and guanylate cyclase ³⁸⁴. Due to its low solubility and potential oxidative toxicity of the iron within haem mammals have developed a number of mechanisms to transport and contain free haem. Examples of these include the formation of stable complexes between free haem with proteins such as albumin, lipoproteins and haemopexin, to deliver the tetrapyrrole to tissues and distribute it within cells ⁶²⁰. The importance of haem and in turn iron homeostasis is illustrated by the wide range of pathological diseases associated with uncontrolled free haem, e.g. sickle cell disease, renal failure, atherogenesis and the porphyrias ⁶²¹.

The principal mechanism for the metabolism of free haem and containment of its iron molecule is via the enzyme haem oxygenase and the iron storage protein ferritin, respectively ^{479,484}. Basal levels of haem are metabolised by the constitutive isoform of haem oxygenase (HO-2), while excessive amounts

of haem are dealt with by the inducible isoform, HO-1. Both isoforms give rise to CO, bilirubin and Fe^{2+} as the end products of haem metabolism. Thus, haem derived from haemoproteins can serve as both a substrate for haem oxygenase and promote the induction of the HO-1 gene, which in turn leads to increased metabolism of haem ³⁶⁴. The cytoprotective characteristics of haem oxygenase, in particular HO-1, stems from the following observations: 1. the HO-1 gene has stress response elements (StREs), which bear similarity to the recognition sequence for the oxidant sensitive transcription factor Nrf2, within its promoter region ⁴⁶⁵; 2. the products of haem metabolism, CO and bilirubin, have the ability to attenuate the adverse effects of oxidative reactions ⁴⁸⁰; and 3. NO has a very high affinity for haem and other haemoproteins, which in turn can promote HO-1 induction ^{617,630}.

Oxidative stress was identified as one of the first mechanisms responsible for the induction of HO-1 ⁶⁸⁰. Recent evidence suggests that HO-1 can also be induced following exposure to elevated levels of NO, NO^+ and NO^- type agents ^{427,481,681}. Collectively, these observations led to the hypothesis that HO-1 is upregulated in the presence of RNS and oxidative type insults to attenuate such cellular stressors and maintain cellular function ³⁷³. However, a direct interaction between the substrate of haem oxygenase (haem) and NO in stimulating HO-1 induction has not been investigated. This chapter will describe experiments to examine how HO-1 expression is modulated by the prooxidant haem (delivered as hemin (ferri-(Fe^{3+})protoporphyrin IX) its oxidised form) and inducers of nitrosative stress, e.g. the NO^- liberator AS and the NO donor DeaNO. To elucidate the signalling pathway(s) relating to the

antinitrosative capacity of HO-1, the involvement of the redox-sensitive transcription factor Nrf2 was examined in parallel. The potential biological implication of these findings will also be discussed.

5.2 Objective

The aim of this chapter is to verify that induction of HO-1 by the combined effects of the prooxidant haem and different types of RNS is an intrinsic mechanism to alleviate redox imbalances caused by different types of cellular stress. The transcriptional mechanism leading to this induction will also be examined.

5.3 Materials and Methods

5.3.1 Preparation of reagents

Stock solutions of AS, DeaNO, NAC and hemin were prepared as described in Section 2.2.3. All of the agents were prepared fresh for each experiment in opaque Eppendorfs, or a 15 ml tube wrapped in foil for hemin, and placed on ice until required.

5.3.2 Maintenance and treatment of cells

Rat H9c2 cardiomyocytes were cultured in DMEM as previously described⁶⁸¹. The agents were applied to cells directly, or following their preincubation either alone or in combination for 1 h at 37°C, in vehicle (DMEM), at the concentrations shown in each graph. Assessment of haem oxygenase activity, isolation of Nrf2, Western blotting of HO-1 and Nrf2, RT-PCR, or cell metabolism assays were carried out at the end of the experiments as

described in Sections 2.3.4, 2.4.2, 2.4.4, 2.4.1 and 2.2.4, respectively. Negative controls, which consisted of vehicle alone, were included in all experiments.

5.3.3 Oxygen tension experiments

Experiments in this chapter were carried out under normoxic (5% CO₂, pO₂ = 150 mmHg) or hypoxic (5% CO₂ and 95% N₂, pO₂ = 2 mmHg) conditions at 37°C. For the hypoxia experiments cardiomyocytes were incubated with the agents within an air tight chamber. A hypoxic environment was established by infusing the gas at a flow rate of 5 L/min for 2 h followed by 1 L/min for the remaining duration of the experiment. Controls consisted of vehicle alone.

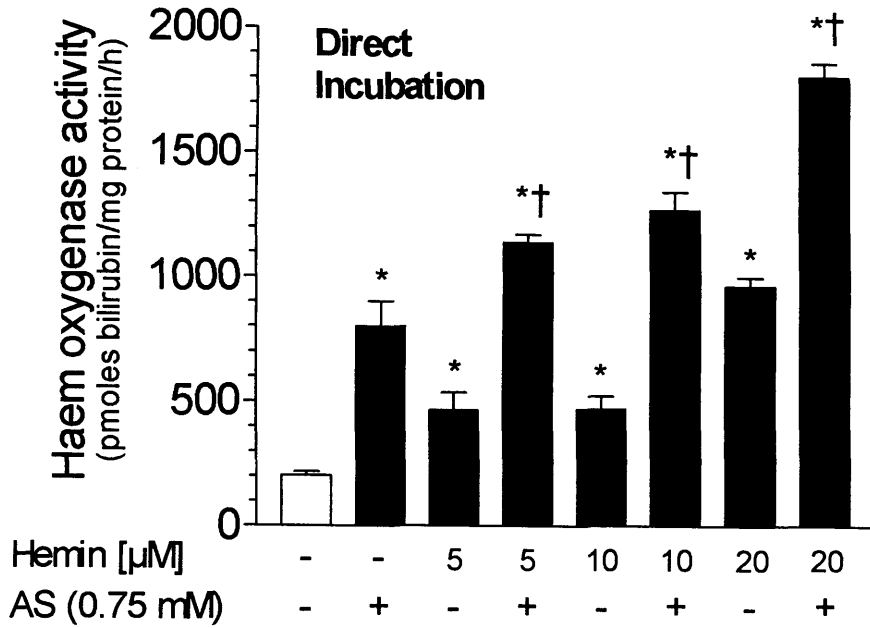
5.4 Results

5.4.1 Synergism between Angeli's salt (AS) and hemin increases haem oxygenase activity, HO-1 protein and mRNA expression

Figure 5.1 reports the effects of hemin, AS or a combination of both agents on haem oxygenase activity (**A**) and HO-1 protein expression (**B**; $n = 1$) in H9c2 cells following direct application of the agents in complete media for 6 h. Akin to previous experiments involving this mode of addition, AS promoted a significant increase in haem oxygenase activity compared to control (797.8 ± 102.3 (AS) vs. 202.9 ± 14.9 (control) pmoles bilirubin/mg protein/h, respectively; $P < 0.05$); this observation was replicated vis-à-vis HO-1 protein expression. Similarly, hemin (5-20 μM) induced a concentration dependent increase in haem oxygenase activity and HO-1 protein levels. Of particular interest is the observation that simultaneous addition of AS and hemin significantly elevates the measured parameters in a synergistic manner ($P < 0.05$ vs. control or hemin alone). When the same experiments were repeated following a 1 h preincubation of the agents at 37°C in media before addition to cells, AS lost its ability to induce either haem oxygenase activity (260.7 ± 37.1 (control) vs. 223.7 ± 6.1 (AS) pmoles bilirubin/mg protein/h) (Figure 5.2 **A**) or HO-1 protein (Figure 5.2 **B**; $n = 1$). This response is similar to that witnessed in earlier experiments involving decomposed AS. However, hemin (5-20 μM) maintained AS's ability to promote an elevation in haem oxygenase activity and intensity of HO-1 protein expression. Surprisingly, coincubation of AS with hemin (5-20 μM) resulted in augmented haem oxygenase activity ($P < 0.05$) and HO-1 protein expression compared to control, an effect similar to direct

application of the agents. Thus, the presence of hemin during the preincubation step synergised with NO⁻ to induce HO-1.

A



B

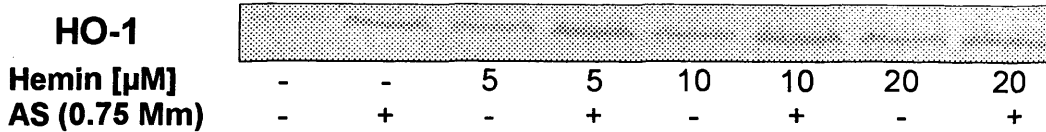
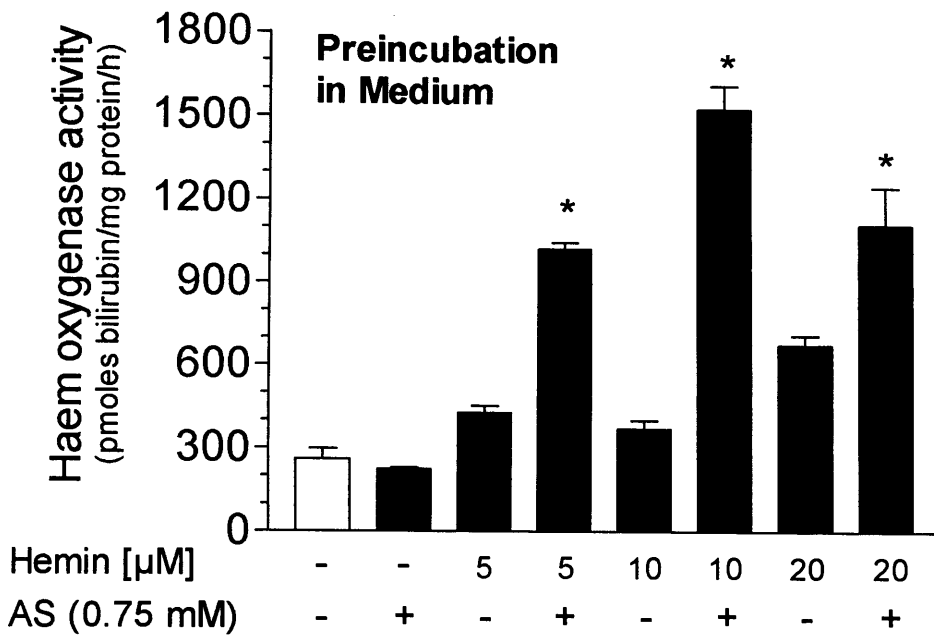


Figure 5.1 Interaction between Angeli's salt and hemin amplifies haem oxygenase activity and HO-1 protein expression.

Cells were exposed directly to Angeli's salt (AS, 0.75 mM) in the absence (-) or presence (+) of hemin (5, 10 or 20 µM) in fresh complete DMEM. After 6 h haem oxygenase activity (**A**) and HO-1 protein expression (**B**) were determined according to Sections 2.3.4 and 2.4.2, respectively. Control (open bar) represents cells exposed to fresh DMEM alone. The Western blot panel is representative of one experiment. Bars represent the mean ± S.E.M. of *n* = 4 experiments. * *P* < 0.05 vs. Control. † *P* < 0.05 vs. hemin alone.

A



B

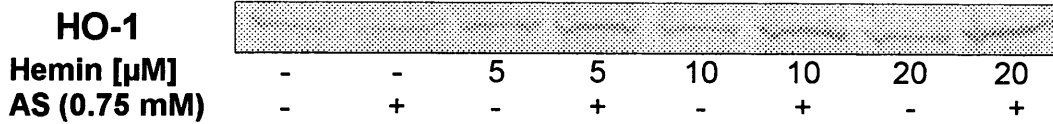


Figure 5.2 Formation of a haem-nitrosyl complex maintains the induction of haem oxygenase activity and HO-1 protein expression.

Cells were exposed to Angeli's salt (AS, 0.75 mM) in the absence (-) or presence (+) of hemin (5, 10 or 20 μM) following their preincubation for 1 h at 37°C beforehand in fresh complete DMEM. After 6 h haem oxygenase activity (**A**) and HO-1 protein expression (**B**) were determined according to Sections 2.3.4 and 2.4.2, respectively. Control (open bar) represents cells exposed to fresh DMEM alone. The Western blot panel is representative of one experiment. Bars represent the mean \pm S.E.M. of $n = 4$ experiments. * $P < 0.05$ vs. Control.

The results of RT-PCR analysis (Figure 5.3; $n = 1$) shows that AS has the ability to intensify the transcriptional activation of HO-1 mRNA in a concentration dependent manner in the presence of low micromolar concentrations of hemin, compared with either agent alone, following direct incubation with cells for 3 and 6 h.

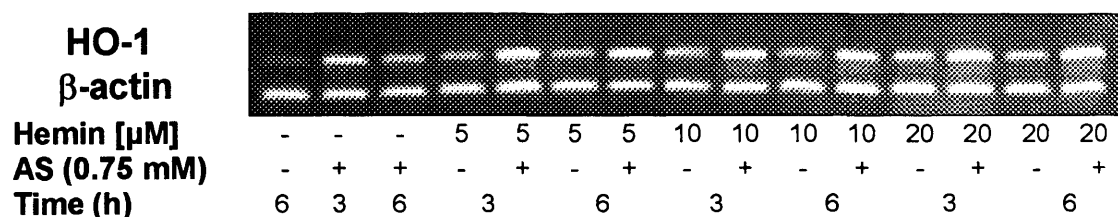


Figure 5.3 Interaction of Angeli's salt and hemin intensifies HO-1 mRNA expression.

Cells were exposed directly to Angeli's salt (AS, 0.75 mM) in the absence (-) or presence (+) of hemin (5, 10 or 20 μ M) in fresh complete DMEM. After 3 and 6 h HO-1 mRNA expression was determined by RT-PCR using rat HO-1 primers (see Section 2.4.4). Control (no treatment) represents cells exposed to fresh DMEM alone. The PCR image is representative of a single experiment with β -actin serving as a loading control.

The data reported in Figure 5.4 reveals that combining 1 mM NAC with AS significantly attenuated the haem oxygenase inducing capacity of NO^- , (279.9 ± 5.1 vs. 863.2 ± 53.4 pmoles bilirubin/mg protein/h; $P < 0.05$), thus supporting the previous observations in Chapter 4, i.e. that thiols can prevent HO-1 induction by nitrosative agents. However, the simultaneous addition of AS and NAC with increasing concentrations (5-20 μM) of hemin was unable to prevent an increase in haem oxygenase activity which was significantly ($P < 0.05$) higher than cells treated with hemin alone. These results suggest that:

1. a strong and specific interaction between NO^- and hemin elicits an induction of HO-1;
2. hemin stabilises the NO^- groups, possibly through formation of a haem-nitrosyl complex, even in presence of high thiol concentrations; and
3. the affinity of NO^- towards haem is stronger and much greater than its affinity for cysteine residues.

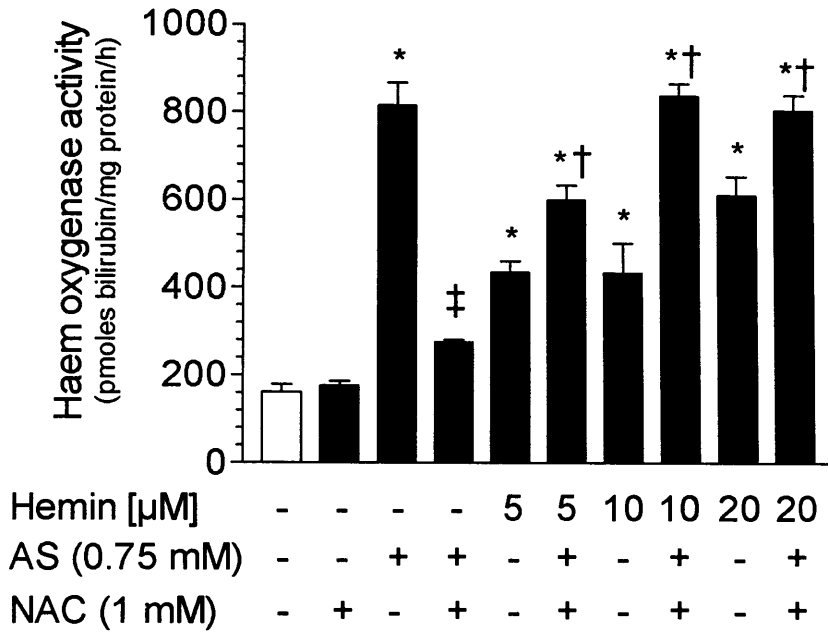
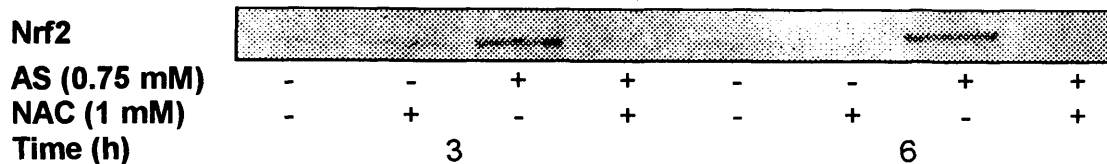


Figure 5.4 Possible formation of a haem-nitrosyl complex maintains the induction of haem oxygenase activity in the presence of thiols.

Cells were exposed directly to Angeli's salt (AS, 0.75 mM) or hemin (5, 10 or 20 μM) in the absence (-) or presence (+) of *N*-acetylcysteine (NAC, 1 mM). After 6 h haem oxygenase activity was determined according to Section 2.3.4. Control (open bar) represents cells exposed to fresh DMEM alone. Bars represent the mean \pm S.E.M. of $n = 4$ experiments. * $P < 0.05$ vs. Control. ‡ $P < 0.05$ vs. AS alone. † $P < 0.05$ vs. hemin alone.

Previous data from Chapters 3 and 4 suggested that NO^- activates HO-1 at the transcriptional level. This observation was reinforced by Western blot data for the redox-sensitive transcription factor Nrf2. Following a 3 and 6 h exposure to the NO^- generator AS, there was a significant increase in the expression of Nrf2 in the nuclear fraction (see Figure 5.5 **A**; $n = 1$), but this response was eliminated completely by the addition of 1 mM NAC. Similarly, the combination of AS and hemin (5-20 μM) greatly intensified Nrf2 expression (Figure 5.5 **B**; $n = 1$), but NAC was unable to attenuate the latter response (data not shown) which is akin to the effect observed in Figure 5.4. Interestingly, hemin alone did not induce any Nrf2 expression. Possible explanations for these observations are that hemin (a prooxidant) may be activating the haem oxygenase pathway via a route that is sensitive to oxidative stress, e.g. the activator protein-1 (AP-1) transcription factors or the haem response element, whereas the haem-nitrosyl complex (a pronitrosant) may mediate enhancement of HO-1 protein via Nrf2 in this model. Collectively, these data suggest that Nrf2 activation is augmented in response to nitrosative stress and haem-nitrosyl complex formation.

A



B

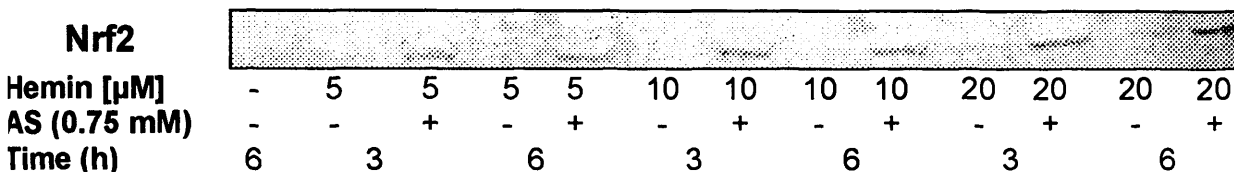


Figure 5.5 Modulation in the expression of the transcription factor Nrf2 in response to Angeli's salt, thiols and hemin.

Cells were treated directly with Angeli's salt (AS, 0.75 mM) in the absence (-) or presence (+) of the thiol generator *N*-acetylcysteine (NAC, 1 mM) **(A)** or with (AS, 0.75 mM) in the absence or presence of hemin (5, 10 or 20 μM) **(B)** in fresh complete DMEM. After 3 and 6 h nuclear extracts were prepared and Western blot detection of Nrf2 protein was performed as described in Sections 2.4.1 and 2.4.2, respectively. Control (no treatment) represents cells exposed to media alone. Each Western blot panel is representative of one experiment.

As noted in Chapter 3, the NO releasing agent DeaNO led to a concentration dependent increase in haem oxygenase activity and HO-1 protein expression. To examine the possibility that DeaNO (0.5 mM) could also complex with hemin (5-20 μ M) the agents were preincubated, either alone or in combination, for 1 h at 37°C in media prior to their addition to cells for a further 6 h. Also, a set of cells treated with DeaNO directly for 6 h was included for comparison. The results in Figure 5.6 reveal that DeaNO promoted a significant increase in haem oxygenase activity when applied directly to cells (1287 ± 62.0 (DeaNO) v. 260.7 ± 37.1 (control) pmoles bilirubin/mg protein/h; $P < 0.05$). In analogy with the AS data, decomposed DeaNO was unable to induce haem oxygenase activity. However, the presence of various concentrations of hemin in the media led to an augmented increase in haem oxygenase activity which was much more substantial ($P < 0.05$) than hemin alone and almost comparable with the direct addition of DeaNO. Therefore, both NO^- and NO have the capacity to directly synergise with hemin to promote an increase in cardiac haem oxygenase activity.

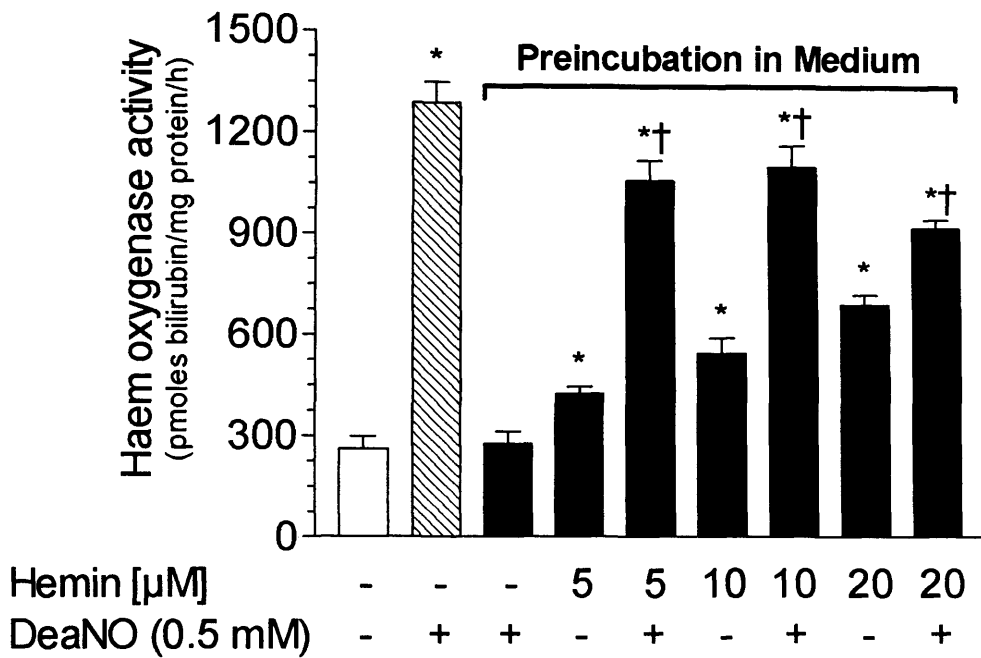


Figure 5.6 Interaction between DeaNO and hemin amplifies haem oxygenase activity.

Cells were exposed to DeaNO (0.5 mM) in the absence (-) or presence (+) of hemin (5, 10 or 20 μM) following their preincubation for 1 h at 37°C beforehand in fresh complete DMEM. The effect of directly applied DeaNO (hatched bar) is shown for comparison. After 6 h haem oxygenase activity was determined according to Section 2.3.4. Control (open bar) represents cells exposed to fresh DMEM alone. Bars represent the mean \pm S.E.M. of $n = 4$ experiments. * $P < 0.05$ vs. Control. † $P < 0.05$ vs. hemin alone.

Exposure of cells directly to AS (0.75 mM), hemin (5-20 μM) or a combination of both agents caused minimal toxicity over a 6 h period, see Figure 5.7. A somewhat similar pattern was observed after a 1 h preincubation of AS with hemin (5 or 10 μM), although AS plus 20 μM hemin resulted in a 25% decrease in cell metabolism. This data correlates with previous findings showing reduced activation of the haem oxygenase system in response to increased cytotoxicity⁶⁸¹.

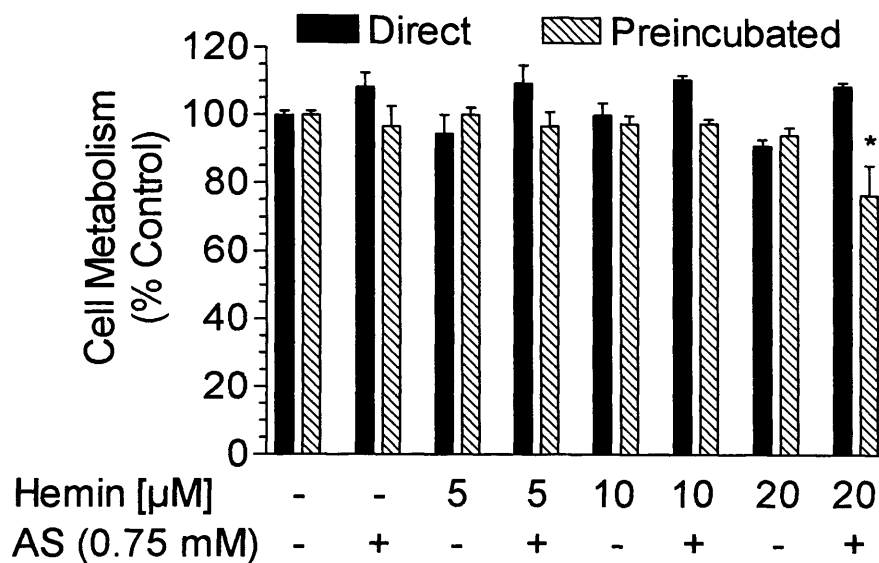


Figure 5.7 Effect of haem-nitrosyl interaction on cell metabolism.

Cardiomyocytes were treated for 6 h with Angeli's salt (AS, 0.75 mM) in the absence (-) or presence (+) of hemin (5, 10 or 20 μM) either directly (filled bars) or after the agents were preincubation (hatched bars) for 1 h at 37°C beforehand in fresh complete DMEM. Cell metabolism, which was determined according to Section 2.2.4, was expressed as a percentage of the control (assigned 100%). Control (no treatment) represents cells exposed to fresh DMEM alone. Bars represent the mean \pm S.E.M. of $n = 6$ experiments. * $P < 0.05$ vs. Control.

The data presented so far in this chapter were obtained from cells incubated under normoxic conditions. A series of experiments were conducted under hypoxic conditions, which is a very potent stimulus for the haem oxygenase pathway in different cell types^{423,495,682,683}, to investigate if the synergism observed between hemin and NO⁻ could be maintained. Hemin (5 μM), AS (0.75 mM) or a combination of both agents were preincubated in media for 1 h at 37°C before their addition to cells under normoxic or hypoxic conditions. The results of a time-course analysis of haem oxygenase activity, expressed as a percentage of the control group, following 0-18 h normoxia or hypoxia are shown in Figure 5.8 **A** and **B**, respectively.

The control (0 h) values (100.3 ± 7.8 pmoles bilirubin/mg protein/h) for normoxia and hypoxia were derived from the same set of flasks as they are equivalent situations. Similarly, the haem oxygenase activity levels did not deviate significantly from 0 h control at the subsequent control time points (6, 12 and 18 h) analysed. Akin to previous observations, decomposed AS was unable to promote a significant increase in haem oxygenase activity, therefore, a pattern similar to control was observed over 18 h at both oxygen tensions. Based on the absolute values of haem oxygenase activity measured in cells during hypoxia or normoxia no difference was observed in cells treated with AS alone for all the time points considered. In the presence of hemin alone there was a significant ($P < 0.05$) increase in haem oxygenase activity over 18 h under both hypoxic and normoxic conditions. However, while the increase in haem oxygenase activity was sustained during normoxia, in hypoxia the pattern consisted of an increase (6 h) followed by a plateau (12 h)

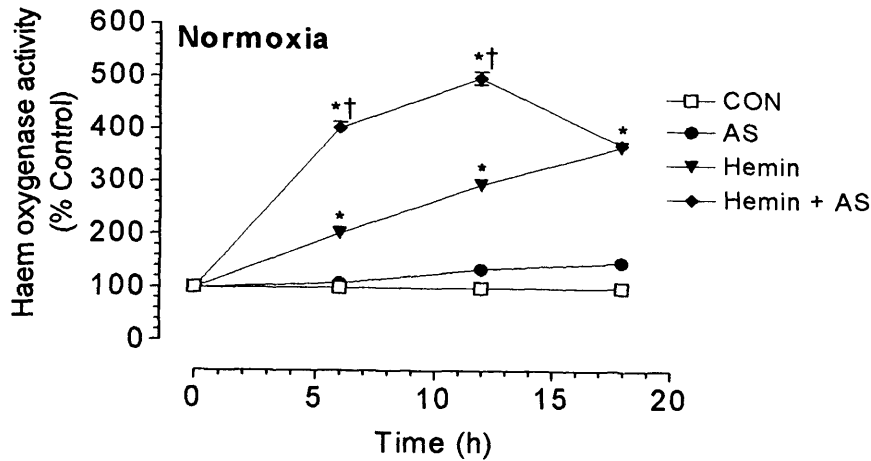
followed by a further increase at 18 h. Comparison of the respective time points of hemin treatment revealed a significant ($P < 0.05$) difference at the corresponding time points especially under hypoxic conditions. The basis for the latter observation could be due to the simultaneous stimulation of the haem oxygenase pathway by two potent stimuli, i.e. hemin and hypoxia. When exposed to a combination of AS and hemin there was a strong amplification in the level of haem oxygenase activity during the first 12 h of hypoxia and normoxia, but no inter group differences in haem oxygenase activity at 6 and 12 h were noted, after which there was a respective decline to levels equal to or below the ones obtained with hemin alone. This decline in haem oxygenase activity was more pronounced ($P < 0.05$) when cells were exposed to AS and hemin for 18 h hypoxia compared with a similar scenario under normoxic conditions. A possible reason for this is that the capacity of the haem oxygenase enzyme was overwhelmed by a triad of events, i.e. the combination of AS and hemin plus the low oxygen tension environment.

Although Western blot data has not been included with these observations our group has previously shown the ability of hypoxia and hemin to induce HO-1 protein expression in a time-dependent manner compared to normoxia⁶⁴⁷. The aforementioned data, together with the previous Western blot data for AS presented in this and preceding chapters of this Thesis strongly support the fact that an increase in haem oxygenase activity under hypoxic and normoxic conditions would be paralleled by a corresponding induction of HO-1 protein expression, thus precluding the need for further Western blot analysis. Taken together, these data reveal that the ability of hemin to complex with NO^- and

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promote a significant augmentation in haem oxygenase activity can be maintained during conditions of high and low oxygen tension.

A



B

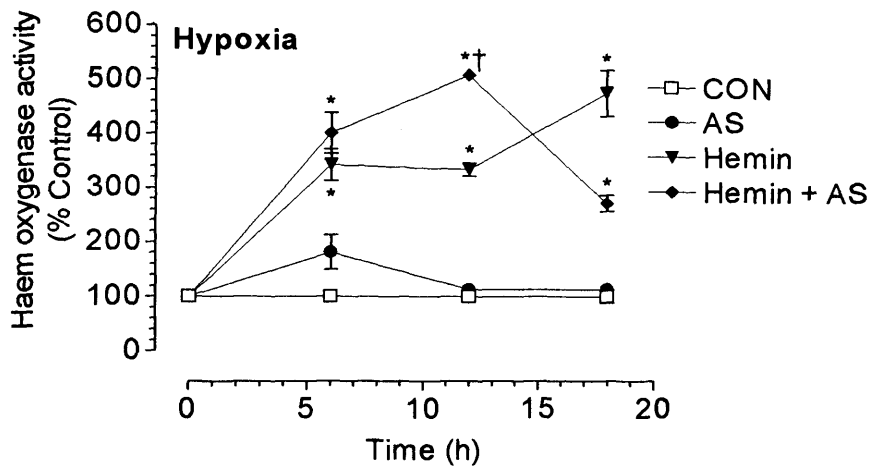


Figure 5.8 Induction of haem oxygenase activity by a haem-nitrosyl interaction is maintained at different oxygen tensions.

Cells were exposed to Angeli's salt (AS, 0.75 mM) in the absence (-) or presence (+) of hemin (5 μ M) following their preincubation for 1 h at 37°C in fresh complete DMEM. After 6, 12 or 18 h exposure to a normoxic (A) or hypoxic (B) environment haem oxygenase activity was determined according to Section 2.3.4. The activity was expressed as a percentage of control (0 h) (assigned 100%), where control represents cells treated with fresh DMEM alone. Each symbol represent the mean \pm S.E.M. of $n = 4$ experiments. * $P < 0.05$ vs. Control. † $P < 0.05$ vs. hemin alone.

5.5 Discussion

Both NO and its congeners, e.g. NO⁺ and NO⁻, are effective inducers of HO-1 in vascular and cardiac cell types^{427,481,681}. The data presented in this chapter demonstrates that a combination of hemin (the oxidised (Fe³⁺) form of haem) and the nitroxyl generator (AS) or NO donor (DeaNO) significantly increased haem oxygenase activity and enhanced HO-1 protein and mRNA expression. These effects were more pronounced than those observed when either agent was applied to cells alone. Furthermore, preincubation of AS or DeaNO with hemin for 1 h in media at 37°C before their addition to cells preserved the biological potency of both the former compounds to activate the HO-1 pathway. This suggests a direct interaction between haem and NO⁻ or NO leads to an amplification of HO-1 induction. The formation of a haem-nitrosyl complex by NO⁻ is a feasible outcome given its affinity for Fe³⁺ complexes^{68,684}. The data also shows that NO (originating from DeaNO) can form a similar complex which is unusual given the greater affinity of this species for Fe²⁺ centres^{19,67,185}, although Juckett *et al.* observed a similar affinity when using DeaNO in endothelial cells⁶¹⁷. Furthermore, the stability of the haem-nitrosyl complex, which can avidly bind with serum proteins such as albumin⁶⁸⁵, is enhanced as the latter protein is present at high levels in the media used in these experiments. Interestingly, the addition of NAC to the medium was only capable of moderately attenuating the induction of haem oxygenase activity and HO-1 protein by AS plus hemin, despite the fact that data in Chapter 4 and from previous groups^{423,668} indicated that NAC can suppress HO-1 mediated induction by NO⁻ or NO. Thus, the strong affinity of the haem-

nitrosyl complex can serve as a potent inducer of the HO-1 system to combat different types of nitrosative stress even in the presence of high concentrations of thiols.

The actinomycin D data from Chapter 3 suggests that induction of HO-1 by NO^- or NO occurs at the transcriptional level. Chen and Maines have reported that HO-1 mRNA expression is upregulation by different NO congeners via the activation of MAPKs, e.g. ERK and p38, but the SAPK/JNK pathway and AP-1 transcription factor levels were unchanged⁶⁸⁶. The possibility that other redox-sensitive transcription factors participate in the induction of HO-1 by NO^- or NO-related species has not been examined. For the first time the current study shows that protein expression of the redox-sensitive transcription factor Nrf2 is significantly increased within the nuclear fraction of cells in response to NO^- stimulation, but this effect is completely eliminated by the addition of NAC. In contrast, a fixed concentration of AS and increasing concentrations of hemin promotes a gradual enhancement in intensity of Nrf2 expression. Taken together, the observations so far indicate that Nrf2 can serve as a sensitive gauge of the extent of nitrosative stress, which in turn leads to HO-1 induction, and these responses are strongly regulated by thiols.

The ability of Nrf2 to exert these effects upon HO-1 is feasible due to the presence of a series of StREs, which resemble the recognition sequence for Nrf2, within the promoter region of the HO-1 gene⁴⁶⁵. Normally Nrf2 lies in an inactive state within the cytosol via binding with its chaperone Keap1⁶⁶⁶. In response to electrophilic agents, or compounds that modify cysteine residues⁶⁸⁷, the link between Nrf2 and Keap1 is broken, thus allowing Nrf2 to migrate

into the nucleus where it can activate phase II detoxifying enzymes and stress inducible genes including HO-1 ⁴⁶⁶. The fact that Keap1 contains many highly reactive sulphhydryl groups ⁶⁸⁷, and NO⁻ and NO possess a strong affinity for such groups ⁶³⁶, it is feasible that activation of the Nrf2/HO-1 sequence by haem-nitrosyl complexes occurs through S-nitrosation ⁶⁸⁸. Further work is required to clearly elucidate the mechanism(s) behind this chemical modification of the HO-1 gene.

As formation of a haem-nitrosyl complex can not only amplify the HO-1 pathway during normoxic, but also hypoxic conditions, suggests that interaction between haem and NO⁻, or NO-related species, could serve as a potent signalling mechanism to augment the cytoprotective aspects of haem oxygenase during situations of reduced oxygen tension. A number of pathological conditions, e.g. sickle cell anaemia or haemorrhagic and subarachnoid shocks, are characterised by increased haemolysis, thus increasing free haem availability, as well as localised hypoxia, both of which serve as potent inducers of the HO-1 pathway ^{365,647,689}. In addition, these pathologies may be associated with excessive NO formation as well as significant vascular dysfunction ^{354,690}. Therefore, further expansion upon the findings in this chapter could serve as a potential therapeutic intervention in a wide range of diseases.

5.6 Conclusion

It is well documented that haem can serve as both a stimulus and substrate for the induction of HO-1 ³⁶⁴. More recently, the gaseous molecule NO and its

reduction product NO^- have joined the long list of potential HO-1 inducers^{373,681}. This chapter details how haem and NO^-/NO can synergistically promote enhanced haem oxygenase activity, HO-1 protein and mRNA expression. The potency of the haem-nitrosyl complex to increase haem oxygenase activity is quite strong as thiols are unable to diminish this response. The data suggests that augmented HO-1 gene expression occurs in response to activation of the redox-sensitive transcription factor Nrf2. Collectively, these observations support the notion that amplification of the HO-1 pathway is an important defence stratagem towards the twin dilemmas of oxidative and nitrosative stress.

6 PROTECTION AGAINST NITROSATIVE STRESS BY BILE PIGMENTS

6.1 Introduction

Oxidative catabolism of haem by the microsomal enzyme haem oxygenase releases the central iron molecule of the tetrapyrrole and produces CO and biliverdin. The biliverdin is subsequently reduced by the serine/threonine kinase biliverdin reductase to bilirubin within the cytosol⁴⁴⁶. Collectively, these two reactions are known as the haem oxygenase pathway and this represents the primary mechanism by which mammals generate bilirubin⁴¹¹. The traditional perception that the bile pigments, biliverdin and bilirubin, were toxic waste products derived from the known prooxidant haem was challenged by compelling evidence revealing their antioxidant potential, particularly by bilirubin, and their ability to modulate cell signalling pathways^{385,525}. Furthermore, a number of publications have highlighted a correlation between plasma levels of bilirubin and the incidence of CAD, i.e. increased serum bilirubin is associated with a reduced risk of cardiovascular morbidity and *vice versa*^{543,546,547}. Although low serum bilirubin is not necessarily a causative factor for CAD development it can occur due to increased oxidative activity⁵⁴³ or via parental history of cardiovascular disease⁵⁴⁹, thus making the individual more prone to CAD and serving as a potential predictive risk marker for this ailment.

The process of haem catabolism, particularly by the inducible isoform HO-1,

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can be augmented by agents that cause oxidative injury⁴⁵⁷. In addition, recent evidence suggests that HO-1 activity is markedly increased by NO and its different redox subtypes, thus highlighting the potential of HO-1 to attenuate nitrosative stress⁶³⁶. As HO-1 is induced by oxidative and nitrosative type stressors and bilirubin and biliverdin decrease the degree of oxidative injury³⁸⁵, the premise that these bile pigments could exert a comparable effect upon NO and its congeners is an enticing prospect. To investigate this hypothesis biliverdin and bilirubin were reacted with the NO⁻ liberator AS and the NO donor DeaNO under *in vitro* conditions and the stability of bilirubin was quantified spectrophotometrically. In addition, the antinitrosative potential of bilirubin was examined in plasma using a NO microsensor.

6.2 Objective

The aim of the study investigated in this chapter is to verify that bilirubin and biliverdin can interact with NO⁻ and NO, thus highlighting the potential antinitrosative capacity of bile pigments.

6.3 Materials and Methods

6.3.1 Preparation of reagents

The bile pigments (bilirubin and biliverdin) and RNS (AS and DeaNO) were prepared as 10 mM stock solutions in 0.1 M (bile pigments) or 0.01 M (AS and DeaNO) NaOH and diluted down to 0.5 mM or 1 mM working solutions, respectively. All the agents were prepared fresh at the time of each experiment in opaque Eppendorfs and placed on ice until required.

6.3.2 Spectrophotometric studies

All spectra were recorded using a UV-visual spectrophotometer (Helios α , Unicam Ltd., Cambridge, UK) according to Section 2.5.1. The absorbance readings between 300 and 750 nm were conducted at 37°C against a blank containing vehicle (DPBS, pH 7.4) and an equal concentration of buffer (0.01 M NaOH). To assess the effect of NO⁻ or NO, increasing concentrations (10-300 μ M) of AS or DeaNO were added to DPBS containing bilirubin or biliverdin (5 μ M final concentration) and absorption spectra recorded after 5 min. Changes in the spectra of the bile pigments over time (30 min) were also determined after their reaction with different concentrations (10 and 50 μ M) of AS or DeaNO. In each experiment the bile pigment was added to the DPBS first, mixed gently by inversion, followed by either AS or DeaNO, remixed and read. The latter sequence was reversed only when AS or DeaNO were allowed to decompose in vehicle for 1 h at 37°C, i.e. bilirubin was added after 1 h. The experimental data and kinetics of these reactions was recorded onto a disk and plotted using the accompanying Convert package, Microsoft Excel and GraphPad Prism 3.0 software. Negative controls were included in all experiments.

6.3.3 Nitric oxide (NO) electrode experiments

The quantity of NO released by the NO donor DeaNO (5 μ M) in the presence or absence of bilirubin (50 μ M) was recorded continuously in a solution (final volume 1 ml) consisting of 50% (v/v) rat plasma, 50% (v/v) DPBS (Control) or a combination of the two, using a NO-sensitive electrode under constant

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stirring at 37°C as described in Section 2.5.2. The rate of NO release was expressed as the area under the curve (AUC) calculated after 15 min of reading ($\mu\text{M} \times \text{s}$). Controls consisted of vehicle alone.

6.4 Results

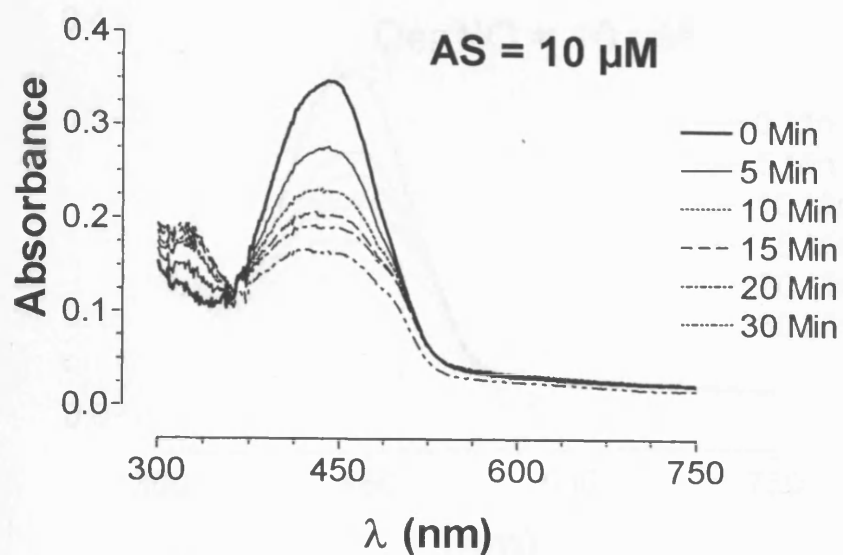
6.4.1 Bilirubin and biliverdin interact with nitric oxide (NO)-related species over time

Figure 6.1 and 6.2 reveal that bilirubin (5 μM) has the capacity to react with variable concentrations (10 and 50 μM) of AS or DeaNO, a source of NO^- and NO, respectively, over a 30 min duration. In the case of 10 μM AS (see Figure 6.1 **A**), bilirubin led to a steady decline in absorbance from a maximum of 0.36 to 0.15 in the 445-450 nm region. Concurrently, a second peak began to appear in the 315-320 nm region. Addition of AS (50 μM) (Figure 6.1 **B**) promoted a rapid consumption of bilirubin, e.g. after 5 min the absorbance maximum was 0.15 compared with 0.28 in the presence of 10 μM AS. In parallel, the absorbance peak in the 315-320 nm region became more pronounced over time.

When 10 μM DeaNO (Figure 6.2 **A**) was reacted with bilirubin the absorbance pattern was somewhat similar to that obtained with the same concentration of AS, but there was little evidence of a second peak developing. In the presence of 50 μM DeaNO (Figure 6.2 **B**) the loss of bilirubin was more gradual; however, there was a change in the absorbance pattern between 315-320 nm which is akin to that observed with 10 μM AS. Figure 6.3 illustrates that biliverdin (5 μM) does not react as rapidly with 50 μM AS (Figure 6.2 **A**) or DeaNO (Figure 6.3 **B**) as bilirubin does over 30 min. In both cases the characteristic double peaks of biliverdin are observed at 410 and 650 nm. However, the appearance of a third peak in the 315-320 nm region of the AS graph suggests that an as yet uncharacterised entity is formed.

ANTINITROSATIVE CAPACITY OF BILE PIGMENTS

A



B

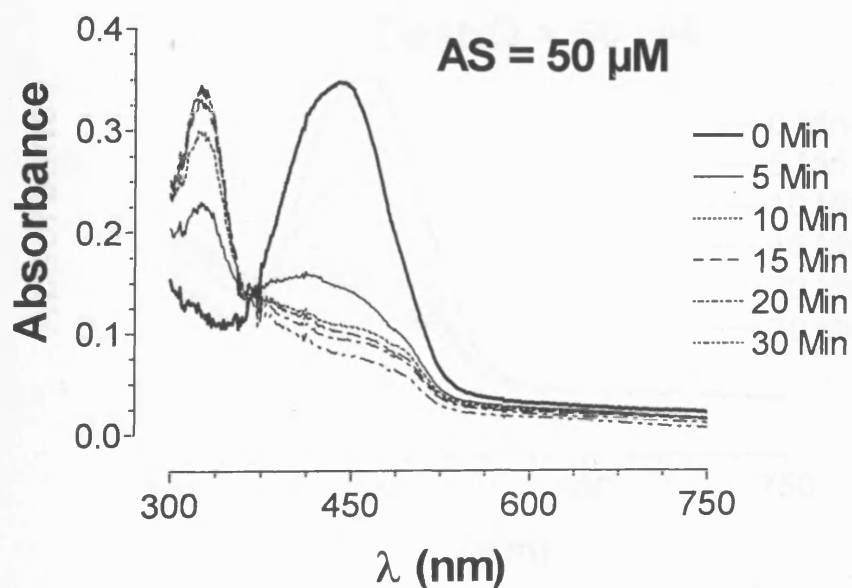
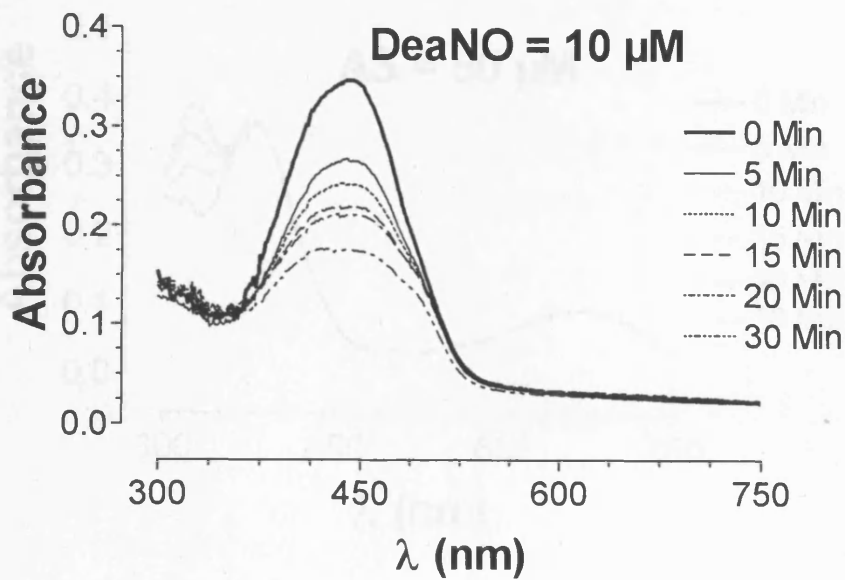


Figure 6.1 Effect of nitroxyl (NO^-) on bilirubin absorption spectra.

Absorption spectra of bilirubin (5 μM) were recorded at different time points (0-30 min) after interaction with Angeli's salt (AS) at 10 μM (A) or 50 μM (B) in DPBS (pH 7.4, 37°C) as described in Section 2.5.1.

A



B

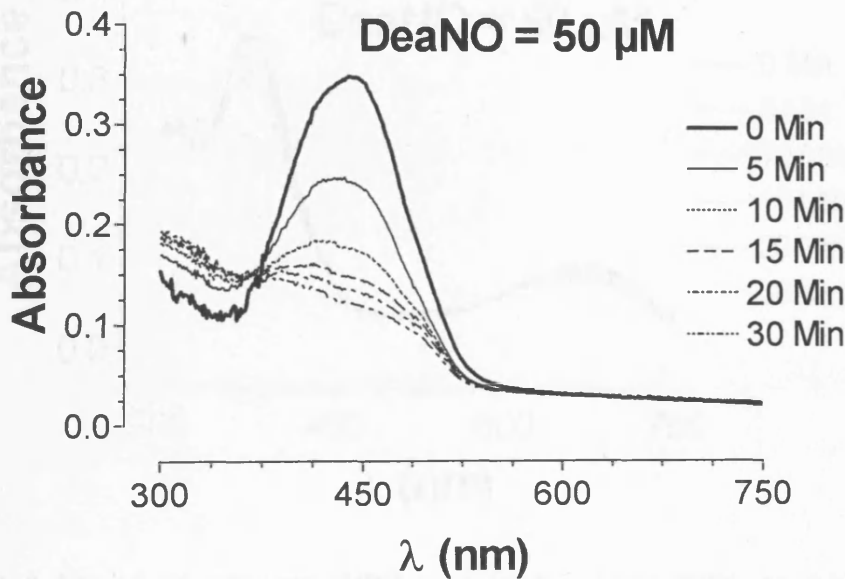
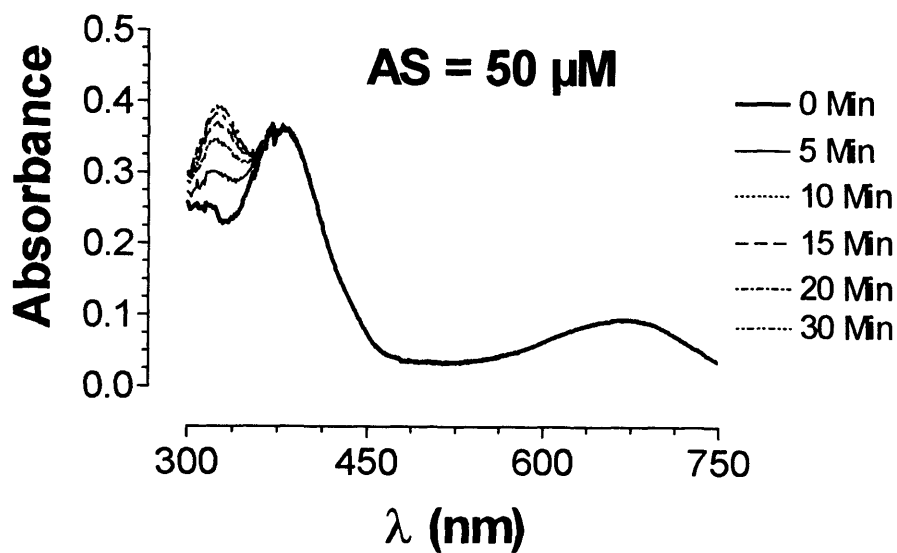


Figure 6.2 Effect of nitric oxide (NO) on bilirubin absorption spectra.

Absorption spectra of bilirubin (5 μ M) were recorded at different time points (0-30 min) after interaction with DeaNO at 10 μ M (A) or 50 μ M (B) in DPBS (pH 7.4, 37°C) as described in Section 2.5.1.

ANTINITROSATIVE CAPACITY OF BILE PIGMENTS

A



B

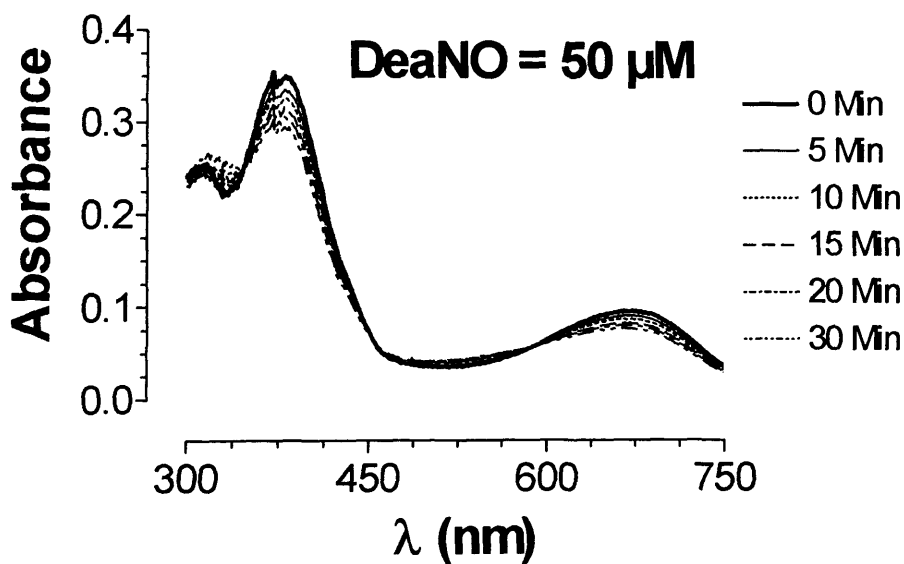


Figure 6.3 Effect of nitroxyl (NO^-) and nitric oxide (NO) on biliverdin absorption spectra.

Absorption spectra of biliverdin ($5 \mu\text{M}$) were recorded at different time points (0-30 min) after interaction with $50 \mu\text{M}$ AS (**A**) or DeaNO (**B**) in DPBS (pH 7.4, 37°C) as described in Section 2.5.1.

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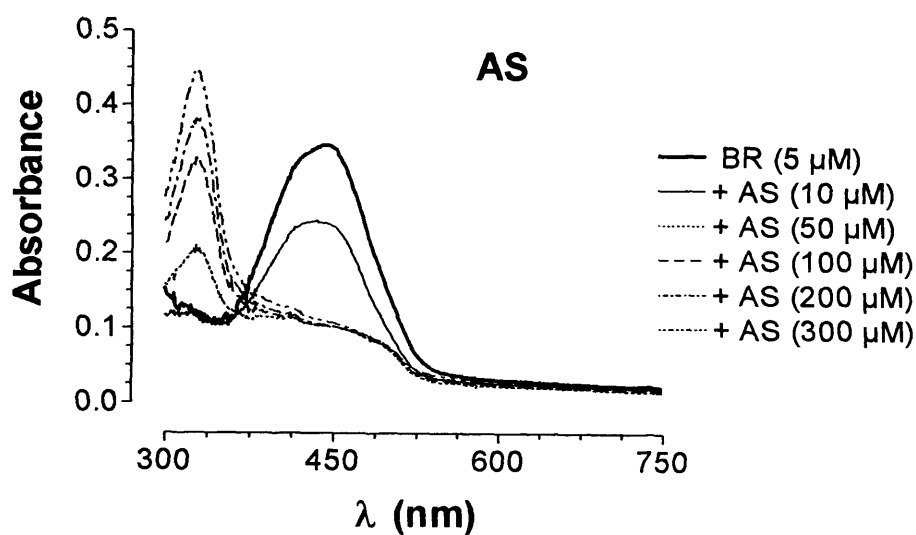
6.4.2 Bilirubin and biliverdin attenuate nitrosative agents in a concentration-dependent manner

The antinitrosative capacity of bilirubin is quite strong as 5 μM of the bile pigment is sufficient to attenuate up to 300 μM of AS (Figure 6.4 **A**) or DeaNO (Figure 6.4 **B**). Increasing concentrations (10-300 μM) of AS promoted the loss of bilirubin over 5 min. As observed in the time-dependent graphs a second peak became more defined in the 315-320 nm region in parallel with a decline in the absorbance maxima in the 445-450 nm zone. In contrast, the change in absorbance spectra observed with DeaNO (10-300 μM) was gradual over 5 min and there was little evidence of a second peak developing. The basis for these diverse results could relate to a stronger affinity between NO^- and bilirubin, thus leading to a more rapid consumption of the latter. The ability of AS and DeaNO to consume bilirubin is lost when both agents have undergone decomposition (i.e. they have lost their biological potency) via a 1 h preincubation in vehicle (see Figure 6.5). This suggests that a direct interaction occurs between the NO congeners and bilirubin leading to degradation of the latter.

The pattern of results obtained with biliverdin in the presence of 10-300 μM AS or DeaNO (Figure 6.6 **A** and Figure 6.6 **B**, respectively) is similar to that with 50 μM of both agents after 30 min. Again the double peak fingerprint of biliverdin is maintained, while the third peak produced by AS within the 315-320 nm region becomes more distinct.

ANTINITROSATIVE CAPACITY OF BILE PIGMENTS

A



B

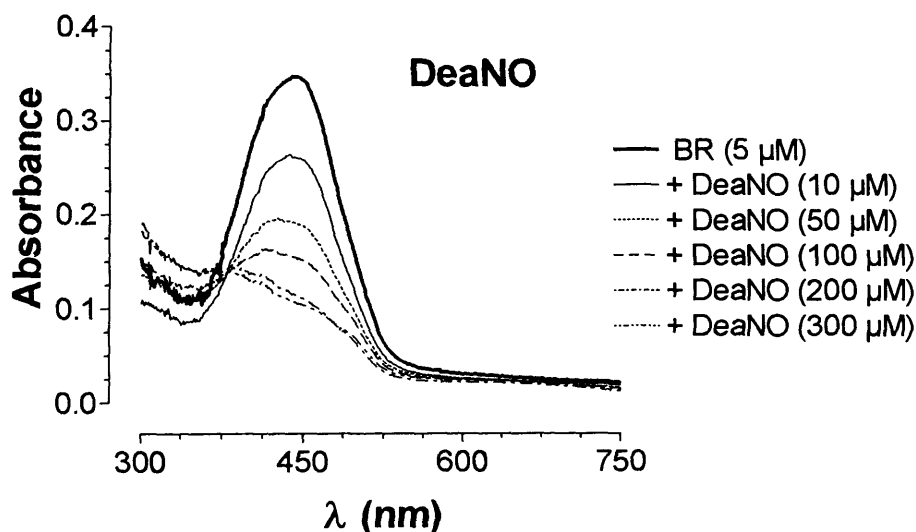
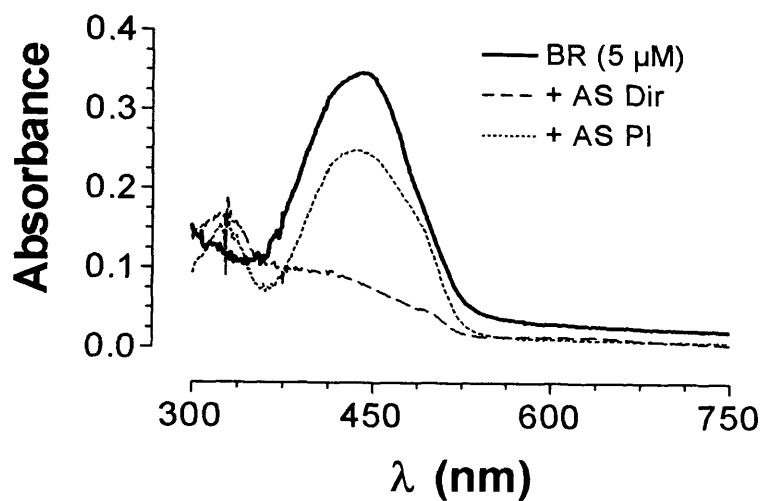


Figure 6.4 Nitroxyl (NO^-) and nitric oxide (NO) promote a concentration-dependent change in bilirubin absorption spectra.

Changes in the absorption spectra of bilirubin (BR, 5 μM) were detected 5 min after reaction with increasing concentrations (10-300 μM) of Angeli's salt (AS) (**A**) or DeaNO (**B**) in DPBS (pH 7.4, 37°C) as described in Section 2.5.1.

ANTINITROSATIVE CAPACITY OF BILE PIGMENTS

A



B

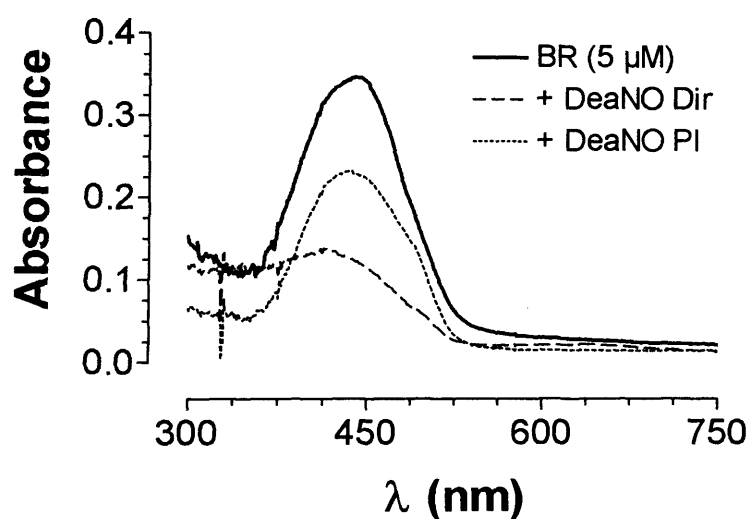
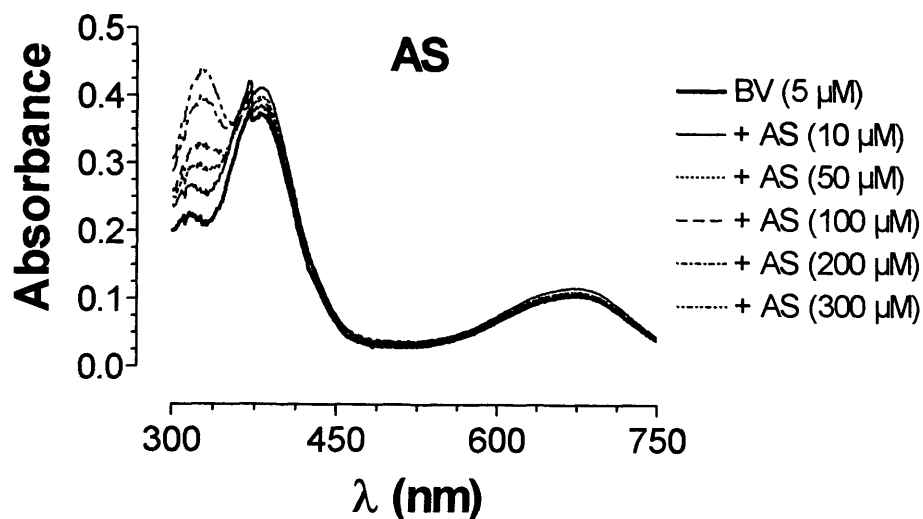


Figure 6.5 Direct interaction between NO congeners and bilirubin.

Changes in the absorption spectra of bilirubin (BR, 5 μM) were detected 5 min after reaction with a fixed concentration (50 μM) of Angeli's salt (AS) **(A)** or DeaNO **(B)** either directly (Dir) or following a 1 h preincubation (PI) in DPBS (pH 7.4, 37°C) as described in Section 2.5.1.

ANTINITROSATIVE CAPACITY OF BILE PIGMENTS

A



B

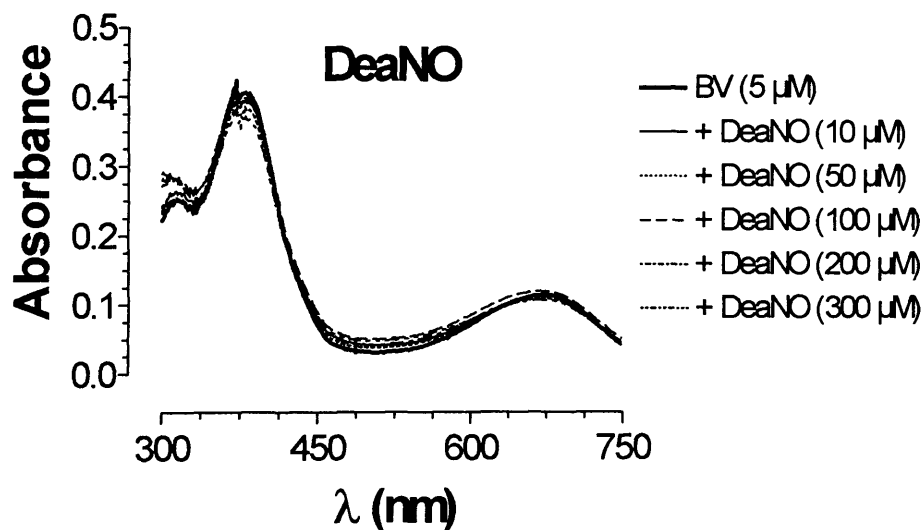


Figure 6.6 Nitroxyl (NO^-) and nitric oxide (NO) promote a concentration-dependent change in biliverdin absorption spectra.

Changes in the absorption spectra of biliverdin (BV, 5 μM) were detected 5 min after reaction with increasing concentrations (10-300 μM) of Angeli's salt (AS) (**A**) or DeaNO (**B**) in DPBS (pH 7.4, 37°C) as described in Section 2.5.1.

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6.4.3 Kinetics of bilirubin interaction with nitrosants

A standard curve of bilirubin (1-20 μM) in DPBS was plotted using the maximal absorbance reading at 440 nm for each concentration (see Figure 6.7). The extinction coefficient ($\epsilon_{440} = 64,000 \text{ M}^{-1} \text{ cm}^{-1}$) was derived from the slope of the latter plot. Using the ϵ_{440} value of the bilirubin standard curve and the absorbance maxima (i.e. $A_{440 \text{ nm}}$) of the time course (Figure 6.1 and Figure 6.2) and concentration (Figure 6.4) data for AS and DeaNO rate graphs were plotted according to Equation 6.1 to illustrate the concentration of bilirubin remaining during a 30 min analysis.

Following its interaction with AS (Figure 6.8 **A**) a 10 μM concentration caused a gradual decline in the rate of bilirubin consumption over the course of 30 min. In contrast, 50 μM AS caused a rapid and near complete consumption of bilirubin within the first 10 min, while the rate remained unchanged for the remaining 20 min. These observations correspond with most of the half-lives of AS ($t_{1/2} = 2.3 \text{ min}$), and therefore its biological potency, being exhausted. The reaction rate for both 10 and 50 μM DeaNO (Figure 6.8 **B**) caused a gradual decrease in bilirubin concentration over 30 min. Despite having similar half-lives (AS $t_{1/2} = 2.3 \text{ min}$; DeaNO $t_{1/2} = 2 \text{ min}$) these results reflect the rapid decomposition of AS and instability of its product NO^- compared with the NO donor DeaNO.

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$$\text{Concentration of bilirubin remaining } (\mu\text{M}) = \frac{\text{OD}_{440\text{ nm}}}{\varepsilon (\mu\text{M}^{-1})}$$

Equation 6.1 How to calculate the rate of bilirubin disappearance

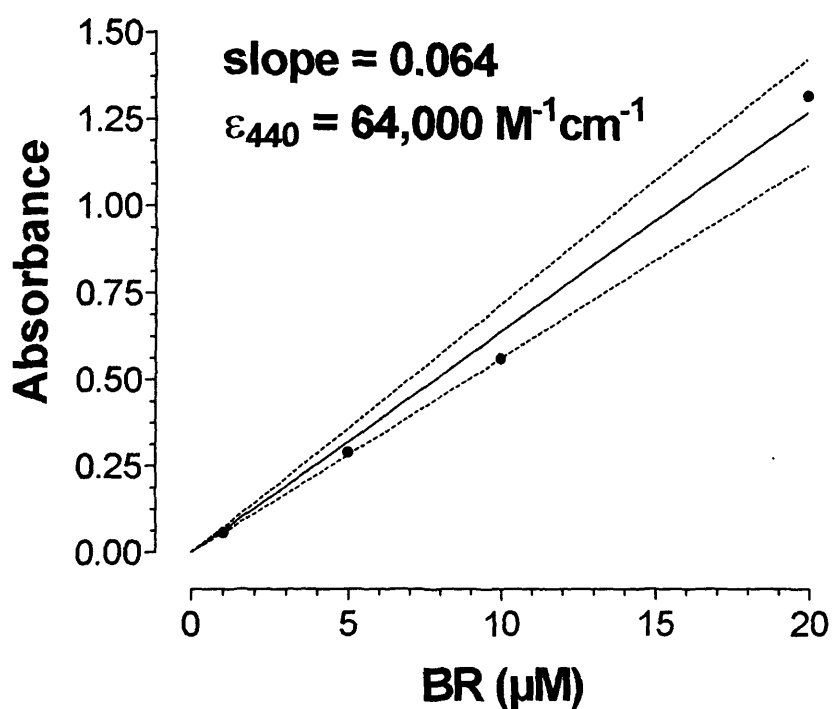


Figure 6.7 Standard curve of bilirubin in DPBS.

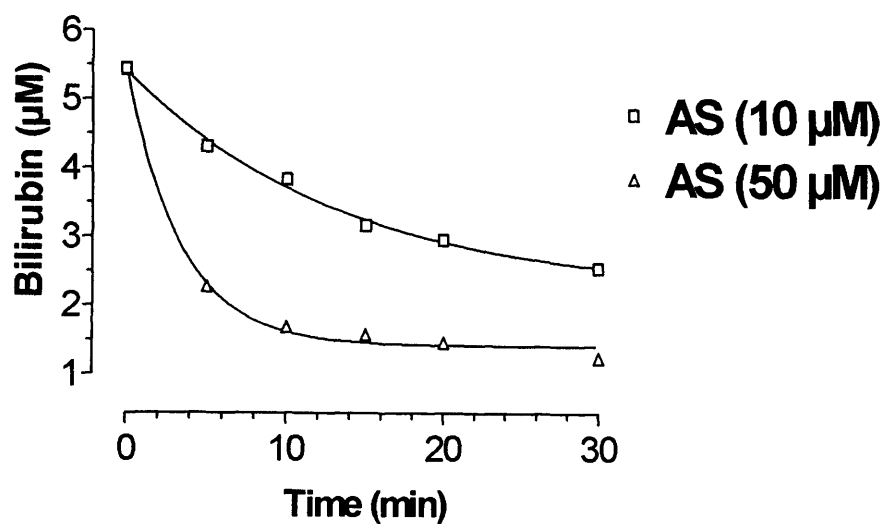
Bilirubin (BR) from stock solutions was added at a final concentration of 1, 5, 10 or 20 μM to DPBS (pH 7.4, 37°C) as described in Section 2.5.1. Absorbance values were read at 440 nm and plotted against the respective BR concentration. Solid line indicates the standard curve line. The dashed lines represent the 95% confidence interval.

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The rate of bilirubin disappearance was also plotted using the A_{440} nm of the concentration (10-300 μ M) graphs for AS and DeaNO, Figure 6.4 **A** and **B**, respectively. Figure 6.9 illustrates that in the presence of a fixed concentration of bilirubin and increasing concentrations of DeaNO the rate of change in the absorbance spectra was uniform over 30 min. However, the reaction between bilirubin and AS had reached completion by 100 μ M AS and remained constant thereafter. Collectively, the data in Figure 6.8 and Figure 6.9 demonstrate that bilirubin has the capacity to combine with different types of RNS for various durations and at numerous concentrations. The pattern of these reactions suggest that bilirubin is consumed more rapidly by NO^- rather than NO type species.

ANTINITROSATIVE CAPACITY OF BILE PIGMENTS

A



B

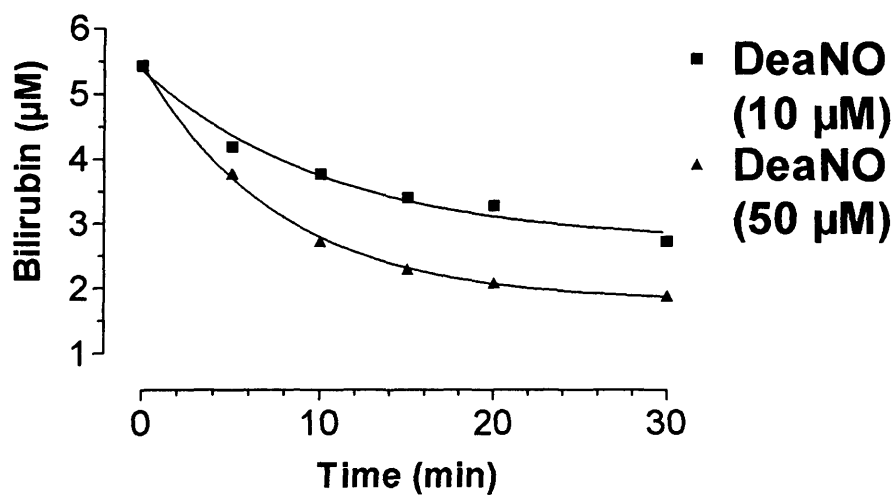


Figure 6.8 Rate of disappearance of bilirubin over time after reaction with Angeli's salt and DeaNO.

Absorption spectra of bilirubin (5 µM) were recorded at different time points (0-30 min) after interaction with Angeli's salt (AS, 10 or 50 µM) **(A)** or DeaNO (10 or 50 µM) **(B)** in DPBS (pH 7.4, 37°C) as described in Section 2.5.1. The concentration of BR at different time points was measured from Figure 6.7 using $\epsilon_{440} = 64,000 \text{ M}^{-1} \text{ cm}^{-1}$.

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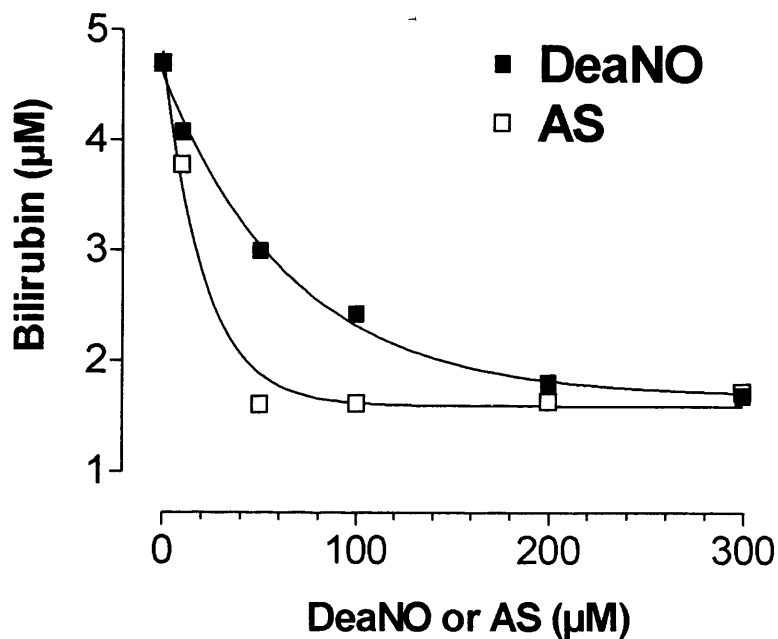


Figure 6.9 Rate of disappearance of bilirubin after reaction with increasing concentrations of Angeli's salt and DeaNO.

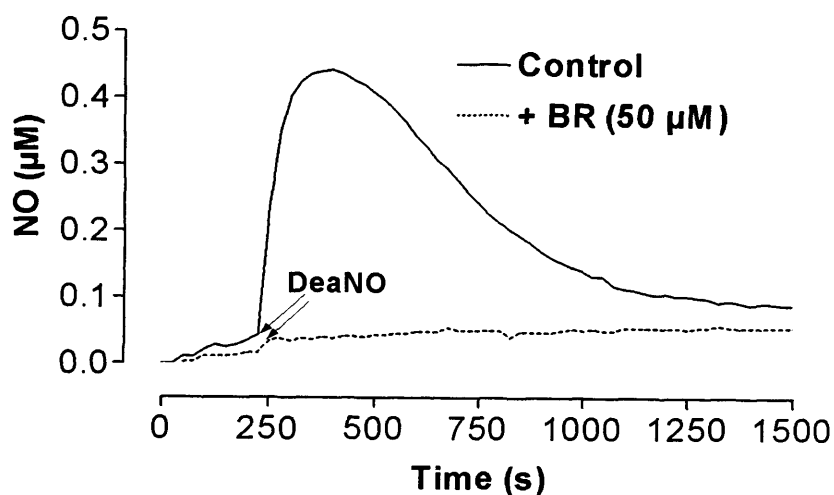
The change in absorption spectra of bilirubin (5 μM) were detected 5 min after reaction with increasing concentrations (10-300 μM) of Angeli's salt (AS) (open boxes) or DeaNO (filled boxes) in DPBS (pH 7.4, 37°C) as described in Section 2.5.1. The concentration of BR was measured from Figure 6.7 using $\epsilon_{440} = 64,000 \text{ M}^{-1} \text{ cm}^{-1}$.

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Having established that bilirubin is capable of reacting with different types of nitrosants in DPBS, we wondered whether the same could occur in a physiological solution. To test for a physiological role for the antinitrosative capacity of bilirubin, rat plasma with or without bilirubin (50 μM) was exposed to DeaNO (5 μM) for 15 min and the concentration of NO release was quantified in both cases using a NO-electrode. Figure 6.10 **A** shows that in the presence of DeaNO a 50% (v/v) mixture of plasma and DPBS releases a much greater concentration of NO compared with a similar mixture that also contains bilirubin. Plotting the area under the curve (AUC) of each trace (Figure 6.10 **B**) clearly demonstrates that bilirubin significantly ($P < 0.05$) attenuates the rate of NO release from DeaNO in plasma. This data suggests that bilirubin has the potential to exert a protective influence *in vivo* against pathologies characterised by increased NO release.

ANTINITROSATIVE CAPACITY OF BILE PIGMENTS

A



B

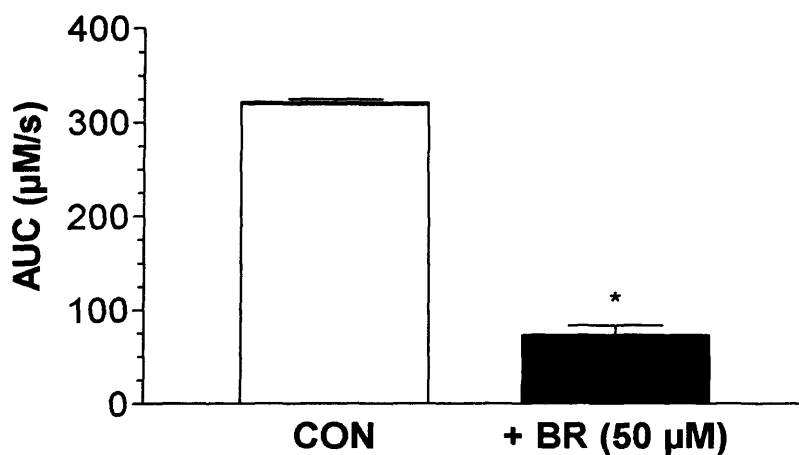


Figure 6.10 Bilirubin attenuates NO release from DeaNO.

A NO electrode was immersed in 1 ml of a solution consisting of 50% (v/v) rat plasma and 50% (v/v) DPBS (Control) or in plasma/DPBS solution containing bilirubin (BR, $50 \mu\text{M}$ final concentration). The NO releasing agent, DeaNO ($5 \mu\text{M}$ final concentration), was then added to the chamber and the NO concentration continuously monitored under constant stirring at 37°C as described in Section 2.5.2 (A). The rate of NO release was expressed as the area under the curve (AUC) calculated after 15 min of reading ($\mu\text{M} \times \text{s}$) (B). Bars represent the mean \pm S.E.M of three independent experiments. * $P < 0.05$ vs. control (CON).

6.5 Discussion

The haem of senescent erythrocytes represents the primary source of linear tetrapyrroles (LTPs) or bile pigments (biliverdin and bilirubin) in mammals. This reaction is catalysed by two enzymes: 1. microsomal haem oxygenase (HO, EC 1.14.99.3) which oxidises haem to biliverdin with CO and Fe^{2+} as by products; and 2. cytosolic biliverdin reductase (BVR, EC 1.3.1.24) which metabolises biliverdin to bilirubin ³⁶⁴. The intermediate biliverdin is water soluble, while bilirubin is insoluble. Within the liver bilirubin is made water soluble via the glucuronyl transferase system and excreted in the bile ⁵⁶⁸. In birds, amphibians and reptiles the reactions involving BVR and liver metabolism do not occur, rather biliverdin is excreted directly ⁴¹¹. Why mammals should expend energy in the generation of a toxic product bilirubin, from a known prooxidant haem, puzzled scientists for many years ³⁸². However, during the last few decades a number of reports have highlighted the potential usefulness of the LTPs, particularly bilirubin, as potential antioxidants ³⁸⁵, immunomodulators ⁵²⁵, a safeguard against different models of ischaemic reperfusion injury ^{430,553}, hypoxic conditions ^{386,647}, as well as an indicator of CAD development ⁵⁴³.

For the first time the results presented in this chapter describes a potentially novel function for the LTPs biliverdin and bilirubin as they could function as antinitrosative agents. In the presence of a fixed concentration of bilirubin and varied concentrations of AS, a source of NO^- , or DeaNO, a source of NO, there was a time- and concentration-dependent loss of bilirubin. This attenuation of different forms of nitrosative stress (a type of cellular threat

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which is analogous to the ability of ROS to cause oxidative stress)³³⁸ demonstrates the robust cytoprotective capacity of bilirubin. Even though AS and DeaNO have similar half-lives ($t_{1/2} = 2.3$ and 2 min, respectively) the rate of change in the bilirubin absorbance spectra was more pronounced in the presence of AS than DeaNO. A possible reason for this is due to a faster rate of interaction between bilirubin and AS. In both cases the NO congeners promoted the degradation of bilirubin through a direct interaction with the bile pigment as there was little, if any change, in the maximal absorption spectra of bilirubin in the presence of decomposed AS and DeaNO. The change in the absorption pattern of biliverdin in the presence of AS or DeaNO was not as dramatic compared with the bilirubin traces, suggesting biliverdin is not as strong an antinitrosative agent as bilirubin.

A common feature in the traces with AS, particularly so in the presence of bilirubin, was the appearance of a peak between 315-320 nm. The absorbance intensity of this peak became more pronounced with increasing time and concentration of exposure to AS. Due to technical limitations it was not possible to identify and characterise the species originating from this peak; one could speculate that it has arisen due to AS forming a 'nitrosated' complex with bilirubin and biliverdin. Further work using more sophisticated techniques, e.g. electron spin (paramagnetic) resonance (ESR, EPR) spectroscopy, could help to elucidate the exact nature of the latter nitrosated complex⁶⁸. Although it has not been included in this chapter, a set of experiments using the NO⁺ donor S-nitroso-N-acetylpenicillamine (SNAP) in the presence of bilirubin revealed a second peak, which became more intense

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with increasing concentration and duration of exposure, within the 315-320 nm region. Parallel with the latter changes the intensity of the bilirubin absorption peak declined (unpublished data, not shown). Together, these results illustrate the ability of bilirubin to exert an antinitrosative influence against a variety of NO congeners. Furthermore, the data measuring NO release from plasma using a NO electrode highlight the potential of bilirubin as a physiological scavenger of RNS *in vivo*. These observations concur with those presented by Kaur *et al.*⁶⁵⁶ with regards the ability of bile pigments to attenuate nitrosative stress caused by different types of RNS.

6.6 Conclusion

Collectively, these results reveal a potential new physiological role for the bile pigments, particularly bilirubin, as a means to assuage the adverse effects of nitrosative stress. The results observed would only become relevant in situations where NO is in excess and the potential scavenging capacity of other targets, e.g. thiols in the form of GSH^{691,692}, are saturated as Kaur and colleagues demonstrated decreased bilirubin degradation by RNS in the presence of NAC⁶⁵⁶. As the level of NO and activation of the haem oxygenase pathway to release bile pigments are intimately linked³⁷³, in situations where NO is limited GSH levels would not be diminished and thus HO-1 would not be activated. Any endogenous bilirubin generated by the haem oxygenase pathway would not necessarily be a threat to NO in situations where the bioavailability of the latter is limited, e.g. due to oxidative stress. Rather, HO-1-derived bilirubin would exert a protective influence where NO is limited due to the fact that bilirubin can attenuate oxidative stress

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386,529,533,535. Given that NO and its congeners have been implicated in a wide range of pathological conditions ²⁵, and in view of the fact that an 'uncontrolled' increased production of NO redox forms can strongly activate the haem oxygenase system leading to enhanced bilirubin production, the findings presented in this chapter merit further exploration both in *in vitro* and *in vivo* models to confirm the antinitrosative properties of bile pigments.

7 EFFECT OF GLYCERYL TRINITRATE ON HAEM OXYGENASE ACTIVITY AND HO-1 PROTEIN IN CARDIOMYOCYTES

7.1 Introduction

Glyceryl trinitrate (nitroglycerin; GTN (MW 227.09), Figure 7.1) is a potent organic nitrate vasodilator that has been used clinically for the past 125 years to treat a number of cardiovascular complications, e.g. angina pectoris, acute and chronic episodes of myocardial infarction and congestive heart failure ⁶⁹³.

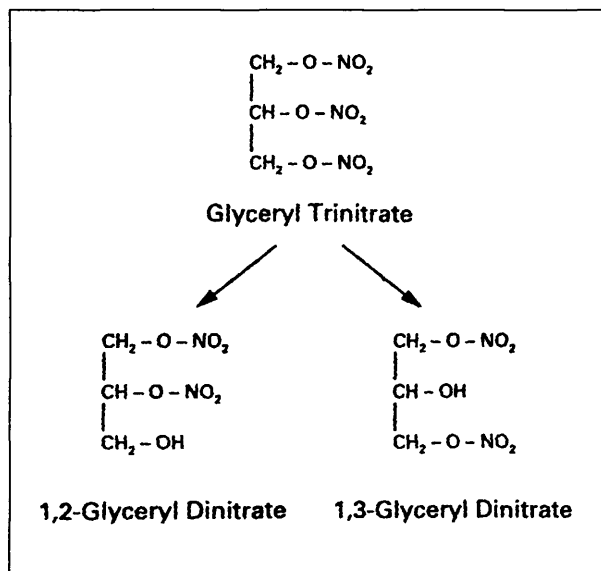


Figure 7.1 Chemical structure of glyceryl trinitrate and its metabolites, 1,2-glyceryl dinitrate and 1,3-glyceryl dinitrate.

Adapted from ⁶⁹³.

EFFECT OF GTN ON HAEM OXYGENASE

Given its long term therapeutic use, facile delivery, variable formulations (tablet, spray and patches) and routes of administration (buccal, sublingual and transdermal) *[Note: oral administration is not feasible due to inactivation via rapid first-pass metabolism] the precise mechanism(s) of GTN action still remains unresolved and controversial^{693,694}. A major drawback of GTN therapy is the development of nitrate tolerance, i.e. loss of clinical efficacy due to its continuous administration¹⁹². Nitrate tolerance is a complex phenomenon possibly involving specific vascular and biochemical responses. The subject is currently of much debate and still unresolved; however, it appears that both aspects of GTN, i.e. its mode of action and nitrate tolerance, are interlinked⁶⁹³.

From a mere pharmacological point of view, GTN is considered a prodrug as it requires cellular metabolism to yield 1,2-glycerol dinitrate (1,2-GDN), 1,3-glycerol dinitrate (1,3-GDN) (see Figure 7.1 for structures), inorganic nitrite (NO_2^-) and NO (or a NO congener e.g. R-SNO) in variable amounts and ratios⁶⁹⁵. In general, the therapeutic effects of GTN are mediated by its biotransformation to NO, the active metabolite, which in turn activates the enzyme sGC, leading to smooth muscle relaxation^{4,693}. Two pathways have been proposed for the latter reaction: 1. clearance-based metabolism or a detoxification pathway which produces NO_2^- that has no direct cardiovascular effect; and 2. mechanism-based biotransformation that requires a chemical mechanism to produce an activator of sGC, e.g. NO. In addition to NO_2^- , both pathways generate the active metabolites 1,2-GDN and 1,3-GDN, although 1,2-GDN predominates *in vivo*^{192,345,693}. So far the factor(s) responsible for

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the metabolic denitration and reduction of GTN to NO has not been identified; however, it is known that the reaction takes place within microsomal membranes, is enhanced by reducing equivalents, e.g. thiols, and is catalysed by nonenzymatic and enzymatic processes ⁸.

Nonenzymatic production of NO by reduced intracellular thiols was the first proposed mechanism of GTN biotransformation, and oxidation or depletion of thiols was associated with tolerance ³⁴. These theories have since been contradicted and thiols have been suggested as cofactors ⁶⁹⁶, despite the fact that thiol containing drugs (e.g. *N*-acetylcysteine) can enhance the effects of GTN in the absence of tolerance ⁶⁹³ or overcome tolerance ³⁴⁶. Four enzymatic pathways have been proposed for GTN biotransformation: 1. glutathione-S-transferase (GST); 2. microsomal CYP-450 or the CYP-450 reductase system; 3. an unidentified membrane-bound pathway; and 4. mitochondrial aldehyde dehydrogenase (mtALDH) which metabolises GTN to 1,2-GDN and NO₂⁻ ^{345,695}. In all these examples, the role of these enzymes in GTN metabolism and nitrate tolerance is ambiguous. Of note is the crucial role of thiols, either as cofactors or part of an enzyme (except in CYP-450s), for the interaction and metabolism of GTN and activation of sGC ⁶⁹⁴, although this (thiol) induction is weak compared with NO ⁶⁹⁷.

So far, the present Thesis has investigated the potential of NO[•]/NO releasing agents (AS, DeaNO and DetaNO) to activate HO-1 and dissected the biochemistry associated with these reactions *in vitro*. However, these agents are not clinically used unlike GTN, a pharmacological agent that exhibits NO bioactivity. Since NO derived from NO releasing compounds is capable of

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interacting with the HO-1 pathway, one aspect of this subject that might be clinically relevant is to examine the effects of GTN on haem oxygenase activity and HO-1 protein expression in cardiomyocytes. Although the concentration of GTN administered in these experiments was greater than clinically observed, i.e. $0.5 \mu\text{M}$ ³⁴⁵, the purpose of this chapter was not to make a comparison with a clinical scenario, rather to examine if GTN has the ability to modulate the haem oxygenase pathway. Indeed, changes in cardiac haem oxygenase activity and HO-1 protein were noted in this model following administration of GTN and were in agreement with data reported by Minamiyama *et al.* in rat hearts exposed to GTN⁶⁹⁸. The findings will be discussed with regards their potential implications in the mechanism required to alleviate vascular dysfunction.

7.2 Objective

The intention of this chapter is to explore a possible link between a NO-generating compound that is used for the treatment of vascular dysfunction (GTN) and its ability to induce haem oxygenase activity and HO-1 protein expression, and briefly discuss the possible clinical implications of these findings.

7.3 Materials and Methods

7.3.1 Preparation of reagents

Ampoules of glyceryl trinitrate (GTN, Nitrocine®, 1 mg/ml, 4 mM) were provided *free gratis* by Schwarz Pharma Ltd (Chesham, Bucks., England). Stock solutions of *N*-acetylcysteine (NAC, 100 mM) and hydroxocobalamin

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(HCB, 50 mM) were dissolved in 5% (v/v) NaOH (2 M) and dH₂O, respectively. The latter reagents were prepared fresh at the time of each experiment in opaque Eppendorfs and placed on ice until required.

7.3.2 Maintenance and treatment of cells

Rat H9c2 cardiomyocytes were cultivated in DMEM as previously described⁶⁴⁷. The agents were applied to cells directly either alone or in combination in DMEM at the concentrations shown in each graph. Assays for haem oxygenase activity and determination of HO-1 protein expression were performed as described in Sections 2.3.4 and 2.4.2, respectively. Negative controls, which consisted of vehicle alone, were included in all experiments.

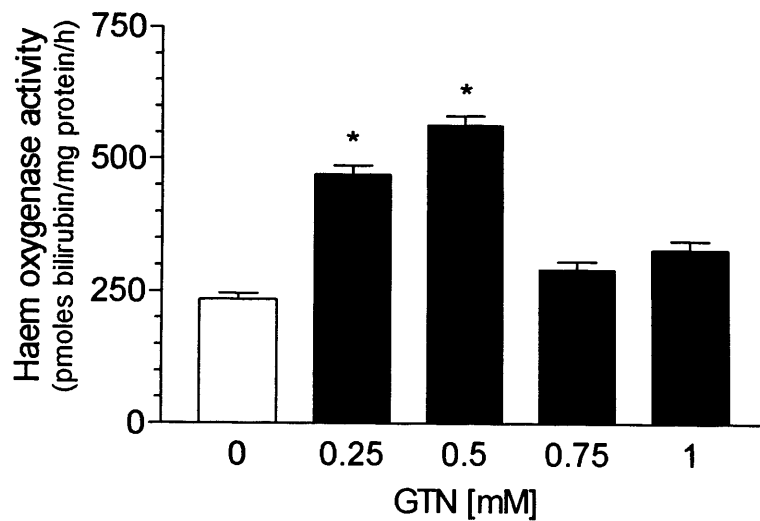
7.4 Results

7.4.1 Concentration-dependent effect of glyceryl trinitrate (GTN) on haem oxygenase activity and HO-1 protein expression in H9c2 cells

To confirm a biochemical link between GTN and the haem oxygenase pathway, cardiomyocytes were exposed to increasing concentrations (0.25-1 mM) of the organic nitrate in complete DMEM. After 6 h incubation cells were collected and assayed for haem oxygenase activity and HO-1 protein expression. As illustrated in Figure 7.2 **A** GTN promoted an increase in haem oxygenase activity up to 0.5 mM relative to control (566.4 ± 17.9 (0.5 mM GTN) vs. 234.7 ± 12.4 (control) pmoles bilirubin/mg protein/h; $P < 0.05$) followed by a decline to near baseline at subsequent concentrations. A corresponding pattern in the expression of HO-1 protein was found using the same concentrations of GTN (Figure 7.2 **B**; $n = 1$).

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A



B

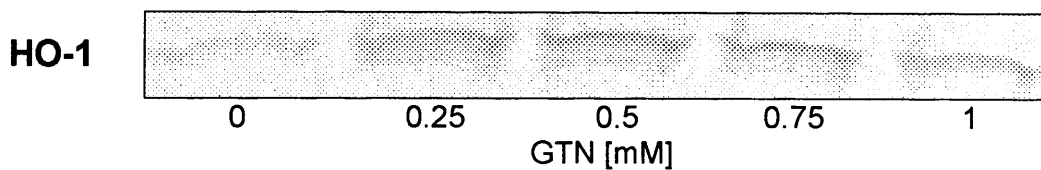


Figure 7.2 Concentration-dependent induction of haem oxygenase activity and HO-1 protein expression by glyceryl trinitrate.

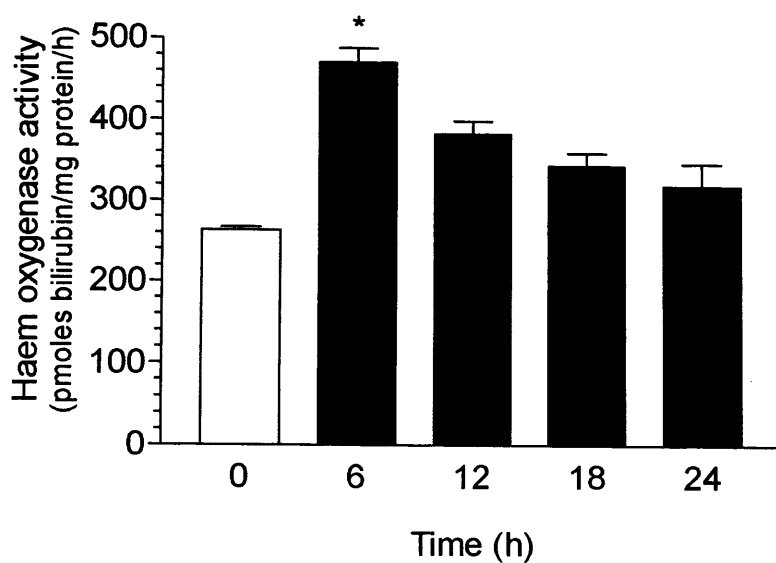
Cells were treated directly with glyceryl trinitrate (GTN, 0, 0.25, 0.5, 0.75 or 1 mM) in fresh complete DMEM. After 6 h haem oxygenase activity (**A**) and HO-1 protein expression (**B**) were determined according to Sections 2.3.4 and 2.4.2, respectively. Control (open bar) represents cells exposed to fresh DMEM alone. The Western blot panel is representative of one experiment. Bars represent the mean \pm S.E.M. of $n = 4$ experiments. * $P < 0.05$ vs. Control.

7.4.2 Time-dependent effect of glyceryl trinitrate (GTN) on haem oxygenase activity and HO-1 protein expression in H9c2 cells

Since 0.5 mM GTN promoted the maximal level of haem oxygenase activity and HO-1 protein induction, this concentration was chosen to assess the effect of GTN on haem oxygenase activity and HO-1 protein over a 24 h period. Figure 7.3 **A** shows that GTN caused a time-dependent increase in haem oxygenase activity with maximal levels attained at 6 h (459.9 ± 35.22 pmoles bilirubin/mg protein/h; $P < 0.05$) and a gradual decrease in activity at successive time points (from 396.6 ± 30.92 (12 h) to 322.4 ± 55.85 (24 h) pmoles bilirubin/mg protein/h; $P > 0.05$). The expression of HO-1 protein (Figure 7.3 **B**; $n = 1$) correlated with the activity data, i.e. strongest intensity at 6 h followed by weaker, but rather similar, intensities for the remaining duration of exposure to GTN. Collectively, the results in Figure 7.2 and Figure 7.3 reveal that GTN has the capacity to promote an increase in haem oxygenase activity and HO-1 protein expression at various concentrations and time points.

EFFECT OF GTN ON HAEM OXYGENASE

A



B

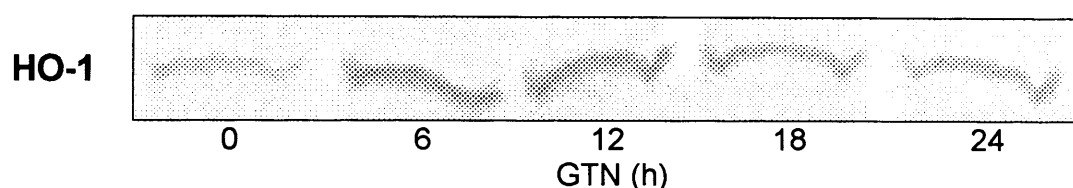


Figure 7.3 Time-dependent induction of haem oxygenase activity and HO-1 protein expression by glyceryl trinitrate.

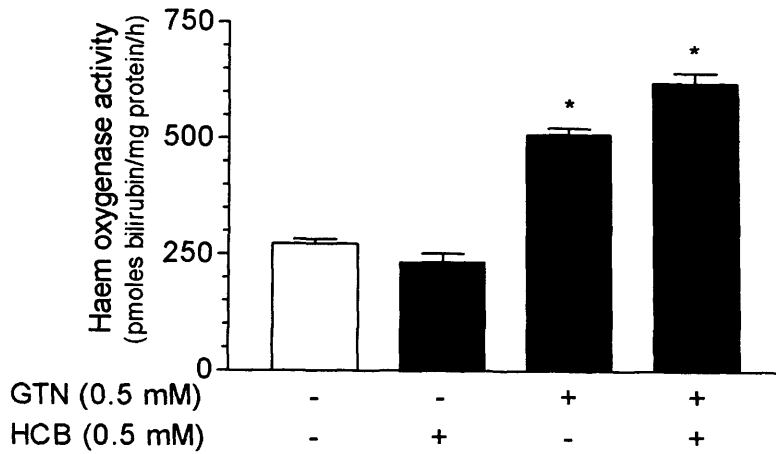
Cells were treated directly with glyceryl trinitrate (GTN, 0.5mM) for 6, 12, 18 or 24 h in fresh complete DMEM. After 6 h haem oxygenase activity (**A**) and HO-1 protein expression (**B**) were determined according to Sections 2.3.4 and 2.4.2, respectively. Control (open bar) represents cells exposed to fresh DMEM alone. The Western blot panel is representative of one experiment. Bars represent the mean \pm S.E.M. of $n = 4$ experiments. * $P < 0.05$ vs. Control.

7.4.3 Interaction of glyceryl trinitrate (GTN) with thiols and a nitric oxide (NO) scavenger

Previous reports have highlighted the ability of thiols and NO scavengers to attenuate the activation of the haem oxygenase pathway, by NO and its congeners, in various cell types^{668,681}. Therefore, the potential of using such agents to modulate the induction of haem oxygenase activity in response to GTN was investigated. Following exposure of cardiomyocytes to a fixed concentration (0.5 mM) of GTN in the presence or absence of the NO scavenger HCB (0.5 mM) there was a significant ($P < 0.05$) increase in haem oxygenase activity (Figure 7.4 A). Of note, is the observation that GTN plus HCB enhanced haem oxygenase activity compared with GTN alone (665.0 ± 46.98 (GTN plus HCB) vs. 510.5 ± 24.96 (GTN alone) pmoles bilirubin/mg protein/h, respectively; $P < 0.05$). As expected, HCB alone did not promote any significant change in haem oxygenase activity. When similar concentrations (0.5 mM) of GTN and the glutathione precursor NAC were coincubated there was no reduction in the level of haem oxygenase activity compared with GTN alone (Figure 7.4 B), i.e. both scenarios promoted a significant ($P < 0.05$), but similar, degree of haem oxygenase induction, whereas NAC alone did not enhance haem oxygenase activity.

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A



B

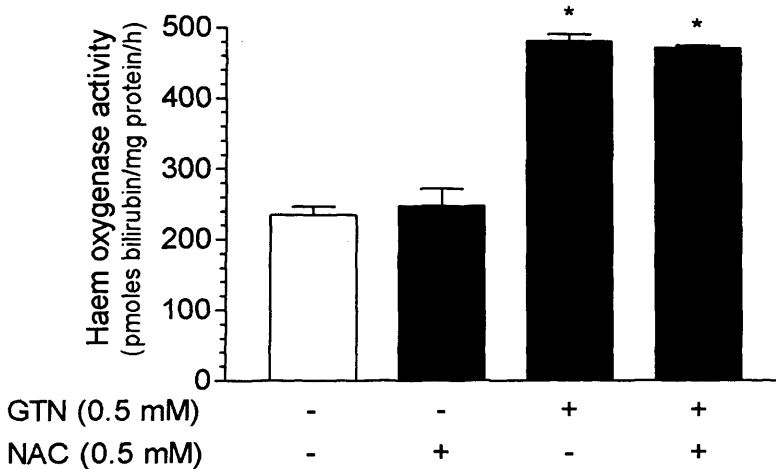


Figure 7.4 Induction of haem oxygenase activity by glyceryl trinitrate is not attenuated in the presence of a nitric oxide scavenger or thiols.

Cells were exposed directly to glyceryl trinitrate (GTN, 0.5 mM) in the absence (-) or presence (+) of an equal concentration of hydroxocobalamin (HCB) **(A)** or *N*-acetylcysteine (NAC) **(B)** in complete DMEM. After 6 h haem oxygenase activity was determined according to Section 2.3.4. Control (open bar) represents cells exposed to fresh DMEM alone. Bars represent the mean \pm S.E.M. of $n = 4$ experiments. * $P < 0.05$ vs. Control.

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7.4.4 Effect of nitrate (NO₃⁻) on haem oxygenase activity

As its name suggests GTN has NO₃⁻ as part of its chemical structure, in fact it has three NO₃⁻ molecules. To investigate the contribution of NO₃⁻ to GTN-mediated activation of the haem oxygenase pathway an experiment was conducted using GTN (0.5 mM) and NO₃⁻ (1.5 mM, as there are three NO₃⁻ molecules per GTN). Following a 6 h incubation with cells the haem oxygenase activity assay (see Figure 7.5) revealed that NO₃⁻ did not influence the latter parameter ($P > 0.05$), but gave a reading comparable to control (293.4 ± 31.14 (control) vs. 284.8 ± 3.82 (NO₃⁻) pmoles bilirubin/mg protein/h). In the presence of GTN there was a significant increase in haem oxygenase activity compared to control (573.8 ± 20.76 vs. 293.4 ± 31.14 pmoles bilirubin/mg protein/h, respectively; $P < 0.05$). Therefore, it is GTN and not NO₃⁻ that activates the haem oxygenase pathway.

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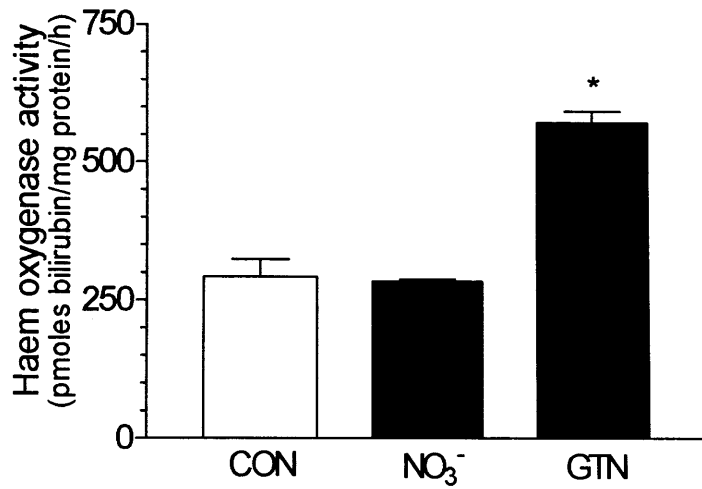


Figure 7.5 Nitrate does not influence haem oxygenase activity.

Cells were exposed directly to glyceryl trinitrate (GTN, 0.5 mM) and nitrate (NO₃⁻, 1.5 mM) in complete DMEM. After 6 h haem oxygenase activity was determined according to Section 2.3.4. Control (open bar) represents cells exposed to fresh DMEM alone. Bars represent the mean \pm S.E.M. of $n = 4$ experiments. * $P < 0.05$ vs. Control.

7.5 Discussion

To date no studies have measured the parameters of haem oxygenase activity and HO-1 protein expression following exposure of cells to GTN. Herein, the clinically used organic nitrate GTN enhanced haem oxygenase activity and HO-1 protein expression in a concentration- and time-dependent manner in cardiac cells. Previous experimental evidence suggests that GTN mediates its pharmacological effect, vasorelaxation of vascular smooth muscle cells, via enzymatic or nonenzymatic biotransformation within the microsomal membrane to specifically yield NO and 1,2-GDN, while loss of this activity may account for tolerance^{8,695}. Therefore, GTN-derived NO enhances haem oxygenase activity and HO-1 protein expression, an observation observed in other cells types using different sources of NO^{374,670}. In this model cells exposed to high concentrations of GTN (0.75 and 1 mM) or a fixed concentration (0.5 mM) for prolonged durations (12, 18 or 24 h) both led to an attenuation in the measured parameters. A possible reason for this is that the cells became tolerant to the effects of GTN under these conditions⁶⁹⁶. A number of theories have been advanced for this phenomenon including decreased formation of NO from GTN, depletion of intracellular thiols and altered sGC activation, enhanced phosphodiesterase activity and production of vascular O₂⁻⁶⁹⁷.

Data from Chapter 4, as well as from previous groups, have highlighted the ability of thiols, particularly NAC, to attenuate haem oxygenase induction by NO, its congeners or other factors^{482,668,699,700}. However, the results in this chapter suggest that NAC is unable to allay an increase in haem oxygenase

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activity caused by GTN. A possible reason for this is that NAC may be promoting the biotransformation of GTN in this model. In Chapter 4 HCB attenuated NO⁻-mediated induction of haem oxygenase activity. The inhibitory aspects of HCB can also be mediated against NO and its redox congeners in various cell types^{49,452,668,678,701}. However, in this model HCB was unable to attenuate haem oxygenase activity, in fact, it enhanced GTN-mediated activation. A recent publication by Chambers *et al.* suggests that B vitamins, e.g. vitamin B_{12a} or HCB, may increase the availability of NO through reduction of free plasma homocysteine (Hyc), thus enhancing vascular endothelial function⁷⁰² and haem oxygenase activity in the current scenario. Using endothelial cells Sawle and coworkers noted that Hyc attenuated haem oxygenase activity induced by the NO donor SNP⁶⁷⁹. Thus, HCB may be thwarting the negative impact of Hyc towards the endogenous cytoprotectant HO-1 which enhances the potency of GTN. Although the potential of the GTN metabolites 1,2-GDN and 1,3-GDN to induce haem oxygenase activity was not investigated the data reveals that NO₃⁻ does not influence the effect of GTN.

7.6 Conclusion

The aim of this chapter was to examine a possible direct link between GTN and the haem oxygenase pathway. The data reveals this hypothesis to be true, although the mechanism by which this is achieved is not entirely clear and differs with respect to the current knowledge of NO donors and HO-1 induction. Given that GTN mediates blood vessel relaxation via bioactivation to NO, and the latter can upregulate the HO-1 gene, which in turn releases the

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vasoactive gas CO and antioxidant bilirubin, the current results suggest that the products of the haem oxygenase pathway may enhance the vasodilatory capacity of GTN. The lengthy (over 125 years) clinical use of GTN for the treatment of certain cardiovascular disorders and the inability to fully explain its mechanism(s) of action makes scientists wonder whether additional biochemical pathways may be involved. The data presented in this Thesis merits further investigation *in vivo*. It is plausible to suggest that the beneficial actions of NO-derived GTN may ultimately involve the generation of a stable signalling factor (i.e. CO) and a potent antioxidant (i.e. bilirubin), both required to restore the function of impaired vascular activities.

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8.1 Background to current work

Each day a myriad of important biological and chemical reactions take place within our cells, tissues and organ systems to sustain life. At the same time potentially toxic waste products are generated; among these are the prosthetic haem moiety(ies) of denatured haemoproteins, e.g. haemoglobin and cytochromes. Due to the potential toxicity of uncomplexed 'free' haem and its central iron atom mammals have developed highly sophisticated and regulatory processes to catabolise, compartmentalise and recycle these molecules³⁶¹. The most effective means of haem catabolism, also known as the haem oxygenase pathway, occurs in two steps: 1. oxidative cleavage of haem's tetrapyrrole ring by the rate-limiting enzyme microsomal haem oxygenase which generates biliverdin concomitant with the release of CO and Fe²⁺; and 2. reduction of biliverdin to bilirubin by the cytosolic serine/threonine kinase, biliverdin reductase^{364,446}. The resulting Fe²⁺ is sequestered by the parallel induction of the iron storage protein ferritin, thus both proteins (haem oxygenase and ferritin) act in unison to prevent iron toxicities and maintain iron homeostasis^{484,485,703}, while CO is expired via the lungs⁵⁸² and bilirubin is made water soluble via glucuronidation in the liver and excreted in the bile

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The existence of the haem oxygenase system was first reported by Tenhunen and colleagues in the late 1960s^{363,413}. Since then three distinct isoforms of the enzyme haem oxygenase have been identified in mammals: the inducible

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isoform haem oxygenase-1, HO-1 (also known as HSP-32)⁴¹⁵; a constitutive isoform, HO-2⁴³⁶; and a weak haem metaboliser, HO-3⁴¹⁸. Of these, HO-1 and HO-2 have been fully characterised and shown to exhibit a number of differences, principally, HO-1 is extremely sensitive to a wide variety of stress-inducing stimuli to which HO-2 is refractory³⁷¹. Therefore, HO-1, rather than HO-2, is the most studied of the two isoforms. A unique feature of haem is that it serves as both a substrate and inducer of HO-1, thus, for two decades the perception of the haem oxygenase pathway was purely as an important, but unassuming, means to degrade and control intracellular haem levels³⁸². However, from the late 1980s this opinion was profoundly changed, and is still being challenged, with the accumulation of convincing evidence showing that induction of the ubiquitous and redox-sensitive protein HO-1 decreases the cellular haem (prooxidant) level and elevates the production of bilirubin, which exhibits potent antioxidant characteristics^{385,529}. Furthermore, CO may exert a positive influence in vasorelaxation³⁷⁷, neurotransmission⁵⁰⁶ and inhibition of platelet aggregation⁵⁹⁴, in a manner similar to the gaseous molecule NO^{504,704,705}. In addition, iron released from HO-1 regulates several genes including: ferritin; transferrin (an iron binding protein); NOS; and HO-1 itself^{368,384}.

The discovery that enhanced *de novo* synthesis of HO-1 could occur in response to an extensive array of chemically and structurally diverse non-haem agents, many of which have the ability to alter the intracellular redox state, led to a deeper interest into the mechanisms controlling HO-1 induction^{381,431,432}. As a result, HO-1 soon gained the reputation as an effective

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endogenous cytoprotectant towards oxidative stress, especially in conditions whereby other cellular antioxidants, e.g. glutathione, become exhausted ⁴²⁶. This led to the notion that induction of HO-1, concomitant with the release of bilirubin and CO, could be exploited as a means to thwart the adverse effects associated with pathologies arising from an oxidative source in the cardiovascular ^{428,430,480}, immune ⁷⁰⁶ and neurological ^{372,483,554} systems. However, HO-1 may also exert a prooxidant influence depending upon the cellular redox potential and the metabolic fate of iron ^{486,707}.

The concept that unabated production of ROS leads to tissue oxidative stress resulting in the development of a variety of pathologies is now universally accepted ⁷⁰⁸. Similarly, compelling evidence exists to support the notion that activation of the HO-1/bilirubin/CO pathway is an effective means to thwart the adverse effects associated with oxidative stress ^{370,386,470}. More recently, the gaseous molecule NO and its redox congeners, known collectively as RNS, have been implicated in another form of cellular damage identified as nitrosative stress ^{338,709}. Recent data has shown an enhancement of HO-1 protein, mRNA and haem oxygenase activity in response to NO and other RNS ^{374,427,481,669,681}. In light of these observations it has been proposed that the haem oxygenase system is an effective stratagem to combat the adverse effects associated with nitrosative stress ^{373,636}.

Since the discovery that NO mediates the vasodilatory actions of EDRF ⁷ this diatomic gaseous molecule has been implicated in a diverse range of physiological and pathophysiological processes within the vascular ²⁰⁹, immune ³⁰⁵, neurological ⁷¹⁰ and reproductive ^{320,323,330} systems. More recent

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research has broadened to take account of the diverse chemistry of the NO group, in particular oxidation (loss of an electron) and reduction (gain of an electron) type reactions, to generate the NO^+ and NO^- ^{13,25,29,58}, respectively (Equation 8.1).



Equation 8.1 Redox reactions of nitric oxide

Understanding how these and other NO related redox products, e.g. NO_3^- , NO_2^- , R-SNO, ONOO^- and nitrosyl metal complexes, are formed enables a better appreciation of how NO biochemistry relates to its biological effects^{13,29}. Using R-SNO and the redox products derived from it, i.e. oxidised thiols (SSR), sulfenic acid (SOH), sulfinic acid (SO_2H) and sulfonic acid (SO_3H), as an example, the importance of associating NO chemistry to its biological outcome is illustrated in Figure 8.1.

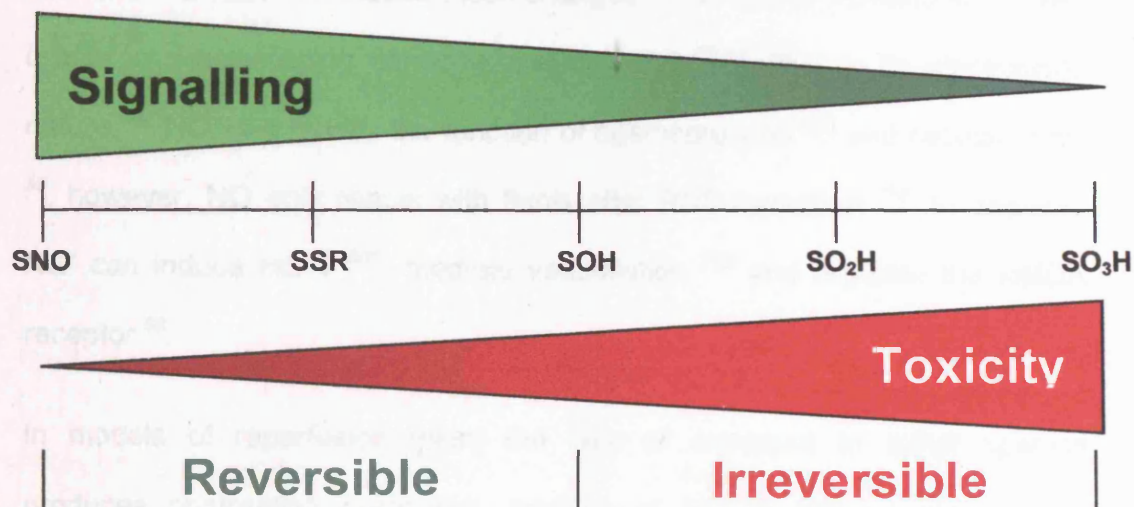


Figure 8.1 Relationship between NO chemistry and its biological effects.

Adapted from ⁷¹¹.

In the above diagram there is an inverse correlation between signalling and toxicity with respect to the redox subtype produced as a result of successive rounds of oxidation. Of significance is the reversible nature of R-SNO-related signalling process, whereas production of stronger acids, i.e. SO₂H and SO₃H, leads to cellular damage due to irreversible toxicity.

Although there is still much debate about the final product of NOS oxidation of L-arginine, i.e. is it NO or NO⁻ ^{43,45,671}, the chemical biology of the latter has assumed greater importance. Despite its short lived nature (i.e. milliseconds) studies have shown that under certain conditions NO⁻ can be generated *in vitro* and *in vivo* ^{25,46}. Furthermore, studies of the biological, toxicological and pharmacological aspects of NO and NO⁻ in these situations reveal distinct features in each case ⁶¹. For example, high concentrations of NO⁻ reacts with O₂ to generate potent oxidising species capable of damaging DNA ^{352,353},

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whereas NO does not induce these changes under similar conditions, instead it acts in a scavenging capacity towards ROS^{57,58}. Due to its electrophilic nature³⁵¹ NO⁻ can modify the function of haemoproteins⁶⁷² and cellular thiols³⁰; however, NO only reacts with thiols after RNS formation⁷¹². In addition, NO⁻ can induce HO-1⁶⁸¹, mediate vasodilation⁷¹³ and regulate the NMDA receptor⁵⁶.

In models of reperfusion injury the time of exposure to either species produces contrasting outcomes. Addition of NO or NO⁻ at the time of reperfusion endows protection against injury⁷¹⁴ or augments infarct area and tissue damage⁵³, respectively. Interestingly, exposure to NO⁻ before ischaemia endowed greater protection against subsequent reperfusion injury compared with NO donors⁴⁶. *In vivo* experiments in healthy and unhealthy dogs revealed that NO⁻ enhanced left ventricular contractility (i.e. it is positively inotropic) and lowered cardiac preload and diastolic pressure, via increasing the release of calcitonin gene-related peptide (CGRP), without altering heart rate. All these aspects of NO⁻ contrast with the effects observed with NO in the same model⁵⁴. These examples illustrate that the orthogonal (i.e. of the same origin but not overlapping) aspects of the redox siblings NO⁻ and NO with regards their pharmacological, physiological and biological effects is highly condition dependent⁶¹.

8.2 Hypothesis and objectives

As noted earlier, the haem oxygenase pathway is extremely sensitive to activation by NO-mediated redox signalling in addition to being an effective means to thwart cellular stress arising from RNS. Due to the complexity of this induction it is not known if it is NO, NO⁺ or NO⁻ that elicits this response.

Therefore, the hypothesis of this Thesis was as follows:

Increased haem oxygenase activity and induction of HO-1 protein and gene expression in response to reactive nitrogen species, particularly NO⁻, attenuates nitrosative stress in cardiac cells.

To verify this theory a number of objectives were set:

- (i) Establish that Angeli's salt (AS), a NO⁻ generator, activates the haem oxygenase pathway.
- (ii) Dissect the mechanism of AS-mediated HO-1 induction.
- (iii) Investigate the mechanism and identify possible molecular targets which lead to increased haem oxygenase activity and HO-1 expression in response to cellular stress.
- (iv) Utilise bilirubin, an end product of the haem oxygenase pathway, as an effective means to counteract nitrosative stress.
- (v) Examine the potential of a clinically used NO donor, GTN, to promote activation of the haem oxygenase pathway.

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The data presented in Chapter 3 illustrated the novel finding that Angeli's salt (AS) was able to promote an increase in cardiac haem oxygenase activity, HO-1 protein and gene expression both in a time- and concentration-dependent manner. This induction occurs at the transcriptional level as actinomycin D completely eliminated AS-mediated activation of HO-1 mRNA. Furthermore, the results related to the transcription factor Nrf2 (detailed in Chapter 5) suggest that the latter is a potential transcriptional target in the response of HO-1 towards RNS. Even though AS has a short half-life ($t_{1/2} = 2.3$ min) sustained stimulation of the haem oxygenase pathway by multiple additions of micromolar concentrations of AS, a scenario likely to occur during prolonged NO^- production under physiological/pathological conditions, led to levels of haem oxygenase activity comparable to a bolus dose of this agent. This suggests a potential clinical technique to maintain continuous stimulation of the haem oxygenase pathway by AS, or other types of nitrosative agents, without causing adverse toxicity.

In addition, only freshly prepared AS had the ability to enhance haem oxygenase activity, either in DMEM or Dulbecco's phosphate buffered saline (DPBS), while 'decomposed' AS (see Section 2.2.3 for preparation) had no inducing potential. Although DMEM promoted a greater degree of induction compared with DPBS one cannot exclude the possibility of the media constituents promoting the formation of other RNS, thus augmenting haem oxygenase activity levels. Comparison between AS and a fast (DeaNO) and slow (DetaNO) acting NO donor revealed that DeaNO ($t_{1/2} = 2$ min) upregulated haem oxygenase activity and induced HO-1 protein in a manner

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similar to that seen with AS, although AS was the most tolerable of the three agents examined via the Alamar blue assay.

Having verified that NO^- derived from AS can directly stimulate an increase in haem oxygenase activity and induce HO-1 expression the intention of Chapter 4 was to explore the mechanism behind these responses. Akin to the diverse biology and complex chemistry of NO its reduction product, NO^- , has its own unique biochemistry^{39,58,61,352}. The latter aspect of NO^- was examined further in the context of AS-mediated HO-1 induction using a number of *in vitro* tools, including: 1. promotion of NO^- oxidation to NO; 2. scavengers of NO and NO^- ; 3. modulation of thiol groups; and 4. measurement of NO release from AS and the NO donors DeaNO and DetaNO. The incentive for these experiments stemmed from data suggesting that transition metals, e.g. Cu^{2+} and Fe^{3+} , can promote the oxidative conversion of NO^- to NO both *in vitro* and *in vivo*^{53,63}, while intracellular thiols, potential targets for NO and NO^- , can influence the magnitude of these redox interconversions^{13,30,47}. In this system copper sulphate (CuSO_4) was employed to oxidise NO^- to NO and thus assess the contribution of these congeners towards the modulation of haem oxygenase activity and HO-1 protein expression. The data revealed that coadministration of AS and CuSO_4 led to an enhancement of haem oxygenase activity and HO-1 protein expression. However, one cannot exclude the possibility that generation of other RNS by media constituents amplified this oxidation.

To eliminate the contribution of NO derived from the metabolism of AS towards the measured parameters, the NO trapping agent carboxy-PTIO (C-PITO, 25 μM) and NO^-/NO scavenger hydroxocobalamin (HCB, 0.75 mM)

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were coincubated with cells. Inclusion of HCB in the medium with AS resulted in a significant attenuation of haem oxygenase activation, but C-PTIO was unable to promote a similar response. The inability of C-PTIO to attenuate haem oxygenase activity suggests that AS-derived NO^- , rather than NO, contributes towards HO-1 induction. This observation was strengthened by comparing the effect of AS and DeaNO as these agents, respectively release either NO^- or NO species at physiological pH and have very similar half-lives (AS $t_{1/2} = 2.3$ min and DeaNO $t_{1/2} = 2$ min). Experiments using a sensitive NO electrode, which can discriminate between NO^- and NO species, revealed that in DMEM, containing comparable concentrations (0.5 mM) of both agents, almost 33-fold less NO was released from AS compared with DeaNO. However, DeaNO (20 μM) releases the same equivalents of NO ($0.31 \pm 0.01 \mu\text{M} \times \text{sec}$) as 0.5 mM AS. When 20 μM DeaNO and 0.5 mM AS were applied to cells, only AS promoted the greatest degree of haem oxygenase induction. Additional experiments into AS metabolism revealed that it is NO^- liberated from AS, rather than nitrite (NO_2^-), the product of NO^- protonation, dimerization, and dehydration, which is responsible for the observed results. Collectively, the above data suggests that NO^- , rather than NO, liberated from the metabolism of AS, is the principal factor responsible for increasing haem oxygenase activity and HO-1 transcription in this model. However, this balance can be shifted towards NO when the extent and rate of oxidation of NO^- to NO is significantly increased.

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It is known that thiols participate in the modulation of HO-1 expression by NO and NO-related species possibly through the formation of endogenous R-SNO^{423,668,679}. In this study, the glutathione precursor *N*-acetylcysteine (NAC), promoted a significant reduction in the AS-mediated induction of haem oxygenase activity and HO-1 protein expression in both DMEM and DPBS. This indicates that redox activation of thiols groups by NO[•] is a plausible mechanism for the transcriptional activation of the HO-1 gene. This idea is reinforced by experiments using the glutathione synthesis inhibitor buthionine sulfoximine (BSO), which decreased the strength of haem oxygenase activity elicited by AS. The results from thiol modulation, along with the data related to actinomycin D from Chapter 3, indicate that thiols, in particular glutathione, which represents the largest pool of intracellular thiols, contribute towards the preservation of NO[•] bioactivity and signalling properties for the transcriptional activation of inducible genes, e.g. HO-1³¹. The importance of thiols in the regulation of the haem oxygenase pathway is emphasised by the fact that they, most notably in the form of GSH, represent the first barrier against cellular stress while their depletion activates the second line of cell defence enzymes, e.g. HO-1^{343,426,692,715,716}.

The relationship between intracellular thiols and the haem oxygenase pathway was further illustrated by the lack of HO-1 induction in the presence of NAC, i.e. the latter provides more GSH, thus diverting NO[•] away from critical thiol groups within transcription molecules, e.g. Nrf2. Although the experiments combining AS with BSO, a known depletor of GSH and inducer of HO-1 mRNA expression⁴²⁶, were expected to reveal enhanced haem

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oxygenase activity this was not the case. An explanation for this finding could be that BSO is blocking glutathione production but not necessarily thiol groups which are constitutive components of the signal transduction molecule Nrf2. To further probe the basis for these observations it would be necessary to employ a specific thiol blocker, e.g. N-ethylmaleimide (NEM) or mercuric chloride (HgCl_2). However, as both these agents are toxic to cells we can speculate that targets other than Nrf2 may be involved in HO-1 amplification.

The principal function of the haem oxygenase pathway is to metabolise the prooxidant haem (ferro-(Fe^{2+})protoporphyrin IX) to bilirubin, CO and Fe^{2+} ³⁶⁴. Using the information obtained from Chapters 3 and 4 it was decided to examine the responsiveness of the haem oxygenase system to simultaneous exposure to oxidative (hemin, the oxidised (Fe^{3+}) form of haem) and nitrosative (AS or DeaNO) stress in rat cardiomyocytes. Also, the signalling pathways leading to transcriptional activation of the HO-1 gene was investigated in parallel. Akin to previously documented reports, the data from Chapter 5 shows that addition of hemin (5-20 μM), AS (0.75 mM) or DeaNO (0.5 mM) directly to cells promoted an increase in haem oxygenase activity, HO-1 protein and mRNA expression ^{374,681}. Furthermore, the intensity of the latter parameters were strongly enhanced when hemin was coincubated with AS or DeaNO. Of note, is the observation that the synergistic capacity of hemin and the nitrosants (AS or DeaNO) to activate the haem oxygenase pathway is maintained following exposure of cells to agents that were preincubated for 1 h at 37°C in complete DMEM beforehand, whereas decomposed AS or DeaNO on their own were ineffective. Although NO^- and

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NO have differing degrees of affinity for haem ^{19,67,185,684} the novelty of these findings is that an interaction is taking place between hemin and NO⁻ or NO and the resulting haem-nitrosyl complex is sufficiently potent to cause an amplification of the haem oxygenase pathway response, even under conditions of reduced oxygen tension (hypoxia).

Moreover, the presence of hemin during the preincubation step maintains the biological potency of AS towards increasing haem oxygenase activity and HO-1 protein expression compared with previous assays in Chapters 3 (Figure 3.5) and 4 (Figure 4.2 and Figure 4.5) where decomposition of this agent was involved. As formation of a stable haem-nitrosyl complex is enhanced by serum proteins, e.g. albumin, which can bind haem ⁶⁸⁵, this detail must be considered in this model as the DMEM contains a high concentration (10%) of serum proteins, i.e. FBS. Recent experiments involving preincubation of AS (20, 50 or 100 μ M) with hemin (1 μ M) for 1 h at 37°C in a low (0.5%) FBS environment revealed that the haem oxygenase inducing capacity of the haem-nitrosyl complex was maintained in H9c2 and Girardi, a human cardiac cell line, cells (unpublished preliminary data, not shown). Taken together, this data illustrates the potential of utilising low micromolar concentrations of both an oxidant (hemin) and a nitrosant (AS) in a clinical scenario to promote increased production of the endogenous cytoprotectant HO-1. More experiments are now required to characterise the eventual haem-nitrosyl complex responsible for this amplification phenomenon.

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Further evidence of the potency and strength of the haem-nitrosyl complex was shown by the inability of high thiol concentrations, provided by NAC, to prevent the synergistic effect of AS and hemin on haem oxygenase activity, even though previous examples showed an attenuation of NO⁻/NO-mediated activation of the haem oxygenase pathway by NAC ^{423,668,681}. The acknowledged function of haem as a substrate for haem oxygenase and a molecular switch promoting the induction of the HO-1 gene in response to redox signalling involving RNS suggests the potential of the active products of haem metabolism, particularly bilirubin and CO, as a means to counteract nitrosative stress ^{354,636}. This concept has been investigated by Foresti *et al.* using aortic endothelial cells exposed to hemin and various NO donors ³⁷⁴. The authors noted enhanced HO-1 protein and haem oxygenase activity following increased haem uptake concomitant with an elevation in intracellular bilirubin production, while Clark *et al.* documented a similar effect with bilirubin production using bovine vascular smooth muscle cells in a model of oxidative stress ⁵³³. Collectively, these observations suggest the haem-nitrosyl complex provokes a similar response with the ultimate aim of neutralizing the prooxidant (haem) and pronitrosative (NO⁻/NO) environment. The participation of HO-2 in this response was not investigated; however, this merits further examination as this isoform has been linked with regulating the intracellular balance of NO ⁴⁵² and can protect against oxidative stress, via production of bilirubin ^{483,554}.

Given that AS and other agents promote the activation of HO-1 mRNA at the transcriptional level ^{457,681} the signalling pathways involved in this process are

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subject to intense investigation. One important mechanism for the regulation of cytoprotective genes such as HO-1 is governed by the MAPK signal transduction pathway^{457,460}. The best characterised MAPK subfamilies are ERK, p38 and JNK, each of which are regulated by different extracellular stimuli, namely, growth and proliferation (ERK) and cellular stress signals (p38 and JNK)⁴⁵⁸. The extracellular stimuli, e.g. cytokines, growth factors, ROS and RNS, initiate a sequential phosphorylation cascade which enables the MAPK subfamilies to target downstream substrates, i.e. transcription factors such as NF- κ B, HIF, AP-1 and -2, that upregulate the HO-1 gene via binding to their respective regulatory elements within the 5'-UTR of HO-1^{457,460}. A number of reports have documented a close association between activation of the MAPKs (ERK, JNK and p38) followed by upregulation of HO-1 protein, mRNA or its products bilirubin and CO in different models of cellular stress^{462,463,467,469,470,473}. In the case of RNS and the haem oxygenase pathway, Chen and Maines suggested that ERK and p38 may be potential targets leading to upregulation of HO-1 by NO⁶⁶⁶. Furthermore, activation of the MAPKs has been forwarded as a mechanism by which RNS mediate their signalling effects^{717,718}.

For the first time this report suggests a link between NO⁻-mediated redox signalling leading to enhanced haem oxygenase activity and increased expression of the redox-sensitive transcription factor Nrf2 within the nuclear fraction of cells. In the presence of AS the normally inactive cytosolic protein Nrf2 is released from its molecular chaperone Keap1⁶⁶⁶ and migrates to the nucleus where it binds to the antioxidant-responsive element (ARE) within the

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HO-1 gene⁴⁶⁵. Although the latter response was completely eliminated in the presence of the thiol donor NAC, coincubation of AS, hemin and NAC maintains the stimulation of Nrf2, in parallel with increased haem oxygenase activity. This suggests: 1. a greater affinity between AS and haem compared with thiols; 2. the participation of Nrf2 in the response to nitrosative and oxidative stimuli via the transcription of the HO-1 gene^{465,719}; and 3. regulation by thiol modification of the sulphydryl groups within Keap1 is mediated by nitrosants⁶⁸⁷.

Although Chapter 5 did not explore the mechanisms leading to the chemical modification of the HO-1 gene, a recent article by Balogun *et al.* using curcumin, a potent plant derived polyphenolic inducer of HO-1 in vascular and neuronal cells, observed enhanced haem oxygenase activity and HO-1 gene expression occurs via the Nrf2/ARE and MAPK pathway in renal epithelial cells. However, this induction was lost when two enhancer regions (E1 and E2), that contain at least three AREs and lie upstream of the transcription-initiation site of the HO-1 gene, were deleted, thus implicating a crucial role for Nrf2 in transcription of the HO-1 gene by electrophilic agents⁷²⁰. Furthermore, Alam *et al.* noted that haem can cause a stabilisation of the Nrf2 protein thus enhancing the latter's stimulation of the HO-1 gene⁷¹⁹. In addition to its activation of HO-1, Nrf2 regulates the expression of a number of cytoprotective genes, e.g. glutathione S-transferases (GSTs), NAD(P)H:quinone oxidoreductase (NQO1), γ -glutamylcysteine synthase⁷²¹ and thioredoxin⁷²². Individually and collectively, these enzymes and proteins mitigate the adverse effects of cytotoxic agents, thus illustrating the central

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role played by Nrf2 in the transcriptional regulation of defensive genes, as deficiency of Nrf2 increases susceptibility to carcinogenesis⁷²³ and oxidant-mediated cell injury⁴⁶⁶. In summary, this work suggests that amplification of the HO-1 pathway by the combined effect of two potent signalling mechanisms (the prooxidant haem and pronitrosants NO⁻/NO) is an effective endogenous cytoprotectant, and may have an application in pathological illnesses characterised by disrupted haem metabolism, increased NO release and localised hypoxia, e.g. sickle cell disease, intravascular haemolysis and haemorrhagic and subarachnoid shock³⁵⁴.

The most important mammalian bile pigments (or LTPs) are biliverdin and bilirubin. In humans the primary route of bile pigment formation is via the oxidative cleavage of the haem tetrapyrrole ring at the α position by haem oxygenase which generates biliverdin followed by reduction of the latter to bilirubin via cytosolic biliverdin reductase³⁶⁴. The negative perception of bile pigments, especially bilirubin, has been known for over a century⁵²³. However, this view changed with the conclusive demonstration that bile pigments, specifically bilirubin, are potent antioxidants against oxidative stress and high serum levels of bilirubin are associated with reduced susceptibility to development of cardiovascular complications^{385,525,529,543,546}. Knowing that HO-1 responds to nitrosative stimuli and bile pigments are capable of attenuating oxidative stress Chapter 6 explored the possibility of utilising bilirubin and biliverdin as a means to counteract nitrosative stress originating from NO⁻ (AS) and NO (DeaNO) species.

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Although AS and DeaNO have rather similar half-lives ($t_{1/2} = 2.3$ and 2 min, respectively) the data revealed that AS, a source of NO^\cdot , consumed bilirubin (5 μM) to a greater extent, in both a time- and concentration-dependent manner, compared with the NO donor DeaNO. Concomitant with the decline of the bilirubin peak at 440 nm the appearance of an as yet uncharacterised peak in the 315-320 nm region became more pronounced with increasing exposure to AS, more so than with DeaNO. In the presence of biliverdin a somewhat similar pattern of results was observed, but to a far lesser extent than with bilirubin. Interestingly, incubation of AS or DeaNO that had undergone a 1 h preincubation before the addition of bilirubin was ineffective at reacting with the latter. Of note, is the observation that high plasma levels of bilirubin can attenuate nitrosative stress, originating from DeaNO, thus illustrating the protective influence of bilirubin in *in vivo* situations characterised by excessive NO release. Recently, Western blot analysis of nitrotyrosine (NT), a marker of nitrosation, using human serum albumin (HSA) treated with AS (10-300 μM) revealed a concentration-dependent increase in NT levels; however, combining HSA with bilirubin resulted in a significant attenuation of NT expression, thus reinforcing the antinitrosative potential of bilirubin (unpublished preliminary data, not shown) and adds to earlier observations that bilirubin protects HSA and both can act against oxidative damage^{526,530,532,534}. Overall these results suggest that bile pigments, particularly bilirubin, can react directly with AS and DeaNO to combat different types of RNS and the rate of these reactions is more rapid in the presence of NO^\cdot . Work is still ongoing within the department to investigate these novel

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features of bilirubin.

In the final chapter the haem oxygenase inducing potential of a clinically used NO donor, GTN, was examined. The data revealed that GTN could enhance haem oxygenase activity and HO-1 protein expression in a time- and concentration-dependent manner. However, overloading the capacity of the HO-1 enzyme led to 'tolerance', a complex phenomenon experienced by patients following prolonged exposure to nitrates such as GTN⁸. Examination of the mechanism by which GTN promotes upregulation of haem oxygenase activity suggests that thiols (in the form of NAC) could enhance GTN biotransformation, a theory which has been suggested in other models where GTN was used³⁴⁵, while reduction of plasma homocysteine (Hyc) levels by HCB may increase the availability of NO^{679,702}. The fact that both NAC and HCB, respectively led to sustained and increased levels of haem oxygenase activity, is in sharp contrast to the results obtained when both agents were incubated with AS (see Chapter 4). Together, these observations merit further investigation into the mechanism of GTN-mediated activation of the haem oxygenase pathway.

8.3 Conclusions and future direction

Although many elegant studies have highlighted the ability of the haem oxygenase enzymes (HO-1 and HO-2) and their products, to attenuate oxidative stress, only a few studies have shown an association between induction of HO-1 and nitrosative stress^{423,427,481,482,669,724}. Due to the diverse chemistry of the NO group, which can exist as different redox subtypes¹³, the

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exact nature of this induction, the molecular targets, as well as the mechanism(s) behind it have not been probed. This Thesis has: 1. described the potential of AS, a NO⁻ generator, to activate the haem oxygenase pathway; 2. explored the biochemistry and identified a possible molecular mechanism responsible for this response; 3. identified the potential of bilirubin to counteract nitrosative stress; and 4. established a link between a pharmacological NO donor and the haem oxygenase pathway. Collectively, these findings strengthen the association between two very important physiological systems, 1. haem oxygenase; and 2. nitric oxide, and reinforces the hypothesis that the haem oxygenase pathway exists as a means to thwart the adverse effects induced by excessive NO production and RNS ^{373,452,636}.

The data reveals that NO⁻ liberated from AS can directly stimulate HO-1 induction via modulation of critical thiol targets within the redox-sensitive transcription factor Nrf2. The latter mechanism of transcription factor activation is analogous to studies in *E.coli* of the redox-sensitive transcription factors OxyR and SoxR ^{32,725,726}. These regulatory proteins are activated by reversible oxidation or nitrosylation at critical thiol and transition metal centres, respectively ⁷²⁷⁻⁷²⁹. Although similar mammalian homologues to OxyR and SoxR have not yet been identified the results in Chapter 5 support a direct link between Nrf2 and HO-1 gene expression and imply a mechanism by which cells sense nitrosative stress ^{465,466}. Recent publications by Buckley *et al.* ⁷³⁰ and Alam and coworkers ⁷¹⁹ involving NO, Nrf2 and haem reaffirm the ability of haem and AS to synergistically stimulate HO-1 via Nrf2. These observations, together with sustained stimulation of the haem oxygenase

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pathway through repeated exposure to low doses of AS (see Figure 3.4, Chapter 3) merits further investigation as a potential therapeutic application.

Not only can HO-1 serve as a sensitive redox-activator towards nitrosative stimuli, but one of its products of haem metabolism, bilirubin, can effectively neutralise nitrosative stress akin to its potent antioxidant capacity³⁸⁶. Further studies are required to examine the chemical and biological reactions taking place between bilirubin and different RNS. The novel finding that GTN activates the haem oxygenase pathway could be pursued further with a view to generating a pharmacological NO⁻/NO donor which can be used for prolonged periods without the development of tolerance. Although it was not the intention of this Thesis to look at the role of CO, recent data from this laboratory suggests that transition metal carbonyls, which exhibit the ability to function as carbon monoxide-releasing molecules (CO-RMs) and elicit distinct pharmacological activities in biological systems⁶¹⁵, exert a cardioprotective influence in *in vitro*, *ex vivo* and *in vivo* models of cardiac dysfunction⁶⁴⁰. Work is ongoing within the department looking into the chemistry of bilirubin and the synthesis of more efficacious CO-RMs with a view to harnessing the cytoprotective effect of these haem metabolism products towards different types of cellular stress and vascular dysfunction. Lastly, the chemistry of NO⁻/NO *in vivo* is quite complex, and not always reflected by the findings under *in vitro* experimental conditions; however, the results presented herein provide a deeper understanding of how HO-1 interacts with and responds to nitrosative stimuli. Additional work is now required to examine the reaction of HO-1 and its products to different types of RNS *in vivo*.

GENERAL DISCUSSION

In this Thesis rat cardiomyocytes exposed to different types of RNS were used as an *in vitro* model to illustrate the protective capacity of the haem oxygenase pathway. The data in this Thesis confirm the hypothesis that HO-1 is a sensitive sensor to different types of nitrosative stress at different oxygen tensions, i.e. normoxia and hypoxia, and this effect is possibly mediated via the antinitrosative effect of bilirubin³⁷³. As the human myocardium contains all three isoforms of NOS (eNOS, iNOS and nNOS)²⁴⁵ and the fact that HO-1 is upregulated in vascular tissues and the cardiovascular system exposed to RNS⁶³⁶, these findings have implications for cardiovascular disease and give a further insight into how cardiac muscle responds to nitrosative stress. Like all cells types, cardiomyocytes are constantly exposed to RNS which can mediate cytostatic (low NO) or cytotoxicity (high NO) effects via the activation of signal transduction pathways^{17,332,641,642}. These dichotomous aspects of NO can be explained by its complex interactions with ROS and the maintenance of a redox equilibrium by antioxidants such as GSH, catalase, SOD and α -tocopherol^{14,642,731}. Physiological and pathological factors such as age, exercise and hypertrophy (increase in cell size) can disturb this balance leading to activation of a second line of defence, i.e. HO-1. Although hypertrophy is considered an adaptive mechanism, over a prolonged period sustained haemodynamic changes (e.g. hypertension, aortic or mitral valve regurgitation) can lead to detrimental outcomes such as cardiac dysfunction, necrosis and apoptosis^{642,643}. Both NO and HO-1 have the ability to exert antiapoptotic and antiproliferative effects^{412,732,733}, as a result data has accumulated to illustrate the beneficial potential of these molecules in a

GENERAL DISCUSSION

number of cardiac pathologies, e.g. atherosclerosis, cardiomyopathies, heart failure, IR injury and myocardial infarction^{171,181,430,499,615,642,647,731,734}. The observation in Chapter 5 that NO⁻, in the presence of haem, acts as a very potent inducer of HO-1 has a potential clinical application as Paolocci *et al.* demonstrated that NO⁻ on its own could exert positive inotropic (contractility enhancing), lusitropic (enhances relaxation) and selective venodilator effects in failing hearts^{54,735}. Therefore, in heart failure administration of a pharmaceutical nitroxylhaem complex could possibly provide enhanced therapeutic advantages via the beneficial aspects of both HO-1 and NO⁻⁷³⁶.

From its humble beginnings as a single function enzyme, i.e. haem metabolism, haem oxygenase, and in particular HO-1, has grown to assume an important position in the league table of crucial endogenous enzymes as a result of the myriad of functions in which its products bilirubin, CO and Fe²⁺, participate. Similarly, NO⁻ and NO have developed into multifaceted redox siblings with their own distinct biology and chemistry. However, there is still a great deal to be learned about the regulatory mechanisms of HO-1, the chemical biology of NO⁻ and NO, and how these molecules interact, so as to understand the physiological function of both systems in the pathogenesis of vascular disease.

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