ROLE OF THE HAEM OXYGENASE PATHWAY IN PROTECTION AGAINST VASCULAR DYSFUNCTION

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"Science becomes dangerous only when it imagines that it has reached its goal"

George Bernard Shaw (1856-1950)
ACKNOWLEDGMENTS

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Impairment of vascular function and tissue injury after ischaemic events is a well-known pathophysiological phenomenon. Enhanced oxidative stress by free radicals appears to be a major contributor to the ischaemic episode which can occur either by progression of a disease state (atherosclerosis or angina pectoris) or after surgical intervention (organ transplantation). As a new approach for the preservation of vascular function after ischaemia, one line of research is considering the exposure of tissues to agents or treatments that lead to the stimulation of a number of natural intracellular defense mechanisms. This phenomenon is generally known as the "stress response" and invariably involves the de-novo synthesis of inducible proteins known as heat shock proteins. One such mechanism is the induction of the haem oxygenase (HO) pathway. Haem oxygenase is the mammalian enzyme which degrades haem to the antioxidant biliverdin and carbon monoxide (CO) releasing ferric (Fe$^{3+}$) iron. Biliverdin is rapidly converted to another potent antioxidant, bilirubin, by the cytosolic enzyme biliverdin reductase. Of the three known isozymes, the inducible isoform (HO-1) has been proposed to act as an effective system to counteract oxidant-mediated cell injury since it is extremely sensitive to up-regulation in a variety of mammalian tissues. The aim of this research project is to investigate the role of this potentially beneficial enzyme system in ameliorating vascular dysfunction following stressful insults. In particular, it aims to elucidate 1) the way in which the HO-1 gene is modulated; 2) the role of CO and bilirubin as important effector molecules and 3) the dynamics of the HO-1/bilirubin pathway are examined.
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<tr>
<td>( \mu l )</td>
<td>microlitre</td>
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<td>( \mu M )</td>
<td>micromolar</td>
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<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5' triphosphate</td>
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<tr>
<td>Ca(^{2+})</td>
<td>calcium ion</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanidine monophosphate</td>
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<tr>
<td>CO(_2)</td>
<td>carbon monoxide</td>
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<td>EIA</td>
<td>enzyme immunoassay</td>
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<td>Fe(^{2+})</td>
<td>ferrous ion</td>
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<td>Fe(^{3+})</td>
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<td>hour(s)</td>
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<td>H(_2)O(_2)</td>
<td>hydrogen peroxide</td>
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<td>i.m.</td>
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<td>i.v.</td>
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<tr>
<td>MW</td>
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<td>NADH</td>
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<td>SEM</td>
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1. **INTRODUCTION**

1.1 The clinical problem

Cardiovascular disease is the biggest cause of adult death in the United Kingdom. There are many different diseases of the heart and blood vessels, some very serious and others relatively benign. A number of well defined disease states have been characterised; including, coronary artery disease, angina, myocardial infarction, hypertension, congestive heart disease, cardiac arrhythmia, heart valve disease and congenital heart disease. All these conditions relate to the inability of the vascular system to maintain correct perfusion of blood to the body.

Current treatments for cardiovascular disease include drug therapy, surgery and lifestyle changes such as low fat diet and exercise habits. However, as our knowledge of the pathophysiology of these diseases increase, new developments for the treatment of vascular dysfunction become apparent. One of the aims of this Thesis is to investigate the possibility to exploit novel therapies for the treatment of cardiovascular disease.

1.2 The cardiovascular system

1.2.1 The blood transport system

Blood vessels are a closed system of tubes that carry blood from the heart to tissues and back to the heart again. This network of conduits ensure that every cell in the body has a supply of nutrients and blood, and a system of
waste-product disposal. Vessels (arteries) leave the heart and smaller branches (arterioles) travel into the deeper tissue where even smaller vessels form (capillaries). Upon leaving the tissue the capillaries empty into larger vessels known as venules and then into veins which return to the heart. Blood flows through the vessels through the action of the pumping heart, although in the case of the systemic circulation, diastolic recoil of the walls of the arteries, compression of the veins by skeletal muscles during exercise, and the negative pressure in the thorax during inspiration also move the blood forward.

![Cut-away section of an artery](image)

**Figure 1.1** Cut-away section of an artery

The wall of the arteries is made up of three layers of tissue: the outer layer, or adventitia; the middle layer of smooth muscle; and the inner layer, the intima, consisting of endothelium and connective tissue (shown above in
Figure 1.1. Arteries are elastic and stretch during each heart beat (systole) and then recoil on the blood between beats (diastole). This is courtesy of a layer of tissue found between the inner and middle walls, known as the \textit{elastica interna}. Beyond the arteries are the arterioles, smaller less elastic vessels containing more smooth muscle. Arterioles have a nervous supply which can initiate relaxation or, in some cases, restriction of the vessel: in this way the arterioles play an important role in the body in controlling localised and peripheral circulation.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{arteriole_venule_capillary.png}
\caption{Diagrammatic representation of a vessel bed showing arteriole leading to capillaries and then returning to the venous circulation through venules}
\end{figure}

The arterioles divide further into smaller vessels, capillaries (see Figure 1.2). The opening of the capillaries is surrounded on the distal side by small muscular sphincters. It is not thought that these muscular sphincters are innervated but they are able to respond to local release of vasoactive compounds. The diameter of the capillaries is such that red blood cells can
squeeze through one by one. Unlike the other vessels, capillaries consist of only one cell type, the endothelial cells which is only one layer thick.

After the blood has passed through the tissue and has released its oxygen in exchange for any waste products, it enters into venules. These are only slightly thicker walled that the capillaries and do not contain any smooth muscle. Beyond the venules the blood enters the veins. Veins differ from the other vessels as they contain valves along their length which are formed when the lining (intima) folds.

1.3 The heart: anatomy and physiology

1.3.1 Anatomy and function of the heart

The heart, a hollow muscular organ, lies in the center of the chest. The right and left hand sides of the heart each have an upper chamber (atrium) which collects blood, and a lower chamber (ventricle), which ejects blood. To ensure that blood flows in only one direction, the ventricles have an inlet and outlet valve.

The heart’s primary function is to supply oxygen to the body; as a consequence of this also rids the body of waste products (such as carbon dioxide). In short, the heart performs these functions by collecting oxygen depleted blood from the body and pumping it to the lungs, where it picks up oxygen and drops off carbon dioxide; the heart then collects the oxygen-enriched blood from the lungs and pumps it to the tissues of the entire body. A schematic
representation of the blood flow through, and anatomy of the heart is shown in Figure 1.3.

![Image of heart anatomy](image)

**Figure 1.3** The internal anatomy of the heart. Cross sectional view of the heart showing direction of normal blood flow and gross anatomy.

The heart muscle (myocardium) itself receives a fraction of the large volume of blood flowing through the atria and ventricles. A system of arteries and veins (coronary circulation) supplies the myocardium with oxygen-rich blood and then returns oxygen-depleted blood to the right atrium. The right coronary artery and left coronary artery branch off the aorta, just distal to the aortic valve to deliver blood; the cardiac veins empty into the coronary sinus, which returns blood to the right atrium. Because of the great pressure exerted in the heart as it contracts, most blood flow through the coronary circulation
takes place while the heart is relaxing between beats (during ventricular diastole)

Figure 1.4  **External anatomy of the heart.**
The right coronary artery and the left coronary artery with its two branches - the circumflex and the left anterior descending artery deliver blood to the heart muscle. The cardiac veins return blood to the right atrium.

1.3.2  **Endogenous control of heart function and coronary perfusion**

The muscular chambers of the normal heart beat in an orderly sequence: Contraction of the atria (atria systole) is followed by contraction of the ventricle (ventricular systole) and then all four chambers are relaxed simultaneously (diastole). This contraction is controlled by an innate system of nerve fibres within the heart. The various parts of the conduction system
are normally capable of spontaneous discharge, and are controlled by the central nervous node, called the sino atrial node (SA node, located near the coronary sinus of the right atrium).

The function of the heart is reliant upon its ability to pump blood around the body at the correct rate to support the body’s demand for oxygen and nourishment. For instance, an exercising muscle requires more energy input to afford a greater energy output. There are a number of ways in which the body can control the output of the heart.

Variations in cardiac output can be produced by changes in cardiac rate or the amount of blood pumped in each stroke (stroke volume). The cardiac rate is controlled primarily by nervous stimulation; sympathetic stimulation increases and parasympathetic decreases the rate. The stroke volume is also controlled in part by neuronal stimuli, sympathetic stimulation making the myocardial muscle fibres contract with greater strength. However, there are a number of physical stimuli that can also control cardiac output. When the resistance in the peripheral vessels is increased, the heart is unable to eject as much blood as usual and the heart volume increases. The distended muscles of the ventricles stimulate the heart to beat more forcefully, and output returns to its normal level. In a normal heart, these controls act continuously to maintain a constant cardiac output at rest.

During exercise venous return is increased by the muscular pumping action and an increase in respiration resulting in an increase in cardiac output to compensate.
The coronary perfusion is controlled by similar stimuli to those which control cardiac output, since the two are closely related. The muscles of the heart, rather like skeletal muscle, compresses its blood vessels when it contracts. When the metabolism of the myocardium increases so does blood flow. Metabolic regulation of coronary perfusion is the major mechanism controlling coronary resistance.

1.4 Cardiovascular disease

The term cardiovascular disease is used to describe many pathophysiological states of the heart and blood vessels.

1.4.1 Angina

When coronary artery flow is reduced to the level that the area of the myocardium it supplies becomes hypoxic, angina pectoris develops. In some cases, however, angina can also become manifested when the muscles controlling the blood flow go in to spasm; this condition is called variant angina. Angina “attacks” and normally last for a few minutes after or during physical exertion.

1.4.2 Myocardial infarction

If myocardial ischaemia is severe and prolonged, irreversible changes occur in the muscle and the result is myocardial infarction. The primary cause of a myocardial infarction usually involves obstruction of at least 75 % of the lumen of a coronary artery by a blood clot or a region blocked by an atherosclerotic lesion. Myocardial infarction is a common cause of death.
1.4.3 Atherosclerosis

In atherosclerosis, the wall of an artery becomes thicker and less elastic when fatty material is accumulated under the lining of the arterial wall. In Europe and the United States, atherosclerosis-related disease is the leading cause of illness and death.

The onset of atherosclerosis begins when monocytes migrate from the lumen into wall of the artery and initiate the accumulation of cholesterol. Over time these “plaques” or “lesions” build up causing patchy thickening of the smooth muscle cell layer. Wherever a plaque is found the artery loses its elastic properties and, as the lesions grow, they begin to cause narrowing of the lumen. Furthermore, these lesions begin to calcify and can rupture leading to formation of thromboses or emboli, which can be fatal.

1.4.4 Hypertension

Hypertension is the term used to describe chronically elevated blood pressure. Normal blood pressure is maintained by a balance between the pumping action of the heart and the elasticity and contractility of the blood vessel system. In 90% of cases, the exact cause of hypertension is unknown; this is described as essential hypertension. Obesity is a major risk factor in hypertension as is high sodium intake. In 10% of cases the disease can be traced to a specific cause such as kidney abnormalities, adrenal gland tumors, or a congenital narrowing of the aorta.
1.5 Ischaemia-reperfusion injury

Normal perfusion describes the condition of an organ receiving adequate blood or fluid flow. If normal perfusion is disrupted by any means, the tissue is said to be ischaemic. Ischaemia often results in a decrease of the cells normal function. The restoration of normal blood or fluid flow is termed reperfusion.

1.5.1 Pathophysiological ischaemia and cardiovascular disease

Periods of ischaemia are associated with a number of cardiovascular disease conditions such as acute myocardial infarction and stroke. The onset of these can be caused by a blood clot or by atherosclerosis. Accidental cessation of perfusion can also be caused by trauma such as crush injury. Although restoration of normal flow in many of these pathophysiological conditions is within a few minutes of the onset of ischaemia, the resulting damage to the heart (or brain) can be irreversible. The degree of damage incurred from ischaemia-reperfusion is dependent on a number of factors including the sensitivity of the tissue, which varies with different tissues and between different species, and storage factors, such as the duration of the ischaemia and the storage temperature.

1.5.2 Iatrogenic ischaemia and surgical intervention

Ischaemia-reperfusion is often the inevitable result of some surgical procedures such as operating under a tourniquet, resection of tumors, vascular reconstruction and the harvesting and redistributing of tissues for transplant or free flap surgery (1).
1.5.3 Ischaemic damage

The first change to occur in tissues deprived of oxygen is a depression in the mitochondrial electron transport chain. These changes lead to a reduction in the formation of ATP and a breakdown of adenine nucleotides to non-phosphorylated metabolites including hypoxanthine. In addition, accumulation of reduced pyridine nucleotides, such as reduced nicotinamide adenine dinucleotide (NADH), can no longer be oxidised by the electron transport chain. Many of the ion-dependent membrane pumps are inactivated resulting in loss of cellular homeostasis. Also an influx of Ca\(^{2+}\), Na\(^{+}\) and Cl\(^{-}\) ions and the osmotic movement of water causes the cell to swell (intracellular oedema). Reduction of Fe\(^{3+}\) to Fe\(^{2+}\) may be promoted by the reductive intracellular conditions during ischaemia which may result in the release of sequestered Fe\(^{2+}\) from ferritin (2) which can catalyse the generation of OH\(^{•}\) radicals from O\(_2^{•-}\) or hydrogen peroxide (H\(_2\)O\(_2\)) formation by the Fenton reaction (see Figure 1.5)(3). Hydrogen peroxide itself can degrade haem proteins (such as Cytochromes), thereby releasing more iron (4).

\[
\begin{align*}
\text{Fe}^{2+} + \text{H}_2\text{O}_2 & \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^- \\
\text{Fe}^{3+} + \text{O}_2^{•-} & \rightarrow \text{Fe}^{2+} + \text{O}_2
\end{align*}
\]

Figure 1.5  The chemical pathways involved in the Fenton reaction

Fe\(^{2+}\) ions react freely with hydrogen peroxide to produce the damaging hydroxyl radical (OH\(^{•}\)). Further reactions between the newly formed Fe\(^{3+}\) ion and superoxide radical also occur to re-forms Fe\(^{2+}\) ion which can go back to react further with hydrogen peroxide to produce more hydroxyl radical, thus perpetuating the reaction.
By this mechanism Fe$^{2+}$ is continuously regenerated with the production of the hydroxyl (OH•) radical. The hydroxyl radical is the most reactive free radical species; its half life in cells is estimated to be $10^{-9}$ sec.

There is also a net increase in toxic catabolites often associated with the anaerobic glycolysis such as lactic acid, H$^+$ and denatured proteins. The inevitable result of sustained ischaemia is cell death; however in most of the pathophysiological conditions referred to above the ischaemic episode is followed by reperfusion, which results in further cellular damage.

1.5.4 Reperfusion damage is mediated by oxidative stress

The reintroduction of oxygen to the cell can also be detrimental leading to the formation of oxygen free radical species. A free radical is a chemical containing one or more unpaired electrons (•), an unpaired electron being one that is alone in an orbital. Having this property makes free radicals very highly reactive; as a consequence of gaining or losing an electron, free radicals become more stable. However, donating or removing an electron from a non-radical molecule produces another radical, thereby setting up a chain reaction. This chain reaction is terminated only by the collision of two radical species.

Free radicals are generated continuously in the cell even under normal physiological conditions by the splitting of water into hydroxyl radicals (OH•) and hydrogen atoms (H•) by electromagnetic radiation, and by the "leakage" of electrons from the respiratory chain into molecular oxygen, during
oxidative phosphorylation, forming superoxide radicals (O$_2^•$)(5). These are normally removed by superoxide dismutase, as one main defence mechanism.

The combination of intracellular changes that occur during ischaemia are believed to culminate in the increased formation of free radicals upon reperfusion and reoxygenation, depleting the cells antioxidant enzymes and co-factors (6). Figure 1.6 shows an outline of the likely sequence of events leading to ischaemia-reperfusion damage.

One of the mechanisms that may result in the formation of oxygen derived free radicals is the enzymatic pathway of xanthine dehydrogenase, which under physiological conditions catalyses the catabolism of hypoxanthine (and xanthine) to uric acid (7). However, during ischaemia, the leakage of Ca$^{2+}$ into the cytosol stimulates Ca$^{2+}$-dependent proteolytic enzymes which convert xanthine dehydrogenase to xanthine oxidase; this oxidase consequently catalyses the same pathway but now using molecular oxygen instead of NADP$^+$ as an electron acceptor, thereby generating O$_2^•$ radicals. These free radicals are highly reactive chemical species, capable of interacting with many biological molecules of the cellular system such as lipids, DNA and proteins.

Lipid peroxidation may lead to breakdown of cellular membrane integrity and, in severe conditions, can lead to membrane permeability. Lipid peroxides can also break down further to produce a number of cytotoxic by-products such as aldehydes (8).

- 28 -
Aerobic metabolism interrupted
Metabolites accumulate
Oxygen available with reperfusion
Aerobic metabolism resumes with generation of $\text{O}_2^*$ and $\text{H}_2\text{O}_2$
Antioxidant system overwhelmed
Oxidative cell damage including lipid peroxidation
Cell damage stimulates synthesis of inflammatory cytokines
Synthesis of IL-8 and adhesion molecules
Neutrophils and macrophages attack reperfused tissue
Cytokines act systemically causing remote injury to lungs and other organs

Figure 1.6 The likely sequence of events leading to ischaemia-reperfusion injury.

Proteins with disulphide bridges and thiol groups are particularly sensitive to free radical damage (9). Following ischaemic damage, these types of modifications may lead to inactivation of important protein-dependent ion pumps. In the nucleus, iron derived from ferritin has been shown to catalyse $\text{OH}^*$ generation (10), which can lead to denaturation of DNA and, inevitably, changes in the integrity of the nucleus and mutagenesis. Such changes as these have been implicated in the pathogenesis of certain types of cancers (11). There is also a body of evidence that free radical damage to the endothelium during reperfusion can lead to loss of endothelium-dependent vasorelaxation (12).
The coronary vasculature plays a crucial role in matching cardiac blood supply with the metabolic/energetic requirements of the myocardium. Since the vascular endothelium actively participates in inflammatory and thrombotic processes associated with ischaemia-reperfusion injury, protection of endothelial functions may result in adequate reperfusion of the tissue or organ, and a decrease in the risk of vasospasm and the prevention of platelet aggregation and tissue inflammation. In the heart, a consequence of this would be increased myocardial perfusion and limited infarct size and post-ischaemic contractile dysfunction (13).

1.6 Endogenous protection against oxidative stress

1.6.1 Constitutive cytoprotective systems - antioxidant defense

An 'antioxidant' is a substance that, when present at lower concentrations than the oxidisable substrate, significantly delays oxidisation of that substrate (14). The term 'oxidisable' refers to almost everything found in living cells (i.e. lipids, DNA, proteins and carbohydrates). There are a number of antioxidants found in most cells that have the ability to reduce damage caused by oxidative stress but the major intracellular antioxidants in the human body are catalase, superoxide dismutase (SOD), and glutathione peroxidase (see Figure 1.7). In addition to these enzymes there are a number of "Chain breaking" antioxidants and co-factors such as vitamin C, α-tocopherol and β-carotene. "Chain breaking" refers to the ability of these compounds to break the chain reaction of free radical formation which may lead to increased cellular damage or cell death.
In the extracellular environment, antioxidants still play an important role in protecting tissues against the attack of free radicals. A major constituent of the plasma antioxidants is α-tocopherol, which is a powerful antioxidant against lipid peroxidation. This is particularly relevant, as it is widely believed that lipoproteins contribute towards the onset of atherosclerosis (15).

In contrast to the intracellular environment, enzymatic antioxidants are much more scarce in the plasma. There have been low levels of an extracellular form of SOD reported in purified human plasma and these may act as a protective coating on the endothelium as they have been shown to have heparin-binding properties (16). Ascorbic acid has multiple antioxidant properties (17), including the ability to regenerate α-tocopherol from α-tocopherol radicals, thus recycling its antioxidant capacity. A number of other...
antioxidant systems in the plasma relate to their ability to scavenge free transition metal ions which are able to accelerate damaging free radical reactions such as lipid peroxidation, including albumin (18).

However, the ability of a cell to avoid oxidative damage from external stimuli by these mechanisms is limited since most of these compounds are only produced at relatively low levels in cells and at times of increased oxidative stress these constitutive defenses can become overwhelmed.

1.6.2 The stress response

The stress response is the cells response to stressful stimuli, such as free radical-oxidative stress or heat shock. The heat shock phenomenon was first demonstrated in the larvae of the fruit fly (Drosophila melanogaster). Simply raising the temperature of Drosophila above its physiological norm resulted in the decreased expression of genes which were active before the temperature shock and increased expression of genes encoding a group of proteins referred to as the heat shock proteins (HSP). Subsequently, other stimuli (e.g., transition metals and anoxia) were shown to induce increased synthesis of these proteins.

It is now widely believed that the stress proteins play an important role in the cell's response to all kinds of stress. The heat shock response has been observed in many different species; moreover, the HSPs from different species appear highly conserved with respect to their primary structure, regulatory mechanisms and biochemical function (19). Since their discovery, HSP up-
regulation has also been associated with other cellular insults such as exposure to heavy metals, amino acid analogues, anoxia and even exercise (20-22).

1.6.3 Protein folding and unfolding - cytoprotection by chaperones

Many of the HSPs are known as molecular chaperones. A “molecular chaperone” was originally defined as a protein that mediates the correct assembly of another protein but is not involved in the final assembly of the protein (23). However, more recently the term “molecular chaperone” has been re-defined to describe a protein that binds and stabilizes another protein and facilitates its correct fate in vivo, be it folding, transport or disposal (24). It is important to note that many molecular chaperone proteins have essential functions under normal conditions and are present in both constitutive and inducible forms.

There are many different groups of HSP molecular chaperones but the family that has probably received the most attention is the ~70KDa, HSP70 family and our understanding of the general role of chaperones in protein folding emerged from research on this family of closely homologous polypeptides. HSP70 plays an important role in stabilizing both newly synthesised polypeptides as well as existing proteins that may denature under stress (25).
1.6.4 Haem oxygenase, a different kind of HSP

Of all the heat shock proteins, there is one which may prove of particular importance in cardiovascular research. Haem oxygenase is the microsomal enzyme responsible for the rate-limiting step of haem degradation to the bile pigments biliverdin and bilirubin and carbon monoxide. Haem oxygenase is a 32 KDa mixed-function oxidase, in that for each mole of O₂ used in the reaction one mole of H₂O is produced (26). There are three known isozymes of haem oxygenase, each being a different gene product. HO-2 is the constitutive form found mainly in the brain and CNS, testes, blood vessels and liver (see Table 1.1). HO-1 is the inducible form and, recently, HO-3 has been shown to be present in the rat brain (27, 28).
Figure 1.8  Schematic of the haem synthesis and degradation pathway
Table 1.1  The distribution of haem oxygenase in the microsomal fractions of rat organs
Taken from Maines, 1988 (29)

<table>
<thead>
<tr>
<th>Organ</th>
<th>Haem oxygenase, nmol/mg.hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>10.62*</td>
</tr>
<tr>
<td>Heart</td>
<td>1.93</td>
</tr>
<tr>
<td>Kidney</td>
<td>3.12</td>
</tr>
<tr>
<td>Liver</td>
<td>7.57</td>
</tr>
<tr>
<td>Lung</td>
<td>2.27</td>
</tr>
<tr>
<td>Spleen</td>
<td>11.59*</td>
</tr>
<tr>
<td>Testes</td>
<td>10.72*</td>
</tr>
</tbody>
</table>

*In the brain, spleen and testes, the predominant form of haem oxygenase is the constitutive form, HO-2.

There are major differences between the two well-characterised isozymes of haem oxygenase, the most striking of these is in their regulatory mechanisms. For instance, HO-1 activity can be increased 100-fold in the liver without effecting HO-2 activity (29). This lead to the discovery that HO-1, HO-2 and HO-3 are products from three distinctly different genes and, in fact, on closer inspection the three proteins possess very different properties. The most recently discovered member of the haem oxygenase family, HO-3, compared to the other haem oxygenases is a poor haem catalyst although it is thought that it still has the ability to bind haem (28).
Chapter 1

Introduction

The HO-1 gene is exquisitely sensitive to many types of stimuli, which have the common ability to cause oxidative stress (30), and the regulation of HO-1 has been extensively studied since its discovery. A very potent inducer of this enzymatic pathway is the substrate, haem (26), but other substances such as heavy metal ions (including Mn, Fe, Ni, Cu, Zn, Au, Hg, Pb, Cd, Sn, Pt and Sb) have also been shown to be powerful modulators of the haem oxygenase gene (31-35). For example, hepatic haem oxygenase activity can be induced up to 45-fold with a single dose of CoCl₂, an induction several times higher than that obtained with the substrate, hemin (36). There has been some evidence to suggest that HO-1 expression can be regulated by hormones, such as thyroid hormone and oestrogens (37, 38). However, the degree of induction seen in these hormonal responses has been varied and there are few reports in recent years.

Increasing evidence suggests an important role for haem oxygenase upregulation after increases in the levels of bacterial endotoxin (lipopolysaccharides, LPS) and interferon in cells (39-43). In addition to LPS, hepatic haem oxygenase activity has been reported to be enhanced after infection of rats with the bacteria Baccillus calmette-guerin and Corynebacterium parvum (44).

In addition to responding to a number of chemical and pharmacological agents, HO-1 induction has also been reported following physical stimuli such as shear stress and other haemodynamic forces (45). Also, Eyssenhernandez and co-workers demonstrated the up-regulation of the HO-1 gene by hypoxia,
thus supporting the theory that HO-1 may play an important role during or after ischaemia (46, 47).

Our group first reported that incubation of endothelial cells with various nitric oxide (NO)-releasing agents resulted in a marked increase in HO activity (48). Since this important finding many others have shown a similar trend in other cell types and tissue such as smooth muscle, skeletal muscle and rat liver (49-53). Upregulation of haem oxygenase activity by NO depends on de novo RNA and protein synthesis and arises predominantly via the transcriptional activation of the HO-1 gene (50).

It appears that the HO-1 gene contains a number of regulatory factors in its promoter region including heat shock factor, activator protein-1 (AP-1), and nuclear factor kB (NF-kB) (54-56). HO-2 on the other hand is relatively unresponsive to stimuli and, to date, only a single glucocorticoid responsive element has been found present on the HO-2 gene (57). There are no data available at present concerning the up-regulation of the HO-3 gene.

1.6.5 Haem oxygenase-mediated protection

How can the haem oxygenase pathway offer any protection to a cell simply by degrading free haem? In general, HO-1 expression increases when cells are exposed to any stressful stimuli or in a disease state; in fact, many have suggested that HO-1 may be a suitable marker of abnormality, indicating a disease state prior to any clinical signs (58, 59). There are a number of pathophysiological conditions where the increased ability to safely catabolise
haem is beneficial to a cell. For example, in a number of haemolytic anaemias, haem oxygenase activity increases in response to an increase in free intracellular haem though erythrocyte lysis. HO-1 activity recruits ferritin, which sequesters iron, thereby helping to avoid iron dependent oxidative stress (60). However, it is believed that the majority of the protective effects elicited by haem oxygenase are as a direct result of the products of haem catabolism, biliverdin, bilirubin and carbon monoxide (61).
1.6.6 The products of haem catabolism: biliverdin, bilirubin and carbon monoxide

When produced during haem catabolism, biliverdin is immediately reduced to bilirubin by the cytosolic enzyme biliverdin reductase. Biliverdin reductase enzyme is present in all cells that constitutively express HO-2 as well as cell types that have the potential to express HO-1 (62). In humans, nearly 80% of the bilirubin excreted in bile per day is derived from haemoglobin haem. However, cytochrome P-450 and other haem proteins also contribute to the production of bilirubin. Bilirubin is usually regarded as a potentially cytotoxic waste product when accumulated at abnormally high concentrations in biological tissues. However, Stocker and colleagues elegantly demonstrated that low concentrations of bilirubin are as effective as vitamin E in inhibiting lipid peroxidation in vitro suggesting that the bile pigment is a physiological, chain-breaking antioxidant (63, 64). In fact, bilirubin is now regarded as the most powerful endogenous antioxidant substance in vitro as well as being a very effective physiological antioxidant in vivo. Despite these promising features, little has been done to elucidate the biological importance of bilirubin in the cardiovascular system. In particular, the inherent cytoprotective characteristics of endogenous bilirubin derived from the haem oxygenase pathways during and after stress situations have yet to be explored.

CO is a small highly diffusible gaseous molecule which is produced in equimolar quantities to bilirubin during the degradation of haem by the haem
oxygenase system (47). Until recently, CO was only considered a toxic byproduct of haem catabolism and as an atmospheric pollutant; however more recently it has been shown to have powerful vasodilator effects through activation of guanylate cyclase (65-67), as well as being a powerful inhibitor of platelet aggregation and a putative neuronal messenger molecule (65, 68-71).

Recently our group reported that induction of haem oxygenase in isolated rat aortas was associated with an increase in CO production which accompanied a marked decrease in vasomotor response to pressor agents and which was reversed by an inhibitor of haem oxygenase activity (52). CO-induced vasorelaxation is not due to the release of prostaglandins or adenosine and occurs independent of any adrenergic effects (72). It appears to control vessel tone by activation of soluble guanylate cyclase (GC) and elevation of intracellular cGMP; in fact, CO has been shown to activate isolated GC by binding to the haem regulatory subunit of the enzyme (73). Although the effect of CO on GC is not as pronounced as that offered by the analogous NO, CO-stimulated increase in cGMP levels has been measured in cultured smooth muscle cells and isolated aortic tissue (68, 74-77). However, CO does not simply act through activation of GC to mediate its vasomotor effects. Recently Wang and colleagues described a mechanism by which CO acts directly on Kca channels (78). In addition to it's vasoactive effects HO-1-derived CO has been attributed to a number of other cellular functions (68). For example, Morita and co-workers reported that HO-1 derived CO can modulate smooth muscle cell proliferation (79).
The exact role of CO in the regulation of vascular cell function is not yet fully understood. CO may simply be acting as a secondary messenger on the surrounding tissues and may serve as regulator of platelet aggregation, muscle tone and smooth muscle cell proliferation. The HO-1-CO pathway may well be fundamental in the control of cellular homeostasis (80).

1.7 Nitric oxide and control of vessel tone

1.7.1 The EDRF revolution

Several years ago, a chance observation led to the discovery that the endothelium plays a key role in vasoregulation. Many different stimuli act on the endothelial cells to produce endothelium-derived relaxing factor (EDRF), a substance that has now been identified as nitric oxide (NO)(81). NO is synthesised from L-arginine by the cytosolic enzyme nitric oxide synthase (NOS) (see Figure 1.7)(82). Similar to haem oxygenase, NOS is found in a number of isozymes. Inducible NOS (iNOS) is analogous to HO-1. It is a highly inducible enzyme induced for example by inflammatory cytokines and bacterial toxins (83, 84). iNOS is found in many cell types including skin, vascular tissue and brain (85) (86, 87). The constitutively expressed isozymes, eNOS (cNOS) and bcNOS, are found in the vascular endothelium and neuronal tissue respectively (88) and are known to participate in signal transduction pathways, neurotransmission as well as acting as part of the host anti-microbial defenses. However, the principal role for NO in the vasculature is in the maintenance of vascular tone (89). This was first demonstrated when inhibitors of NOS increased regional vascular resistance.
and raised blood pressure (90). In a similar manner to CO, NO activates soluble GC to elicit vasorelaxation by production of cGMP (91).

![Nitric oxide synthesis pathway](image)

**Figure 1.9** The nitric oxide synthesis pathway

**1.7.2 The dual role of NO: cytoprotection vs. cytotoxicity**

Nitric oxide has been shown to have a multiplicity of cellular functions (89). It has often been described as a “two-edged sword” - indicating that there is mixed opinion about the possible beneficial vs. toxic functions of NO (92). When considering the cytotoxic vs. cytoprotective nature of the NO molecule in biological systems, one has to consider the synthesis pathways involved in each circumstance. According to some calculations, the major differences between cNOS and iNOS activities are not in the concentrations of NO produced, but rather in the duration over which NO is generated (93). In addition to this, when induced, iNOS may be present in much higher amounts than the constitutive form. Therefore, cytotoxicity of NO is often associated
with the product of iNOS and not of the two cNOS isozymes. It is in fact, the iNOS form that is found in macrophages involved in the host defense system, and it is the constitutive forms that are primarily involved in the maintenance of the vasculature and in neurotransmission (89).

NO has a relatively long half life, depending on the presence of other molecules in the solvent such as glutathione or proteins containing reduced cysteine moieties, which can be regarded as NO-storage molecules (94). It is, therefore almost impossible to determine the life-span of NO in biological systems. NO has been shown to be cytoprotective through a reaction with lipid radicals as well as iron (95), and in low concentrations NO has been shown to be protective against $H_2O_2$ and other reactive oxygen species (96). Protective effects of NO have also been reported during cerebral or myocardial ischaemia-reperfusion injury, although the protective effect in these situations is probably the result of its vasodilator function and it's ability to inhibit platelet aggregation and prevent cell adhesion rather than a direct effect of NO (97).

However, NO is a highly reactive molecule, and it has been proposed that NO can enhance free radical toxicity due to its rapid reaction with superoxide to form the highly reactive free radical peroxynitrite (98). The formation of peroxynitrite radical could dramatically increase the toxicity associated with the presence of superoxide or NO alone.

The factors that dictate the protective versus damaging effect of NO on oxidative injury remain poorly defined. NO may be cytoprotective directly as a
terminator of radical propagation or as an inducer of defense responses (such as the haem oxygenase pathway). However, at higher concentrations, in conditions when iNOS is up-regulated such as septic shock or a severe inflammatory response, NO would most probably be cytotoxic.

1.8 Modulation of the stress response: the preconditioning phenomenon

The term preconditioning has been used to characterise a variety of strategies that involve the pre-treatment of cells or tissue with an agent (be it chemical or physical) and then subsequent exposure to a potentially lethal stress. Many variations on this theme of preconditioning have been examined and can, essentially, be divided into three primary groups.

1.8.1 Ischaemic preconditioning

Until recently it was generally believed that repeated ischaemic episodes would have, if anything, a cumulative adverse effect (99). However, studies carried out since that early work have proven that this is not actually the case and that the heart, for instance, is capable of rapid adaptation following brief ischaemic episodes which leads to a marked increase in resistance to myocyte injury following a prolonged ischaemic challenge (100). This phenomenon, termed "ischaemic preconditioning" demonstrates one of the best forms of protection against myocardial necrosis and has since been shown in many other organs as well as in patients with cardiovascular disease (100-102).
Ischaemic preconditioning can be divided into two very distinct phases, based on the mode of protection. These are referred to as early- and late phase preconditioning. The first phase is short lived in tissues. The transient nature of this type of protection is attributed to its mode of action. The mechanisms underlying early preconditioning are not completely understood; however there are several lines of evidence suggesting a role for a number of endogenous paracrine mediators released during the brief period of ischaemia and acting on local receptors as triggers of preconditioning. Such suggested paracrine activators are adenosine (103); acetyl choline (104); catecholamines (105); angiotensin II (106); bradykinin (107); endothelin (108); and opioids (109, 110).

Late preconditioning appears to be an adaptive response that, unlike the acute, early response, is dependent on altered gene expression as well as the synthesis of new proteins, including antioxidant enzymes, HSPs and NO synthase (111).

1.8.2 Heat shock pretreatment

Heat shock was the first form of preconditioning described when heat shock proteins were first characterised. This type of preconditioning utilises the cells de novo synthesis of heat shock protein to elicit cytoprotection. Both heat stress and ischaemic preconditioning can induce the synthesis of stress proteins (112). The protection afforded by heat shock has an initial delay in onset by a number of hours (to allow mRNA production and protein synthesis) and tends to be long lasting, up to 48 h (113).
Several studies have shown the ability of heat stress to offer a degree of protection against further potentially lethal stresses. Gbur and colleagues showed that heat stressed vascular endothelial cells (42 °C for 1hr) had a greater degree of resistance to H$_2$O$_2$-mediated damage up to 18 h after heat treatment (measured by LDH release into the medium) than untreated cells (114). Other studies have used whole body heat shock in rats: for example, by raising the core body temperature to 42 °C for 15 min, Safford and colleagues showed that arterial balloon injury could be significantly suppressed (115).

1.8.3 Pharmacological preconditioning

Without understanding of the mechanisms involved in either preconditioning phase (early or late), attempting to try and chemically manipulate this response would be almost impossible. However, there are a number of approaches that would seem feasible for pharmacological manipulation of the stress response.

- mimic the mediator (such as adenosine, which has been found to be protective in the 'early window' after ischaemic preconditioning)

- enhance the effects of endogenous protector molecules by modulating their release, transport, uptake or metabolism

- modulate the expression of the HSP family of proteins which are well established cytoprotective agents
There is increasing evidence that adenosine is involved in both early and late 
preconditioning (103, 116, 117). This has lead to a search for selective A1 
adenosine receptor agonists. However, there are practical difficulties with this 
approach especially as the half lives of many adenosine analogues are very 
different from their endogenous counterpart (118), and site-specific targeting 
of a drug such as adenosine is not always possible except in situations such as 
thrombolysis where there is the possibility of localised administration. This 
drawback may lead to side-effects of the compound such as fall in heart rate 
and a reduction of arterial pressure (119). To date it has not been shown that 
A1 receptors, adenosine or synthetic adenosine agonists have any beneficial 
effect on ischaemic injury in humans.

The advantage of enhancing existing protective mechanisms is that their 
action can be specifically targeted to the site of interest. For example, 
administration of angiotensin converting enzyme (ACE) inhibitors potentiates 
bradykinin release in the myocardium. There is increasing evidence to suggest 
that bradykinin plays a role in ischaemic preconditioning and that it does 
indeed contribute to the protective effects of ACE inhibitors (107, 120).

Probably, the protective mechanisms that has attracted the most interest in 
recent years is the modulation of the stress response. It would seem more 
relevant to attempt to manipulate these protective mechanisms rather than 
the short-lived early-preconditioning response since the protection is longer 
lasting and therefore likely to be more relevant to the clinical situation. There 
is some evidence that this can be achieved using physiological stimuli such as
a period of increased temperature (115) or transient ischaemia (100). More importantly, there is also evidence that many chemical/pharmacological agents such as free radicals (121, 122), heavy metals (22, 123), and nitric oxide have the ability to modulate this stress response \textit{in vitro} and \textit{in vivo} in a variety of models (124). Another advantage of this type of protection is that the expression of proteins is modulated by gene expression. This opens up the opportunity for gene manipulation and, therefore, engineering of protection into organs by gene therapy (22). In fact, genetic manipulation of the stress response has recently been demonstrated in a model of mouse-to-rat cardiac xenograft transplant (125), and isolated myogenic cells (126). It seems that the long term use of preconditioning for transplant surgery may well use these kinds of treatments to elicit the stress response specifically in tissue.

\textbf{1.9 Preconditioning in man}

It is not known whether preconditioning can protect against myocardial infarction in man. There is very little evidence for ischaemic preconditioning in man (119). There have been a number of studies to investigate the effect of long term angina prior to infarction but none of these provide conclusive evidence although some have reported better short term outcomes after severe myocardial infarction following episodes of angina pectoris (127, 128). However, these studies can not exclude the involvement of other protective effects such as collateral blood flow and the long term administration of anti-ischaemic medication such as NO-donors (SNP and GTN). It is therefore important to validate any pharmacological treatments believed to offer protection against ischaemic damage in animal models with patient studies.
1.10 Aims and hypothesis

Haem oxygenase appears to have an important role to play in the maintenance of cellular homeostasis in vascular tissues and, by virtue of its products, bilirubin and carbon monoxide (CO) may be an important enzyme in the prevention of cellular damage during oxidative challenge (80). Hence, the studies reported in this Thesis aim to investigate: (i) the mechanisms controlling haem oxygenase-1 gene expression in vascular cells and tissue with the ultimate goal of finding pharmacological agents that can be used for preconditioning; (ii) to determine the effect of increased haem oxygenase activity, bilirubin and CO on cell function and survival during oxidative challenge and, finally; (iii) to evaluate the effect of increased haem oxygenase expression and bilirubin/CO generation on short-term survival of myocardial tissue after ischaemia-reperfusion injury.

The unifying hypothesis of these studies is:

Upregulation of the haem oxygenase-1 gene and increased generation of bilirubin and carbon monoxide offers protection to vascular tissue against oxidative damage.
2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents

All chemical reagents, unless otherwise stated were obtained from Sigma Chemical Company (Poole, Dorset, UK). All cell culture plastic-ware, including cell culture flasks, pipettes and multi-well plates, unless otherwise stated, was from Nunc (Costar, Cambridge, MA). Hemin (ferriprotoporphyrin IX chloride) and tin protoporphyrin IX dichloride were purchased from Porphyrin Products Inc. (Logan, Utah, USA). Bilirubin was obtained from Fluka Chemicals (Gillingham, Dorset, UK). Polyclonal HO-1 and HO-2 antibodies were from Stressgen (Victoria, Canada).

2.1.2 Scientific instruments

The spectrophotometer was a Helios-α (Unicam, USA). The peristaltic pump was a Minipulse 2 (Gilson, UK). The polygraph recorder was a GRASS Model 7D polygraph recorder (Astra-Med Inc, UT, USA). The computer was supplied by MSL computers (London, UK) and the P-100 data acquisition hardware and Aquaknowledge™ Software were purchased from Biosoft (Cambridge, UK). Re-usable pressure transducers model P23-XL were supplied by Astra-Med Inc. (UT, USA). The electron microscope was a JEOL 1200EX STEM and the inverted light microscope was an Olympus CK.
2.2 Cell culture

2.2.1 Culture and maintenance of cell lines

Bovine aortic smooth muscle cells (AG08595A) were purchased from the N. I. A Aging cell culture repository, Coriell Institute for Medical Research (Camden, NJ, USA). Human epidermal keratinocytes were kindly provided by Dr. Claire Linge of The RAFT Institute of Plastic Surgery, Northwood, Middlesex, HA6 2RN. Each cell type was grown in specific culture medium, outlined below.

Table 2.1 Composition of culture media for the cell types used

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<th>Human epidermal keratinocytes:</th>
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<td><strong>Additives:</strong></td>
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Cells were grown in 75cm² tissue culture flasks (Costar, Cambridge, MA) and maintained in an incubator at 37°C in an atmosphere of 5 % CO₂ balanced
with air. Plate 2.1 and Plate 2.2 show a light micrograph and scanning electron micrograph of a vascular smooth muscle cell line.

Plate 2.1  Phase-contrast light micrograph of bovine smooth muscle cells.
Bovine smooth muscle cells were grown to confluence in culture medium in a 75cm² flask (magnification: x 400; *indent* magnification: x 1200).

After reaching confluence, cells were washed twice with 5 ml ice cold 10 x Dulbecco's phosphate buffered saline (DPBS) and then incubated for 5 min with 2.5 ml 0.25 % Trypsin-1 mM EDTA solution until cells were detached from the surface of the flask. A further 7.5 ml of culture medium was added to each flask and the contents transferred into a sterile 13 ml sterile centrifuge tube and centrifuged at 1000 x g for 5 min in a bench-top centrifuge (Beckman Avanti 30, Beckman, UK). The supernatant was discarded, cells were resuspended with 10 ml fresh culture medium, placed in a 75 cm² tissue culture flask and returned to the incubator to allow the cells to grow (This procedure is termed *passaging* or *sub-culturing*).
2.2.2 Prim ary culture of vascular smooth muscle cells

Male Lewis rats weighing 280-320 g were euthanased by cervical dislocation. The pulmonary artery was isolated after thoracotomy and a 5 mm section of the artery dissected out. The vessel was transferred into a sterile 80 mm Petri dish containing 2 ml Dulbecco's Modified Eagles Medium (DMEM) containing 10 % (v/v) foetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin (growth media). The Petri dish was transferred under a Class II sterile culture hood (ESB, UK) and cleaned of any fatty and connective tissue ensuring that the tissue was kept wet with saline at all times. The vessel was then cut along its length using a pair of micro-dissection scissors to expose the

Plate 2.2 Scanning electron micrograph of vascular smooth muscle cell
Smooth muscle cells were grown to 80% confluence on Lux thermanox™ cover-slips and then processed for SEM as described in Materials and Methods (magnification: x 720, bar = 10 μm)
lumen surface. Using a sharp, sterile scalpel the endothelium lining was scraped with enough force to remove the single layer of endothelial cells but trying to avoid damaging the underlying smooth muscle cells and fibrous connective tissue.

The tissue was washed thoroughly with culture medium and transferred into a clean sterile Petri dish. The vessel was then cut into small (1 mm x 1 mm) sections which were placed, lumen side down, on the base of a 25 cm² tissue culture flask and left to adhere. After 5 min, 3 ml of culture medium was added to each flask and allowed to settle gently before placing in an incubator (37°C, 5% CO₂) for at least 7 days.

After 7 days, or once a mono-layer of outgrowing cells surrounded each explant, the tissue was carefully removed from the flask with sterile tweezers and discarded. After a further 5 days, once the cells had become confluent in the 25 cm² flasks, cells were passaged without a dilution step into 75 cm² flasks and returned to the incubator.

2.2.3 Immunological determination of smooth muscle cell type

Cells were allowed to grow to confluence in 75 cm² flasks. They were washed twice with 10 ml ice cold DPBS and then removed from the base of the 75 cm² flask by incubation with 5 ml 0.25 % (v/v) Trypsin-1 mM EDTA solution. Once the cells were all detached from the plastic, a further 5 ml of culture medium was added and the cells centrifuged at 1000 x g for 5 min. The supernatant was discarded and the pellet was shaken out onto a sterile gauze.
Cells were fixed for 1 ½ h in 5 ml formalin solution. The formalin was carefully removed and the pellet was transferred into 0.5 ml of melted agar solution (2 % (w/v)) in a 1.5 ml Eppendorf centrifuge tube and allowed to cool in a water bath at room temperature. The pellet, and surrounding agar, was carefully removed by needle from the Eppendorf tube held under running water. Specimens were fixed in 4 % (w/v) paraformaldehyde in PBS, dehydrated in graded alcohol, cleared in toluene and embedded in paraffin wax. Sections were cut at 4 µm across the centre of the cell pellet, floated onto cold and warm water baths and collected onto glass microscope slides. The slides were then incubated at 37°C for 1-2 days in order to allow them to dry.

Prior to staining, the paraffin embedded sections were warmed for 10 min at 37 °C in an incubator. The sections were immersed in two xylene baths for 5 min each consecutively to remove wax. This was followed by sequential immersion in absolute alcohol, 90 % alcohol and 70 % alcohol for 5 min each to remove the xylene. The alcohol was removed by immersing the slides in distilled water for 5 min.

Endogenous peroxidase activity was blocked by immersing the slides in methanol containing 0.3 % (v/v) hydrogen peroxide in a coplin jar for 15-20 min, followed by washing in running water. Slides were incubated for 1 h with PBS containing 1 % (w/v) bovine serum albumin, 10 % (v/v) normal goat serum and 1 % (v/v) Tween-20 to reduce non-specific labelling. Sections were then incubated with anti-CD40 (1:100 dilution, DAKO, UK) or anti-smooth muscle actin antibody (1: 50 dilution, DAKO, UK) overnight at 4°C.
The slides were then washed three times in PBS containing 1 % (w/v) BSA (5 min each wash) and incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories) at a dilution of 1:500 for 1 h at room temperature. After two washes in PBS the slides were incubated with avidin-biotin complex (ABC Elite kit, Vector Laboratories) for 1 h at room temperature. After washing with PBS once more, slides were treated with 3-3' diaminobenzidine (DAB substrate kit for peroxidases, Vector Laboratories) for 4 min. The slides were then washed in PBS, distilled water and counter-stained by washing with Mayer's haematoxylin for 5 min followed by washing in tap water, acid alcohol (1 % (v/v) hydrochloric acid in 70 % (v/v) alcohol) and tap water again for 5 min.

The sections were then mounted in DPX (a mixture of distrene, plasticizer and xylene) and a glass coverslip applied taking care to exclude bubbles from the specimen.
Figure 2.1 Immunohistochemical staining of primary cultured rat aortic smooth muscle cells. Cells were harvested and processed as described above; A) staining for CD40 (x 200) a marker of endothelial cells, B) staining for smooth muscle actin (x 200) , a marker of smooth muscle cells and C) x 600 magnification of smooth muscle actin staining
2.2.4 Preparation of cell culture reagents

Stock solutions or reagents were prepared freshly every day just prior to cell culture incubations. Stock solutions of both hemin and tin protoporphyrin (SnPPIX) were prepared by dissolving the compounds in 0.05 M NaOH and then adjusting the pH to 7.4 by addition of 0.01 M phosphate buffer. Bilirubin was solubilised in 0.05 M NaOH prior to adjusting the pH to 7.4 with 0.01 M phosphate buffer. Hemin, bilirubin and SnPPIX are all light sensitive molecules; therefore special care was taken to avoid exposing them to bright light by keeping all tubes and bottles covered in tin foil. The metal carbonyl complex molecule (manganese carbonyl) was solubilised in dimethyl sulfoxide (DMSO).

S-nitroso-acetyl penicillamine (SNAP), the nitric oxide donor, was dissolved in DMSO and stored at -20 °C until required. Sodium nitroprusside (SNP) was dissolved directly into the culture medium prior to incubation.

2.2.5 Incubating cells with agents

Before each experiment, cells were washed with 10 ml DPBS at 37°C to remove cell debris, detached cells or unwanted chemical factors in the culture. Treatment agents were prepared as described in Section 2.2.4. and diluted in culture medium to the required final concentration for incubation with cells. 10 ml of incubation media was added to each flask which were returned to the incubator for the time-course of the experiment.
At the end of the incubation period the medium was aspirated and cells washed 2 x with 10 ml ice cold DPBS. Cells were then scraped from the flask in 5 ml DBPS with a plastic policeman (Thomas Scientific, Swedesboro, NJ) before being washed into a 12 ml centrifuge tube and centrifuged for 5 min at 1000 x g. The supernatant was discarded and the cell pellet re-suspended in a suitable volume of buffer (500μl PBS containing 2 mM MgCl₂ for haem oxygenase activity or 300μl PBS containing 1% (v/v) Triton X-100 for Western blot).

2.2.6 Determination of cell number and cell proliferation assay

Confluent cells in 75 cm² flasks were washed with warm (30 °C) 10 x DPBS and harvested with 5 ml 0.25 % (v/v) trypsin - 1 mM EDTA, transferred into a 13 ml centrifuge tube and spun at 1500 x g for 5 min. The supernatant was discarded and the pellet re-suspended in 5 ml culture medium. The density (number of cells/ml medium) was determined by counting cells in suspension on a haemocytometer using an inverted light microscope. The cell suspension was diluted with a suitable amount of culture medium to give 2 x 10⁵ cells/ml and then 1 ml of medium was transferred into each well of a 6-well plate.

Cells were allowed to settle and grow for 24 h prior to incubation with various agents. After the time-course of the experiment, cells were washed with warm (37°C) PBS and removed from the plate using 1 ml 0.25 % (v/v) trypsin - 1 mM EDTA. Aliquots from each well were taken and the number of cells was counted using a haemocytometer as before.
2.2.7 Cell viability assay: trypan blue exclusion method

After treatment of cells with various agents, confluent cells were washed with warm (37°C) 10 x DPBS, and 2 ml DPBS containing 0.5 % (v/v) trypan blue was added to each well and returned to the incubator for 2 min. The flasks were placed under an inverted light microscope and the total number of cells in each high powered field (HPF) were counted. If a cell’s membrane is damaged trypan blue dye enters the cell and has a blue appearance under the microscope. Therefore, the number of blue cells in each HPF was also counted and the number of blue cells as a percentage of total cells per HPF was calculated.

2.2.8 Cell metabolism assay: the MTS method

Cells were grown to confluence in 96-well tissue culture plates and were incubated for various times with a number of agents. Cell metabolic activity, and therefore an indication of the viability of the cells, was assessed by using the Cell Activity Assay™ (Promega, USA). The MTS tetrazolium stock solution was diluted 1:6 (v/v) according to the manufacturers' instructions in culture growth medium; 200 μl were transferred into each well and left to incubate for 3 h in the incubator. The absorbance at 490 nm was read using a plate reader (VersaMax, Molecular Devices, UK). The assay uses a novel MTS tetrazolium compound (3 - (4, 5 - demethylthiazol - 2 - yl) - 5 - (3 - carboxymethoxyphenyl) - 2 - (4-sulphophenyl) - 2H - tetrazolium salt) which is bio-reduced by cells into a coloured formazan product that is soluble in medium. This conversion is accomplished by NADPH or NADH produced by
dehydrogenase enzymes in metabolically active cells. The amount of formazan measured gives an indication of the metabolic activity of cells and the results are expressed as % increase in activity above control.

![Reductive transformation of MTS to insoluble formazan product](image)

**Figure 2.2** Reductive transformation of MTS to insoluble formazan product

### 2.2.9 Cell metabolism assay: the alamar blue method

Another method of determining cellular viability is the alamar blue cell viability assay (Serotec, UK). Like the MTS method, the alamar blue assay incorporates a fluorometric/colourmetric growth indicator based on detection of metabolic activity; however, the indicator used in the alamar blue assay both fluoresces and changes colour in response to chemical reduction of growth medium resulting from cell growth. Cells were treated as described in Section 2.2.8. The alamar blue stock solution was diluted 1:10 (v/v) according to the manufacturers' instructions in culture growth medium; 200 µl transferred into each well and left to incubate for 3-5 h in the incubator or until a colour change was seen. After incubation the absorption at 570 and
600 nm was read in a microtitre plate reader (VersaMax, Molecular Devices, UK). Relative metabolic activity was determined by subtracting the background absorbance measured at 600 nm from the absorbance at 570 nm. Data were represented as percent of viability compared to untreated control using the following formula:

\[
\frac{OD_{570} - OD_{600} \text{ of unknown sample}}{OD_{570} - OD_{600} \text{ of untreated control}} \times 100
\]

Equation 2.1 Calculation of cell viability (percentage of control)

2.3 Biochemical assays

2.3.1 Preparation of liver and myocardial microsomal fraction

At the end of the experimental protocol the animals were euthanased by cervical dislocation. The liver and heart were quickly dissected out and placed on a gauze soaked with cold (4 °C) saline (0.9 % (w/v) NaCl). Using a 50 ml syringe, saline was slowly passed through the heart, via the aorta, until all the blood had been removed and the heart appeared blanched. The liver was dissected further into its separated lobes and ice cold saline was perfused through each lobe vascular bed via the exposed vessels. The organs were then homogenised in 5 volumes of 0.25 M sucrose-0.05 M Tris-HCl buffer (pH 7.4). The homogenates were centrifuged at 27,000 x g for 10 min at 4°C. The supernatants were removed, transferred into a new centrifuge tube and re-centrifuged at 105,000 x g for 90 min to yield the microsomal pellet. The
microsomal fraction was re-suspended in 100-500 μl 0.1 M PBS - 2 mM MgCl₂ (pH 7.4) and a small aliquot was taken for determination of protein content.

2.3.2 Protein determination of cells and tissue

The Bio-Rad DC Protein assay protocol was followed according to the manufacturers recommended procedure. Cell culture samples were prepared in PBS containing 10 % (v/v) Triton-X100 and kept on ice. Tissue homogenates and microsomal fractions were diluted 1:20 in PBS before protein determination. A 100 μl aliquot of the sample was taken and added to 500 μl Solution A’ in a 10 ml polystyrene test tube and vortexed thoroughly making sure not to agitate the solution too much and cause frothing.

![Sample standard curve for protein assay](image)

**Figure 2.3** Sample standard curve for protein assay

Four ml of Solution B was then added to the test tube, mixed and left for 15 min. Then 2 ml of each solution was transferred into a spectrophotometer
cuvette and the absorption at 750 nm was read and compared to a standard curve made with bovine serum albumin (see Figure 2.3).

2.3.3 cGMP Enzyme immunoassay (EIA)

For measurement of cell cGMP an EIA in kit form from Amersham Life Sciences (p/n RPN 266) was used.

![Diagram of EIA system](image)

**Figure 2.4** The principal of the EIA system:
1) wells are coated with anti-rabbit Ab; 2) the rabbit anti-cGMP antibody binds to the primary antibody forming a stable complex; 3) The assay is based on the competition between unlabelled cGMP (unknown samples or standards - top) and a fixed quantity of peroxidase-labelled cGMP (bottom), for the limited number of binding sites on cGMP specific antibody; 4) the substrate (3, 3', 5, 5' - tetramethylbenzidine/hydrogen peroxide, in 20% (v/v) dimethylformamide) reacts with the antibody-bound peroxidase-labelled cGMP causing a change in colour in the well.

All reagents were prepared according to the manufactures' instructions and the procedure followed according to the manufacturers' protocol. The protocol for the non-acetylation assay was followed.
Sample preparation: Cells were grown to confluence in 6-well culture plates (Costar, CA) in culture medium. Cells were incubated with various agents according to the experimental protocol. The incubation was terminated by washing the cells two times with 700 µl ice cold PBS. Cells were scraped from the wells in 1 ml ice cold PBS using a plastic policeman (Thomas Scientific, Swedesboro, NJ). Then 1.3 ml ice-cold absolute ethanol was added to the cell suspension to give a final suspension volume of 2.0 ml containing 65 % (v/v) ethanol. Samples were left on ice in 2.5 ml Eppendorf tubes to settle.

The supernatant was drawn off using a glass Pasteur pipette and transferred into a 5 ml polycarbonate centrifuge tube (Beckman). The remaining precipitate was washed further with 2 ml ice cold 65 % (v/v) ethanol and the washings added to the appropriate glass test tubes. The extracts were centrifuged at 2,000 x g for 15 min at 4°C and the supernatant transferred into clean 7 ml glass test tubes. The combined extracts were dried under a stream of nitrogen at 60°C for approximately 3 h, or until all the 65 % (v/v) ethanol had been evaporated. Samples were stored on ice until required for analysis. The dry samples were re-constituted in 250 µl assay buffer prior to analysis.

Reagent preparation: All reagents were prepared according to the manufacturers' recommendations. The contents of the supplied assay buffer powder bottle was diluted into 500 ml distilled H₂O in a 500 ml graduated cylinder and mixed thoroughly. The contents of the wash buffer bottle was transferred into a 500 ml graduated cylinder and 500 ml distilled H₂O was
added. Eleven ml of freshly prepared assay buffer was added to the supplied Antiserum bottle and mixed by careful inversion and swirling. Care was taken to avoid foaming. Ten ml of freshly prepared assay buffer was added to the non-acetylation standard bottle and mixed until completely dissolved. Eleven ml of assay buffer were added to the cGMP conjugate bottle and mixed well.

Protocol: Eight tubes were labelled 50 fmol, 100 fmol, 200 fmol, 400 fmol, 800 fmol, 1600 fmol, 3200 fmol and 6400 fmol. 500 µl of assay buffer was added to each tube. Into the 6400 fmol tube 500 µl of stock standard was added (128 pmol/ml) and mixed well on a vortex mixer. 500 µl were taken from the 6400 fmol tube and added to the 3200 fmol tube and vortexed well. This serial dilution was continued with the remaining tubes ensuring correct mixing each time with a vortex mixer. Aliquots of 100 µl from the 8 serially diluted tubes were then used as the 8 standard levels of cGMP for the range 50-6400 fmol/well. One hundred µl of reconstituted stock standard served as the top (12800 fmol/well) standard.

Before the assay was performed, all reagents were allowed to equilibrate to room temperature. The micro-titre plate was set up as described below with sufficient wells to enable the running of all blanks, standards and samples as required. Into the wells labelled non specific binding (NSB), 200 µl assay buffer were pipetted. One hundred µl of assay buffer was pipetted into the 0 fmol/well standard well and 100 µl of each standard was transferred into the appropriate wells. Standard were run in duplicate to ensure a good standard curve is produced. One hundred µl of each unknown was transferred into the
remaining wells. All unknown samples were run in triplicate to reduce any error in the assay. The antiserum was diluted 1:2 in assay buffer and 100 µl was pipetted into each well, except the blank and NSB wells. The plate was covered with the provided lid, gently mixed and incubated at 3-6°C for 18 h.

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Figure 2.5 Layout of 96-well titre plate for cGMP EIA

After 18 h the cGMP peroxidase conjugate was diluted 1:2 in assay buffer and 50 µl was carefully added to all the wells except the blank. The plate was covered and gently mixed and incubated at 3-5°C for exactly 3 h. After this time, all the content of the wells were aspirated with a vacuum pump connected to a glass Pasteur pipette and each well was washed 4 times with 400 µl wash buffer. The plate was blotted on tissue paper between each wash to ensure that the whole volume was removed.
Immediately after the final wash, 200 μl of enzyme substrate was added to each well and the plate was covered and mixed on a microtitre plate shaker (Dynatech, Switzerland) for exactly 30 min at room temperature (15-30°C). Into each well, a further 100 μl 1.0M sulphuric acid was pipetted and the content of the wells mixed thoroughly on the shaker. The optical density at 450 nm was read on a plate reader (VersaMax, Molecular Devices, CA, US) within 30 min after a yellow colour developed.

**Calculation of results** - The average optical density was determined for each set of replicate wells. The percentage bound for each of the standards and samples was calculated using the following formula:

\[
\frac{\% B}{B_0} = \frac{(\text{standard or sample OD} - \text{NSB OD})}{(B_0 \text{ OD} - \text{NSB OD})} \times 100
\]

**Equation 2.2 Equation for the calculation of %age antigen bound**

Where \(B_0\) OD is the mean OD reading at 450 nm from the 0 fmol/well standards and NSB OD is the mean OD reading at 450 nm from the non-specific binding wells. A standard curve was generated by plotting the percent \(\frac{B}{B_0}\) as a function of the Log cGMP concentration. The plot of % \(\frac{B}{B_0}\) (y axis) against fmol/well cGMP standard (x axis). The fmol/well was read directly from the graph. Below is an example of a standard curve:
2.3.4 Preparation of rat liver cytosol

Male Sprague-Dawley rats weighing between 200-250g were euthanased by cervical dislocation and after thoracotomy the liver was removed and placed on a saline-soaked gauze. The lobes of the liver were dissected and perfused (using a 21-gauge Teflon intravenous catheter) with ice cold 1.15 % KCl solution. The liver was then finely minced using surgical scissors in 2-3 volumes of ice cold 1.15 % KCl containing 20 mM Tris-HCl (pH 7.4).

The samples were kept on ice and transferred to 21 ml polycarbonate centrifuge tubes and centrifuged in a Beckman LE-80 Ultracentrifuge (Beckman, UK) at 5,000 x g for 20 min. After centrifugation, the turbid lipid layer was removed from the top of the homogenate and the supernatant was
transferred into a new tube and re-centrifuged at 105,000 x g for 60 min. The cytosol was then removed with a Pasteur pipette taking care not to disturb the microsomal pellet at the bottom of the tube. Two ml aliquots were made in 2.0 ml Eppendorf tubes and stored at -70°C until required for the haem oxygenase assay.

2.3.5 Measurement of haem oxygenase activity

Haem oxygenase activity was measured as described previously (129, 130). Briefly, cells were grown in 75 cm² flasks until confluent and incubated with various agents according to the experimental protocol. At the end of the incubation, cells were washed with 10 ml ice-cold 10 x DPBS, scraped with a plastic policeman (Thomas Scientific, Swedesboro, NJ) in 5 ml 1 x DPBS and the cell suspension centrifuged at 10,000 g for 10 min at 4°C. The cell pellet was re-suspended in 2 mM MgCl₂ - 100 mM phosphate buffer (pH 7.4), frozen at -70°C and then freeze-thawed three times.

The protein content of the cells was measured using a total protein assay kit (Bio-Rad, Herts, UK) as described in Section 2.3.2. The cell suspension (400 µl) was added to the reaction tube containing 20 µM NADPH, 2 mM glucose 6-phosphate, 0.5 U glucose-6-phosphate 1-dehydrogenase, 2 mg protein of rat liver cytosol as a source of biliverdin reductase, potassium phosphate buffer (100 mM, pH 7.4) and hemin (20 µM) in a final volume of 1 ml. The reaction was allowed to run for 1 h at 37°C in the dark and terminated by the addition of 1 ml chloroform.
Chapter 2

Materials and Methods

The extracted bilirubin was calculated by the difference in absorption between 464 and 530 nm using a quartz cuvette (extinction coefficient, 40 mM⁻¹cm⁻¹ for bilirubin). Haem oxygenase activity was calculated as picomoles of bilirubin formed/mg protein/h.

\[
\frac{\text{pmole Bilirubin}}{\text{mg protein at 60min}} = \left( \frac{\Delta OD(OD_{464} - OD_{530})}{40} \right) \times 10^6
\]

Equation 2.3  Equation for the calculation of haem oxygenase activity

2.3.6 Measurement of cell-derived bilirubin in vitro

Cells were grown in 6-well cell culture plates in 2 ml culture medium to confluence. The wells were incubated with a number of agents as described in the experimental protocol then 0.5 ml of each culture supernatant was collected and 250 mg BACl2 was added. After vortexing (10-15 s), 0.75 ml benzene was added and the tubes were vortexed vigorously (50-60 s) leading to the formation of a relatively stable milky-white emulsion. After centrifugation (13,000 x g, 30 min at room temperature), the upper benzene layer was collected and the absorbance at 450 nm was measured with a reference at 600 nm. In a separate tube, 0.5 ml of fresh medium was processed in the same way and the benzene layer was collected and used as a blank. The quantity of bilirubin produced was calculated using the molar extinction coefficient of bilirubin dissolved in benzene \( \varepsilon_{450} = 27.3 \text{ mM}^{-1}\text{cm}^{-1} \).
2.4 Molecular biology techniques

2.4.1 Maintenance of an RNase-free environment

In order to preserve an RNase-free environment the following precautions were taken: all glassware was cleaned and baked at 200 °C for 6 h; all plastic-ware was new and used specifically for RNA extraction; and all water was treated with diethyl pyrocarbonate (DEPC). To treat water with DEPC, distilled water was poured into previously baked glass bottles and diethyl pyrocarbonate (DEPC) was added (0.5 ml/litre). The water was vigorously shaken and left in an oven at 40 °C for 24 h. The water was then autoclaved at 121 °C for 25 min to eliminate any residual DEPC.

The method used for RNA extraction from both cell culture and solid tissue was based on that originally described by Chomczynski and Sacchi (131).

2.4.2 RNA extraction from solid tissue

Heart tissue was collected and snap-frozen in liquid nitrogen (-170 °C). It was stored at -80 °C until further processing. Frozen tissue was crushed and ground to a powder under liquid nitrogen using a mortar and pestle. The ground tissue was immediately added to 2 ml of lysis buffer (4 M guanidinium thocyanate, 25 mM sodium citrate, 0.5% (v/v) sarcosyl pH 7.0, 0.1 M β-mercaptoethanol). Further processing of solid tissue and cell culture samples was the same, as described below.
Samples were transferred to 12 ml tubes and left on ice for 5 min. Then, 0.2 ml of 2 M sodium acetate (pH 4.5) was added, followed by 2.5 ml phenol (pH 4.3), saturated with 0.1 M citrate buffer and finally 0.4 ml chloroform:isoamylalcohol (in the ratio of 49:1 v/v). Tubes were then vortexed thoroughly to mix all layers and left on ice for 15 min. Samples were centrifuged at 10,000g for 20 min at 4 °C in a bench-top centrifuge (Avanti 30 Centrifuge, Beckman Instruments Inc., Palo Alto, California, U.S.A.). The top, aqueous layer was transferred into a clean tube and an equal volume (~ 2 ml) of cold (-20 °C) 2-propanol was added. The tube was inverted three times to mix the aqueous layer with the 2-propanol and then left for 2 h at -20 °C. Samples were centrifuged at 10,000g for 30 min at 4 °C. The supernatant was discarded and the pellet re-suspended in 300 μl GT buffer. To this, 300 μl of cold 2-propanol was added. The tube was inverted three times to mix the contents and left at -20 °C for 1 h. Samples were centrifuged at 10,000g for 30 min at 4 °C. The supernatant was discarded and the pellet was washed by adding 500 μl of 75 % ethanol. The tube was vortexed briefly to loosen the pellet and once again centrifuged at 10,000g for 15 min at 4 °C. The washing step was repeated and the sample re-centrifuged. The supernatant was discarded and the pellet was left to air-dry by inverting the tube and leaving it on the bench top for 30 min. The RNA pellet was dissolved in (20-60 μl) DEPC-treated water.

RNA concentration in each sample was determined spectrophotometrically. One μl of sample was diluted in 99 μl of DEPC-treated water and the absorbance against a DEPC-treated water blank was determined at 260 and
280 nm using a spectrophotometer (Helios α, Unicam Ltd., Cambridge, U.K.). Total RNA concentration of the sample was calculated according to the following equation:

\[ \text{OD}_{260} \times 4 = \text{RNA mg/ml} \]

The quality of the RNA was determined by the ratio (good quality RNA has an \( \text{OD}_{260}/\text{OD}_{280} \) ratio of 1.7-2.0. A ratio of < 1.7 suggests a significant degree of residual protein in the sample):

\[ \frac{\text{OD}_{260}}{\text{OD}_{280}} \]

To check the quality of the RNA, 2 μl of the sample were mixed with 5 μl of loading dye (15 % (w/v) Ficoll 400; 20 % (v/v) glycerol; 0.5 % (w/v) bromophenol blue; 0.5 % (w/v) xylene cyanol) and loaded into the wells of a 1 % (w/v) agarose gel (1 g agarose in 100 ml 1x TBE buffer). Electrophoresis was performed in 1x TBE buffer (90 mM Tris; 90 mM boric acid; 2 mM EDTA; pH 8) containing ethidium bromide (0.5 mg/ml) at 90 V until the bromophenol blue marker band had migrated a distance of 5 cm. The gel was inspected using an ultraviolet transilluminator (UV Dual-intensity Transilluminator TM 20, UVP, Genetic Research Instrumentation Ltd., Dunmow, Essex, U.K.) to check the quality of the RNA. Photographs were taken when necessary using a Polaroid DS 34 camera with a DS H-8 hood (Polaroid UK Ltd., St. Albans, Hertfordshire, U.K.) and Polaroid film (Type 667) with a No. 15, deep yellow filter.
2.4.3 PCR analysis of HO-1 mRNA

Total RNA was isolated as described above. The RT reaction was carried out in a 20 µl reaction volume, using GeneAmp® in a thin walled PCR reaction tube (Perkin Elmer) by adding 2.5 µM random hexamer primers, 1mM DNTP mixture (equal amounts dATP, dCTP, dGTP, dTTP), 1U/µl of RNase inhibitor, 2.5µMU/µl MuLV reverse transcriptase in 1 x PCR buffer, 5 mM MgCl₂. The reaction was carried out for 25 min at 42°C followed by 6 min of heating at 99°C to denature the enzyme.

The RT reaction was amplified in a 100µl reaction volume in a PCR reaction tube in 1 x PCR buffer using 2.5 units AmliTaq polymerase (Perkin Elmer), 0.5 mM sense and anti-sense primers, and 2.5 mM MgCl₂. The PCR reaction was carried out in a PCR machine (UNO II, Biometra®, Jencons-PLS., U.K.) Cycle parameters consisted of a 60 s denaturing step at 95°C, a 90 s annealing step and a 7 min extension step at 72°C. 36 cycles were used per amplification and the annealing temperature for HO-1 and β-actin was 60°C. PCR products were electrophoresed on a 2 % (w/v) agarose gel, stained with 0.5 µg/ml ethidium bromide and visualised and photographed using a UV dual-intensity transilluminator TM 20 (UVP, Genetic Research Instrumentation Ltd., Dunmow, Essex, U.K.) and a Polaroid DS 34 camera with a DS H-8 hood (Polaroid UK Ltd., St Albans, Hertfordshire, UK). A base pair (bp) molecular weight ladder was used as the DNA standard. The primer sequences for HO-1 and β-actin are given in Table 2.2 (overleaf).
### Table 2.2 RT-PCR primer sequences for HO-1 and β-actin

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5' — 3'</th>
<th>Product size (bp)</th>
<th>Primer length</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sense</td>
<td>CTT TCA GAA GGG TCA GGT GTC CA</td>
<td>309</td>
<td>23</td>
</tr>
<tr>
<td>anti-sense</td>
<td>CTG AGA GGT CAC CCA GGT AGC GG</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sense</td>
<td>CGT GGC CGC CCT AGG CAC CA</td>
<td>246</td>
<td>21</td>
</tr>
<tr>
<td>anti-sense</td>
<td>CGT GGC CTT AGG GTT CAG AGG GG</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>

#### 2.4.4 Northern blot analysis of HO-1 mRNA

The method used for Northern blot was performed as previously described by one of my colleagues (Martin Vesely, MD Thesis: “The role of haem oxygenase-1 in the prevention of ischaemia-reperfusion injury in skeletal muscle”) (132). This method was also adapted from that published by Tyrell and Basu-Modak (133). Total RNA (10-20 µg/lane), extracted as above, was taken and 10 µl of denaturing buffer (1x MOPS buffer; 2.2 M formaldehyde; 50 % (v/v) deionised formamide; 0.5 mg/ml ethidium bromide) added. The sample was heated in a water-bath at 65 °C for 10 min to denature the RNA. It was cooled rapidly on ice for 2 min and 2 µl of loading dye (50 % (w/v)

---

1 All Northern blots were kindly performed by Rekha Bassi, Dept. Surgical Research, NPIMR.
glycerol; 0.4 % (w/v) bromophenol blue; 0.4 % (w/v) xylene cyanol; 1 mM EDTA) was added. Samples (10-20 µg RNA/lane) were loaded on a 1.3 % (w/v) denaturing agarose gel (1.3 g agarose-LE (Ambion Inc., Austin, Texas, U.S.A.) per 100 ml, 1x MOPS buffer, 2.2 M formaldehyde) which had been pre-run at 120 V for 10 min. Electrophoresis was performed in 1x MOPS buffer (20 mM 3-(N-morpholino)propanesulfonic acid; 5 mM sodium acetate; 1 mM EDTA; pH 6.0) containing ethidium bromide (0.5 µg/ml) at 120 V, until the bromophenol blue marker band had migrated a distance of 5 cm from the wells (Fig. 2.4.1). The gel was checked on an ultraviolet transilluminator (UV Dual-intensity Transilluminator TM 20, UVP, Genetic Research Instrumentation Ltd., Dunmow, Essex, U.K.) before being rinsed briefly in DEPC-treated water and then washed in rinse solution (75 mM NaOH; 100 mM NaCl) for 45 min on an orbital shaker, followed by a wash in 100 mM Tris-HCl (pH 7.5) for 60 min. 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, Massachusetts, U.S.A.) using phosphate buffer (25 mM Na₂HPO₄; 25 mM NaH₂PO₄; pH 6.5). Transfer was checked by viewing the membrane and the gel on an ultraviolet transilluminator. The membrane was baked at 80 °C for 30 min, wrapped in Saran™ film (Dow Chemical Co., U.S.A.) and stored at -20 °C until hybridisation.

The membrane was first prehybridised using 0.1 ml/cm² Rapid-hyb buffer (Amersham International Plc., Little Chalfont, Buckinghamshire, U.K.) for 1 h at 65 °C in a rotisserie (SI 20H Hybridisation Oven/Shaker, Stuart Scientific...
Co. Ltd., Redhill, Surrey, U.K.). cDNA probes to the rat and human HO-1 genes (both kindly donated by Professor Shibahara, Tohoku University, Japan) were labelled with Redivue™ [α³²P]-dCTP (Amersham International Plc., Little Chalfont, Buckinghamshire, U.K.) using a commercial nick translation kit (Boehringer Mannheim UK Ltd., Lewes, East Sussex, U.K.). Unincorporated nucleotides were removed using a commercial kit (QIAquick™ Nucleotide Removal Kit, Qiagen Ltd., Dorking, Surrey, U.K.). The labelled probe was added to the pre-hybridisation buffer and the membrane was hybridised for 24 h at 65 °C in a rotisserie. The membrane was then 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; 1x SSC/0.1 % (w/v) SDS for 15 min at 65 °C and 0.1x SSC/0.1 % (w/v) SDS for 15 min at 65 °C in a rotisserie. The membrane was then laid down against radiographic film (BioMax MR, Eastman Kodak Co., Rochester, New York, U.S.A.) at -80 °C and exposed for 3-7 days. The film was developed and the image was scanned using a densitometer and the accompanying imaging software (Model GS-700 Imaging Densitometer and Molecular Analyst respectively, Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, U.K.).

The membrane was then re-probed for a control gene (either GAPDH or b-actin). The membrane was first stripped of the previous probe by heating in 0.1x SSC/0.1 % (w/v) SDS for 60 min in a boiling water-bath. Prehybridisation and hybridisation, using cDNA probes for either rat GAPDH gene (kindly donated by Dr. S. Mohammed, Royal Free Hospital Medical School, London) or human β-actin gene (kindly donated by Hazel Truman, RAFT Institute of
Liposuction and Plastic Surgery, Mount Vernon Hospital, Northwood, Middlesex, U.K.), were performed as previously described.

To determine changes in HO-1 gene expression, the densitometric value obtained from the film probed for HO-1 was normalised to its respective value from the film probed for the control gene (rat GAPDH or human b-actin), as described in Tyrrell & Basu-Modak (133). Briefly, the densitometric values for the control gene were averaged and the actual control gene value for each sample was divided by this mean to get a ratio. The actual densitometric value for the HO-1 gene of each sample was then multiplied by its calculated ratio to arrive at a corrected value for the HO-1 mRNA level. This was done to prevent variations in the loading of RNA samples onto the gel or in the spectrophotometric quantification of RNA from causing apparent changes in HO-1 gene expression.

2.4.5 Western blot protein analysis

Total protein was calculated using a Total Protein Assay Kit (Bio-Rad). The correct amount of protein (according to the table below) was taken from each sample and mixed with Laemmli (loading) buffer (0.5M Tris-HCL, pH 6.8, Glycerol, 10 % (w/v) SDS, 2 B-mercaptoethanol, bromophenol blue) to give a total volume of 30 μl. The samples were heated to 100°C for 10 min on a dry block heater (Techne DB2A, Techne GmbH, Germany) to denature the proteins.
Table 2.3  protein concentrations used for Western blot for HO-1, HSP-70 and iNOS

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Sample type</th>
<th>Total protein loaded / 30 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO-1</td>
<td>Cultured cells</td>
<td>20 µg</td>
</tr>
<tr>
<td></td>
<td>Heart tissue</td>
<td>60-100 µg</td>
</tr>
<tr>
<td>HSP-70</td>
<td>Cultured cells</td>
<td>20 µg</td>
</tr>
<tr>
<td></td>
<td>Heart tissue</td>
<td>60-100 µg</td>
</tr>
<tr>
<td>iNOS</td>
<td>Cultured cells</td>
<td>10 µg</td>
</tr>
<tr>
<td></td>
<td>Heart tissue</td>
<td>30 µg</td>
</tr>
</tbody>
</table>

Samples were loaded into the wells of 4 % SDS-polyacrylamide stacking gel with a 15 % SDS-polyacrylamide resolving gel (Novex pre-cast gels, Novex, Germany) and run at room temperature in a tank containing running buffer (0.025 M Tris buffer, 0.192 M glycine, 0.1 % (v/v) SDS, pH 8.2) for about 45 min at 180V (Mini Protean II System, Bio-Rad, Herts, UK). Care was taken not to allow the bromophenol blue markers to run off the bottom of the gel.

The gel casting supports were removed and the gel allowed to equilibrate in transfer buffer (0.025 M Tris buffer, 0.192 M glycine, 0.1 % (v/v) SDS, 20 % (v/v) methanol, pH 8.2) Proteins were transferred onto a nitrocellulose membrane overnight at room temperature with a transfer voltage of 30V at a variable current not exceeding 250 mA.
All further procedures were done on an orbital shaker (Janke and Kunkel GmBH, Germany) at room temperature. Non specific binding of antibodies was blocked with 3 % (w/v) non-fat dried milk in PBS (0.01 M phosphate buffer, 0.0027 M KCl, 0.137 M NaOH, pH 7.4), for 2 h at room temperature. Following this the membranes were washed for 5 min with 10 ml PBS (pH 7.4). Membranes were then probed with a polyclonal rabbit anti-HO-1 antibody diluted 1:1000 in TBS (0.05M Tris-HCl, 0.0037 M KCl, 0.7137 M NaOH, pH 7.4) for 2 h at room temperature or polyclonal anti-iNOS or cNOS (Affiniti Bioreagents, York, UK) diluted 1:2000 in TBS for 1 h.

The primary antibody was poured into waste and the membranes washed three times for 5 min each with PBS containing 0.05 % (v/v) Tween-20 (PBS-T). Blots were visualised using an ExtrAvidin™ amplified Alkaline Phosphatase kit (Sigma, UK). Secondary antibody (whole biotinylated anti rabbit IgG) was diluted 1:2000 in TBS and applied to the membrane for 1 h. The secondary antibody was washed away with 3 x 5 min washes with 10 ml TBS followed by a 1 h incubation with ExtrAvidin Alkaline phosphatase conjugate diluted in Tris buffered saline (1:2000). After a final 3 x 5 min wash with 10 ml PBS-T, substrate (9.8 ml 0.1M Tris Buffer, pH 8.2 containing 200μl of 1 % (w/v) naphthol AS-BI phosphate in N-N-N-dimethyl formamide and 50 mg Fast Red DT tablet) was then incubated with the membrane until red bands showing the presence of antigen / antibody complex developed.
Figure 2.7  The sequence of antibody-substrate incubations for Western blot:
1) the nitrocellulose membrane will contain a number of antigens; 2) a specific polyclonal (raised in rabbit) antibody to the required antigen is bound; 3) anti-rabbit IgG with a biotin tail is then bound to the 1° antibody; 4) Extravidin alkaline phosphatase specifically binds to the biotin and, finally; 5) addition of the substrate gives a read precipitate indicating the presence of the antigen.

The membrane was washed for a further 10 min in ddH₂O and stored in foil until analysis. Membranes were scanned using a GS-700 Densitometer (Bio-Rad, UK) and relative band densities were calculated using Molecular Analyst™ Software (Bio-Rad, UK).
2.5 *In vitro* measurement of CO

2.5.1 Measurement of CO release by metal-carbonyl complexes

CO is a highly diffusible gaseous molecule and this property was utilised to develop a system to measure CO released from an organic compound that contains carbonyl groups. For this purpose we used iron pentacarbonyl (Fe(CO)\(_5\)) and manganese carbonyl (Mn\(_2\)(CO)\(_{10}\)).

To determine the levels of CO actually released by the metal-carbonyl complexes a system was set up to measure the conversion of deoxy myoglobin (Mb) to carbonyl-myoglobin (MbCO). MbCO has a distinctive absorption spectrum between 500 and 600 nm wavelength (see Figure 2.8A and B) and the increase in the 540 nm peak was used to measure the MbCO concentration in the myoglobin solution (shown as a red dotted line in Figure 2.8). Mb solutions were prepared freshly for each experiment by dissolving 17 mg horse cardiac myoglobin in 15 ml 0.04M phosphate buffer (pH 6.8) and mixing well.

To convert the Mb solution to ~100 % deoxyMb, a pinch (3-5 mg) of sodium hydrosulphite (a powerful reducing agent) was added to the solution just before the spectrum was read.

Before each reading was made over the time-course of the experiment, sodium hydrosulphite was added to the spectrophotometer cuvette to reduce any dissolved oxygen in the myoglobin solution. It is important to note that
addition of these small amounts of reducing agent did not change the characteristics of MbCO formation.
MbCO formation after exposure of a deoxy-myoglobin solution to CO gas.
A) The absorption spectrum of myoglobin between 500 and 600 nm was measured before and after bubbling for 2 min. with 1% CO gas. The generation of carboxy-myoglobin can be seen by the distinctive peak at 540 nm. B) Using the calculations described below, nmoles MbCO formed over time were calculated and plotted.
1.5 ml (~66 μM) deoxyMb was placed in the upper chamber in the anapore membrane cell culture insert instead of cells. In order to calculate the total myoglobin in the solution, deoxyMb solution was bubbled for 10 min with 1% CO gas until the absorbance at 540 nm reached a maximum. This OD value was taken as the maximum MbCO concentration and therefore the total myoglobin concentration of the mixture. Although, in theory, the solution of myoglobin was converted to ~100% MbCO the measured amount was never as high as 66 μM (the initial calculated concentration of Mb) because of impurities such as salts and, possibly, denatured myoglobin in the original
myoglobin solutions. Total myoglobin concentration was calculated using the equation shown below.

\[ \text{MbCO}_{\text{max}} = \left( \frac{\text{OD}_{540}}{\varepsilon} \right) \times 1000 \]

**Equation 2.4**  
Equation for calculating total myoglobin concentration in a saturated solution of carmoxy-myoglobin (MbCO).  
\( \varepsilon = \text{extinction coefficient of MbCO} = 15.4 \text{ mM}^{-1}\text{cm}^{-1} \)

Although the maximum absorbance at 540 nm after saturation with CO gas gives the total MbCO concentration (\( \text{MbCO}_{\text{max}} \)), any intermediate values of MbCO (between \( \sim 100\% \text{ deoxyMb in and } \sim 100\% \text{ MbCO} \)) have to be calculated by mathematical iteration from the \( \text{OD}_{540} \) reading. This is performed as follows:

Firstly, it must be assumed that in the deoxyMb solution there is \( \sim 0\% \text{ MbCO} \), so the \( \text{OD}_{540} \) at this point (always a positive value) indicates 0 μM MbCO. Assuming this, a new extinction coefficient (\( \varepsilon_2 \)) must be calculated to take into account the change in absorbance at 540 nm (\( \Delta\text{OD}_{540} \)). To aid in the accuracy of this calculation, another wavelength is used as a constant reference point. Conveniently, the deoxyMb and MbCO spectra share four isosbestic (\( \text{OD}_{\text{iso}} \)) points between 500 and 600 nm (\( \sim 510, \sim 550, \sim 570 \) and \( \sim 585 \text{ nm} \)). The value at 510 nm (\( \text{OD}_{\text{ISO510}} \)) was taken for these set of experiments. Thus, the new extinction coefficient was calculated:
Equation 2.5  **Equation needed to calculate an unknown COMb extinction coefficient.**

Taking into account the change in absorbance at the isosbestic point ($\Delta \text{OD}_{\text{iso}}$) and the change in absorbance at 540 nm ($\Delta \text{OD}_{540}$) a new extinction coefficient ($\varepsilon_2$) can be calculated.

From these calculations, the concentration of MbCO in a solution of myoglobin can be calculated if three things are known about the solution: i) the change in $\text{OD}_{540}$ between deoxy- and saturated MbCO (by saturating the Mb solution with CO gas); ii) the change in the $\text{OD}_{\text{iso}}$ in the sample from original deoxyMb solution and; iii) the $\text{OD}_{540}$ and $\text{OD}_{\text{iso}}$ of the sample solution. The following equation is therefore used to calculate the [MbCO] of the unknown sample:

$$\text{MbCO} = \left( \frac{\text{OD}_{540} - \text{OD}_{\text{exo}}}{\varepsilon_2 \times 1000} \right)$$

Equation 2.6  **Calculation unknown MbCO concentrations.**

where $\Delta \text{OD}_{540}$ is the change in absorbance at 540 nm, $\Delta \text{OD}_{\text{iso}}$ is the change in absorbance at the isosbestic point, MbCO$_{\text{max}}$ is the MbCO concentration calculated after saturation of the Mb with CO gas and $\varepsilon_2$ is the calculated absorption coefficient.

After all the time points have been calculated, the data is converted from a concentration ($\mu$M) to absolute MbCO values (nmoles) by multiplication of the $\mu$M value by the volume of myoglobin in the solution (in l). The plotted standard curve (Figure 8.2A, below) shows MbCO (nmoles) vs. time (min). From this graph, the incubation time required for any MbCO concentration

- 89 -
can be calculated; therefore cells can be exposed to a known concentration of CO using this system.

2.5.2 Exposure of cells to CO using the MCC system

Smooth muscle cells were grown to confluence on 0.4 μm anapore membrane cell culture inserts (Costar, Cambridge, MA) in 6-well plates at 37 °C in a 5 % CO₂ environment. 0.5 ml CO-RM was pipetted into the top space of the inverted 20 ml bottle and the cells culture inserts were transferred from the 6-well plates onto the top of the organic MCC reservoir, still being kept in the dark (see Figure 2.9). The cells were washed with medium and then 1.5 ml fresh medium was pipetted into the wells. The upper cell culture chambers were secured to the lower chamber using lab tape to ensure a tight junction between the two surfaces. Furthermore a sheet of parafilm™ was stretched over the top of the upper chamber to ensure containment of any gases within the cell culture environment. To start the experiment, the light source was turned on and the cells exposed to the MCC for pre-determined time points to expose to the cells to a known concentration of CO in the culture medium.

To terminate the experiment, the light was turned off and the cell culture well removed from the top of the MCC reservoir and processed according to the experimental protocol.
2.6 Extraction and measurement of tissue bilirubin

2.6.1 Spectrophotometric measurements

Hearts were perfused according to the Langendorff technique (see Section 2.8). After re-circulating the buffer for 2 hr, hearts were removed from the Langendorff apparatus and dried in an oven (60 °C) overnight, until completely desiccated and the dry weight calculated. The re-circulated Krebs buffer (100 ml) was cooled on ice and shaken vigorously for 10 min with 25 ml chloroform in the dark at 4°C. The solution was allowed to settle and the chloroform layer removed using a 250 ml fractionation column. This procedure was repeated 4 times and the pooled chloroform (100 ml) was slowly evaporated under a stream of nitrogen gas in the dark at room temperature.

The tubes were washed with 1 ml spectrophotometric grade chloroform and the extracted bilirubin was measured by the difference in absorption between 464 and 530 nm using a quartz cuvette (extinction coefficient, $\varepsilon$, 40 mM$^{-1}$cm$^{-1}$ for bilirubin). Bilirubin production was calculated as picomoles of bilirubin formed/mg dry weight/h.

2.6.2 HPLC analysis

Bilirubin was extracted from myocardial tissue and quantified by high performance liquid chromatography (HPLC). Because bilirubin is highly sensitive to light, all the extraction procedures were performed in tubes covered with aluminum foil. Briefly, hearts were homogenised in 5 volumes of 0.25 M sucrose/0.05 M Tris-HCl (pH 7.4), mixed with 2 volumes of chloroform
and shaken vigorously for 5 min. The chloroform phase, containing the extracted bilirubin, was separated from the aqueous phase by centrifugation at 1000 x g for 20 min. This extraction step was repeated twice and the extracts pooled. The organic phase was transferred to a dry tube, evaporated under a stream of nitrogen gas, and the residue re-suspended in 1 ml of DMSO and stored at 4 °C until analysis. All HPLC equipment and software were purchased from Gyncotek Ltd. (Macclesfield, Cheshire, UK). Chromatographic separations of bilirubin were performed using a Techsphere™ 5 μm C18 column (15 cm x 4.6 mm). The eluent consisted of 40 % ammonium acetate (100 mM, pH 5.0)/60 % methanol running a linear gradient over 25 min to methanol (100 %) at a flow rate of 1 ml/min. Peak identity for bilirubin was confirmed by measuring the retention time, spiking the sample with commercially available standards and determination of absorbance spectra using a UV photo-diode array detector (Gyncotek, Model VVD-340) set at 450 nm. Authentic standards of bilirubin were prepared in DMSO.

2.7 Histological analyses

2.7.1 Immunohistochemical staining of rat heart muscle for HO-1

Animals were killed by cervical dislocation and hearts removed after thoracotomy. The hearts were perfused with 25 ml ice cold (4 °C) saline or until completely flushed of blood. The left ventricle portion of the hearts was dissected, attached to cork tiles with CryoEmbed™ and immediately plunged
into liquid nitrogen. Sections of 5-10 μm were cut onto Vectabond™ (Vector Laboratories, Burlingame, USA) coated glass slides.

Cryostat sections were treated with 0.3 % hydrogen peroxide in methanol for 30 min at room temperature to block endogenous peroxidases. After rinsing in phosphate buffered saline (PBS), slides were incubated with PBS containing 1 % bovine serum albumin, 10 % normal goat serum and 1 % Tween-20 to reduce non-specific labelling. Slides were incubated with anti-HSP 32 (haem oxygenase-1) antibody (1: 1000) (Stressgen, Victoria, Canada) overnight at 4 °C. The slides were then washed three times in PBS containing 1 % (w/v) BSA for 5 min in each wash and incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories) at a dilution of 1:500 for 1 h at room temperature. They were then washed twice with PBS and incubated with avidin-biotin complex (ABC Elite kit, Vector Laboratories) for 1 h at RT. After washing with PBS, slides were treated with 3-3’ diaminobenzidine (DAB substrate kit for peroxidases, Vector Laboratories) for 4 min. The slides were finally washed first in PBS and then distilled water and counter stained with heamotxylin.

2.7.2 Immunocytochemistry for HO-1

Cells were cultured to confluence in Lab-Tek (Nunc, IL, USA) chamber slides (8 chambers per slide). Cells were incubated with various agents in culture medium for predetermined times according to the experimental protocol then washed twice with ice cold DPBS for 5 min without shaking. The upper media chambers of the slides were carefully removed and the slides transferred into 10 cm plastic Petri dishes for the wash and incubation steps. Cells were fixed
to the slides with ice cold 95 % (v/v) ethanol in water for 10 min in ice and washed a further twice more with ice cold DPBS. Samples were incubated with ice cold 3 % (v/v) H$_2$O$_2$ in DPBS for 5 min after which the H$_2$O$_2$ was discarded and the cells incubated in 5 % normal goat serum in DPBS at room temperature for 20 min.

The serum was removed and 10 ml PBS containing diluted HO-1 antibody (1:2000) was added. This was left for 18-20 h in the refrigerator on ice (3-6°C). All following incubations were performed at room temperature on an orbital shaker (Janke & Kunkel GmbH, Germany). After this time the antiserum was removed and the cells washed 2 x 15 min 10ml with DPBS. The secondary antibody was applied to the slides (goat anti rabbit whole IgG diluted 1:100 in PBS) and allowed to incubate for 60 min. The ABC complex was made according to the following protocol: 5 µl sol A and 5 µl sol. B was added to 990 µl PBS, the two solutions being added separately to avoid an insoluble complex. The slides were washed 2 x 10 min 10ml DPBS before application of the ABC complex (tertiary antibody) and left for 60 min. Finally, cells were washed twice with PBS (5 min each wash) prior to the activated DAB substrate (5.0 ml dH$_2$O containing 2 drops buffer stock solution, 4 drops DAB stock solution and 2 drops hydrogen peroxide solution) being added and allowed to develop over 10 min. Cells were washed with distilled water and allowed to dry in the dark before application of a cover slip.
2.7.3 Transmission electron microscopy

Heart muscle biopsies were fixed in 0.1 M phosphate buffer containing 3% glutaraldehyde (Agar Scientific Ltd., Essex, England) for 2 h. After two washes, a second fixation with 1% osmium tetroxide in 0.1 M phosphate buffer was carried out for 1 h at room temperature. The specimens were then dehydrated through an increasing acetone series, infiltrated with acetone-araldite CY212 resin (1:1) overnight in the specimen rotator, embedded in araldite CY212 resin and finally, these blocks were polymerised at 60°C for 18 hrs. Ultra-thin sections (70-100 nm) were cut, collected on copper grids and stained with 2% uranyl acetate (10 min) followed by Reynolds lead citrate (10 min). Mitochondrial integrity was analysed in the stained sections using a Jeol 1200CX electron microscope.

2.7.4 Scanning electron microscopy of vascular smooth muscle cells

Vascular smooth muscle cells were grown on Lux thermanox™ cover-slips (Miles Scientific, IL) until 80% confluent. Cells were fixed in 3% glutaraldehyde (Agar Scientific Ltd., Essex, England) in 0.1 M phosphate buffer for 2 h. After two buffer washes, the secondary fixation with 1% osmium tetroxide in 0.1 M phosphate buffer was carried out for 1 h at room temperature. The dehydration was accomplished by serial immersion of the samples (20 minutes) in 25%, 50%, 75%, 95% and 100% ethanol. Specimens were then critical point dried and mounted on copper stubs using silver dag (Agar Scientific Ltd). They were gold coated in sputter coater and kept in a
vacuum dessicator until viewed in a JEOL 1200 EX electron microscope in SEM mode.

2.8 Isolated heart preparation

2.8.1 The Langendorff isolated heart technique

Male Lewis rats weighing 300 to 350 g were killed by cervical dislocation. Hearts were removed after thoracotomy and immediately immersed in ice cold saline (0.9 % NaCl in distilled H₂O). The aorta was exposed, cleaned and mounted on a metal cannula, with the base of the cannula a few millimetres from the tricuspid valve. Hearts were perfused by the non-re-circulating Langendorff technique, with a modified Krebs-Henseleit solution at a constant flow rate of 15 ml/min (see Table 2.4).

The perfusate was equilibrated at 37°C with 5 % CO₂ ad 95 % O₂. The heart was enclosed in a temperature controlled, water-jacketed, chamber allowing the ventricles to remain immersed in warm Krebs-Henseleit buffer.
<table>
<thead>
<tr>
<th>Solute</th>
<th>Concentration (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>Potassium chloride</td>
<td>4.70</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>2.50</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>1.66</td>
</tr>
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</tr>
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<td>Potassium phosphate</td>
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<tr>
<td>Glucose</td>
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</tr>
<tr>
<td>Sodium pyruvate</td>
<td>2.00</td>
</tr>
<tr>
<td>EGTA</td>
<td>0.50</td>
</tr>
<tr>
<td>pH</td>
<td>~7.4 (after bubbling with 95% O₂-5% CO₂)</td>
</tr>
</tbody>
</table>
Plate 2.3  Langendorff heart apparatus showing Grass™ polygraph recorder (centre) and data acquisition computer (right)

Plate 2.4  The Langendorff isolated heart apparatus
Coronary perfusion pressure (CPP) was measured with a pressure transducer (Grass Instruments) installed above the aortic cannula. A latex balloon filled with saline, connected by a catheter to a pressure transducer, was inserted into the left ventricle through an atriotomy and secured by a suture around the atrio-ventricular groove. The balloon was inflated to provide an end-diastolic pressure of 10 mm Hg. Both transducers were connected to a microcomputer running data acquisition software (Aqknowledge™, Biopac Systems, UK) and data were continuously recorded over the course of the experiment.
The heart rate (HR) was mathematically derived on the computer from the peak rate of the left ventricular developed pressure (LVDP).

In most cases, hearts were allowed to beat spontaneously throughout the experiments. However, in those experiments when pharmacological treatment of the heart in situ was performed (L-NAME - induced contraction) hearts were paced using a Grass SD-88 (Astro-Med, RI, USA) stimulator at 5 V at 300 beats per min (bpm) The pacing electrodes were positioned as shown in Figure 2.10.
Equation 2.7  Calculation of cardiac performance

2.8.2 Perfusion protocols

In all experiments, isolated hearts were allowed to equilibrate, paced or un-paced, at normal flow for 20 min before any treatment was applied.

In ischaemia-reperfusion experiments, hearts were made globally ischaemic by interrupting the flow. Ischaemic hearts were totally immersed in Krebs-Henseleit solution at 37 °C in the water-jacketed chamber for 30 min. After the ischaemic period, the flow was restarted and the heart was raised so that, as before, only the ventricles remained immersed in buffer. After 60 min of normal perfusion, hearts were removed from the aortic cannula, freeze-clamped in liquid nitrogen (with tongs) and stored at -70 °C or perfused with tetrazolium red for 15 min for tissue viability staining.

In some experiments, drugs were administered to the hearts during normal perfusion. Drugs were delivered from a 50 ml syringe mounted in a syringe pump (Vickers Medical, UK) connected via a pressure tight intravenous catheter into a side arm of the aortic cannula. Flow of the drug into the heart could be tightly controlled by adjusting the rate of flow in the syringe pump so that the desired final concentration was reached upon dilution into the
perfusate. Hearts were kept under normal perfusion conditions in the jacketed chamber throughout the experiment.

2.8.3 Macro-staining for tissue viability

A representative number of hearts from each experimental group were not removed from the cannula immediately after the 60 min reperfusion. These hearts were further perfused through a side arm of the aortic cannula for 20 min with 3 % 2-3-5-triphenyltetrazolium-chloride in modified Krebs Henseleit buffer at a constant pressure of 150-200 mmHg. This allowed time for the colourless tetrazolium salt to enter the cells of the myocardium and coronary bed. In cells where there was active reduction occurring (i.e. cells that were still viable following the ischaemia - reperfusion event) the colourless dye was reduced to an insoluble red precipitate. Thus, at the end of the treatment viable tissue appeared brick red whereas infarcted areas (damaged cells) remained colourless.
Figure 2.11  Schematic diagram of the Langendorff apparatus
After the staining period hearts were cut from the aortic cannula and stored in the dark in 2% formal saline solution (0.9% NaCl containing 2% formalin v/v) prior to analysis.

When required for processing, hearts were removed from the formalin and dried. Tissues were then placed in a Perspex block with guides for a microtome blade spaced 2 mm apart. The sample was carefully cut into serial sections 2 mm thick and the resulting tissue slices stored in formal saline.

![Heart block used for sectioning](image)

Figure 2.12  Heart block used for sectioning
Hearts were fixed in formal saline for 24 h before being mounted on the heart block and sliced into 2 mm slices using a microtome blade.

Slices were placed on a flatbed colour image scanner (Bio-Rad GS-700 Densitometer) and the images captured at high resolution on a microcomputer. The volume of ischaemic tissue (% of total) in each heart was calculated by the following formula:

\[
\text{% Ischaemic Volume} = \left( \frac{\sum \text{ischaemic area per slice} \times \text{number of slices}}{\sum \text{total slice area} \times \text{number of slices}} \right) \times 100
\]

Equation 2.8  Calculation of ischaemic volume in isolated hearts
2.9 Chronic catheterisation of anaesthetised rats

Male Lewis rats (280-350 g) were anaesthetised with an injection (i.m.) of 0.6 ml Hypnorm® (10mg/ml fluanisone - 0.315 mg/ml fentanyl citrate, Janssen, UK) and injection (i.p.) of 0.4 ml diazepam (5 mg/ml, Phoenix Pharmaceuticals Ltd, UK). The upper inner thigh on the left and right side of the animal was shaved and the animal secured onto a dissection board under an operating microscope (Zeiss, Germany).

A lateral incision was made into the groin space of the left leg. After separating the fascia from the panniculus carnosus muscle, the femoral artery and vein were exposed. The surrounding skin and muscle was held away from the field of view by means of elasticated retractors. The femoral vein was
carefully blunt dissected away from the femoral artery and nerve and three 4/0 silk sutures were loosely tied around the vessel at equal intervals along its length. After clamping the proximal end of the vein, the distal suture was tied tight. A small incision was made in the vessel and a saline-filled intravenous catheter (Portex, UK) was inserted into the vein and then the clamp removed. Once approximately 1.5 cm of catheter had been inserted, the three sutures were tied and knotted (see Plate 2.6A & B). The operating area was flushed with sterile saline and the wound closed with a continuous stitch with 2/0 silk suture. The catheter was connected to a 3-way tap and 5 ml saline-filled syringe and flushed (approximately 0.3 ml) through to remove any blood from the catheter.

This procedure was repeated on the opposite leg, except that the femoral artery was cannulated and once secure and flushed with saline was connected to a primed and calibrated pressure transducer (Astra-Med, UT, USA) for measurement of MAP. The side arm of the pressure transducer was connected to a saline-filled syringe to enable flushing of the arterial line. This allowed precise measurement of the MAP throughout the experiment. At the end of experimentation, the animals were euthanased by infusion with 2.5 ml Lethobarb® (200 mg/ml sodium pentobarbital, Fort Dodge, UK).
Plate 2.6  Positioning of the femoral arterial and venous cannulae
A) The cannulae (c) were tied in place by 3 sutures (s). The arterial cannula is shown here before tying. B) Complete cannulation of both femoral vein (v) and artery (a) on the same side. This is shown as a demonstration of the surgical procedure; in the experimental model described here left femoral vein and right femoral artery were used.
2.10 Statistical analysis

Statistical analysis was performed by the Student's two-tailed $t$-test, and an analysis of variance (ANOVA) was performed where more than two treatments were applied. $P$ values $< 0.05$ were considered statistically significant.
3. Regulation of the Haem Oxygenase System in Vascular Tissues

3.1 Introduction

Haem oxygenase-1 (HO-1) has emerged as an important component of the mammalian stress response, since it has been shown that its gene can be up-regulated by a number of stimuli (59, 134). Since HO-1 protein expression can be induced by a number of pro-oxidants and it appears to be ubiquitously expressed in mammalian tissues, it has been suggested that HO-1 plays a crucial role in cellular antioxidant defense mechanisms (48, 135, 136). This hypothesis is further supported by evidence showing that biliverdin and bilirubin, products of the enzyme's action, have significant antioxidant properties and carbon monoxide, also produced by HO-1-mediated haem degradation is an important signalling molecule in both the vasculature and central nervous system (61, 63, 68, 137-139).

It is important, therefore, that we further understand the regulation of this important pathway in cells and tissue if we are to exploit the cells' endogenous defense mechanisms in the prevention or reduction of a number of pathophysiological conditions. Hence, in the studies described in this chapter the regulation of HO-1 expression was investigated in a variety of mammalian vascular tissues evaluating a number of pharmacological and chemical agents.
3.2 Objective

The purpose of this study was to determine haem oxygenase activity and HO-1 protein expression in vascular tissues, including vascular smooth muscle and rat vascular tissues such as heart and liver in response to various agents believed to have a stimulatory effect on the haem oxygenase system.

The ultimate aim of this study is to determine suitable agents for further study into the role of the haem oxygenase system (specifically HO-1) in vascular tissues.

3.3 Methods

3.3.1 Preparation of reagents

The nitric oxide donor, sodium nitroprusside (SNP) was prepared in culture medium at the desired concentration in the dark immediately before incubation with cells. Hemin (ferriprotoporphyrin IX chloride) was prepared at a stock concentration (normally 20 x maximum final concentration) in 5 ml 0.05M phosphate buffered saline (pH 7.4) by the addition of 50 μl 2M NaOH.
3.3.2 Treatment of cells with various agents

Bovine aortic smooth muscle cells (Cell line AG08595A - Corel Cell Repository, NJ) were grown in minimal essential medium (MEM) supplemented with 20 % (v/v) fetal calf serum, 2 x MEM amino acids, 2 x MEM non-essential amino acids, 2 x MEM vitamins and penicillin (5 mg/l) and streptomycin (5 mg/l). Cells were passaged by trypsinisation (0.25 % trypsin EDTA) with a 1:3 or 1:4 dilution. All experiments were performed on cells between passage number 8 and 24. Cells were allowed to grow until confluent before incubation with the substrate, hemin (0 - 500 μM, 0 - 48 h), cadmium chloride (CdCl₂, 0 - 10 μM), and the nitric oxide donor, SNP (0 - 500 μM, 6 h), which have been shown previously to be potent up-regulators of haem oxygenase activity and HO-1 protein expression in various cell types (140-144). After the incubation time, cells were collected for analysis of haem oxygenase activity and HO-1 protein expression as described in Materials and Methods.

3.3.3 Cytotoxicity studies

A complementary set of experiments were carried out to determine the possible cytotoxic effects of each of the compounds found to induce haem oxygenase activity and HO-1 expression. Cells were grown in wells of a 24-well tissue culture plate until confluent and treated with the same agents as described above. At the end of the experiment, each well was processed to determine cell viability using a cell metabolism assay (see Section 2.2.8).
3.3.4 In vivo tissue methodology

Male Lewis rats (280-350 g) were administered (i.p.) with various agents thought to be inducers of tissue haem oxygenase-1 including hemin (10-50 mg/kg body weight), the nitric oxide donor, SIN-10 (molsidomine, 0-20 mg/kg)(145), and bacterial lipopolysaccharides (LPS) from E. coli and S. typhimurium all of which have been shown previously to have HO-1 inducing properties (40, 42, 46, 49). After the predetermined time-course (0 - 24 h) animals were sacrificed by cervical dislocation and the hearts and livers carefully removed and flushed with ice-cold (4 °C) saline. Tissue was processed as described in Materials and Methods for microsomal membrane collection (see Section 2.3.1). Haem oxygenase activity and HO-1 protein expression in the microsomes were assessed by haem oxygenase activity assay and Western blot using a specific polyclonal antibody against HO-1 (as described in Materials and Methods).

3.4 Results

3.4.1 Regulation of HO-1 expression in primary cultured vascular smooth muscle cells by hemin

To determine whether hemin is able to up-regulate haem oxygenase activity in vascular smooth muscle cells, primary cultured rat aortic smooth muscle cells and the bovine smooth muscle cell line were treated for 2 h with complete medium containing hemin followed by 4 h with normal growth medium to allow up-regulation of the haem oxygenase enzyme system.
The effect of hemin on the haem oxygenase activity of primary cultured smooth muscle cells is shown in Figure 3.1 (above). A concentration-dependent increase in haem oxygenase activity was observed after treatment of primary cultured cells with hemin with a maximum, 2.5-fold increase in activity, measured after treatment of cells with 200 µM (P<0.05). However, increasing the concentration of hemin further to 400 µM did not further increase the haem oxygenase activity attained, although the activity was still significantly above control levels (P<0.05).

Cultured vascular smooth muscle cells (a cell line) treated with hemin for 2 h showed a time- and concentration-dependent increase in HO-1 expression and haem oxygenase activity. Maximal protein levels and activity were detected
4 h after treatment with hemin at 100 μM (Figure 3.2 and Figure 3.3). Although HO-1 protein expression was more prominent at 200 μM compared to 100 μM hemin, haem oxygenase activity peaked at 100 μM and started to decrease at higher concentrations of hemin. Figure 3.2 illustrates the change in haem oxygenase activity after incubation with a range of concentrations (0-200 μM) at two different time points (6 and 24 h) after the start of the hemin incubation. After 4 h, haem oxygenase activity was significantly elevated ($P<0.05$) when cells were treated with 50 μM hemin and continued to rise to a peak at 100 μM hemin where there was an approximate 5-fold increase in haem oxygenase activity compared to control. At still further increased concentrations of hemin (200 μM) there was a significant decrease in activity from the maximum but it still remained significantly higher than control levels.

At 22 h after exposure of cells to hemin HO-1 expression and haem oxygenase activity were considerably diminished at all concentrations used (Figure 3.2), indicating a gradual return of cells to basal conditions after the initial response to hemin. However, there was a significant, dose-dependent increase in haem oxygenase activity up to 100 μM hemin with a 3-fold increase in activity at the maximum when compared to control values. However, similar to the 6 h group, a concentration of 200 μM hemin 22 h after exposure resulted in a decrease in haem oxygenase activity.

HSP70 is considered a sensitive marker of cellular stress and so was used as an indicator of hemin-induced toxicity in the cells (146). However, changes in
HSP70 protein expression were only found after treatment of cells with hemin concentrations of 200 μM, or above, indicating that at concentrations of hemin below 200 μM there was no evident cellular toxicity. However this is discussed in more detail in Section 3.4.2 (below).

In a further set of experiments, the time-course of haem oxygenase-1 protein expression was determined at a fixed concentration (100 μM) of hemin; this can be seen in Figure 3.4. After incubation with 100 μM hemin it took between 1 and 3 h to find detectable amounts of HO-1 protein in the cell lysates. There was maximal HO-1 expression at 6 h which remained elevated for a further 6 h before dropping again after 18 and 24 h incubation. However, HO-1 protein levels did not return to baseline until after 24 h.
Figure 3.2 Effect of hemin on smooth muscle cell haem oxygenase activity.
Haem oxygenase activity was measured after incubation of cells with hemin (50 - 200 μM) for 2 h and then normal growth medium for a further 4 and 22 h. Control experiments are represented by cells exposed to complete medium alone. Each value represents the mean ±SEM of 4-6 experiments. *, P<0.05 compared with control; **, P<0.01 compared to control.
**Figure 3.3** Western blot analysis of HO-1 and HSP70 expression in vascular smooth muscle cells after treatment with hemin
Vascular smooth muscle cells were exposed to hemin for 2 h and then allowed to incubate for a further 4 h in normal growth medium. Polyclonal antibody against HO-1 and HSP70 was used to determine relative HO-1 and HSP70 protein expression.

**Figure 3.4** Western blot analysis of HO-1 in vascular smooth muscle cells after hemin treatment
Vascular smooth muscle cells were exposed to hemin for 1, 3, 6, 12, 18 and 24 h (2 h exposure to hemin and then 1, 4, 10, 16, 22 h incubation with growth medium). After incubation, cells were scraped from the flasks and Western blot performed on the cell lysates using a polyclonal antibody against HO-1.
Figure 3.5  **Haem oxygenase-1 expression in vascular smooth muscle cells after treatment with hemin**

Cells were incubated for 2 h with 100 μM hemin. After a further 4 h the cells were processed for HO-1 immunocytochemical staining. A) control cells, incubated with culture medium alone; B) cells after being treated with 100 μM hemin.

To support these data, cells were also grown in multi-well cell culture slides and the cells treated with hemin for 2 h, as before. After a further 4 h the samples were processed for immunocytochemistry using a polyclonal antibody against HO-1. Figure 3.5 shows the results of this staining. There is a definite increase in the intensity of positive staining for HO-1 proteins, indicated by a red/brown colouration in the cells.

### 3.4.2 Effect of hemin on vascular smooth muscle cell viability

To determine the effect of hemin on the viability of smooth muscle cells, cells were incubated with hemin for 2, 6 or 24 h and cell viability after the period of incubation was ascertained using the MTS tetrazolium cell metabolism assay.
The results of this experiment is shown in Figure 3.6 (below). Up to 300 μM hemin there were no statistically significant changes in cell viability at any time point. However at 400 μM hemin after 24 h there was a significant ($P<0.05$) decrease in cell viability in cells compared to 0 μM hemin.

**Figure 3.6** Effect of hemin on vascular smooth muscle cell viability
Cell viability was assessed using the MTS tetrazolium cell metabolism assay after incubation with hemin (0-400 μM) for 2, 6 or 22 h normal growth medium. Each value represents the mean ±SEM of 5-6 experiments. *, $P<0.05$ compared with 0 μM hemin.
3.4.3 Regulation of HO-1 expression in vascular smooth muscle cells with nitric oxide donors and heavy metal ions

It has been reported that nitric oxide (NO) is a potent inducer of haem oxygenase activity in aortic endothelial cells (147). To determine whether NO has a similar effect on smooth muscle cell haem oxygenase-1 expression, vascular smooth muscle cells were incubated with the NO-donor, SNP. A concentration-dependent increase in haem oxygenase activity was observed in cells incubated for 6 h with SNP; this is shown in Figure 3.7. Haem oxygenase activity was significantly increased at 0.125 mM SNP (P<0.05) and continued to rise to a maximum (3.6-fold increase in activity) at 0.25 mM SNP (P<0.05). Treatment of cells with a higher concentration of SNP (0.5 mM) did not result in a further increase in haem oxygenase activity.

Interestingly, when cell viability was assessed in cells treated with SNP by the MTS tetrazolium metabolic activity assay (Figure 3.8), there was a significant decrease (P<0.05) in cell viability at concentrations of SNP above 0.25 mM. However, at concentrations below 0.25 mM SNP there was no significant effects of the nitric oxide donor on the viability of the cells.
Figure 3.7  **Effect of the NO donor SNP on BASMC haem oxygenase activity**
Haem oxygenase activity was measured after incubation of cells with SNP (0.125 - 0.5 mM). Control experiments are represented by cells exposed to complete medium alone. Each value represents the mean ±SEM of 4-6 experiments. *, P<0.05 compared with control.

Figure 3.8  **Effect of the NO donor SNP on BASMC viability**
Cell viability was measured after incubation of cells with SNP (0.125 - 0.5 mM) by the MTS assay (see Materials and Methods). Control experiments are represented by cells exposed to complete medium alone. Each value represents the mean ±SEM of 4-6 experiments. *, P<0.05 compared with control.
Heavy metal ions, such as cadmium, stannic ions, copper and iron, have all been reported to increase the expression of HO-1, presumably through their relative toxicity at high concentrations (31, 46, 143, 148). To test this hypothesis in vascular smooth muscle cells, cells were incubated with 10 μM cadmium chloride (CdCl₂) in growth medium for 18 h. Figure 3.9 shows a significant (P<0.05) increase in haem oxygenase activity in cells treated with CdCl₂ compared to control cells; this is also reflected in the intense band seen by Western blot analysis (Figure 3.9, Inset).

Figure 3.9 Haem oxygenase activity and HO-1 expression in smooth muscle cells after treatment with CdCl₂
Cells were incubated in normal medium for 18 h with 10 μM CdCl₂. Cells were harvested and the haem oxygenase activity determined. Also Western blot analysis for HO-1 was performed on the samples (Inset). Data represent means (± SEM) or 4 experiments. *, P<0.05 vs. 0 μM CdCl₂.
### 3.4.4 Regulation of HO-1 expression in vascular tissue (heart and liver) by hemin, LPS and nitric oxide donors

In order to determine the effects of a number of possible inducers of the haem oxygenase system *in vivo*, male Lewis rats (280-350 g) were injected (i.p.) with a number of compounds, previously reported to have possible HO-1 inducing ability. The compounds used were:

1) Hemin, a substrate for the haem oxygenase enzyme and a known inducer of HO-1 in vascular cells *in vitro* (149, 150)

2) SIN-10, a precursor of the NO-donor SIN-1. NO has been shown to be a potent inducer of HO-1 in many *in vitro* and *in vivo* systems and (49, 50, 147, 151, 152);

3) Lipopolysaccharides (LPS), which has been shown to induce the stress response in a number of *in vitro* and *in vivo* models (40, 153).

For the purpose of this study it was important to try and maximise HO-1 induction with minimal toxic effects of the drug.

Hemin has been shown previously to be a potent inducer of HO-1 in vascular smooth muscle cells and vascular endothelial cells. A number of male Lewis rats (280-250 g) received an i.p. injection of hemin at a single dose of 40 mg/kg body weight. At 0, 6, 12, 18 and 24 h the animals were euthanased and the hearts surgically removed and flushed with ice cold (4 °C) saline. For mRNA analysis (Northern blot), the ventricular cardiac tissue was immediately
frozen in liquid nitrogen ("snap" frozen) and processed for RNA extraction as described in Materials and Methods. Northern blot analysis was performed on the tissue, a representative result of which is shown below in Figure 3.10

Figure 3.10  **HO-1 mRNA after treatment with hemin**  
Rats were injected with 50 mg/kg body weight hemin (i.p.) and after 0, 1, 3, 6, 12, 18 and 24 h the hearts were removed and processed for Northern blot analysis according to Materials and Methods. A) mRNA probe for HO-1; B) RNA probe for GAPDH, as a housekeeping gene.

From Figure 3.10 a slight increase in HO-1 mRNA at 6 h after the injection can be seen followed by a dramatic increase in HO-1 mRNA after 12 h, which appears to drop back to baseline levels 18 h after administration.

In addition to HO-1 mRNA measurement cardiac tissue samples were also collected from animals at later times (1, 2, 3, 4 and 5 days) after i.p. injection of 50 mg/kg body weight hemin. Cardiac tissue was processed for microsomal...
preparation as described in Materials and Methods and haem oxygenase activity was measured in the microsomes.

Figure 3.11  **Haem oxygenase activity in the heart after administration of hemin**

Male Lewis rats (280 - 350 g) received an i.p. injection of hemin (50 mg/kg body weight). After 1, 2, 3, 4 and 5 days the hearts were removed and processed for microsome collection and HO-1 activity measured by haem oxygenase activity assay. Data represent means (± SEM) of 3-4 experiments. *, P<0.05 vs. time 0.

There was a clear, significant (P<0.05) increase in HO-1 activity in hearts 1 day after hemin administration but the activity had returned to baseline (0 day) levels by the third day. This is shown in Figure 3.11.

Clinically, an increase in systemic LPS is associated with the condition of sepsis; however in this study LPS was simply used as a possible up-regulator of the haem oxygenase pathway, although the data may give an indication of the effects of sepsis on haem oxygenase activity and expression (42, 154, 155).
Figure 3.12  **Haem oxygenase activity in heart microsomes 18h following administration of bacterial LPS**

Male Lewis rats were injected (i.p) with LPS (0-1.5 mg/kg body weight) derived from two different bacteria (*S. typhymurium* and *E. coli*). After 18 h the hearts were removed, flushed with ice-cold saline and the microsomes were prepared as described in Materials and Methods. Haem oxygenase activity was measured. Data represent means (± SEM) or 4-6 experiments. *, P<0.05 vs. 0 mg/kg body weight LPS.

Figure 3.12 (above) shows the effect of two bacteria-derived LPS (from *Salmonella typhymurium* and *Escherichia coli*) on cardiac haem oxygenase activity. A concentration-dependent increase in haem oxygenase activity can be seen in microsomes taken from animals treated with either of the LPS compounds. It is interesting to note that in this model, both types of bacterial endotoxin have a similar effect on haem oxygenase activity.

Induction of the haem oxygenase pathway by hemin and LPS, however, may not be as straightforward as these data suggest. In these experiments only HO-1 mRNA, protein expression or haem oxygenase activity was determined. There are a number of other pathways known to be affected by LPS, including
the nitric oxide synthase pathway (iNOS) (156-158). It is important, therefore, to consider these pathways when choosing a drug suitable for HO-1 induction in *in vitro* and *in vivo* studies.
Figure 3.13  Differential expression of HO-1 and iNOS in hearts and livers from rats treated with hemin or LPS

Rats received an i.p. injection of either A) LPS (1.25 mg/kg body weight) or B) hemin (50 mg/kg body weight). After 24 h, hearts and livers were removed and flushed with ice-cold saline. Organs were homogenised and samples were analysed by Western blot for HO-1 and iNOS using polyclonal antibodies against each protein.
The data show that the administration of *S. typhymurium*-derived LPS not only increased HO-1 expression but also the inducible form of nitric oxide synthase (iNOS) in the rat heart and liver. Figure 3.13 shows the differential expression of HO-1 and iNOS measured by Western blot in the heart and liver 24 h after administration of either hemin (50 mg/kg body weight) or LPS (1.25 mg/kg body weight). It can be observed from the figure that both hemin and LPS up-regulate HO-1 protein expression in both heart and liver tissue. However, LPS also had an effect on the expression of iNOS in both heart and liver, greatly increasing the amount of iNOS protein found in the samples. This contrasts with the effect of hemin on both liver and heart iNOS expression, where no change was seen from the vehicle control.

Nitric oxide (NO) has been shown to have marked effects on haem oxygenase activity and expression in vascular tissue. In order to demonstrate the effects of NO on the vascular tissue of a rat, animals were injected with the nitric oxide donor SIN-10. Figure 3.14 shows the effect of a single injection of SIN-10 (0 or 20 mg/kg body weight) on heart and liver haem oxygenase activity.

The nitric oxide donor SIN-10 had a significant ($P<0.05$) effect on haem oxygenase activity in both the heart and liver after 24 h. In both cardiac and liver tissue there was an approximate 2-fold increase in HO activity in both tissues.
Figure 3.14  **Haem oxygenase activity in heart and liver 18h following administration of the NO donor SIN-10**

Male Lewis rats were injected (i.p) with SIN-10 (20 mg/kg body weight) or saline vehicle. After 24h the hearts and livers were removed, flushed with ice-cold saline and the microsomes were prepared as described in Materials and Methods, and haem oxygenase activity was measured. Data represent means (± SEM) of 4-6 experiments. *, P<0.05 vs. 0 mg/kg body weight SIN-10.

3.5 Discussion

This study demonstrates that the HO-1 gene, HO-1 protein and haem oxygenase activity are readily up-regulated by a number of chemical agents including hemin, NO-donors and endotoxin.

3.5.1 In vitro studies

Treatment of vascular smooth muscle cells with hemin stimulates HO-1 protein expression and haem oxygenase activity in a time- and concentration-dependent manner. This was demonstrated in this report by direct
measurement of HO-1 expression in smooth muscle cells by immunolocalisation as well as biochemical measurement of haem oxygenase activity and intracellular HO-1 protein expression. This finding is not altogether surprising since hemin is the substrate for haem oxygenase and it has previously been shown to induce haem oxygenase activity in vascular smooth muscle cells and the ductus arteriosus in the lamb (159, 160).

It is interesting to note that at higher concentrations of hemin (400 μM), haem oxygenase activity began to fall. This effect is presumably a consequence of the cytotoxic nature of the hemin moiety. In fact, by virtue of its iron content, hemin can act as a promoter of reactive oxygen species formation and thus mediate cell injury (53, 161, 162). This concept is also sustained by our findings showing that at 200 μM hemin, cells started to exhibit increased levels of inducible heat shock protein 70 (HSP70) (Figure 3.3), a chaperone involved in refolding of damaged and denatured proteins; at higher concentrations still (400 μM), there was a discernable decrease in cell viability measured by an index of cellular metabolism.

In this Chapter we have also demonstrated the ability of NO to induce haem oxygenase activity in vascular smooth muscle cells. Similar to the observations made after hemin treatment, SNP-mediated HO activity was concentration-dependent. Likewise, at the highest concentration of SNP used in this study (0.5 mM) the nitric oxide donor appeared to be cytotoxic. The pro-oxidant actions of nitric oxide are well documented and it can participate
in a wide spectrum of biological reactions by virtue of its free radical nature (92).

The ability of NO to stimulate haem oxygenase activity in vascular smooth muscle cells complements a study by our group demonstrating NO-mediated activation of haem oxygenase activity in vascular endothelium (147). Takahashi and co-workers recently showed that sodium nitroprusside (SNP) also increased the levels of HO-1 protein in human cervical cancer cells, human melanoma and neuroblastoma cells as well as smooth muscle cells (163). In a similar study, Hartsfield and co-workers demonstrated induction of the HO-1 gene in smooth muscle cells by the NO-donor, SNN (51). Interestingly their study also showed that NO-mediated induction of HO-1 was cGMP independent, using the selective guanylate cyclase inhibitor ODQ. Thus, the observed induction of heme oxygenase-1 in this study may represent an important response to NO or NO-related oxidative stress in vascular tissue (80).

Induction of the haem oxygenase system in the presence of the substrate should result in an increase in the production of its two catabolites, biliverdin and carbon monoxide (CO). Many studies have over the last decade looked at the effects of NO on cellular systems. In the light of these present data, perhaps serious consideration should now be given to the role of CO in mediating the responses observed, since this molecule has also been shown to possess similar functions to those of NO (74, 137, 164). There is an increasing body of evidence to suggest that there is a correlation between NO and CO.
Both NO and CO share the unique ability to diffuse freely through membranes, independent of transporters or channels. It is interesting, therefore that NO should up-regulate the CO production pathway in such a striking manner. In fact it has been suggested that CO may act as an inhibitory modulator for the NO-cGMP signaling system in the brain (76). However, since in this present study haem oxygenase activity was measured in smooth muscle cells after only brief exposure to a single NO-donor (SNP) it is really impossible to speculate on the regulatory mechanisms of this pathway or on the exact nature of the relationship between the two messenger molecules in the vasculature.

Cadmium chloride (CdCl$_2$) had a similar effect on smooth muscle cells. Our findings that incubation of smooth muscle cells with CdCl$_2$ resulted in a 3-fold increase in haem oxygenase activity and increased HO-1 protein expression are in keeping with previous studies that have shown an increase in HO-1 mRNA after incubation with similar concentrations of CdCl$_2$ (140, 143). Since CdCl$_2$ contains transition metal ions it can behave as a reactive oxygen intermediate (ROI) generator. This can, in turn lead to an imbalance in the redox status of the cell and lead to oxidant damage. However, it is possible that the heavy metal ions are able to activate directly, or indirectly, putative metal elements in the HO-1 gene promoter (165).

3.5.2 In vivo studies

This study demonstrated that administration of hemin (50 mg/kg, i.p.) to rats resulted in a time-dependent increase in myocardial HO-1 mRNA expression
as well as HO-1 protein expression. The data show that there was a brief upregulation of the gene at 12 h after treatment which was completely abolished before 18 h. The peak in HO-1 mRNA corresponded with a transient increased HO-1 protein expression in the myocardium 24 h after hemin administration. It is well established that hemin is a potent inducer of the HO-1 gene since it is the substrate of haem oxygenase enzymatic activity (129), and HO-1 gene transcription (29, 54).

HO-1 induction by endotoxin has been described previously in the lung, spleen, liver and kidney (40, 41, 43). This is the first demonstration of HO-1 induction by LPS endotoxin in the rat myocardium. Rat heart HO-1 expression was increased 2.2-fold after a single bolus administration of endotoxin (1.5 mg/kg). Previous studies suggest that liver and spleen cells treated with LPS activate an NF-κB-like factor (43). The HO-1 gene has an NF-κB-binding motif in its promoter region, and oxidative stress has been shown to be a strong activator of NF-κB. LPS is an oxidative reagent, thus the transcriptional activation of the HO gene might well be activated via the NF-κB site (166).

The differential expression of HO-1 and iNOS in rat myocardium and livers after treatment with LPS further demonstrate the actions of both hemin and LPS on the HO system. Although a recent report demonstrated that hemin was able to induce both HO-1 and iNOS activity in cultured smooth muscle cells, this was not apparent in the current study (167). Although, this appears to be the first demonstration of co-induction of HO-1 and iNOS by endotoxin,
previous studies have demonstrated endotoxin-induced iNOS expression and activity in the rat heart, hypothalamus and pituitary in a similar model (157, 168).

Molsidomine (SIN-10), the precursor to the NO-donor, SIN-1, is used clinically for the treatment of angina and associated myocardial ischaemia by virtue of its ability to release NO (145). This study also demonstrated the ability of SIN-10 to induce the haem oxygenase system in both heart and liver. Perhaps further clinical studies may demonstrate a similar trend in human patients. A number of previous studies have demonstrated the ability of SIN-10 and SIN-1 to induce HO-1 in the rat liver and isolated endothelial cells and our data support these findings (147, 169).

3.6 Conclusions

In this Chapter we have demonstrated some of the wide variety of compounds that can induce the HO-1 gene and consequently increase HO-1 protein expression and haem oxygenase activity. The aim of the study was to find a suitable inducer of HO-1 to investigate the role of this enzyme in vascular tissues. Although endotoxin (LPS) has been shown to up-regulate the HO-1 gene, the fact that iNOS expression is also enhanced introduces the possibility of the involvement of other biochemical pathways which may make interpretation of the data confusing. Therefore, LPS will not be used in further studies to up-regulate HO-1.
In conclusion, therefore, in the following Chapters we will investigate the effects of hemin- and NO-induced HO-1 expression in vascular tissues, since these two compounds are potent inducers of HO-1.
4. **HAEM OXYGENASE-MEDIATED PROTECTION AGAINST OXIDATIVE STRESS IN VASCULAR SMOOTH MUSCLE CELLS**

4.1 *Introduction*

Because of its anatomical location, smooth muscle is particularly exposed to oxidative stress-mediated injury and vascular wall dysfunction is associated with the pathology of a number of common cardiovascular diseases. In previous studies, our group have reported that exogenously applied bilirubin attenuates hydrogen peroxide-induced damage in vascular endothelial cells (48). More recently, our research group has demonstrated that HO-1-derived bilirubin is involved in reduction of endothelial apoptosis mediated by peroxynitrite (170) and improvement of post-ischaemic myocardial function in the isolated perfused rat heart (171). Doré and co-workers have also demonstrated that enhanced bilirubin production following activation of haem oxygenase-2 (HO-2), the constitutive isoform of haem oxygenase, is protective against the neurotoxicity elicited by hydrogen peroxide (172). However, further studies on the physiological action of bilirubin generated by HO-1 are needed to be able to fully account for the cytoprotection conferred by HO-1 induction. In this Chapter, the dynamics of HO-1 expression and bilirubin production after stimulation of vascular smooth muscle cells with hemin, a potent inducer of the HO-1 gene, were examined. The response to an oxidant
generating system was then evaluated in control cells and cells producing increased amounts of bilirubin.

4.2 Objective

The aims of this project were two-fold: 1) to investigate the dynamics of bilirubin production in response to HO-1 up-regulation; and 2) to determine the role of haem oxygenase-derived bilirubin in cytoprotection of vascular smooth muscle cells (SMC) against oxidant-mediated injury.

4.3 Methods

All experiments were performed on cells between passages 7 and 17 and between 90-100% confluence (estimated by visual inspection).

4.3.1 Western blot analysis for HO-1 expression and haem oxygenase activity.

Confluent cells were exposed to increasing concentrations of hemin (50-400 μM) for 2 h followed by incubation with medium alone for different times (1-22 h). At the end of the incubation, cells were collected and analysed for HO-1 protein expression and haem oxygenase activity according to the protocol described in Materials and Methods (Section 2.4.5 and 2.3.5 respectively).
4.3.2 Determination of bilirubin in cell culture medium.

Haem oxygenase-derived bilirubin was determined as described in Materials and Methods (Section 2.3.6). Cells were pretreated with hemin (100 and 200 μM) for 2 h and exposed to medium alone or medium containing the inhibitor of haem oxygenase activity, tin protoporphyrin (SnPPIX, 40 μM), for an additional 4 or 22 hrs. Data were expressed as nmoles bilirubin/well.

4.3.3 Cytotoxicity assay.

Cells were pretreated with hemin (25-400 μM) for 2 h followed by 4 or 22 h incubation with medium alone or medium containing SnPPIX (40 μM). In another set of experiments, different concentrations of exogenous bilirubin were applied to cells for 2 h. At the end of the incubation, cells were washed and exposed for 2 h to fresh culture medium containing various concentrations (10-120 mU/ml) of glucose oxidase (GOX), a flavoenzyme which catalyses the conversion of D-glucose to D-gluconolactone producing hydrogen peroxide at constant rate (see Equation 4.1). This oxidant-generating system has been previously used in cell culture in vitro to promote oxidative injury (161).

\[
\begin{align*}
\text{a) } & \beta\text{-D-glucose} + \text{GOX}_{\text{FAD}} \leftrightarrow \text{GOX}_{\text{FADH}_2} + \delta\text{-D-gluconolactone} \\
\text{b) } & \text{GOX}_{\text{FADH}_2} + \text{O}_2 \rightarrow \text{GOX}_{\text{FAD}} + \text{H}_2\text{O}_2
\end{align*}
\]

**Equation 4.1**  The catabolic reaction of glucose oxidase (GOX)  

a) D-glucose is oxidised to D-gluconolactone producing the reduced form of the enzyme. As soon as this comes into contact with molecular oxygen it is rapidly oxidised to release H2O2
After exposure to GOX, cells were washed and cell viability was assessed using the alamar blue kit according to Materials and Methods (Section 2.2.9).

4.3.4 Experimental groups

For analysis of haem oxygenase activity and HO-1 and HSP70 protein expression, cells were grown in 75 cm² flasks to confluence before being exposed to hemin (0 - 400 μM) for various times (0 - 22 h).

For the GOX toxicity and preconditioning experiments, cells were grown in 24-well (for preliminary data) and 96-well plates to confluence before being washed several times with fresh medium and treated according to the protocol described above.

4.4 Results

4.4.1 Increased HO-1 expression correlates with increased bilirubin production in smooth muscle cells

Increased HO-1 expression following treatment of cells with hemin was accompanied by augmented bilirubin production, as shown in Table 4.1. However, and in accordance with the haem oxygenase activity results described previously (see Section 3.x), the majority of the bile pigment (86 and 68 %) was generated within the first 4 h after exposure to 100 and 200 μM hemin, respectively. Notably, after this initial period of incubation, cells treated with 100 μM hemin did not exhibit a significant further elevation in bilirubin levels.
Table 4.1 Production of bilirubin in control and hemin-treated cells

Cells were exposed for 2 h to medium alone (control) or medium supplemented with hemin (100 or 200 μM). Bilirubin released into the culture medium was measured 4 or 22 h after hemin treatment as described in Experimental Procedures. Bilirubin was also measured in the medium of cells treated with 100 μM hemin for 2 h followed by incubation with 40 μM tin protoporphyrin IX (SnPPIX) for 4 h. Results represent the mean (± SEM) of 6 experiments for each group. *P < 0.05 vs. control; †P < 0.05 vs. 4 h.

<table>
<thead>
<tr>
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<th>Bilirubin (nM)</th>
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<tbody>
<tr>
<td></td>
<td>4h</td>
</tr>
<tr>
<td>Control</td>
<td>375 ±38</td>
</tr>
<tr>
<td>100 μM hemin</td>
<td>540 ±33*</td>
</tr>
<tr>
<td>100 μM hemin + 40 μM SnPPIX</td>
<td>302 ±35</td>
</tr>
<tr>
<td>200 μM hemin</td>
<td>769 ±54*</td>
</tr>
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</table>

These data suggest that most of the hemin up-taken by cells is consumed by haem oxygenase in a relatively short period of time and that the tetrapyrrole ring subsequently becomes the limiting factor in the generation of bilirubin despite HO-1 protein expression remaining elevated. This hypothesis is substantiated by the fact that smooth muscle cells exposed to higher concentrations of hemin (200 μM) continued to release bilirubin into the medium both during the earlier and later times of incubation.
4.4.2 HO-1 up-regulation decreases glucose oxidase-mediated oxidative damage in vascular smooth muscle cells

Having established that HO-1 up-regulation is accompanied by increased bilirubin production, it was important to verify whether this inducible pathway could provide protection against oxidative stress. Several previous reports have revealed a direct correlation between HO-1 induction and high resistance to oxidant-mediated damage (173-176); this cytoprotective effect has always been attributed to augmented levels of the antioxidants biliverdin and bilirubin although actual measurements of any of these HO-1 products were lacking. This is an important point to address since 1) up-regulation of the HO-1 gene does not necessarily imply up-regulation of the products and 2) specific investigations on the availability of haem as a substrate for increased HO-1 expression have yet to be performed. In this study, cells exposed to GOX (80 mU/ml) for 2 h showed a considerable decrease in viability (Figure 4.1A). In contrast, pre-treatment with hemin resulted in a concentration-dependent reduction in GOX-mediated cell injury.

Highest protection was seen 4 h after treatment with 100 µM hemin, the concentration that resulted in maximal HO activity; a similar decrease in cell injury was also observed with 200 µM hemin. Significantly less protection was found 22 h following exposure to hemin (P < 0.05 vs. 4 h); however, pre-treatment with 200 µM hemin still resulted in considerable reduction of cell damage caused by oxidative stress.
These data are best explained when considering the experimental protocol employed. Four or 22 h after exposure of smooth muscle cells to hemin, the medium was replaced with fresh medium containing GOX (2 h) prior to assessment of cell viability. Therefore, the medium in which bilirubin accumulated was removed before the addition of GOX. In view of this finding and the bilirubin data presented in Table 4.1, cytoprotection afforded 4 h after treatment with 100 μM hemin was likely a result of endogenous bilirubin actively generated by HO-1 at the time of oxidant challenge. Accordingly, a smaller production of bilirubin can account for the decreased ability of hemin (100 μM) pre-treatment to protect cells at 22 h. Also at 22 h, the effect of 200 μM hemin could reflect a sustained bilirubin generation over a longer period of time with a possible contribution of other inducible defensive systems such as HSP 70.
Figure 4.1  Effect of hemin pre-treatment on glucose oxidase-mediated cell injury.

A) Cells were pretreated for 2 h with increasing concentrations of hemin (0-400 μM) and were then incubated with fresh medium for 4 or 22 h prior to exposure to 80 mU/ml glucose oxidase (GOX) for 2 h. Cell injury was assessed as reported in Experimental Procedures. B) Cells were pretreated for 2 h with 100 μM hemin, incubated with fresh medium for 4 or 22 h and then exposed to increasing concentrations of GOX (20-120 mU/ml) for 2 h. Control experiments (untreated) were performed by exposing cells to medium alone prior to GOX challenge. Each bar represents the mean (± SEM) of 6 experiments performed independently. *P < 0.05 vs. 0 μM hemin; †P < 0.05 vs. 4 h; **P < 0.01 vs. untreated.
Injury following exposure to increasing concentrations of GOX (10-120 mU/ml) was also significantly diminished in cells pretreated with 100 μM hemin; once again this protection was evident at 4 but not at 22 h (Figure 4.1B) and is consistent with the dynamics of HO-1 expression, haem oxygenase activity and bilirubin production in relation to haem availability.

4.4.3 Tin protoporphyrin IX attenuates hemin-mediated cytoprotection against oxidative stress

A direct contribution of the haem oxygenase pathway in minimizing oxidative damage was ascertained in our study by using an inhibitor of its activity, tin protoporphyrin IX (SnPPIX). As shown in Table 4.1 and Figure 4.2B, SnPPIX (40 μM) reduced bilirubin formation (302±35 nmoles/well) to levels comparable to control (375±39 nmoles/well) and attenuated the cytoprotection conferred by hemin treatment against GOX-induced cell injury. It is important to note that SnPPIX per se did not cause any evident cytotoxic effect at the concentrations tested (Figure 4.2A). The participation of additional defense mechanisms can not be excluded since SnPPIX did not completely reverse the effect mediated by hemin.
Figure 4.2  **Tin protoporphyrin IX (SnPPIX) attenuates hemin-mediated cytoprotection against oxidative stress.**

A) Effect of various concentrations of SnPPIX on cell viability. Cells were incubated for 4 h with 0-40 μM SnPPIX and cell viability was assessed as described in Materials and Methods. B) Cells were pretreated with 100 μM hemin for 2 h and then incubated for 4 h to medium alone or medium containing 40 μM SnPPIX. After incubation, cells were exposed to increasing concentrations of glucose oxidase (GOX) for 2 h and cell viability was assessed as described in Experimental Procedures. Control experiments (untreated) were performed by exposing cells to medium alone prior to GOX challenge. Results represent the mean (±SEM) of 6 experiments for each group. *P < 0.05 vs. untreated; †P < 0.05 vs. hemin-treated.
4.4.4 Exogenously applied bilirubin directly protects smooth muscle cells against glucose oxidase-mediated oxidative damage

To confirm the important role of bilirubin in protection against oxidative stress, cells were incubated with various concentrations of the bile pigment prior to GOX challenge. A marked decrease in cell damage was observed with bilirubin at 500 nanomolar and above (Figure 4.3A). Similarly, 1 μM bilirubin significantly reduced the toxic actions of increasing concentrations of GOX (Figure 4.3B). Although our data are in agreement with recent published observations by Doré and co-workers in cultured neurons (172), the concentrations of bilirubin which provided cytoprotection in the present work are higher. However, differences in cell types and experimental conditions used can explain differences in results. For instance, we found that perfusion of isolated rat hearts with serum-free buffer containing bilirubin at concentrations as low as 100 nanomolar significantly ($P<0.05$) reduced infarct size and improved myocardial function following ischaemia-reperfusion (171).
Figure 4.3  Effect of exogenous bilirubin on glucose oxidase-mediated cell injury in smooth muscle cells.
A) Cells were incubated for 2 h with increasing concentrations of bilirubin (0-5 μM) and cell injury was assessed 2 h after exposure to 80 mU/ml glucose oxidase (GOX). B) Cells were incubated for 2 h with medium alone (untreated) or medium containing bilirubin (0.25 or 1 μM) and cell viability was assessed 2 h after exposure to increasing concentrations of GOX (0-120 mU/ml). *P < 0.05 vs. 0 μM bilirubin; **P < 0.05 vs. untreated.
4.5 Discussion

This study demonstrates that increased bilirubin, as a consequence of HO-1 induction contributes to cytoprotection of smooth muscle cells against oxidant-mediated damage. In addition, these results indicate how the dynamics of haem oxygenase activity and bilirubin production determine the adaptive response of cells to oxidative stress. The important issues concerning the availability of haem as a substrate for augmented HO-1 levels and the generation of bilirubin in sufficient amounts to provide resistance to stressful stimuli are also raised. These findings strongly support a role for bilirubin derived from HO-1 in cytoprotection; nevertheless, the participation of the other products of haem degradation by HO-1 is likely since carbon monoxide, biliverdin and iron have all been shown to modulate biological processes (60, 63, 176-178). Accordingly, Otterbein and co-workers have recently reported that exposure of rats to low concentrations of CO increases tolerance to hyperoxic lung injury (179). The fact that bilirubin can counteract oxidative injury caused by hydrogen peroxide (48, 172), peroxynitrite (80, 170) and peroxyl radicals (63) indicates a wide spectrum of antioxidant activities for this bile pigment. It is tempting to suggest that these properties of bilirubin can be extended to nitric oxide and other reactive nitrogen species (80, 180).

4.6 Conclusions

This study demonstrates that increased haem oxygenase activity and the consequent increase in bilirubin production can offer cytoprotection to vascular smooth muscle cells against an in vitro model of oxidative challenge.
The protection is partially blocked by the inhibitor of haem oxygenase activity, tin protoporphyrin. The evidence presented here does not preclude the involvement of other defense mechanisms such as other heat shock proteins although at the concentrations used, hemin did not induce HSP70.

The notion that the bile pigment is directly involved in cytoprotection is substantiated by the results showing that exogenously applied bilirubin at physiological concentrations also offered protection against GOX-mediated oxidative challenge, further supporting the antioxidant role of haem oxygenase-derived bilirubin in cellular systems.
5. HAEM OXYGENASE AS A MODULATOR OF CELLULAR FUNCTION IN HUMAN CULTURED KERATINOCYTES

5.1 Introduction

During the course of wound healing in the skin, epidermal keratinocytes are exposed to a number of agents including inflammatory macrophage-derived free radicals which play a critical role in the control of microbial invasion of the skin. Nitric oxide (NO), is also produced by macrophages as well as granulocytes and is involved in host defense (181). NO is produced by NO synthase (NOS) when converting L-arginine to L-citrulline. The enzyme exists in two isoforms: a constitutively expressed protein (cNOS) and an inducible protein (iNOS). It has been shown that both constitutive and inducible nitric oxide synthase proteins are expressed in human keratinocytes (182, 183).

Physiological relaxation of vascular smooth muscle cells, inhibition of platelet aggregation and neurotransmission are examples of the effects of NO that is continuously synthesised by cNOS (81, 184, 185). In contrast, the inducible form of NOS can produce considerable amounts of NO (100-1000 times more than cNOS) and it is through this production pathway that NO appears to act in host defense (181).
Another recently discovered effect of NO is its ability to up-regulate the expression of vascular haem oxygenase-1 (HO-1) (50, 186). Haem oxygenase plays a key role in the maintenance of cell function, as biliverdin is a potent antioxidant and CO has been shown to elicit vasodilatation through the activation of guanylate cyclase (48, 63). HO-1 has been shown to be present in cultured human keratinocytes and recently our group has reported that NO donors increase HO-1 expression and activity in cultured transformed human keratinocytes (187, 188).

5.2 Objective

Since NO also has the ability to stimulate increased keratinocytes proliferation (189), the present study was designed to investigate a possible link between NO, HO-1 expression and keratinocyte metabolism.

5.3 Methods

5.3.1 Haem oxygenase activity and HO-1 expression

A human epidermal keratinocyte cell line (SKV14) was incubated at 37°C in a humidified atmosphere of 5% CO₂ - 95% air in 75cm² tissue culture flasks in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum and hydrocortisone (5 mg/l) until confluent. Cells were incubated with; 1) hemin (0-300 μM) for 2 h, washed and then incubated with normal medium for a further 4 h or; 2) the nitric oxide donor sodium nitroprusside (SNP, 0-1 mM) for 6 h and then collected for analysis of haem oxygenase activity and HO-1 protein expression as described in Materials and Methods.
5.3.2 Proliferation studies

In a second set of experiments, keratinocyte cells were seeded in 6-well tissue culture plates at a density of \(2 \times 10^5\) cells per well in growth medium and allowed to grow for 2 days under normal conditions. Cells were then incubated with normal medium containing: 1) 0.5 mM SNP; 2) 0.5 mM SNP and hydroxocobalamin, a NO scavenger or; 3) 0.5 mM SNP and tin protoporphyrin IX (SnPPIX), a potent inhibitor of haem oxygenase activity (190), and allowed to grow for a further 24 h. After this time, cells were removed from the wells by trypsinisation and then cell number was determined by counting the cells using a haemocytometer.

5.4 Results

5.4.1 Effect of hemin and SNP on haem oxygenase activity and HO-1 expression in keratinocytes

Hemin is a potent inducer of haem oxygenase in a number of cell types in culture and was therefore used as a positive control in this study to show the ability of keratinocytes to express HO-1 (29, 135). Addition of SNP (0.5 mM final concentration) to the culture medium for a period of 6 h caused a 2.5 fold increase (p<0.05) in haem oxygenase activity (Figure 5.1A and B). However, at a higher concentration of SNP (1 mM) haem oxygenase activity was significantly decreased (p<0.05) compared to 0.5 mM but was still significantly higher than control. The elevated haem oxygenase activity by SNP was reflected in a significant increase in HO-1 expression as shown by Western blot analysis (Figure 5.1B Inset).
Effect of hemin and sodium nitroprusside (SNP) on HO activity induction in keratinocytes

Haem oxygenase activity was measured 4 h after a 2 h incubation with hemin (100 and 300μM) (A) and SNP (0.5 and 1 mM) (B). HO-1 expression after 0.5 mM SNP treatment was assessed by Western blot (B Inset). Control experiments are represented by cells exposed to complete medium alone. Each value represents the mean ±SEM of 6 experiments. *P<0.05 compared with control; †P<0.05 compared with SNP 0.5 mM.
5.4.2 Effect of SNP on keratinocyte metabolism and proliferation

Keratinocyte cell metabolism, as determined by the MTS tetrazolium assay, was markedly increased in a concentration-dependent manner when cells were incubated with SNP (0.5, 0.75 and 1 mM) (Figure 5.2). Similarly, incubation of cultured keratinocytes with 0.25 and 0.5 mM SNP for 24 h in culture medium resulted in 2.5 and 3-fold increase in cell proliferation, respectively (p<0.05). However, when incubated with a higher dose of SNP (1 mM) for 24 h, keratinocytes proliferation was not significantly different from control (Figure 5.3).

There is evidence that hydroxocobalamin has NO binding properties (191). Therefore, the use of hydroxocobalamin enabled us to determine the specific
role of NO released from SNP in keratinocyte proliferation. As shown in Figure 5.3, addition of hydroxocobalamin (0.5 mM final concentration) to the culture medium containing SNP resulted in a significant decrease in cell proliferation. In addition, incubation of keratinocytes with the haem oxygenase inhibitor SnPPIX (0.5 mM) also resulted in a significant reduction of the proliferative effect mediated by SNP.

**Figure 5.3** Effect of hydroxocobalamin (HCB) and tin protoporphyrin IX on sodium nitroprusside (SNP) mediated keratinocyte proliferation.

Keratinocyte proliferation was determined by counting the number of cells after 24 h incubation with SNP (0.25-1 mM), SNP + HCB (0.5 mM) and SNP + SnPPIX (0.5 mM). Control experiments are represented by cells exposed to complete medium alone. Each value represents the mean ±SEM of 6 experiments. *, P<0.05 compared with control; †, P<0.05 compared with SNP alone.
5.5 Discussion

The main finding of this study is that the expression of HO-1 is involved in keratinocytes proliferation mediated by NO. Our data show that incubation of transformed human keratinocytes with SNP, a well-known NO donor, stimulates HO-1 expression and haem oxygenase activity resulting in increased cellular proliferation. Both blockade of SNP-derived NO with hydroxocobalamin and inhibition of the enzymatic action of haem oxygenase with SnPPIX were found to abolish the proliferative effect of NO.

The expression of HO-1 with the consequent increase in haem oxygenase activity has been described in a variety of tissues as a general response to stressful stimuli (58). It is believed that the products of this enzymatic pathway have a crucial biological role, since biliverdin is a potent antioxidant and CO has been shown to activate the cGMP pathway, thus being an important factor in the regulation of vascular tone (48). The induction of HO-1 in response to various NO donors has been recently reported both in vitro and in vivo systems, but the exact physiological significance of this effect has yet to be elucidated (86, 169, 186, 192). In this study we found that increased haem oxygenase activity by SNP correlates with a higher proliferation of human keratinocytes in culture. The effect was more pronounced at relatively low concentrations of the NO donor (0.25-0.5 mM), whereas at a higher concentration of SNP (1 mM), haem oxygenase activity was reduced and cell proliferation significantly suppressed. Interestingly, the cell viability assay (MTS tetrazolium) indicated that the metabolic activity of keratinocytes

- 157 -
gradually increased in a concentration-dependent manner following incubation with SNP, even at concentrations as high as 1 mM. Taken together, these results suggest that, under our experimental conditions, SNP does not promote cellular damage and that the decrease in haem oxygenase activation observed at higher concentrations of SNP can not be related to the potential cytotoxic action of NO. It can not be excluded that by increasing their metabolic activity, cells can catabolise NO much faster resulting in a higher decomposition of SNP over time. In fact, an increased formation of nitrite from SNP has been shown to correlate with a much lower induction of haem oxygenase by the NO donor (186).

In a previous report we have demonstrated that increased haem oxygenase activity in vascular endothelial cells by SNP was markedly suppressed in the presence of the NO scavenger hydroxocobalamin (186). The results of the present study showing that hydroxocobalamin markedly reduced the number of growing keratinocytes support the hypothesis that NO itself is an important regulator of keratinocyte proliferation. The fact that SnPPIX suppressed the proliferative effect mediated by SNP suggests a direct involvement of HO-1 in keratinocyte metabolism. Although SnPPIX has been shown to partially inhibit haem-dependent proteins other than haem oxygenase (guanylate cyclase, NOS) (193), recent data from our laboratory indicate that SnPPIX becomes a more selective and competitive inhibitor when the level of HO-1 is significantly increased (194). To our knowledge this study provides the first evidence that activation of the haem oxygenase pathway is associated with increased cellular metabolism and growth.
The exact mechanism by which HO-1 induction promotes keratinocyte proliferation remains to be established. It is possible that the NO/HO-1 system elicits cell proliferation by increasing the endogenous production of CO, which has been implicated in intracellular communication pathways (195). As represented in the schematic diagram of Figure 5.4, increased HO-1 protein expression by NO would result in a higher catabolism of haem to CO, which either directly or via a second messenger (cGMP) could trigger the cascade of events required for the production of growth factors. The other product of haem oxygenase, biliverdin, has antioxidant properties but no data are available concerning the potential proliferative effect of this molecule. Janes and coworkers (196) have shown that bilirubin, which is formed during the reduction of biliverdin by biliverdin reductase, decreases cellular proliferation in osteoblasts without affecting cell viability. Those results were, however, obtained from jaundiced patients in conditions of extreme hyperbilirubinemia.
Figure 5.4  Schematic diagram representing a possible mechanism of NO-mediated keratinocyte proliferation
Both iNOS and eNOS have been shown to be present in cultured human keratinocytes and NO can increase cell proliferation. In our experiments an exogenous source of NO was used (SNP). NO mediated HO-1 induction leads to an increase in the production of biliverdin and the messenger molecule CO. We propose that one of these molecules either directly, or through a secondary messenger system (?) is responsible for mediating an increase in keratinocyte proliferation.

The inducible form of NOS (iNOS) has been found in skin cells including keratinocytes, langerhans cells and fibroblasts, and iNOS-derived NO is also produced in activated macrophages as part of their anti-microbial defense (182, 197). As a consequence of this, a traumatic wound site should have high iNOS activity and, therefore, considerable levels of NO being released. Benrath and coworkers demonstrated that a topically administered NOS inhibitor (N^6-nitro-L-arginine methyl ester, L-NAME) to a UVB-induced wound area in rat skin resulted in increased necrosis (198). In addition, UVA/UVB radiation has been reported to stimulate NO release in human
keratinocytes and to up-regulate HO-1 mRNA in skin fibroblasts (199, 200). In view of the data presented here, we reason that elevated NO production might lead to increased keratinocytes proliferation resulting in acceleration of wound healing processes. Although in the present study we used an exogenous form of NO (SNP), the possibility that endogenously produced NO in the epidermis as part of the inflammatory response could influence the HO-1/CO pathway cannot be excluded.

5.6 Conclusions

In summary, we have reported the ability of a NO donor to increase HO-1 expression in human keratinocytes postulating a possible mechanism that involves the haem oxygenase/CO pathway in cell proliferation.
6. HAEM OXYGENASE IN THE VASCULATURE (I):

THE ROLE OF BILIRUBIN

6.1 Introduction

Impairment of myocardial function and tissue injury following ischaemic events is a well-known pathophysiological phenomenon (201). Enhanced oxidative stress by free radicals generated during the reperfusion phase appears to be a major contributor to this effect (202); therefore up-regulation of the intracellular defensive pathways (such as HSPs and HO-1) may represent a plausible strategy to combat the cell death caused by ischaemia-reperfusion injury. Recent studies on cardiac muscle have revealed a direct correlation between elevated expression of the 70-kD stress protein (HSP70) and improved contractile function after ischaemia (203). Recent work has confirmed the ability of HSP70 to decrease infarct size in hearts of transgenic HSP70 mouse models (204).

Ischaemic preconditioning elicits a very powerful form of myocardial protection. However, one of the major problems of classical "ischaemic" preconditioning is that, it is rather short-lived. The finding that a "second window" of protection manifests itself some time after the preconditioning stimulus (18 - 48 h) has generated considerable interest in the delayed effect of sub-lethal ischaemia on the tolerance to vascular dysfunction. The delayed effect of preconditioning involves the production of adenosine (116), the induction of cardioprotective proteins or enzymes (205), and enhancement in
the levels of myocardial antioxidant enzymes such as catalase (206). So far, there are no studies which have investigated the possible role of the haem oxygenase-bilirubin system in myocardial preconditioning despite some promising supporting data on the antioxidant properties of bilirubin. For example, Stocker and colleagues demonstrated that physiological concentrations of bilirubin are as effective as vitamin E in inhibiting lipid peroxidation in vitro suggesting that the bile pigment is a naturally occurring, chain-breaking antioxidant (63, 64). Little has been done to elucidate the biological importance of bilirubin in the cardiovascular system. Specifically, the inherent cytoprotective characteristics of endogenous bilirubin derived from HO-1 during stress situations have yet to be explored.

In Chapter 4, a role for HO-1-derived bilirubin in cytoprotection against oxidative stress in vascular smooth muscle cells was described. However, to further understand the role of this important pathway and the potential implications for organ preservation and transplant surgery it is important to investigate the involvement of the HO-1-bilirubin pathway in maintenance of organ function after an ischaemic episode.
6.2 Objectives

A series of experiments were designed to characterize the HO-1 expression and haem oxygenase activity in cardiac tissue after stimulation with hemin. A pharmacological preconditioning protocol was established to investigate the induction of the HO-1-bilirubin pathway in cardiac tissue and its potential involvement in mitigating ischaemia-reperfusion injury in the isolated Langendorff rat heart.

6.3 Methods

6.3.1 Haem oxygenase activity, expression and intracellular bilirubin quantification

In order to assess the expression of haem oxygenase and the changes in haem oxygenase activity in the cardiac tissue, rats (n=6 for each treatment) were injected (i.p.) with 50 mg/kg body weight hemin or vehicle (control). At different time points (1 to 5 days), hearts were removed, perfused with ice-cold (4 °C) saline and either: 1) the microsomal fraction was prepared as described in Materials and Methods for HO-1 and HSP70 Western blot analysis and haem oxygenase activity; or 2) the tissue was processed for bilirubin measurement by HPLC as described in Materials and Methods.

6.3.2 Bilirubin release from isolated hearts

For determination of bilirubin release from the cardiac tissue, hearts were perfused according to the Langendorff technique with a re-circulating buffer system. The Langendorff apparatus was primed with buffer (120 ml) and a
heart mounted on the cannula. Once the heart had been allowed to equilibrate for 20 min the outflow from the jacketed chamber (see Figure 2.11) was connected to the inflow of the peristaltic pump with a 50 ml Krebs buffer reservoir so that the same 170 ml of buffer was re-circulated for 2 h in the dark. At the end of the experiment, the 50 ml in the reservoir were processed for the extraction of bilirubin as described in Materials and Methods.

6.3.3 Ischaemia-reperfusion studies

A number of experiments were designed to determine the effect of up-regulated haem oxygenase activity and bilirubin production on the tolerance to ischaemia in the isolated heart. Hearts previously treated as described below were removed from male Lewis rats (280-350 g) and perfused according to the Langendorff technique. All hearts in these studies were perfused at a constant flow of 15 ml/min for 20 min and then subjected to 30 min global warm ischaemia followed by 1 h of normal perfusion (reperfusion).

All haemodynamic parameters [CPP, HR, EDP and cardiac performance (LVDP x HR)] were monitored throughout the time-course of the experiment. Figure 6.1 shows representative polygraph traces taken before, during and after 30 min global warm ischaemia in the isolated heart.

During baseline perfusion samples of Krebs Henseleit buffer were taken from the arm of the aortic cannula and the coronary sinus outflow (pulmonary artery) using a 5 ml gas syringe and the pO₂, pCO₂, and pH measured using a clinical blood gas analyser. Table 6.1 shows the changes in pO₂, pCO₂ and pH
of the buffer after passing through the coronary circulation. These readings were taken to determine the oxygenation of the perfusion buffer having passed through the oxygenator and to ensure that hearts were consuming oxygen. As expected, the pO₂ decreased and the pCO₂ was elevated in the perfusate having passed through the coronary circulation, the pH remained statistically unchanged in both samples taken.
Figure 6.1  
Representative polygraph traces showing LVDP and CPP in hearts subjected to 30 min global ischaemia
These graphs show that upon the onset of ischaemia, hearts continue to beat, subdued by lack of substrate, for about 30 seconds until both CPP and LVDP reach the baseline (0 and 10 mm Hg respectively). Upon reperfusion, the CPP and LVDP immediately rise and at development of a "normal" coronary pressure the hearts begin to beat again (The traces shown above were from hearts pretreated with hemin (A) and hearts treated with exogenous bilirubin (B), see Experimental Procedures, below).
Table 6.1 pH, pO\(_2\) and pCO\(_2\) measured in the perfusate before entering and after leaving the isolated heart

Samples of perfusion buffer were taken from the side arm of the aortic cannula and from the pulmonary artery of the heart using a gas syringe. The syringe was fitted with a gas-tight luer lock cap, after collection, and samples were immediately taken for measurement. Values are means of 4 independent experiments (±SEM)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Before</th>
<th>After</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>pO(_2) (kPa)</td>
<td>79.10 (± 8.86)</td>
<td>53.53 (± 2.7)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>pCO(_2) (kPa)</td>
<td>5.03 (± 0.11)</td>
<td>5.67 (± 0.14)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>pH</td>
<td>7.37 (±0.04)</td>
<td>7.34 (± 0.03)</td>
<td>NS</td>
</tr>
</tbody>
</table>

At the end of the experiments, a representative number of hearts (n=3-4) were stained with a tetrazolium dye for measurement of ischaemic volume (see Materials and Methods Section 0). The remaining hearts (n=2-3) were processed for analysis of mitochondrial integrity by electron microscopy (Section 2.7.3).

Since both tin protoporphyrin IX and bilirubin are sensitive to light, all the experiments requiring these compounds were performed in the dark.

6.3.4 Experimental groups

Group 1: Hearts were removed from rats (n=6-8) 24 h after treatment with vehicle (600 µl PBS pH 7.4 1 % (v/v) NaOH (2 M)). Hearts were then subjected to ischaemia-reperfusion as described above and physiological parameters recorded.
Group 2: Rats (n=6) were injected as in Group 1 except that 1 h prior to heart removal, animals received an injection of SnPPIX (40 μmol/kg body weight). Hearts were then subjected to ischaemia/reperfusion as described above and physiological parameters recorded.

Group 3: Hearts were removed from rats (n=6) 24 h after receiving an injection (i.p) of hemin (50 mg/kg body weight in PBS pH 7.4 1 % (v/v) NaOH (2 M)). Hearts were then subjected to ischaemia/reperfusion as described above and physiological parameters recorded.

Group 4: Rats (n=6) were treated as in Group 3 except that 1 h prior to heart removal, animals received an injection of SnPPIX (20 or 40 μmol/kg body weight). Hearts were then subjected to ischaemia/reperfusion as described above and physiological parameters recorded.

Group 5: Rats (n=6) were treated as in Group 1 except that 10 min prior the ischaemic event, hearts were perfused with 50 or 100 nM bilirubin in buffer. Hearts were then subjected to ischaemia/reperfusion as described above and physiological parameters recorded.
6.4 Results

6.4.1 HO-1 expression in the myocardium in response to hemin

We first examined whether treatment with hemin (50 mg/kg body weight, i.p.), a substrate and a potent inducer of the haem oxygenase pathway, stimulates the HO-1 gene and protein expression in rat cardiac tissue.

Figure 6.2  PCR analysis of rat myocardium after treatment of animals with hemin

Rats were injected with hemin (50 mg/kg body weight) and after 1, 2, 6, 12, 18 and 24 h the hearts removed, flushed with saline, 'snap' frozen in liquid nitrogen and processed for PCR analysis of HO-1 gene expression.

In a previous Chapter (Chapter 3, Section 3.4.4), haem oxygenase activity was measured in hearts following treatment of rats with 50 mg/kg body weight hemin (i.p. injection). In this study, we investigated whether the increase in haem oxygenase activity correlated with increased HO-1 protein and gene expression in cardiac tissue.
There was little or no change in HO-1 mRNA levels in cardiac tissue during the first 6 h following hemin administration. However, 12 h after hemin treatment there was a dramatic increase in HO-1 mRNA which subsided by 18 h (Figure 6.2). Figure 6.3A and Figure 6.3B show the time course of HO-1 protein expression measured by Western blot analysis. Hemin markedly increased HO-1 expression (4-fold) in the microsomal fraction of hearts taken from animals 1 day after injection. The HO-1 protein levels gradually returned to control values within 4-5 days after treatment. Induction of HO-1 observed at 1 day following hemin administration was associated with increased haem oxygenase activity; this effect was suppressed by treatment with tin protoporphyrin IX (PP, 40 μmol/kg body weight i.p.), a competitive inhibitor of haem oxygenase enzymes (Figure 6.3A, Inset). These data indicate that maximal up-regulation of the HO-1 pathway occurs in the myocardium at 1 day after treatment of animals with hemin. Therefore, this time point was chosen to examine the possible involvement of the HO-1 pathway in protection against myocardial ischaemia-reperfusion injury.

Also, the expression of HSP70, an inducible heat shock protein, was measured in cardiac tissue from hemin-treated animals. HSP70 has been shown to be up-regulated by ischaemic preconditioning in the myocardium resulting in protection of tissues against ischaemia-reperfusion damage (205). We found that the level of HSP70 did not change following hemin treatment as myocardial protein expression was comparable at all the time points examined (Figure 6.3B).
Figure 6.3  Hemin pre-treatment stimulates HO-1 activation in cardiac tissue
A) Time-course of myocardial HO-1 expression at various time points after treatment of animals with an intra-peritoneal (i.p.) injection of hemin (50 mg/kg body weight). HO-1 protein was measured in the microsomal fraction of hearts by Western blot using a polyclonal antibody. Data points are mean values ± SEM of n=3 independent experiments; *P<0.05 vs. control. B) Representative Western blots showing HO-1 and HSP70 protein expression in heart samples at different time points after treatment of animals with hemin. Results shown are mean values ± SEM of n=4-6 independent experiments; *P<0.05 vs. 0 day.
These results show that a functional role of HSP70 in myocardial protection in hemin-treated animals can be excluded \textit{a priori} and that this model provides a useful tool for investigating the potential effect of HO-1 in cardiac tolerance to ischaemia.

\subsection{6.4.2 Increased bilirubin production in hearts expression high HO-1}

Haem oxygenase-mediated haem degradation results in the production of carbon monoxide (CO) and biliverdin. Biliverdin is promptly converted to bilirubin by the cytosolic enzyme biliverdin reductase, an enzyme present in most cell types. Biliverdin reductase activity was determined using a similar reaction mixture to that of the haem oxygenase activity assay. The reaction tube contained glucose-6-phosphate dehydrogenase (0.5 U), biliverdin (25 \text{ µ M}), NADPH (20 \text{ µ M}), phosphate buffer (100 mM, pH 7.4) and 400 \mu l (2 mg protein) of unknown sample in a total volume of 1 ml. A considerable biliverdin reductase activity was detected in both control and hemin-treated hearts (see Figure 6.4).

The bilirubin IX isomer was measured in cardiac tissue by HPLC as described in Materials and Methods (Section 2.6.2). A representative chromatogram and absorbance spectrum of bilirubin extracted from cardiac tissue of haem-treated animals are shown in Figure 6.5.
Figure 6.4  
**Biliverdin reductase activity in cardiac tissue after hemin treatment**
Rats were injected (i.p.) with 600 μl saline (control) or 50 mg/kg body weight hemin. Hearts were removed immediately or 1, 2 or 3 days after injection. Cytosolic fractions were obtained as described in Materials and Methods and the sample assayed for biliverdin reductase activity. Results shown are means ± standard error of the mean of n=3 independent experiments.

Figure 6.5  
**Representative elution curve from the HPLC analysis of heart chloroform extract**
Chromatograms show peak and absorption spectra at 450 nm typical of bilirubin.
Figure 6.6 Rate of bilirubin released into the circulating buffer of isolated perfused hearts

(A) Hearts were removed from animals 1 day after pre-treatment with vehicle (control) or hemin (i.p. 50 mg/kg body weight) and perfused according to the Langendorff technique as described in Materials and Methods. After 20 min equilibration, 100 ml of buffer was re-circulated through the system for 2 h to allow bilirubin released from the heart to accumulate in the perfusion buffer. Bilirubin was extracted with chloroform at the end of the perfusion and measured spectrophotometrically as a difference in absorbance between 460 and 530 nm (B). Tissue bilirubin content in hearts removed from animals 1 day after treatment with vehicle or hemin (50 mg/kg body weight). Tissue bilirubin was extracted with chloroform and measured by HPLC as described in Materials and Methods. Results shown are means ± standard error of the mean of n=5-7 independent experiments; *P<0.05 vs. saline treatment; **P<0.05 vs. saline treatment.
An increase of 23% in bilirubin was measured in hearts from animals treated with hemin compared to the vehicle-treated rats (Figure 6.6A). Bilirubin was also measured in the heart perfusate which was circulated continuously through the isolated organ for 2 h. The rate of bilirubin released into the perfusion buffer from hemin-treated hearts was also markedly increased by 55% ($P < 0.05$) compared to hearts removed from vehicle-treated animals (Figure 6.6B).

6.4.3 Increased recovery of post-ischaemic myocardial function in hearts expressing high HO-1

To determine the effect of HO-1 induction on the functional recovery of hearts subjected to ischaemia-reperfusion, cardiac performance ($LVDxHR$) and other haemodynamic parameters were evaluated in the Langendorff preparation of hearts from vehicle-treated (control group) and hemin-treated animals. As shown in Figure 6.7, cardiac performance decreased to 65% of baseline following ischaemia-reperfusion in hearts from control group, whilst hearts removed after hemin treatment showed an improved recovery (81% of baseline, $P < 0.05$).

Coronary (CPP) and end diastolic pressure (EDP) were also better maintained in hearts expressing high levels of HO-1 compared to control (Figure 6.8). Heart rate was unchanged over the time-course of the experiment (Figure 6.9). The increase in CPP over the baseline value ($ACPP$), which is indicative of a rise in coronary vessel contractility, was more pronounced in hearts from
vehicle-treated animals (41 mmHg) compared to those treated with hemin (14 mmHg).

Figure 6.7  **Hemin pre-treatment improves myocardial function following ischaemia-reperfusion (I/R)**
Myocardial function (% cardiac performance (LVDPxHR)) was determined at 5, 30 and 60 min reperfusion following 30 min ischaemia in hearts isolated from animals pretreated with hemin (H, 50 mg/kg body weight) or hemin plus tin protoporphyrin IX (PP). Animals received hemin or vehicle 1 day prior to removal of the hearts. PP (20 or 40 μmole/kg), an inhibitor of haem oxygenase activity, was given to the animals 1 h before heart isolation. Hearts were perfused according to the Langendorff technique as described in Materials and Methods. Results shown are means ± standard error of the mean of n=6-8 independent experiments; *P<0.05 vs. Control.
Figure 6.8  **Hemin pre-treatment improves the maintenance of myocardial haemodynamic function following ischaemia-reperfusion (I/R)**

ΔCPP and ΔEDP were determined at 5, 30 and 60 min reperfusion following 30 min ischaemia in hearts isolated from animals pretreated with hemin (50 mg/kg body weight) or hemin plus tin protoporphyrin IX (PP). Animals received hemin or vehicle 1 day prior to removal of the hearts. SnPPIX (20 or 40 μmole/kg), an inhibitor or haem oxygenase activity, was given to the animals 1 h before heart isolation. Hearts were perfused according to the Langendorff technique as described in Materials and Methods. Results shown are means ± standard error of the mean of n=6-8 independent experiments; *P<0.05 vs. Control.
Interestingly, the use of tin protoporphyrin IX (40 μmol/kg body weight), a potent inhibitor of haem oxygenase activity, completely abolished the post-ischaemic recovery of cardiac function of hemin-treated hearts (Figure 6.7) resulting in reduction of cardiac performance (70% of baseline) and increase in both ΔEDP (31 mmHg) and ΔCPP (33 mmHg) (Figure 6.8).

![Graph showing heart rate (HR) at different reperfusion times](image)

**Figure 6.9** Hemin pre-treatment improves maintenance of heart rate following ischaemia-reperfusion (I/R)

Heart rate (HR) was determined at 5, 30 and 60 min reperfusion following 30 min ischaemia in hearts isolated from animals pretreated with hemin (50 mg/kg body weight) or hemin plus tin protoporphyrin IX (PP). Animals received hemin or vehicle 1 day prior to removal of the hearts. SnPPIX (20 or 40 μmol/kg body weight), an inhibitor of haem oxygenase activity, was given to the animals 1 h before heart isolation. Hearts were perfused according to the Langendorff technique as described in Materials and Methods. Results shown are means ± standard error of the mean of n=6-8 independent experiments; *P<0.05 vs. Control.

The doses of tin protoporphyrin IX used in this study (20 and 40 μmol/kg body weight) did not have any apparent effect on the functional recovery of post-ischaemic hearts removed from vehicle-treated animals (Figure 6.10).
Figure 6.10  Effect of tin protoporphyrin on myocardial haemodynamic parameters
Cardiac performance (LVDP x HR), ΔCPP and ΔEDP were determined at 60 min reperfusion following 30 min ischaemia in hearts isolated from animals pretreated with tin protoporphyrin IX (PP). PP (20 or 40 μmole/kg), an inhibitor of haem oxygenase activity, was given to the animals 1 h before heart isolation. Hearts were perfused according to the Langendorff technique as described in Materials and Methods. Results shown are means ± standard error of the mean of n=6-8 independent experiments; *P<0.05 vs. Control.
6.4.4 Perfusion with exogenous bilirubin offers protection against myocardial ischaemia-reperfusion injury

Since bilirubin has been shown to have cytotoxic properties it was important in this study to determine the effects of long term exposure of the isolated heart system to increased bilirubin levels. Hearts were removed from untreated animals and mounted on the Langendorff apparatus. After 20 min baseline perfusion the hearts were perfused with Krebs' buffer containing different levels of bilirubin (0 - 400 nM). After 1 h perfusion the haemodynamic parameters, HR, LVDP, CPP and EDP were plotted.

As shown in Figure 6.11, at bilirubin concentrations of up to 100 nM, there was no change in any of the haemodynamic parameters measured. However, at concentrations above 120 nM bilirubin there was a dramatic increase in EDP (~650% of baseline), signifying a change in the basal tone of the heart muscle which is indicative of damage. There was also a significant increase in the CPP after 1 h perfusion with 400 nM bilirubin.

The effect of exogenously applied bilirubin on this ex-vivo system was investigated after having demonstrated that up-regulation of the haem oxygenase enzymatic pathway in cardiac tissue results in increased bilirubin production and enhanced myocardial function after ischaemia-reperfusion. A therapeutic window ( ) was determined from the data presented in Figure 6.11 to assess the concentration of bilirubin to be used for pre-treatment of isolated hearts prior to the ischaemic event.
Figure 6.11  **Effect of bilirubin on myocardial CPP, HR, CP and EDP**

Hearts were removed from untreated animals and perfused according to the Langendorff technique as described in Materials and Methods. After 10 min of equilibration, hearts were perfused with Krebs-Henseleit buffer containing bilirubin (0-400 nM final concentration), CPP, HR, CP and EDP were measured throughout the perfusion. Results shown are means ± standard error of the mean of 6-7 independent experiments; *P<0.05 vs. 0 nM bilirubin.
Chapter 6  

HO-1 in the Heart - Bilirubin

Figure 6.12  Exogenously applied bilirubin increases functional recovery of the myocardium after ischaemia-reperfusion (I/R)  
Hearts were removed from untreated animals and perfused according to the Langendorff technique as described in Materials and Methods. After 10 min of equilibration, hearts were perfused with Krebs-Henseleit buffer containing 50 or 100 nM bilirubin (Br) for 10 min and then subjected to 30 min ischaemia plus 60 min reperfusion. Myocardial function (cardiac performance (LVDPxHR)) was measured at 5, 30 and 60 min of the reperfusion period and compared to untreated hearts (control). Results shown are means ± standard error of the mean of 6-7 independent experiments; *P<0.05 vs. Control.

Hearts removed from untreated animals and perfused for 10 min with buffer containing low levels of bilirubin (100 nM) prior to ischaemia showed an 87 % recovery in cardiac performance at 60 min reperfusion as opposed to 65 % recovery in untreated hearts (P < 0.05, Figure 6.12). The increased myocardial performance in hearts pretreated with bilirubin was associated with a significant reduction in ΔCPP (25 mmHg vs. 41 mmHg in untreated hearts) and ΔEDP (19 mmHg vs. 44 mmHg in untreated hearts) at 60 min reperfusion (Figure 6.13).


Figure 6.13  Exogenously applied bilirubin preserves myocardial
haemodynamics after ischaemia-reperfusion (I/R)
ΔCPP and ΔEDP were determined at 5, 30 and 60 min reperfusion
following 30 min ischaemia in hearts pretreated with bilirubin (50 or
100 nM). Hearts were perfused according to the Langendorff
 technique as described in Materials and Methods. Results shown are
means ± standard error of the mean of n=6-8 independent
experiments; *P<0.05 vs. Control.
Figure 6.14  Changes in heart rate (HR) in ischaemic hearts after endogenous bilirubin treatment

Hearts were removed from untreated animals and perfused according to the Langendorff technique as described in Materials and Methods. After 10 min of equilibration, hearts were perfused with Krebs-Henseleit buffer containing 50 or 100 nM bilirubin for 10 min and then subjected to 30 min ischaemia plus 60 min reperfusion. Heart rate was measured throughout the reperfusion period and compared to untreated hearts (0 nM). Results shown are means ± standard error of the mean of 6-7 independent experiments; *P<0.05 vs. Control.

Similarly to what was found after pretreatment with hemin (Figure 6.9), the heart rate (HR) of the isolated organs remained remarkably well preserved throughout the experiments after bilirubin treatment (see Figure 6.14).

These data demonstrate that, at concentrations as low as 100 nM, exogenously applied bilirubin partially prevents reperfusion-mediated impairment of myocardial contractility.
6.4.5 Reduction of infarct size and preservation of mitochondrial integrity in hemin pretreated hearts

In order to assess the damage caused by ischaemia-reperfusion in cardiac tissue, hearts were stained with 2-3-5-triphenyltetrazolium-chloride (tetrazolium red) or fixed for electron microscopy analysis at the end of the reperfusion period (see Materials and Methods, Sections 2.8.3 and 2.7.3 respectively).

Figure 6.15A shows the ischaemic volume (infarct size) measured at 60 min reperfusion in the experimental groups examined as well as representative heart sections from each group. Hearts from vehicle-treated animals that were subjected to ischaemia-reperfusion showed a significantly \( P < 0.05 \) larger infarct size compared to non ischaemic, control hearts (11.7 vs. 0.4 %, \( P < 0.05 \)). The infarct size was reduced to 6.3 % in hearts from animals treated with hemin.

The protective effect of hemin was abolished by treatment with tin protoporphyrin IX (infarct size =10.5 %), which has been previously shown to inhibit haem oxygenase activity. Perfusion of the hearts with bilirubin (100 nM) significantly \( P < 0.05 \) reduced the infarct size to 3.9 %.

Plate 6.1 shows four electron micrographs of mitochondria from the left ventricular cardiac muscle. Compared to non ischaemic tissue (control), mitochondria from hearts subjected to ischaemia-reperfusion appeared swollen and the cristae structure was disrupted. However, both haem
treatment and exogenous bilirubin perfusion preserved mitochondrial integrity after ischaemia-reperfusion.

Figure 6.15 Protective effect of hemin pre-treatment and exogenously applied bilirubin against myocardial ischaemia-reperfusion (I/R) injury.

(A). Hearts perfused according to the Langendorff technique were removed from animals pretreated with vehicle, hemin (50 mg/kg body weight) or hemin plus tin protoporphyrin IX (PP, 40 µmol/kg body weight). Bilirubin (Br, 100 nM) was infused in the isolated heart for 10 min prior to ischaemia-reperfusion (I/R). (B). The ischaemic area was determined at 60 min reperfusion following 30 min ischaemia using the tetrazolium red assay. Control group (Con) is represented by hearts perfused for 20 min at constant flow without I/R. The area of unstained white tissue is indicative of cellular damage and the ischaemic size was calculated as a percentage of the total tissue volume. Results are expressed as the means ± s.e.m., n=4; *P<0.05 vs. Control; †P<0.05 vs. I/R.
Plate 6.1  Effect of ischaemia-reperfusion (I/R) on the integrity of cardiac mitochondria. Representative electron micrographs of hearts from each of the treatments described in Fig 6.13 are shown. Compared to control hearts, cardiac tissue subjected to I/R resulted in severe injury with swollen mitochondria and disrupted cristae. Pretreatment of animals with hemin or perfusion of isolated hearts with bilirubin showed intact mitochondria with well preserved double membrane and parallel cristae (bar, 235 nm).
6.5 Discussion

In isolated perfused hearts, it was found that reperfusion following 30 min ischaemia caused a significant decrease in myocardial contractility and impairment of haemodynamic function, as well as tissue damage and mitochondrial disruption. Myocardial ischaemia-reperfusion injury is a well documented pathological condition characterised by depletion of high energy phosphates, loss of cellular integrity and, ultimately, irreversible injury and cell death (201). It is also well established that oxidative stress resulting from increased production of reactive oxygen species plays a pivotal role in the progression of cell damage in post-ischaemic tissue (202). Paradoxically, increased resistance to reperfusion injury has been shown to occur when brief ischaemic episodes precede a prolonged ischaemic insult, a phenomenon termed “preconditioning” (100). Two temporally distinct phases distinguish the cardioprotective effect of ischaemic preconditioning: an early phase, which develops immediately after preconditioning and wanes within a few hours, and a late phase associated with increased expression of inducible stress proteins or HSPs (205). In several experimental models of myocardial ischaemia, cardioprotection directly correlates with the over-expression of either HSP70, HSP90 or HSP60, the most widely studied molecular chaperones in the heat shock protein family (207). Since diverse stimuli can enhance HSPs levels, the possibility to pharmacologically or genetically manipulate the expression of these proteins to limit ischaemic injury is attainable (208).
This study shows that maximal up-regulation of the stress protein HO-1 (or HSP32), the inducible isoform of haem oxygenase, occurs in cardiac tissue 24 h following treatment of animals with hemin, both a substrate and inducer of HO-1. Interestingly, the recovery of post-ischaemic myocardial function was markedly increased in hearts isolated after treatment of animals with hemin compared to hearts from vehicle-treated rats. Moreover, hearts displaying high HO-1 levels showed a considerably reduced infarct size and preserved mitochondrial integrity after ischaemia-reperfusion. These data are indicative of a major role for HO-1 in cardioprotection. Although such a role for HO-1 has not been described in the context of ischaemia-reperfusion yet, recent reports highlighted the importance of this stress protein in cardiac xenograft survival and prevention of chronic rejection in heart allografts (125, 209).

The direct involvement of the haem oxygenase pathway in minimizing reperfusion damage was ascertained in our study by using an inhibitor of its activity, tin protoporphyrin IX. Predictably, blockade of haem oxygenase activity completely abolished the improved post-ischaemic myocardial performance observed after hemin treatment. Likewise, cardiac tissue injury was exacerbated by treatment of animals with tin protoporphyrin IX.

How can haem oxygenase activation provide protection against the cytotoxicity caused by ischaemia-reperfusion? Since haem oxygenase is the rate limiting step in haem degradation, increased HO-1 protein is expected to result in augmented production of its catabolites, bilirubin and CO. Bilirubin is regarded as a waste product but has been known to possess antioxidant
properties for a long time (210). This peculiarity of bilirubin has been examined by Stocker and colleagues who showed that physiological levels of the bile pigment suppress the oxidation of lipid membranes more than α-tocopherol, which hitherto has been regarded as the best antioxidant against lipid peroxidation (125). Bilirubin is normally present in plasma either as free bilirubin or bound to serum albumin to form a bilirubin-albumin complex. Interestingly, free bilirubin protects low density lipoproteins from oxidation more efficiently than albumin-bound bilirubin (64). It has also been recently reported that both exogenous and endogenously produced bilirubin can effectively prevent cell death mediated by hydrogen peroxide and peroxynitrite, respectively in cultures of aortic endothelial cells (147, 170).

Dore and colleagues demonstrated that accumulation of bilirubin due to enhancement of HO-2 catalytic activity by phosphorylation is also protective against hydrogen peroxide-induced cytotoxicity in neuronal cultures (172).

The results of this study provide strong evidence for a direct involvement of HO-1-derived bilirubin in reducing reperfusion injury. Indeed, consistent with the significant recovery of post-ischaemic myocardial function and preserved tissue viability, that hearts displaying high levels of HO-1 and increased haem oxygenase activity exhibited elevated intracellular bilirubin content. In addition, the rate of bilirubin released into the circulating buffer was higher in hearts showing increased HO-1 expression. The notion that bilirubin is most likely a candidate for cardioprotection was further corroborated by the findings showing that bilirubin, exogenously delivered to isolated hearts prior
to ischaemia, significantly restored myocardial function and minimised both infarct size and mitochondrial damage upon reperfusion.

The findings on increased intracellular bilirubin as a consequence of HO-1 induction imply that production of CO, the other catabolite of haem degradation, is also enhanced. CO, as a vasorelaxant molecule, could be partially responsible for the beneficial effects mediated by activation of the haem oxygenase pathway. Notably, the rise in coronary perfusion pressure, a parameter indicative of coronary vessel contractility, was less pronounced in post-ischaemic cardiac tissue expressing high levels of HO-1. These results suggest that increased endogenous CO may play a role in the maintenance of vascular tone during the reperfusion of ischaemic hearts. Two recent reports sustain this hypothesis by demonstrating that HO-1-derived CO is a major regulator of pressor responses in vitro and in vivo (52, 176, 211). This concept is addressed in the next Chapter.

6.6 Conclusions

This study delineates a plausible mechanism by which HO-1 protein confers protection against oxidant injury in the myocardium. The induction of this stress-sensitive enzyme, which is triggered by a range of oxidant-related stimuli, has been postulated as a ubiquitous defensive system in a variety of cell types (58). In addition the fundamental role of haem oxygenase in the maintenance and restoration of cellular homeostasis has been recently postulated (80). The results presented here substantiate this concept by direct assessment of the cardioprotective action of bilirubin. That the antioxidant
defense provided by bilirubin in the ischaemic myocardium could be physiologically significant is indicated by reports showing an inverse correlation between plasma bilirubin levels and the risk of coronary artery disease (212, 213). Therefore, manipulation of the HO-1 pathway to raise endogenous bilirubin levels may represent a feasible strategy to counteract oxidative stress and, ultimately, have relevant clinical implications in the prevention of cardiovascular disease.
7. HAEM OXYGENASE IN THE VASCUlATURE (II):

THE ROLE OF CARBON MONOXIDE

7.1 Introduction

Although some of the pharmacological affects of CO on the systemic circulation have been reported decades ago, the physiological importance of this response has only recently come to light (214). In analogy with nitric oxide (NO), constitutively generated CO by haem oxygenase has been suggested both to act as a neurotransmitter (137, 215), to inhibit platelet aggregation (216), and to participate in the control of vasorelaxation (217).

Heme oxygenase is believed to modulate vessel tone and blood pressure in an endothelium-independent mechanism, presumably by virtue of the ability of CO to increase cGMP by activating soluble guanylate cyclase (218, 219). Like nitric oxide, CO binds to the ferrous atom of the haem moiety of guanylate cyclase (GC), and forms a six-coordinate bond to activate the enzyme. Activated GC increases cGMP, decreases cytosolic Ca^{2+}, and produces vasorelaxation (220). Endogenous production of CO has been shown to play a role in the maintenance of normal systemic vessel tone (218), as well as in organs such as the liver (221). More recently, our group has shown that increased CO production by HO-1 in vascular tissue contributes to the suppression of acute hypertensive responses under stress conditions in vivo (176). Similarly, we have reported that HO-1-derived CO significantly suppresses phenylephrine-mediated contraction in isolated aortic rings (52).
However, the specific role of HO-1-derived CO in the control of vessel contractility in the coronary circulation remains to be established.

7.2 Objective

The aim of this study was to determine the involvement of haem oxygenase-1-derived CO on the control of coronary perfusion pressure in the isolated heart and mean arterial pressure in the whole animal.

7.3 Methods

7.3.1 Preparation of reagents

Stock solutions of L-NAME were prepared by dissolving the drug in pre-warmed (37 °C) perfusion buffer at a final concentration of 750 μM. L-NAME solution was loaded into a 50 ml syringe and attached to a syringe pump. The pressure tubing loading from the syringe was primed and connected to the side arm of the aortic cannula as described in Materials and Methods.

Tin protoporphyrin (SnPPIX) and hemin were solubilised in PBS (pH 7.4) with the addition of 20 μl 2M NaOH and stored wrapped in tin foil (in the dark) at 4°C until required for injection. Prior to injection, the solutions were warmed in the palm of the hand before loading into a 1 ml syringe and administered (i.p.) to the rat.

7.3.2 Immunohistochemical staining for HO-1 in myocardial tissue

Six male Lewis rats (280 - 350 g) were injected with hemin (50 mg/kg body weight, i.p.) and returned to their cages. At the designated time (0, 18 and 24
h, n=2 for each time-point), the animals were euthanased by cervical
dislocation and hearts removed and processed for immunohistochemical
staining for HO-1 according to the protocol described in Materials and
Methods (see Section 2.7.1).

7.3.3 Effect of augmented HO-1 expression on the maintenance of coronary
perfusion pressure ex vivo

The ability of haem oxygenase-1-derived CO to control coronary vascular tone
was tested *ex vivo* using the isolated Langendorff heart preparation. Changes
in coronary perfusion pressure, an index of vessel contractility, was
continuously measured during perfusion of buffer at constant flow (15 ml/min)
containing the nitric oxide synthase inhibitor N^G^-nitro-L-arginine methyl ester
(L-NAME). L-NAME has been extensively used to produce vasoconstrictor
effects in various species *in vivo* by a mechanism involving the blockade of the
NO synthesis pathway (89); a decrease in endogenous NO production in the
vascular endothelium and smooth muscle leads to an increase in vessel
contractility. Therefore using this *ex vivo* system, we were expecting to
observe an increase in coronary perfusion pressure. Interestingly, perfusing
hearts with L-NAME has also been shown to have a regulatory effect on their
heart rate (222), To discriminate any effects of L-NAME on the heart rate in
this system, hearts were connected to an electronic stimulator as described
previously (Section 2.8.1) and paced at a constant heart rate of 300 beats per
min (bpm). Heart rate was closely monitored throughout the experiments.
Hearts were removed from male Lewis rats (280-350 g), after treatment according to the protocol described below, and perfused according to the Langendorff technique (see Section 2.8.1). After 20 min equilibration, hearts were perfused with buffer containing L-NAME (25 µM final concentration) or PBS (Control group). Haemodynamic parameters were measured throughout the time-course of the experiment.

7.3.3.1 Experimental groups

Group 1: Hearts were removed from rats (n=6) 24 h after treatment with vehicle (600 µl PBS pH 7.4 1 % (v/v) NaOH (2M). After 20 min of normal perfusion, the syringe pump containing PBS (pH 7.4) was turned on and the heart allowed to perfuse for a further 1 h. All haemodynamic parameters were measured throughout the experiment.

Group 2: Rats (n=4) were treated as in Group 1 except that 1 h prior to removal of the heart, animals received an injection (i.p.) of SnPPIX (40 µmol/kg body weight). After 20 min of normal perfusion, hearts were perfused with 25 µM L-NAME and allowed to perfuse for a further 1 h. All haemodynamic parameters were measured throughout the experiment.

Group 3: Hearts were removed from rats (n=4) 24 h after treatment with hemin (50 mg/kg body weight i.p. in PBS pH 7.4 1 % (v/v) NaOH (2M)). After 20 min of normal perfusion hearts were perfused
with 25 μM L-NAME and allowed to perfuse for a further 1 h. All haemodynamic parameters were measured throughout the experiment.

Group 4: Rats (n=4) were treated as in Group 3 except that 1 h prior to heart removal, animals received an injection (i.p) of 40 μmol/kg body weight SnPPIX. After 20 min of normal perfusion hearts were perfused with 25 μM L-NAME and then allowed to perfuse for a further 1 h. All haemodynamic parameters were measured throughout the experiment.

7.3.4 Effect of increased HO-1 expression on the maintenance of mean arterial pressure in vivo

In addition to examining the effect of high HO-1 expression and haem oxygenase activity in the maintenance of vascular tone in the isolated Langendorff heart, the effect of the HO-1 system on vessel tone in vivo was also examined.

Anaesthetised rats (n=6) were catheterised as described in Materials and Methods (see Section 2.9) and the arterial catheter line was connected to a calibrated pressure transducer. After an equilibration period of 10 min, by which time the mean arterial pressure had stabilised, 500 μl sterile saline containing treatment agent (see below) was slowly injected into the venous catheter port. After a further 20 minutes, all animals received a bolus injection of L-NAME (30 mmol/kg) and were then left for 1 h. Mean arterial
pressure (MAP) and core body temperature was measured continuously throughout the experiment.

7.3.4.1 Experimental groups

Group 1: Rats (n=6) were anaesthetised and catheters implanted 24 h after treatment with vehicle (PBS, pH 7.4 1 % (v/v) 2M NaOH). Following 10 min equilibration, animals received an infusion (via the femoral vein cannula) of vehicle (1 ml sterile saline). After a further 20 min, the animal was infused with a bolus of L-NAME (30 μmol/kg body weight). MAP was monitored for a further 60 min.

Group 2: Rats (n=6) were anaesthetised and catheters implanted 24 h after treatment with vehicle (PBS, pH 7.4 1 % (v/v) 2M NaOH). Following 10 min equilibration, animals received an infusion of vehicle (1 ml sterile saline). After a further 20 min the animal was infused with a bolus of L-NAME (30 μmol/kg body weight). MAP was monitored for a further 60 min.

Group 3: Rats (n=4) were treated as in Group 2 except that 1h prior to surgery, animals received an injection (i.p.) of SnPPIX (40 μmol/kg body weight). Following 10 min equilibration, animals received an infusion of vehicle (1 ml sterile saline). After a further 20 min, the animal was infused with a bolus of L-NAME
(30 μmol/kg body weight). MAP was monitored for a further 60 min.

Group 4: Rats (n=4) were treated as in Group 1 except that following 10 min equilibration, animals received an infusion of 1 ml sterile saline saturated with CO gas. After a further 20 min, the animal was infused with a bolus of L-NAME (30 μmol/kg body weight). MAP was monitored for a further 60 min.

7.4 Results

7.4.1 Immunohistochemical localisation of HO-1 expression in rat ventricular tissue

In Chapter 6, myocardial haem oxygenase activity, mRNA and protein expression and a subsequent increase of bilirubin production in the heart was observed after hemin treatment. This Chapter is focusing on the specific pharmacological action of haem oxygenase-1-derived CO in biological systems. Therefore, to further understand the biological role of CO in the myocardium, it was important to determine the localisation of HO-1 expression in cardiac tissue after stimulation with hemin.

Plate 7.1(A-C) shows HO-1 protein localisation in left ventricle tissue at various times after the animals received a single bolus (i.p.) injection of hemin (50 mg/kg body weight). The uppermost figure (A) shows background staining of untreated tissue. The circular vessel (artery) is seen surrounded by a layer of smooth muscle cells. Eighteen h after hemin administration (Plate 7.1B)
the smooth muscle cells (SM) surrounding the lumen (L) are heavily stained positive for HO-1 protein expression. It is interesting to note that areas of positively stained tissue, identified as capillaries (C), also stained positive for HO-1. After a further 6 h (24 h after treatment) high HO-1 expression was still seen in the vascular tissue (Plate 7.1C). This corresponds with elevated HO-1 expression and activity measured 24 h after treatment with hemin (50 mg/kg body weight) previously described in Chapter 3 and Chapter 6. It is interesting to note that there is very little, if any, positive staining in the cardiomyocyte cells and connective tissue within the myocardium after treatment with hemin.
Plate 7.1 Immunohistochemistry for HO-1 in rat left ventricular tissue
Rats were injected (i.p.) with 40 mg/kg body weight hemin. After 0, 18 or 24 h (A, B and C respectively) rats were sacrificed and hearts removed and processed for immunohistochemistry as described in Materials and Methods. Brown areas depict cells expressing HO-1. Key - L = vessel lumen; C = capillary; SM = smooth muscle (magnification - x 150).
7.4.2 Effect of hemin-induced HO-1 expression in the myocardium on vessel tone after ischaemia-reperfusion.

In the previous Chapter (Chapter 6) a series of experiments were undertaken to determine a possible role of bilirubin in protection against ischaemia-reperfusion injury in the isolated rat heart. In this study, using the same model of ischaemia-reperfusion the changes in coronary perfusion pressure were examined before and after the ischaemic event in the presence or absence of high HO-1 expression or bilirubin. Although CO production could not be measured in the heart using current techniques the increased production of bilirubin (as reported in Chapter 6) in hearts expressing elevated HO-1 is likely to be mirrored by increased CO generation.

Hearts taken from animals 24 h after hemin treatment (50 mg/kg body weight) exhibited a greater resistance to increases in CPP (ACPP), after I/R, when compared to vehicle-treated control organs (see Figure 7.1). To determine any direct involvement of the HO pathway on the ACPP, rats were treated with tin protoporphyrin (SnPPIX), the inhibitor of haem oxygenase activity 1 h prior to heart removal. Twenty μmol/kg SnPPIX had no effect on hemin-mediated reduction in ACPP; however, this effect was completely abolished by treatment with 40 μmoles/kg of the inhibitor. Perfusion of the hearts with 50 nM bilirubin prior to the ischaemic event had no effect on CPP after reperfusion, whereas higher concentrations of bilirubin (100 nM) significantly reduced the ACPP. However, this reduction was less than the reduction measured in hearts expressing high amounts of HO-1.
Figure 7.1  **Hemin pre-treatment improves maintenance of myocardial perfusion pressure function following ischaemia-reperfusion**

ΔCPP was determined at 60 min reperfusion following 30 min ischaemia in hearts isolated from animals pre-treated with hemin (50 mg/kg body weight) or hemin plus tin protoporphyrin IX (PP). ΔCPP was also measured in untreated hearts perfused with 50 or 100 nM bilirubin (Br) for 10 min before ischaemia. Animals received hemin or vehicle 1 day prior to removal of the hearts. SnPPIX (20 or 40 μmole/kg), an inhibitor or haem oxygenase activity, was given to the animals 1 h before heart isolation. Hearts were perfused according to the Langendorff technique as described in Materials and Methods. Results shown are means ± standard error of the mean of n=6-8 independent experiments; **P<0.01 vs. untreated control; *P<0.05 vs. untreated control; †P<0.05 vs. hemin treatment.
7.4.3 Effect of HO-1 over-expression on L-NAME-mediated increase in coronary pressor responses

Upon perfusion with 25 \( \mu \)M L-NAME, the coronary perfusion pressure of control (untreated) isolated hearts slowly rose to reach a maximum (300 \% baseline) after approximately 30 minutes. Hearts from hemin-treated animals showed significantly \((P<0.01)\) decreased responsiveness to L-NAME-mediated vasoconstriction. Although, after 30 min perfusion with 25 \( \mu \)M L-NAME all hearts ultimately reached the same CPP (~300 \% of baseline), the rate at which hemin-treated hearts reached this maximum was greatly reduced \((P<0.05\ vs.\ control)\) (Figure 7.2).

Hemin-mediated unresponsiveness was reversed if animals were treated with SnPPIX (40 \( \mu \)mole/kg body weight) 1 h prior to heart removal. As seen in previous studies in the isolated heart, treatment with the same concentration of SnPPIX alone did not have any effect on L-NAME-mediated contractility.
Figure 7.2 Effect of HO-1 induction on the maintenance of coronary vessel tone in the isolated heart
Hearts were isolated from animals pretreated with hemin (50 mg/kg body weight) or hemin plus tin protoporphyrin IX (PP). Animals received hemin or vehicle 1 day prior to removal of the hearts. SnPPIX (20 or 40 μmole/kg), an inhibitor of haem oxygenase activity, was given to the animals 1 h before heart isolation. Hearts were perfused according to the Langendorff technique as described in Materials and Methods. Hearts were perfused with 25 μM L-NAME Results shown are means ± standard error of the mean of n=6-8 independent experiments; *P<0.05 vs. vehicle.
In a second set of experiments, anaesthetised and cannulated animals were infused with a bolus of L-NAME (30 μmole/kg body weight) and mean arterial pressure (MAP) measured. In control rats, infusion of L-NAME resulted in a rapid increase in MAP from a baseline of 70 mmHg to a maximum, after 40 min, of 108 mmHg. In contrast, animals which had been treated with hemin 24 h prior to surgery firstly, had a significantly lower basal MAP than control animals (57 mmHg vs. ~70 mmHg respectively) and secondly, the resulting increase in MAP in response to L-NAME was very much reduced with a maximum of 77 mmHg being reached after 60 min. This effect was abolished in hemin-treated animals if SnPPIX (40 μmole/kg body weight) was injected (i.p.) 1 h prior to surgery.

A fourth group of animals were treated with an infusion of saline saturated with CO gas. These animals had a slightly reduced basal MAP although, not significantly lower than control animals. Upon L-NAME infusion, these animals had a significantly reduced MAP rise compared to control animals. A maximum MAP of 82 mmHg was reached 60 min after infusion of the NOS inhibitor.
Figure 7.3  Effect of increased HO-1 expression on changes in pressor responses mediated by L-NAME
Rats were anaesthetised 24 h after treatment with 50 mg/kg hemin or vehicle. 1 h before surgery, animals were treated with 40 μmoles/kg SnPPIX (PP(40)). Left femoral vein and right femoral artery were cannulated and connected to an i.v. line and pressure monitor as described in Materials and Methods. Animals received a bolus i.v. injection (600 μl) of either saline or saline-saturated with CO gas. After 20 min mean arterial pressure changes in response to 30 μmoles/kg L-NAME administration were measured.
7.5 Discussion

This study demonstrates a role for the HO-1/CO system in the control of vascular contractility. Coronary perfusion pressure (CPP) in the isolated heart is a direct indication of the contractility of the vascular bed in the coronary micro-circulation. The isolated heart preparation used for these studies were perfused at a constant flow rate (15 ml/min); therefore, any change in perfusion pressure indicate increased resistance in the coronary circulation, and the presence of any modulators of smooth muscle contractility in the vascular tissue would effect CPP.

In this Chapter, we have shown that by pre-treating rats with a substrate and inducer of haem oxygenase-1, hemin, HO-1 expression was highly increased in the vessel walls of the coronary circulation. HO-1 has been located in the myocardium of every species investigated so far including rat, mouse, rabbit, dog and pig (34, 125, 171, 223, 224). Although it is not new to demonstrate HO-1 protein expression in the myocardium, it is novel to demonstrate the localisation of the HO-1 induction specifically by the substrate hemin. Interestingly, hearts from hemin-treated animals did not show any HO-1 expression in the cardiomyocytes. Although others have shown expression of HO-1 in cardiomyocytes in vitro, and HO-1 mRNA expression in the myocardium 60 min following a 30 min ischaemic episode, this is the first demonstration of localised HO-1 induction by hemin (225, 226). In fact, the data shown here is supported by Balla and coworkers who showed that after infusion with haemoglobin haem oxygenase was localized by in situ
hybridisation co-expressed with ferritin in the pulmonary endothelium (227). Therefore, it is possible that there is a differential induction and expression of the HO-1 gene depending upon the stimuli and the location.

It is well known that, under normal conditions, control of vessel contractility is primarily attributed to NO, and it has been shown that blockade of the NO synthesis pathway in isolated vessels results in a potentiation of the contraction elicited by phenylephrine (228). However under conditions characterised by a drop in normal cellular metabolism such as ischaemia-reperfusion it is possible that the role of haem oxygenase, specifically HO-1, derived CO may play an important role in maintenance of normal vascular perfusion. In the previous Chapter, the effect of HO-1 upregulation and HO-1-derived bilirubin on post ischaemic myocardial function was investigated using the Langendorff isolated heart model. Here, data from the same series of experiments are described, however in this instance the focus is on the coronary perfusion pressure (CPP) rather than the cardiac performance. Increased haem oxygenase activity would be associated with increased haem catabolism (29); in fact, we have demonstrated in the previous Chapter that there was an increase in bilirubin production in hemin-treated animals. Thus, we can reason that up-regulation of HO-1 in hearts from hemin-treated animals would also result in increased CO production. This concept supports works by Horvath et al who have demonstrated that raised levels of exhaled CO are associated with increased expression of heme oxygenase-1 in airway macrophages (229, 230). The majority of published reports that refer to increased CO production in tissue or cells use an indirect measurement of CO,
such as an increase in cGMP production (50, 231, 232). Although very few studies have reported actual CO levels, our group have demonstrated, in rat thoracic aorta, that increased HO-1 expression is correlated with enhanced CO production by direct measurement (52).

Consequently, our data show that after 30 min ischaemia and 60 min reperfusion, hearts from hemin-treated animals showed a significant (P<0.01) reduction in ΔCPP. To test the direct contribution of HO-1-derived products (either bilirubin or CO) in this effect, hemin-treated animals were also treated with tin protoporphyrin (SnPPIX), a potent competitive inhibitor of haem oxygenase activity (190). As predicted, addition of 40 μmoles/kg body weight SnPPIX completely inhibited the protective effect of hemin on CPP. A number of reports have suggested that SnPPIX may have a non-specific inhibitory effect on NO synthesis and cGMP production, both involved in vasorelaxation (233, 234). However, in the previous Chapter we demonstrated that 20 and 40 μmoles/kg (the same doses used in this study) did not have any effects on either basal or post ischaemic haemodynamic parameters, including CPP. Interestingly, although bilirubin (100 nM) treatment alone also had a significant (P<0.05) effect on CPP, this was not as pronounced as the reduction observed after hemin pre-treatment, indeed there was also a significant (P<0.05) difference between the two treatments.

The data presented in this Chapter demonstrate that under conditions of normal perfusion, HO-1 upregulation modulates the ability of hearts to respond to L-NAME-mediated vasoconstriction. Infusion of hearts from
untreated rats with 25 μM L-NAME resulted in a time-dependent increase in CPP. This is in agreement with others who have reported L-NAME-mediated vasoconstriction in rat and rabbit hearts (222, 235). However, hearts from hemin-treated animals did not display a similar response. In these hearts, although the final CPP reached was the same as in control hearts (~300% of baseline), the CPP rose slowly for the first 15 min of perfusion but then rose faster towards the end of the experiment. It is postulated, based on this evidence that, in the experimental conditions when NO production begins to decrease (during L-NAME perfusion), hemin-treated hearts contain high levels of guanylate cyclase-stimulatory CO which has a compensatory effect. Therefore, in the absence of NO, under conditions of high haem oxygenase activity, guanylate cyclase-stimulatory CO maintains the perfusion pressure. Although this effect did not persist throughout the perfusion it could be that the concentration of L-NAME in the myocardium rose to such a level after 15 min perfusion that the levels of CO were no longer effective in maintaining vessel tone.

In the anaesthetized animal, a single bolus of L-NAME (30 μmoles/kg) had a similar vasoconstrictor effect to L-NAME in the heart. Within the first 20 min after injection, mean arterial pressure had reached the maximum. This supports previous work by our laboratory using a similar method to induce vasoconstriction in conscious, cannulated rats (176). L-NAME-mediated vasoconstriction was significantly attenuated in animals treated 24 h prior to surgery with 50 mg/kg hemin. Interestingly, basal blood pressure was also lowered in these animals suggesting HO-1-derived CO may play a part in
regulation of basal vessel tone when haem oxygenase activity is increased, such as after surgical stress (236). Two recent studies have demonstrated that halogenated volatile anaesthetics inhibit CO-stimulated soluble guanylate cyclase activity in rat brain and isolated aortic rings (237, 238). However, there is no evidence to suggest that the anaesthetics used in this study, Hypnorm and diazepam, have any effect on CG.

It has been demonstrated previously that a single bolus injection of hemin is enough to induce HO-1 in heart, liver, kidneys and skeletal muscle (171, 239, 240). In this study, therefore, 50 mg/kg hemin would induce HO-1 in a similar manner, although only heart HO-1 was measured. The fact that the pressor response to L-NAME was abolished after HO-1 upregulation implies a direct involvement of HO-1 expression and blood pressure regulation. This is further supported by the data presented that treatment with SnPPIX, a potent inhibitor of heme oxygenase activity, totally restored the hypertensive response caused by L-NAME. These data are in agreement with previous reports showing that treatment of spontaneously hypertensive rats with haem oxygenase substrates resulted in lowering of blood pressure which was reversed by treatment with an inhibitor of haem oxygenase (241).

It is not unreasonable to suggest that HO-1-derived CO is playing an important role in the maintenance of vessel tone in this model. It has been demonstrated recently that raised expression of HO-1 is associated with an increase in CO production in tissues (178, 229, 231, 239), and cultured cells (50). CO is undoubtedly an important signalling molecule in the major organs
(137, 221, 232, 242), and is readily up-regulated in vascular cells (50, 243, 244). Therefore, in this study, we propose that the HO-1/CO signalling pathway is also involved in the regulation of systemic blood pressure after treatment with hemin. To confirm the ability of CO to modulate L-NAME-mediated hypertension, animals were treated with saline saturated with CO gas (by bubbling) immediately prior to administration. The resultant drop in L-NAME-mediated hypertension supports this view. In fact, it is interesting to note that the decrease in L-NAME-mediated hypertension brought about by hemin treatment was greater than that caused by saline saturated by CO gas. This may be to do with the actual resulting plasma concentrations of CO developed but is more likely to involve the localisation of endogenous CO production by HO-1 induction in the endothelial and smooth muscle cells, the site of its action.

7.6 Conclusions

This study demonstrates an important role for the HO-1/CO pathway in the regulation and maintenance of vessel tone. The data presented here suggest that in circumstances where HO-1 levels are increased such as sepsis and haemorrhagic shock (42, 154), atherosclerosis (245) and post surgical stress (176), HO-1-derived CO may act as a compensatory mechanism to NO.

In summary, in this Chapter, a role for HO-1-derived CO in the regulation of vessel tone has been demonstrated, emphasizing the importance of this enzyme system and the physiological role haem oxygenase has in the vascular system.
8. **In Vitro Carbon Monoxide Studies - Methodology Development**

8.1 Introduction

The discovery of compounds that release or “donate” nitric oxide (NO) enabled research to focus on the pharmacological and physiological processes of NO as well as providing useful compounds for clinical treatment of cardiovascular disease (246, 247). To examine the heme oxygenase (HO) system fully, it is important to be able to segregate each part of the biochemical pathway to determine their function.

The five essential molecules in the HO pathway are haem, biliverdin, bilirubin, carbon monoxide (CO) and ferrous iron. Haem, isomers of bilirubin and biliverdin and ferrous iron are all commercially available and their ease of storage and handling makes them useful for both *in vivo* and *in vitro* laboratory studies. CO, however, is only currently available as gas, which limits the use of CO in scientific studies, since not all experimental models will allow the infusion of a gas or gas-saturated liquids.

Transition metal-carbonyl compounds (MCCs) are metal complexes that contain both a carbon-carbon double bond and a carbon-oxygen double bond (in other words, they contain C=O). A property of a number of MCCs is that they dissociate to release molecules of CO under certain *in vitro* experimental conditions, such as exposure to light (248, 249). However, to date, no work has
been carried out to investigate the release of physiologically active CO by
transition metal carbonyl complexes.

8.2 Objectives

The aim of this study was to establish an in vitro method for the
measurement of CO dissociation from metal-carbonyl complexes (MCC) and to
determine the potential for CO to be released from iron pentacarbonyl
(Fe(CO)₅) and manganese carbonyl (Mn₂(CO)₁₀).

Once CO dissociation was determined, the biological activity of these
compounds was assessed by exposing smooth muscle cells to MCC and
measuring guanylate cyclase activity.

8.3 Methods

8.3.1 Reagent preparation

Two MCC molecules were used for these experiments: 1) Iron pentacarbonyl
(Fe(CO)₅) is a volatile dense liquid at room temperature which spontaneously
degrades rapidly to release molecular iron and carbon monoxide gas; and 2)
manganese carbonyl (Mn₂(CO)₁₀), which is found as solid crystals at room
temperature and is soluble in most organic solvents. For this study dimethyl
sulphoxide (DMSO) was used as a solvent. 5 mg manganese carbonyl
(13 μmoles) was solubilised in 1 ml DMSO by gentle agitation shortly before
being required and stored in the dark until experimentation. Horse heart
myoglobin was solubilised in 0.04 M phosphate buffer (pH 6.8) prior to use.
8.3.2 Measurement of CO dissociation for MCCs

To determine the potential CO dissociation for the two studied MCCs, the apparatus in Plate 8.1 was set up. Manganese carbonyl dissolved in DMSO and 1 ml of the liquid iron pentacarbonyl was pipetted in to the lower chamber. One and a half ml of 66 µM deoxy-myoglobin (deoxyMb) was pipetted into the top chamber before the two chambers were joined together using a 0.8 µm syringe filter. The apparatus was left in the dark, or exposed to a cold light source (10 mW/cm²) for 60 min after which the myoglobin was removed and the [MbCO] was calculated as described in Materials and Methods (see Section 2.5).

In later experiments, the apparatus was modified to that described in Section 2.5.1. The modified apparatus enabled sampling of the myoglobin solution at various time points as well as being able to expose living cells to MCC-derived CO. A time-course of CO dissociation could be calculated from the MbCO measurements taken.

8.3.3 Exposing cells to MCC-derived CO

Smooth muscle cells were grown to confluence in 24 mm Transwell™ plates (Costar, MA) placed in 6-well culture dishes in normal growth medium. Manganese carbonyl was prepared as described in reagent preparations (Section 8.3.1) and placed in the lower chamber of the apparatus (see Plate 8.2). Cells were washed and the medium was replaced with 1.5 ml fresh medium. The Transwell™ plates were placed on the top of the lower reservoir
with a seal of silicone grease and covered with Parafilm™. A cold light source was shone on the lower reservoir for a predetermined time to allow CO to dissociate from the carbonyl and diffuse through the gas-permeable membrane into the cells. After exposure, the Transwell™ plate was removed and the cells were processed for cGMP EIA as described in Materials and Methods (Section 2.3.3).
Plate 8.1  **Apparatus for exposing myoglobin solution to MCCs**  
A) 5 ml sterile syringe containing 1.5 ml 66 μM myoglobin; B) 0.8 μm filter; C) light source; D) inverted syringe containing MCC.
Plate 8.2  **Apparatus for exposing cells to MCCs**  
A) Parafilm™ covered inverted 50 ml centrifuge tube lid; B) 24 mm Transwell™ cell culture insert containing 1.5 ml 66 μM myoglobin; C) cold light source; D) lower reservoir containing 1 ml MCC; E) inverted 20 ml universal centrifuge tube.
8.4 Results

8.4.1 CO dissociation from iron pentacarbonyl

One ml iron pentacarbonyl, when incubated in the dark at room temperature, dissociated to release measurable amounts of CO using the myoglobin-CO apparatus shown in Plate 8.1. After 60 min, a total of 75 nmoles MbCO were formed in the upper chamber (see Figure 8.1A). In a second set of experiments, MbCO was calculated at 10 min intervals. MbCO measured in the upper chamber gradually increased in a sigmoid curve until the maximum of 75 nmoles was reached after 60 min (Figure 8.1).

However, the release of CO from the iron carbonyl complex was associated with the release of metallic iron into the solution. Therefore, after 60 min incubation in the dark, the carbonyl complex had completely broken down leaving an insoluble precipitate of iron metal, making this molecule an unsuitable candidate for a physiological CO-releasing compound.

8.4.2 CO dissociation from manganese carbonyl

Unlike iron pentacarbonyl, manganese carbonyl did not dissociate to release CO without the cold light source (Figure 8.2A). However, when exposed to the cold light source, MbCO was produced in the upper chamber at a faster rate than was seen by 1 ml iron pentacarbonyl. In fact, the maximum amount of MbCO (76 nmoles) was formed after 45 min exposure. DMSO alone did not have any effect on the Mb in the upper chamber and after 60 min exposure there was no change in the baseline [MbCO] measured.
Figure 8.1  Release of CO from Iron Pentacarbonyl in the dark
A) 1 ml Fe(CO)₅ was placed in the bottom of an upturned syringe according to Material and Methods using the CO measurement apparatus. B) Deoxy-myoglobin was placed in a Transwell™ plate and exposed to 1 ml iron pentacarbonyl. MbCO production was determined at timed intervals (each data point is a mean ±SEM of n=2 experiments).
Figure 8.2  CO dissociation from manganese carbonyl
A) 5 mg Mn$_2$(CO)$_{10}$ (13 μmoles) was dissolved in 1 ml DMSO and exposed to light in the apparatus described in Materials and Methods. MbCO concentration was determined at 10 min intervals. The curve for Mn$_2$(CO)$_{10}$-CO release is shown. B) Calibration data showing times required to produce specific amounts of MbCO in this system.
Since Figure 8.2A shows the amount of MbCO produced in the upper chamber over time, the amount of CO diffusing into the upper chamber can be estimated at any time point required. Figure 8.2B shows a table indicating the times required to expose the upper chamber to various amounts of CO. These data are important when using the MCC to expose cultured cells to various concentrations of CO.

### 8.4.3 CO-mediated activation of guanylate cyclase in vascular smooth muscle cells

In order to determine the possible use of MCC-derived CO in biological systems, vascular smooth muscle cells were exposed to MCC. To verify that the cells used were able to produce cGMP, and by means of a positive control, cells were first exposed to a nitric oxide-donor (SNP) which can activate endothelial and smooth muscle cell guanylate cyclase and increase intracellular cGMP (250, 251). Following a 30 minute incubation with SNP (0 - 1000 µM) vascular smooth muscle cGMP levels increased in a concentration-dependent manner (see Figure 8.3A). Maximal cGMP (18 fmol/well) was attained at a concentration of 500 µM SNP. A further two-fold increase in SNP concentration did not further increase cGMP levels.

Interestingly, exposure of vascular smooth muscle cells to manganese carbonyl (13 µmoles, MCC) for 30 min (estimated 70 nmoles CO) resulted in an increase in intracellular cGMP levels from 12 fmol/well (DMSO alone) to 17 fmol/well (manganese carbonyl) (Figure 8.3A). YC-1, a benzylindazole derivative, has been shown to sensitize the affinity of CO to guanylate cyclase...
In fact, studies have shown that in the presence of YC-1, CO can bind to GC with equal affinity to that of NO (252). In this study, smooth muscle cells were exposed to CO in the presence of YC-1 (100 μM). In order to eliminate the possibility of involvement of endogenous NO in this model, cells were exposed to MCC in the presence of the nitric oxide synthase (NOS) inhibitor, L-NAME (500 μM). However, in this study, co-stimulation of smooth muscle cells with YC-1 (100 μM) and CO (~70 nmoles) did not result in further amplification of the cGMP levels when compared with the cGMP in cells exposed to MCC-derived CO alone (Figure 8.3B). Similarly, inhibition of the NO synthase pathway with L-NAME did not result in any change in the cGMP production observed.
**Figure 8.3** Effect of MCC on guanylate cyclase activity in vascular smooth muscle cells after exposure to NO and MCC

Vascular smooth muscle cells in Transwell™ cell culture inserts in wells of a 6-well culture dish. **A)** cGMP levels were measured after a 30 min treatment with SNP (0-1000 μM), manganese carbonyl (MCC, 0(--), 13 μmoles (++)). **B)** cells were exposed to MCC (13 μmoles manganese carbonyl) in the presence or absence of the NOS inhibitor, L-NAME (500 μM) and YC-1 (100 μM) (All values are means ±SEM of n=4-6 independent experiments. *P<0.05 vs. 0 μM SNP; **P<0.01 vs. 0 μM SNP; ‡P<0.05 vs. – MCC)
8.5 Discussion

Because of the limited utility of NO gas in many experimental systems and the short half life of NO in vivo, chemicals that have the capacity to release NO (with or without a requirement for enzymatic metabolism) have been widely employed as pharmacological tools to investigate the role of NO in many areas of cardiovascular physiology and pharmacology (246, 247). However, the overall elegance of these molecules is that the NO released from these compounds can be controlled over a long period of time and, in fact, a number of NO donors selectively release NO in certain tissues (253). In order to develop a suitable CO releasing compound, these important features should be considered. A number of studies have investigated the effects of CO on vascular smooth muscle cell GC using purified GC and CO gas where it has been shown to have only a small effect on cGMP production (254). In cellular systems, however, HO-1-derived CO has been demonstrated to stimulate vascular smooth muscle cell cGMP as well as to inhibit platelet aggregation (45, 243). The data in this Chapter support the possibility of developing CO-releasing compounds that could have similar pharmacological actions to HO-1-derived CO.

In this study we have demonstrated that manganese carbonyl and iron pentacarbonyl dissociate to release CO. In turn, vascular smooth muscle cells were exposed to SNP and MCC and an increase in intracellular cGMP was measured. It is interesting that smooth muscle cells exposed to DMSO and light alone also exhibited elevated intracellular cGMP. However, this was not
significantly higher than cells treated with 0 μM SNP and may simply be increased because of the culture conditions. Cells were grown in Transwell™ cell culture inserts, which provides a different environment for cell growth than normal tissue culture flasks or multi-well plates. There is evidence that cells exposed to shear stresses and other mechanical stresses in culture may have increased nitric oxide synthase activity and possibly increased haem oxygenase activity by activation of a Ca^{2+} independent mechanism (45, 255, 256). This could, by the evidence discussed previously, result in an increase in cGMP production by the cell. However, the explanation may be simpler; cGMP measurements were represented as fmol cGMP per culture well or Transwell™ culture insert irrespective of cell number/well. Smooth muscle cells were grown in 6-well plates or Transwell™ inserts at approximately equal density and allowed to reach confluence (visual estimation) before experimentation. However, since cells were grown in slightly difference environments it is possible that there were more cells in the wells treated with MCC or DMSO compared to SNP-treated samples, therefore the basal cGMP levels/well may be greater.

The principal disadvantage of using these transition metal carbonyl compounds is that they are all only sparingly, if at all, soluble in aqueous buffers. For example, of the two compounds used in this study, manganese carbonyl would seem the more likely candidate for use in an in vivo system. However, it is only soluble in organic solvents such as DMSO or methanol after a period of gentle agitation and even when completely dissolved will not mix well with aqueous solutions. This study has also shown that the more
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stable MCCs may have an energy requirement before CO-liberating dissociation can occur. In in vitro studies, this may not pose such a problem. However, if this compound, or similar compounds were to be used in an in vivo system, providing an energy source, such as light would pose obvious practical problems.

8.6 Conclusions

This study demonstrates a novel in vitro use for the dissociation of CO from transition metal carbonyl complex molecules. There are a great number of transition metal carbonyl complexes commercially available and, although a number were purchased for the purposes of this preliminary study, only two were chosen for the experiments described in this Chapter. In this study, manganese carbonyl appeared to be less volatile than iron pentacarbonyl and using the methods described we were able to control the amount of CO liberated from the molecule. It is clear, however, that if CO-releasing molecules are to become feasible pharmacological agents in the future more work must be carried out to find compounds that are less volatile and, perhaps, are able to dissolve/mix with aqueous solutions and buffers to enable further in vitro and in vivo studies to be executed.
9. GENERAL DISCUSSION

9.1 Analysis of methodology

This Thesis has been structured according to the topic of each chapter rather than by the methodology used. Therefore, the chapters are not presented in chronological order of completion rather, upon completion of the whole work, they have been arranged to best introduce each aspect of the project.

9.1.1 Cell culture methodology

Cell culture is often used for studying the specific effects of environmental changes or chemical mediators on particular cell types. The smooth muscle cell line used for this study was derived from bovine aortic tissue. Visually, the cells maintained smooth muscle cell morphology (described in Chapter 2), and, for the purposes of this study, responded in a similar manner to primary cultured rat aortic smooth muscle cells with respect to haem oxygenase activity and expression (see Chapter 3). Equally, the data obtained using bovine smooth muscle cells is supported by a number of reports using smooth muscle cells from other species of mammal (47, 51, 159, 257). The cell line was passaged when cells reached confluence (determined visually) and maintained until passage 24 at which point cells were discarded and a new cell line acquired. Cells were maintained in a sufficient volume of culture medium at all times and kept in an incubator at 37°C with an atmosphere of 5% CO₂ balanced with air.
9.1.2 Biochemical and molecular biology techniques

The major biochemical and molecular biology techniques used were: (i) the haem oxygenase activity assay and; (ii) Western blot assay for HO-1 protein expression.

The haem oxygenase activity assay was first described by Tenhunen et al in 1970 and has been used extensively by our laboratory to measure intracellular and tissue haem oxygenase activity (49, 129, 130). The assay used in this Thesis reflects the relative haem oxygenase activity in the sample as “pmol. bilirubin/mg prot./h”. Although the value obtained is not directly comparable to the amount of bilirubin measured in the culture medium after stimulation of the HO-1 pathway (see Section 3.4.1 and Section 4.4.1), the fact that the substrate (hemin) and the cofactors NADPH, glucose-6-phosphate and glucose-6-phosphate-1-dehydrogenase and biliverdin reductase were all added in excess and the procedure was carried out in controlled conditions must be considered when interpreting the data.

Western blot is an established molecular biology technique for measuring relative protein expression in cells and tissue (258); therefore every assay was run with both negative and positive controls (untreated cells/tissue and recombinant HO-1 protein respectively). Since Western blot analysis of HO-1 expression is only used in conjunction with haem oxygenase activity in this study, the actual levels (µg, µmoles etc) of HO-1 protein are not necessary; each experiment is presented by a representative blot which has been scanned into a microcomputer, cropped to the right size and printed.
9.1.3 Langendorff isolated heart model

The isolated perfused heart model used in the studies in Chapter 6 and Chapter 7 is based on the original model first described by Oskar Langendorff in 1895 (259). The original model devised by Langendorff used cat and dog hearts, but for the purpose of our studies rat hearts were used. Since this model has been used over the past 100 years for studying cardiac physiology and has been described in some detail it was considered the best available model for studying the effects of haem oxygenase in the myocardium.

When considering the complications that could interfere with data interpretation when using an in vivo model of myocardial ischaemia-reperfusion injury the Langendorff isolated heart offers a greater degree of control of important physiological parameters which are essential for correct interpretation of the result:

- coronary flow rate including rapid onset of ischaemia and reperfusion,
- perfusate oxygenation and temperature,
- infusion of exogenous agents
- close monitoring of coronary perfusion pressure (CPP), heart rate (HR) and left ventricular developed pressure (LVDP)

The ability to rapidly change the flow rate enabled us to establish a model of ischaemia-reperfusion (described in Chapter 6). After a number of preliminary experiments a time course of ischaemia and reperfusion was
established to result in 40-50% stable reduction in coronary performance after the reperfusion period (described in Section 6.3.3). This was used as a “benchmark” to improve upon through preconditioning or exposure of the whole heart to exogenous agents.

The interpretation of the data derived form this model of the isolated heart is straightforward. At a constant coronary flow rate, an increase in CPP (ΔCPP) over time is indicative of a rise in coronary vessel contractility. Of course, this could also mean the presence an obstruction in one of the vessels of the coronary circulation but, since the perfusion system was kept free of particulate matter through filtration, this is unlikely in our studies. A rise in coronary vessel contractility probably is a result of endothelial cell dysfunction, a common consequence of ischaemia-reperfusion injury (260). The endothelial cell dysfunction is manifested as impaired endothelium-dependent, NO-mediated relaxation of smooth muscle (261). Likewise, changes in LVDP (ΔLVDP) reflects changes in the contractility of the myocardium, disruption of the muscle fibres and damage to the mitochondrial integrity is also a direct effect of ischaemia-reperfusion injury. This was also demonstrated in Chapter 6.
9.1.4 In vivo studies

The method for chronic cannulation of the femoral artery and vein was adapted from a recent report using a similar method in conscious rats. Although recent studies reported that some anaesthetics inhibit guanylate cyclase in rat brain and isolated aortic rings (237, 238), there in no evidence in our studies that the anaesthetic had any adverse effects on the animals except to lower their basal mean arterial pressure to about 50% of conscious resting pressures previously reported by our group (236).

9.2 Hypothesis and aims

The hypothesis behind the work carried out for this Thesis is:

**Upregulation of the haem oxygenase-1 gene and increased generation of bilirubin and carbon monoxide offers protection to vascular tissue against oxidative damage.**

The data presented in this Thesis are consistent with the hypothesis, although the mechanisms behind the protection offered by haem oxygenase have not been completely explored. However, this will be addressed in the next section (Section 9.3).
The Aims of this Thesis were stated at the close of Chapter 1 and were:

(i) To investigate the mechanisms controlling haem oxygenase-1 gene expression in vascular cells and tissue with the ultimate aim of finding pharmacological agents that can be used for preconditioning.

Haem oxygenase activity and HO-1 expression was measured in vascular smooth muscle cells, rat liver and heart tissue after treatment with a number of agents believed to stimulate the HO-1 gene. In Chapter 3 we demonstrated induction of HO-1 protein expression and haem oxygenase activity in vascular smooth muscle cells after stimulation with the substrate, hemin, nitric oxide donors, cadmium chloride (a source of heavy metal ions) and bacterial endotoxin. The molecular mechanisms of HO-1 induction are, as yet, unknown although there have been many speculations as to the pathways involved. For example the evolvement of both activator protein-1 (AP-1), and nuclear factor kB (NF-κB) have been implicated in HO-1 gene transcription (54-56). However, in the context of this study this was not examined.

Selecting an agent for use in the preconditioning experiments described in Chapter 4 and Chapter 6 was based on the data obtained. Nitric oxide donors, although potent inducers of the HO-1 system introduce a new variable in the form of NO. NO itself shares some of the physiological and possibly biochemical effects of CO and therefore may confuse the interpretation of any data obtained with these compounds. A similar effect is seen after treatment of cells or animals with bacterial endotoxin Although comparable levels of
HO-1 induction to those obtained using hemin were obtained after endotoxin treatment, we also observed considerable induction of the iNOS pathway by these compounds. The substrate, hemin, was the most suitable compound since at the concentrations used it showed no signs of cytotoxicity in *in vitro* experiments and in the *in vivo* administration to rats there were no ill effects visible at the time of sacrifice. Therefore, in further studies, hemin was used as an inducer of the HO-1 system.

(i) *To determine the effect of increased haem oxygenase activity, bilirubin and CO on cell function and survival during oxidative challenge*

Chapter 4 described a series of experiments that provided evidence to show that elevated HO-1 in cultured vascular smooth muscle cells offers a degree of protection against a form of oxidant challenge. The experimental protocol used the enzyme glucose oxidase to catalyse the formation of \( \text{H}_2\text{O}_2 \) in the cell culture medium and, therefore, expose the cells to oxidant stress. \( \text{H}_2\text{O}_2 \) has previously been shown in a number of studies to cause oxidative damage in cells (48). Vascular smooth muscle cells showed increased levels of bilirubin production when treated with hemin and we demonstrated that treatment of cells with hemin prior to exposure to the oxidant system rendered the cells protected against concentrations of the oxidant that would normally cause damage to smooth muscle cells. This was, however, only apparent 4h after hemin treatment, if cells were allowed to “recover” for 22 h after incubation with hemin, there was little, or no, protection observed. The protection was demonstrated to be HO-1-dependent since an inhibitor of haem oxygenase
activity, tin protoporphyrin reversed the effect. Interestingly, treating cells with exogenous bilirubin, the end product of haem oxygenase activity, also afforded protection against glucose oxidase-mediated cell death.

In Chapter 5, a possible role for the haem oxygenase-CO pathway in human skin cells (keratinocytes) was addressed. Skin is particularly susceptible to oxidative damage since it is regularly exposed to UV\textsubscript{A} and UV\textsubscript{B} irradiation from the sun. Although, in initial experiments no protection was observed after induction of HO-1 by hemin (preliminary data not shown) in further studies it was observed that cells expressing high levels of HO-1 were also seen to be proliferating at an increased rate. However, this is not the first time that HO-1 has been shown to be involved in cellular proliferation. Indeed, bilirubin and CO have been shown to modulate the proliferation of osteoblasts and hypoxic smooth muscle cells respectively (79, 196). Our group have also demonstrated that smooth muscle cell proliferation is impaired after upregulation of HO-1 by treatment with hemin (unpublished preliminary data, not shown), a process that is blocked by incubation with myoglobin, which is known to bind CO. So, perhaps the proliferative effects of HO-1 are mediated by CO. Recently, Siow and colleagues postulated that both bilirubin and CO have anti-atherogenic properties (262).
(i) To evaluate the effect of increased haem oxygenase expression and bilirubin/CO generation on short-term survival of myocardial tissue after ischaemia-reperfusion injury.

Bilirubin has been described as one of the most powerful naturally occurring antioxidants known to man (63). Based on this supposition it is not surprising that our studies demonstrated that hearts expressing increased HO-1 were significantly protected against 30 min ischaemia followed by 60 min reperfusion. The data presented in Chapter 6 and Chapter 7 describes the protective effect of both HO-1-derived bilirubin and CO and exogenous bilirubin on a number of myocardial physiological parameters as well as macro- and microscopic examination of the survival of the myocardial cells and mitochondrial integrity.

All the data shown support the hypothesis that the products of haem oxygenase are protective in the isolated rat heart. Although no direct measurements were made, from these data, we have hypothesised that HO-1-derived CO in the coronary arteries is able to activate a vasodilator mechanism and, in our study, reduce the coronary perfusion pressure after the ischaemic event. Whether this occurs through a cGMP-dependent pathway or through activation of K+ channels is a current topic of much debate (68, 75, 77, 78, 159, 263). Nevertheless, in Chapter 8 we demonstrated the ability of exogenous CO generated by a novel metal carbonyl complex (MCC) to stimulate vascular smooth muscle cell cGMP.
The final chapter of this Thesis described a series of experiments designed to test the ability of novel MCC molecules to release biologically active CO for use in in vitro studies. Although preliminary data were encouraging, in that the MCC compounds tested were able to release CO in a time-dependent, and controlled, manner there were obvious drawbacks in the methodology needed to carry out such experiments. The primary concern for the long term use of these compounds is their apparent bio-incompatibility since both the compounds tested were insoluble in aqueous solutions. The development of NO-releasing compounds has made dramatic improvements in the way in which we can investigate the role of NO in biological systems and has opened up new strategies in the treatment of chronic cardiovascular disease in the clinic (246, 247). Further development of these MCC compounds may reveal molecules that will release CO in a controlled manner and exhibit better compatibility with cellular systems.

9.3 Conclusions, reflections and future perspectives

The potential for protecting vascular tissue against oxidant injury is highlighted by recent reports suggesting that HO-1 gene transfer confers vascular protection. Studies by Abraham and co workers have shown that transfection of the human HO-1 gene into coronary endothelial cells attenuates the damaging effects of free haem and modulates the angiogenic potential of the cell (174, 264) and a recent study by Soares and co workers elegantly demonstrated that in mouse-rat xenografts the HO-1 gene is functionally associated with xenograft survival, and that rapid expression of
HO-1 in cardiac xenografts can be essential to ensure long-term graft survival (265).

These reports, and others, have highlighted the potential for genetic manipulation of vascular genes to provide new insights into the physiological roles of the haem oxygenase isozymes. Perhaps, future studies using gene transfer and transgenic animals would enable us further insights into the protective role of haem oxygenase against vascular dysfunction. Although current studies using exogenous agents such as CO and bilirubin can simulate to some extent the effects of haem oxygenase activation, the exact conditions of HO-1 induction can not be simulated under experimental conditions. Hypothetically, transfection of the HO-1 gene, with a suitable promoter, into a cell will emulate the effects of HO-1 induction (266). There are too a number of co-factors that appear to be upregulated by certain stimuli at the same time as HO-1, such as ferritin (267-269),

The importance of CO in the vasculature in normal and pathophysiological conditions has remained notably understated since this crucial role was first discovered. In normal physiological conditions it is understood that one of NO's functions lies in control of vessel tone by stimulation of the guanylate cyclase pathway leading to vasorelaxation by movement of Ca^{2+} within smooth muscle cells (81, 228). However, it is believed that in stress situations, such as after an ischaemic episode, NO production may be down-regulated in the presence of high HO-1 expression and in this situation CO may play a crucial role in retaining control of vessel tone (80) Therefore, we believe that
further understanding of the exact function of CO in the vasculature in these situations is vital if we are to understand the full extent of the physiological functions of the haem oxygenase system. In Chapter 8, we described the development of a CO-releasing compound (a metal carbonyl complex). Within the limited scope of application (due to their bio-incompatibility) of the two compounds examined in this Thesis we have demonstrated upregulation of cGMP production in smooth muscle cells. If more compounds such as these are developed it will enable the isolation of any effects of CO on biological system from the additive effects of bilirubin in situations where HO-1 expression is enhanced.

In 1999 the first known case of HO-1 deficiency was presented in Japan (270). This patient suffered from a multitude of chronic pathophysiological conditions including retarded growth, persistent haemolytic anaemia, abnormally high cholesterol levels and a very low tolerance to cellular oxidant stress, which is not surprising since it has already been shown that HO-1 plays a vital role in clearing iron from cells and bilirubin is both a powerful antioxidant and can scavenge degraded LDL cholesterol (29, 63, 64, 271). Although this is the only known case of HO-1 deficiency it has certainly demonstrated that the HO-1 system is vital for normal human vascular physiology.

In fact, it is this apparent multiplicity of functions of the haem oxygenase family of proteins, or products of their actions, that have helped to widen the field of vascular research especially with respect to host antioxidant defense
and vasoregulation (52, 76, 170, 176, 221, 272, 273). Despite this, however, to date only a few studies have been carried out to elucidate the exact role of the products of the haem oxygenases in vascular dysfunction in humans. For example, Ahmed and colleagues have previously demonstrated the involvement of CO in myometrial contractility in human subjects and a number of others have measured HO-1 expression in human peripheral blood leukocytes and cultured human endothelial cells (263, 274, 275). Although there appears to be conserved homology between the haem oxygenase isozymes in rodents and HO-1 in humans, it is vital that we understand the regulation of haem oxygenase in human tissues if the development of therapeutic strategies for human disease are to be established.

In conclusion, there have been considerable advances in the biochemical and molecular characterisation of haem oxygenase isozymes and the control of the genes encoding these proteins. However, there is a great deal remaining to be understood about this important enzyme such as the exact regulatory mechanisms of the genes by exogenous and endogenous agents in order to further understand the mechanisms of its physiological function and the pathogenesis of vascular disease.
10. LIST OF PUBLICATIONS

PUBLISHED ARTICLES


PUBLISHED ABSTRACTS


CONGRESSES


11. REFERENCES


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