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NO- AND CO-MEDIATED INHIBITION OF MITOCHONDRIAL RESPIRATION IN ACTIVATED MACROPHAGES

Submitted by

FRANCIS KING WAN LAM

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University of London

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The Wolfson Institute for Biomedical Research

University College London

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ABSTRACT

Nitric oxide (NO) is a gaseous signalling molecule that is synthesised by nitric oxide synthases (NOSs) and has a variety of physiological and pathophysiological roles. Some of its physiological functions are primarily mediated via the activation of soluble guanylate cyclase. However, NO is also able to potently inhibit mitochondrial respiration at complex IV of the electron transport chain (ETC) in a manner that is reversible and in competition with oxygen (O_2). Following prolonged exposure to NO, the ETC undergoes an NO-dependent modification at complex I, which results in a persistent inhibition of respiration.

Activated macrophages were found to produce NO, via upregulation of the inducible isoform of NOS (iNOS), in sufficient quantities to inhibit respiration. This inhibition was initially reversible but became persistent with time. In addition, haem oxygenase-1 (HO-1) is upregulated in these cells, producing carbon monoxide (CO), which is also known to inhibit complex IV. The ability of exogenous and endogenous CO to inhibit respiration was investigated. Although less potent than NO, CO was shown to cause significant inhibition of respiration, particularly under hypoxic (1% O_2) conditions. Furthermore, hypoxia was found to attenuate significantly the synthesis of NO from iNOS but not CO from HO-1.

The consequences of inhibition of respiration were investigated under hypoxia, where the availability of O_2 may be limiting for O_2-dependent cellular processes. Specifically, the effects of respiratory inhibition were investigated with regards to the stability of hypoxia-induced hypoxia inducible factor 1α (HIF1α). Hypoxia resulted in the stabilisation of HIF1α protein, a phenomenon that was prevented by inhibition of the ETC at various complexes.

This destabilisation of HIF1α was found to be due to a redistribution of intracellular O_2 from mitochondrial consumption, resulting in an increased intracellular O_2 concentration and the reactivation of the O_2-dependent degradation of HIF1α protein.

ABBREVIATIONS

ATP	Adenosine 5'-triphosphate
BAT	Brown adipose tissue
BH_4	Tetrahydrobiopterin
cGMP	Cyclic guanosine 3',5'-monophosphate
CO	Carbon monoxide
DEANO	2,2-diethyl-1-nitroso-oxyhydrazine
DETA	(Z)-1-[2-aminoethyl-N-(2-ammonioethyl)amino] diazen-1-ium-1,2-diolate
DNA	Deoxyribonucleic acid
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated protein kinase
ETC	Electron transport chain
FAD	Flavin adenine dinucleotide (oxidised)
$FADH_2$	Flavin adenine dinucleotide (reduced)
FIH	Factor inhibiting hypoxia inducible factor
FMN	Flavin mononucleotide
GSH-EE	Glutathione ethyl ester
H_2O_2	Hydrogen peroxide
HIF	Hypoxia inducible factor
HO	Haem oxygenase
HRE	Hypoxia response elements
HRP	Horse radish peroxidase
HSP	Heat shock protein

IFNγ	Interferon γ
IMM	Inner mitochondrial membrane
I/R	Ischaemia-reperfusion
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
KO	Knock-out
L-NIO	N-(1-iminoethyl)-L-ornithine
L-NMMA	N^{ω}-monomethyl-L-arginine
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
mRNA	Messenger ribonucleic acid
mtNOS	Mitochondrial nitric oxide synthase
NAC	N-acetylcysteine
NAD^+	Nicotinamide adenine dinucleotide (oxidised)
NADH	Nicotinamide adenine dinucleotide (reduced)
NF-κB	Nuclear factor κB
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO_2^-	Nitrite
NOS	Nitric oxide synthase
O_2	Oxygen
O_2^-	Superoxide
OMM	Outer mitochondrial membrane

$ONOO^-$	Peroxynitrite
OxyHb	Oxyhaemoglobin
PCR	Polymerase chain reaction
PHD	Prolyl hydroxylase
PPM	Parts per million
pVHL	Von Hippel Lindau protein
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sGC	Soluble guanylate cyclase
SOD	Superoxide dismutase
TGF	Transforming growth factor
TNFα	Tumour necrosis factor α
UCP	Uncoupling protein
WT	Wild-type

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1 INTRODUCTION

1.1 Mitochondria

Mitochondria are small cellular organelles, typically 2μm in length and 0.5-1μm in diameter. They are mobile and plastic structures with the ability to fuse with and separate from each other (Bossy-Wetzel *et al.*, 2003). Their highly membranous structure is related to their primary function, which is to synthesise usable energy in the form of adenosine 5'-triphosphate (ATP; Brand *et al.*, 2004).

The synthesis of ATP involves the electron transport chain (ETC) which resides in the inner mitochondrial membrane. It is composed of a series of enzyme complexes that sequentially transport electrons, derived from respiratory substrates, coupling their passage through the chain with the pumping of protons from the mitochondrial matrix into the intermembrane space. The localisation of protons in the intermembrane space creates an electrical and chemical gradient across the mitochondrial membranes, and can be considered as a store of energy which may be used to synthesise ATP. The last of these enzymes is complex IV (cytochrome c oxidase, cytochrome aa_3). It passes the electrons to the final electron acceptor, molecular oxygen (O_2), which is reduced to water. It is this reaction that is responsible for approximately 90% of a cell's oxygen consumption (Brown, 1999; Piantadosi, 2002).

In addition to energy production, mitochondria have important roles in thermogenesis (Sell *et al.*, 2004) and apoptosis (Parone *et al.*, 2002). They may also be able to produce reactive oxygen species (ROS) which have been proposed to be involved in cellular signalling pathways (Chandel *et al.*, 2000b). The overproduction of ROS by mitochondria may be important in the pathogenesis of certain diseases, such as Parkinson's and Alzheimer's

diseases and atherosclerosis. Furthermore, mitochondria-derived ROS may be involved in the process of cellular ageing (Brand *et al.*, 2004).

1.1.1 Mitochondrial Structure

Mitochondria possess both inner and outer mitochondrial membranes (IMM and OMM respectively; Figure 1). The IMM is highly convoluted, forming structures called cristae. These provide a larger surface area to contain the components of the ETC and associated proteins. In addition, the IMM contains a high proportion of cardiolipin. This phospholipid renders the IMM impermeable to solutes. In contrast, the OMM is relatively permeable. It contains many copies of the protein porin, which provides aqueous channels for solutes of up to 5kDa in size to pass through.

Between the IMM and OMM is the intermembrane space. This region is acidic, containing a high concentration of protons. This localisation of protons creates both an electrical and chemical gradient across the mitochondrial membranes. The steepness of this electrochemical gradient constitutes the mitochondrial membrane potential ($\Delta\Psi_m$; approximately $^{-}$150-$^{-}$180mV). The higher the concentration of protons in the intermembrane space, the steeper the electrochemical gradient is, and the higher the $\Delta\Psi_m$. The $\Delta\Psi_m$ has an important role in driving the synthesis of ATP, ROS generation (Brand *et al.*, 2004) and apoptosis (Green & Kroemer, 1998).

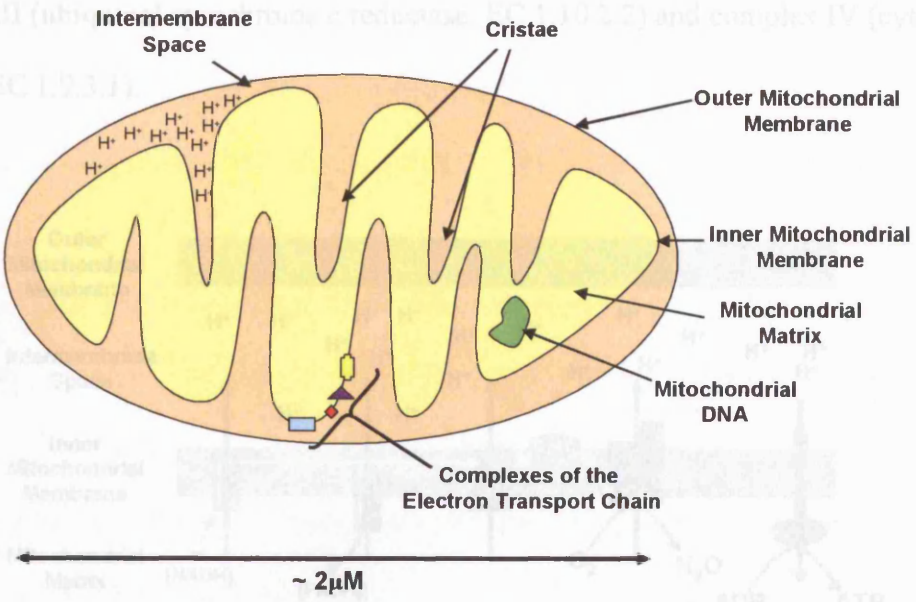

Figure 1. The structure of a mitochondrion. The folding of the inner mitochondrial membrane (IMM) into the mitochondrial matrix forms structures called cristae. Between the IMM and the outer mitochondrial membrane is the intermembrane space containing a high concentration of protons (H^+).

Contained within the IMM is the mitochondrial matrix (Figure 1). This compartment contains mitochondrial DNA and many enzymes, including those involved in the Krebs cycle. It is from the Krebs cycle that most of the electrons are obtained from metabolic substrates and passed onto the ETC. The carrier molecules that transport electrons are either pyridine nucleotides (nicotinamide adenine dinucleotide; NAD^+) or flavins (flavin adenine dinucleotide; FAD).

1.1.2 The Electron Transport Chain

The ETC (Figure 2) is composed of a series of enzyme complexes: complex I (NADH ubiquinone reductase; EC 1.6.5.3), complex II (succinate ubiquinone reductase; EC 1.3.5.1),

complex III (ubiquinol cytochrome c reductase; EC 1.10.2.2) and complex IV (cytochrome c oxidase; EC 1.9.3.1).

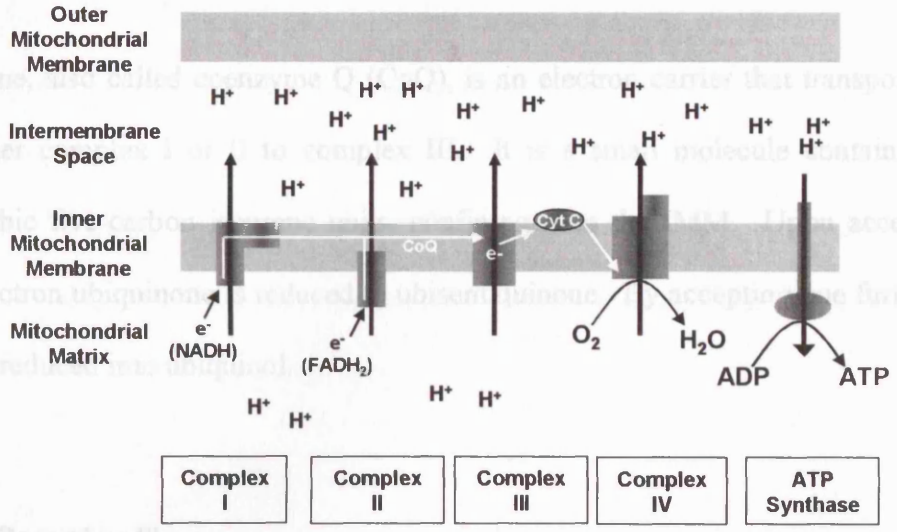

Figure 2. The mitochondrial electron transport chain (ETC). The ETC consists of a series of enzyme complexes (I-IV) which sequentially transport electrons from reducing equivalents (NADH and $FADH_2$) to the final electron acceptor O_2, which is reduced to water. As the electrons pass through the ETC, their transport is coupled to the pumping of protons from the mitochondrial matrix into the intermembrane space. The flow of protons back into the mitochondrial matrix, through ATP synthase, releases free energy which is used to synthesise ATP from ADP. The transport of electrons to complex III is performed by coenzyme Q (CoQ), and from complex III to complex IV by cytochrome c (cyt c).

1.1.2.1 Complex I

The majority of electrons that enter the ETC are passed onto complex I by reduced NAD^+ (NADH). Complex I is a large enzyme of approximately 850kDa and is composed of about 25 polypeptide chains. The electrons donated by NADH are accepted by the flavin mononucleotide (FMN) prosthetic group and then passed onto the iron-sulphur (Fe-S) clusters, where the iron atoms are reduced into the ferrous state (Fe^{2+}). As the electrons pass

through the complex, their transport is coupled to the pumping of protons from the mitochondrial matrix into the intermembrane space. Ubiquinone then receives the electrons from complex I, oxidising the Fe-S clusters back into the ferric state (Fe^{3+}).

Ubiquinone, also called coenzyme Q (CoQ), is an electron carrier that transports electrons from either complex I or II to complex III. It is a small molecule containing 6 to 10 hydrophobic five-carbon isoprene units, confining it to the IMM. Upon acceptance of a single electron ubiquinone is reduced to ubisemiquinone. By accepting one further electron it is fully reduced into ubiquinol.

1.1.2.2 Complex III

Complex III contains two types of cytochromes: cytochrome b and cytochrome c_1. These are electron-transferring proteins that contain a haem prosthetic group. In contrast with ubiquinone, cytochromes can only accept one electron which reduces the haem iron to Fe^{2+}. Ubiquinol donates its two electrons to complex III via a two-step process. The first electron is transferred to an Fe-S cluster, oxidising ubiquinol to ubisemiquinone. Ubisemiquinone is then fully oxidised into ubiquinone by donation of its remaining electron to cytochrome b. This electron is then 'recycled' back to another ubisemiquinone molecule to reform ubiquinol. This recycling process is called the Q cycle. Upon acceptance of an electron the Fe-S cluster of complex III passes it onto cytochrome c_1 which then reduces cytochrome c.

Cytochrome c is another electron carrier. It is loosely bound onto the outer surface of the IMM and shuttles electrons from complex III to complex IV.

1.1.2.3 Complex IV

Complex IV is the terminal enzyme of the ETC and passes the electrons to the final acceptor, O_2. It contains two cytochromes, cytochromes a and a_3, and two copper centres, copper a (Cu_A) and copper b (Cu_B). Cytochrome a and Cu_A are paired together on the same subunit and are the initial acceptors of the electrons from cytochrome c. The electrons are then transferred to the binuclear centre composed of haem a_3 and Cu_B located in subunit I of complex IV. The haem is reduced to Fe^{2+} and the Cu_B centre is reduced to the cuprous (Cu^+) form. In this reduced state the binuclear centre donates these electrons, along with four protons, to O_2, reducing it into water.

1.1.2.4 ATP Synthesis

As the electrons pass through the ETC, and protons are pumped into the intermembrane space, the electrochemical gradient, which constitutes the proton motive force and the $\Delta\Psi_m$, is formed. This gradient can be considered as a store of free energy that may be used to drive chemical reactions. In this case the release of free energy is used to drive the synthesis of ATP (Hinkle and McCarty, 1978). ATP synthase is an enzyme that is also located in the IMM. It provides a pore through which protons can flow down their electrochemical gradients from the intermembrane space back into the mitochondrial matrix. When this occurs, the free energy that is released is coupled to the phosphorylation of adenosine 5'-diphosphate (ADP) to form ATP. This chemiosmotic coupling forms an essential part of the conversion of energy derived from metabolic substrates into a form that is usable by the cell, a process called oxidative phosphorylation (Mitchell, 1961).

1.1.2.5 Complex II

Complex II is also part of the ETC. However, unlike the other complexes, it does not pump protons into the intermembrane space. It receives electrons from reduced FAD ($FADH_2$) that is also produced from the Krebs cycle and passes them initially to its Fe-S clusters, and then onto the ubiquinone pool.

1.1.3 Other Functions of the Mitochondria

In addition to ATP synthesis, mitochondria are also involved in other cellular processes including thermoregulation (Sell *et al.*, 2004) and apoptosis (Parone *et al.*, 2002; Green and Kroemer, 1998).

1.1.3.1 Mitochondria and Thermogenesis

Thermogenesis is the production of heat. It increases in cold climates and is an important adaptive response. One of the mechanisms by which thermogenesis may occur is through dissipation of the free energy that is stored by the proton electrochemical gradient in mitochondria.

Brown adipocytes are lipid storage cells characterised by numerous mitochondria containing many cristae. They are located in brown adipose tissue (BAT) which is often found in small and newborn mammals. The function of brown adipocytes is to generate heat. During cold acclimatisation, BAT is stimulated by the sympathetic nervous system resulting in the upregulation and activation of uncoupling protein 1 (UCP1; Ricquier *et al.*, 1983). UCP1 is located on the IMM and allows the passage of protons from the intermembrane space into

the mitochondrial matrix. Since UCP1 provides an alternative path for the protons to re-enter the mitochondrial matrix, it uncouples the oxidation of respiratory substrates from the synthesis of ATP via ATP synthase. The free energy that is released from the protons passing through UCP1 is dissipated as heat (Sell *et al.*, 2004).

1.1.3.2 Mitochondrial Role in Apoptosis

Apoptosis is a form of cell death that has been shown to be important in the development and tissue homeostasis of multicellular organisms. It involves the activation of caspases, a family of cysteinyl proteases, which may occur via a mitochondrial-dependent or -independent pathway.

Mitochondria contain a number of proapoptotic agents, including cytochrome c, smac/diablo, apoptosis-inducing factor and endonuclease G, and the release of these agents into the cytosol promotes apoptosis. For example, the apoptosome is formed when cytochrome c is released, which is then able to activate caspases-3 and -9. Evidence exists that suggests that the release of cytochrome c and other proapoptotic agents is regulated by the Bcl-2 family of proteins acting at the mitochondria.

The Bcl-2 proteins contain a number of homology domains (BH-1, -2, -3 and -4) and can be divided into two groups; those that are antiapoptotic (e.g. Bcl-2 and Bcl-X_L) and those that are proapoptotic (e.g. Bax and Bak). It has been suggested that the proapoptotic Bcl-2 proteins are able to form channels within the mitochondrial membranes to allow the passage and release of cytochrome c. The antiapoptotic Bcl-2 proteins, however, are able to prevent the formation of these channels.

Thus, regulation of the release of proapoptotic agents from the mitochondria by the Bcl-2 family of proteins may promote or prevent apoptosis.

1.1.4 Mitochondrial Generation of Reactive Oxygen Species

Mitochondria have also been implicated in a number of signal transduction pathways. These pathways include the activation of nuclear factor kappa-B (NF-κB; Palacios-Callender *et al.*, 2004; Zhang & Chen, 2004), stabilisation of hypoxia-inducible factor-1α (HIF1α; Chandel *et al.*, 2000) and the activation of c-Jun NH_2-terminal kinase (JNK; Zhang and Chen, 2004). Mitochondria have been proposed to modulate these pathways through the generation of ROS.

At certain points in the ETC, the electrons that are being transported may be passed onto O_2 to produce superoxide anion (O_2^-). In fact, it has been suggested that 1-3% of the O_2 consumed by mitochondria may be converted into O_2^- by the ETC (Carreras *et al.*, 2004). This free radical is very reactive and unstable, with a half-life ($t_{1/2}$) of about a microsecond, and may react with and damage proteins, DNA and lipids. In addition to O_2^-, ROS includes hydrogen peroxide (H_2O_2) and hydroxyl radicals. O_2^- can be metabolised by enzymes called superoxide dismutases (SOD) to produce H_2O_2, which is less reactive but has a longer $t_{1/2}$. There are two types of SOD: MnSOD, which is found in the mitochondrial matrix, and Cu/ZnSOD, which is found in the cytosol. H_2O_2 itself can be converted into hydroxyl radicals in the presence of ferrous iron via the Fenton reactions.

The production of O_2^- is correlated with the $\Delta\Psi_m$, with a high $\Delta\Psi_m$ favouring the production of O_2^- (Brand *et al.*,2004). Once produced, O_2^- may be directed into the cellular cytosol or into the mitochondrial matrix. Various antioxidant defense mechanisms, such as glutathione, SOD (Raha *et al.*, 2000), thioredoxin (Winyard *et al.*, 2005) and catalase (Radi *et al.*, 1991), may scavenge or metabolise the ROS, thus preventing the possibility of cellular damage caused by these reactive species.

Conditions which cause the components of the ETC to be in a more reduced state may also increase the likelihood of ROS generation by the mitochondria. Such situations may arise when downstream complexes (e.g. complex IV) become inhibited, such as in hypoxia (Duranteau *et al.*, 1998; Palacios-Callender *et al.*, 2004). The effect of this would be to decrease the rate of electron transport through the chain and to increase the risk of the electrons 'leaking' to O_2.

1.1.4.1 Mitochondrial Sites of ROS Production

The precise locations of ROS generation in the ETC are still unclear. However, accumulating evidence, much of it based on *in vitro* experiments using either isolated mitochondria or submitochondrial particles, suggests that both complex I and complex III may be sites of significant ROS production.

1.1.4.1.1 Production of ROS at Complex I

Complex I has been suggested to be a major site of O_2^- production (Brand *et al.*, 2004). ROS can be detected in submitochondrial particles (Turrens and Boveris, 1980) inhibited by

rotenone, an inhibitor of complex I near the ubiquinone binding site (Okun *et al.*, 1999). However, in intact mitochondria rotenone does not generate detectable ROS production (Chen *et al.*, 2003). It has been suggested that O_2^- is released by rotenone-inhibited mitochondria into the matrix side where it is dealt with by antioxidant mechanisms (Grigorieff, 1999). Since O_2^- cannot traverse lipid membranes, it is effectively localised and eliminated in this compartment. In submitochondrial particles, however, it has been shown that antioxidant mechanisms are depleted and the mitochondrial membranes may be inverted; such conditions permit ROS to be detected. This could explain the contradictory results observed by Chen *et al.* and Turrens and Boveris.

From these observations it has been inferred that in cells with intact mitochondria, ROS produced by complex I are eliminated within the matrix. As a result, any effects exerted by the ROS are confined to the inside of the mitochondrion and subsequently have no extramitochondrial actions.

1.1.4.1.2 Generation of ROS at Complex III

Complex III may also be a site of significant ROS generation (St-Pierre *et al.*, 2002). Inhibitors (e.g. antimycin a) that block the distal end (Q_i centre) of complex III prevent electron donation to cytochrome c but not electron acceptance from ubiquinol, resulting in the production of detectable ROS (St-Pierre *et al.*,2002; Chen *et al.*, 2003). However, myxothiazol, which inhibits the proximal end (Q_o centre) of complex III and thus prevents electron acceptance from ubiquinol, does not result in detectable ROS production in intact isolated mitochondria.

Since the ROS produced from complex III has been detected extramitochondrially, it has been suggested that ROS produced from this site may be involved in cellular signalling transduction mechanisms (Chandel *et al.*, 2000b).

1.2 Nitric Oxide

Nitric oxide (NO) is a gaseous free radical molecule that is produced enzymatically within cells. It is both water and lipid soluble (approximately 8 times more soluble in membranes; from Shiva *et al.*, 2001) and, as with O_2^-, has a short $t_{1/2}$ (seconds; Moncada *et al.*, 1991). Nevertheless, NO has been shown to be capable of traversing biological membranes to exert effects in neighbouring cells (Brown *et al.*, 1998).

Since its initial identification as an endogenous vasodilator molecule (Palmer *et al.*, 1987), NO has been shown to play important roles in the cardiovascular, nervous and immune systems (Moncada *et al.*, 1991). Most of the physiological effects of NO are mediated by activation of its receptor soluble guanylate cyclase (sGC; Bellamy & Garthwaite, 2002). More recently, however, NO has been demonstrated to interact with complex IV of the ETC in a reversible manner (Brown & Cooper, 1994; Cleeter *et al.*, 1994; Schweizer and Richter, 1994). Furthermore, NO may react with, and persistently inhibit, complex I of the ETC (Beltran *et al.*, 2000). These interactions may have both physiological (Clementi *et al.*, 1999) and pathophysiological (Bolanos *et al.*, 1994) consequences for the cell.

1.2.1 Nitric Oxide Biosynthesis

Nitric oxide is produced endogenously by a group of enzymes called nitric oxide synthases (NOSs; Figure 3). There are currently three established isoforms of this enzyme: neuronal NOS (nNOS, type I NOS), inducible NOS (iNOS, type II NOS) and endothelial NOS (eNOS, type III NOS), each of which are products of separate genes (Alderton *et al.*, 2001).

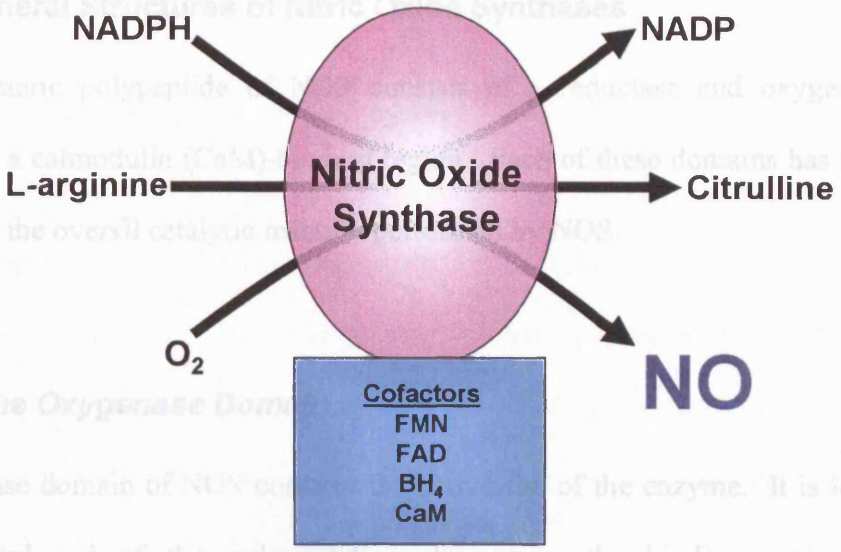

Figure 3. The substrates, cofactors and products of nitric oxide synthase (NOS). NOSs use NADPH, L-arginine and O_2 as substrates to produce NADP, citrulline and NO. In addition, the enzyme requires the presence of a number of cofactors including flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), tetrahydrobiopterin (BH_4) and calcium-calmodulin (CaM).

Nitric oxide synthases use a number of common substrates and cofactors to produce NO. The amino acid L-arginine, NADPH and O_2 are the substrates for NO synthesis, with the nitrogen atom derived from the guanidino nitrogen of L-arginine (Palmer *et al.*, 1988). The enzymes catalyse the sequential oxidation of L-arginine, using electrons donated by NADPH, to produce stoichiometric quantities of NO and citrulline (Bush *et al.*, 1992), via a transient intermediate, N^{ω}-hydroxy-L-arginine (Stuehr *et al.*, 1991).

A number of cofactors are necessary for NOS activity. These cofactors are generally involved in transferring electrons from NADPH to the substrates (FMN, FAD), or they are involved with the formation of the active enzyme (tetrahydrobiopterin, calmodulin).

1.2.1.1 General Structures of Nitric Oxide Synthases

Each monomeric polypeptide of NOS consists of a reductase and oxygenase domain, separated by a calmodulin (CaM)-binding region. Each of these domains has functions that contribute to the overall catalytic reaction performed by NOS.

1.2.1.1.1 The Oxygenase Domain

The oxygenase domain of NOS contains the active site of the enzyme. It is located on the amino-terminal end of the polypeptide and contains the binding regions for haem, tetrahydrobiopterin (BH_4) and L-arginine, which are required for the functional enzyme. Only the oxygenase domain is apparently involved in the formation of the active homodimeric enzyme (Ghosh *et al.*, 1997). In addition, this region contains binding motifs for caveolins which may be involved in the localisation of NOS to membranes.

1.2.1.1.2 The Reductase Domain

The reductase domain of NOS is situated at the carboxy-terminal of the polypeptide chain. It bears a remarkable homology with the cytochrome P450-reductase family of enzymes (approximately 50%; Wang *et al.*, 1997) and, like these enzymes, its principal function is to transport electrons from NADPH to the substrates located in the oxygenase domain. The electron carriers involved in NOS include FMN and FAD. In fact, the rate at which the active enzyme synthesises NO depends upon the rate at which electrons can be shuttled to the oxygenase domain. This was shown through the use of chimeric enzymes containing combinations of the oxygenase and reductase domains from different NOS isoforms (Nishida *et al.*, 1998).

1.2.1.1.3 The Calmodulin Binding Region

Separating the oxygenase and reductase domains of NOS is an approximately 30 amino acid sequence which forms the binding region for the cofactor CaM. Calmodulin is able to incorporate itself into NOS when it is activated by bound calcium. Calmodulin binding to the enzyme triggers the flow of electrons from the reductase to the oxygenase domains and thus synthesis of NO (Abu-Soud *et al.*, 1994). Thus, calcium may also be a necessary requirement for NOS activity (Moncada *et al.*, 1991).

1.2.1.2 Nitric Oxide Synthase Isoforms

The three established isoforms of NOS may be described as either constitutive (eNOS and nNOS) or inducible (iNOS). Constitutively expressed NOS enzymes are principally involved with the physiological actions of NO, including neurotransmission and vasorelaxation (Moncada *et al.*, 1991). They produce NO in picomolar quantities and their activites are transient, regulated by increased intracellular calcium concentrations and activation of CaM (Bredt & Snyder, 1994). Inducible NOS is mainly involved in immunological responses. It is not normally expressed and is induced in certain cells by particular stimuli. This isoform has activity that is CaM-independent, already containing activated CaM that is tightly bound, and therefore, once expressed, produces NO constantly in micromolar concentrations. As a result of its high NO generation, iNOS has been associated with the pathophysiological effects of NO.

1.2.1.2.1 Endothelial NOS

Endothelial NOS was named according to the cell in which it was originally discovered. However, eNOS is also present in non-endothelial cells including platelets (Radomski *et al.*, 1990) and some neurones (Dinerman *et al.*, 1994). It is a 133kDa protein and is one of the constitutive NOS enzymes. Unlike the other NOS isoforms it is normally found in the particulate fraction of a cell. It has been shown that eNOS can be localised onto membranous structures, particularly to the caveolae (Shaul *et al.*, 1996; Michel *et al.*, 1997). These are microdomains of the plasma membrane that contain caveolins, the protein to which eNOS may be bound. Recently, it has been shown that eNOS may also be sublocalised onto mitochondria via a pentabasic amino acid sequence (Gao *et al.*, 2004).

In addition to CaM regulation, eNOS can also be activated by phosphorylation (Dimmeler *et al.*, 1999). Endothelial cells experience shear stress from the drag exerted by the flow of blood in vessel lumen. This force can activate the phosphatidylinositol-3-OH kinase (PI3K)/Akt pathway. The subsequent activation of the Ser/Thr kinase Akt results in the phosphorylation of eNOS on Ser^{1177} resulting in its activation. In this activated state, eNOS shows activity that is CaM-independent (Dimmeler *et al.*, 1999).

1.2.1.2.2 Neuronal NOS

As with eNOS, nNOS is also a constitutive NOS isoform. It is a larger protein of 161kDa in size and is found mainly in the brain and nitrergic neurones of the peripheral nervous system. It is a soluble enzyme that is located in the cytosol.

In the brain glutamate, released from glutamatergic neurones, activates NMDA receptors on the postsynaptic neurone. Once ligated, the NMDA receptor opens an ion channel to allow the influx of calcium into the cell. This calcium can then activate CaM, which in turn stimulates nNOS activity. Like eNOS, nNOS can also be phosphorylated (Hayashi *et al.*, 2000). This reaction is catalysed by CaM-dependent protein kinases and targets Ser^{847} of nNOS. In contrast with eNOS, however, this phosphorylation event results in a decrease in nNOS activity (Hayashi *et al.*, 2000).

1.2.1.2.3 Inducible NOS

Inducible NOS is unlike the other NOS isoforms in that it is not constitutive and produces significantly greater quantities of NO. It too is a soluble enzyme that is located in the cytosol and is a protein of 131kDa in size. It was originally identified in activated macrophages that displayed anti-tumour activity, a property which is attributed in part to its production of NO. However, the large quantites of NO produced by iNOS have also been associated with pathological conditions such as septic shock, arthritis and inflammatory bowel syndrome.

As active iNOS already binds CaM tightly, the enzyme is insensitive to changes in intracellular calcium concentrations. Its activity is therefore regulated by other means, principally by expression at the mRNA level. Inducible NOS expression is stimulated by bacterial endotoxin (lipopolysaccharide; LPS) as well as by a number of pro-inflammatory cytokines including tumour necrosis factor-α (TNFα), interferon-γ (IFNγ) and interleukin-1 (IL-1). In addition, its expression may also be inhibited by certain cytokines such as interleukin-4, -8 and -10 (McCall *et al.*, 1992). The induction of iNOS expression leads to

an increase in iNOS mRNA whose levels may be modulated. Post-transcriptional regulation of the iNOS mRNA has been reported to be modulated by transforming growth factor-β (TGFβ; Vodovotz *et al.*, 1994) which destabilises the mRNA. In addition, NO itself has also been shown to reduce the mRNA levels of iNOS. The inhibition of NOS activity in activated macrophages causes iNOS mRNA levels to increase, whereas treatment with NO from an NO donor dose-dependently reduces iNOS mRNA levels (Hinz *et al.*, 2000). Nitric oxide has also been shown to inhibit iNOS protein activity (Assreuy *et al.*, 1993) by targeting the haem group in the enzyme. This effect is reversible and has been proposed to be a feed-back mechanism to prevent excessive NO production. Inducible NOS protein activity may also be inhibited by the lack of availability of substrates, such as L-arginine, or of cofactors, such as BH_4 (Mori and Gotoh, 2000).

1.2.1.2.4 Mitochondrial NOS

In addition to the three distinct isoforms of NOS described above, there may exist a fourth mitochondrial isozyme. This mitochondrial NOS (mtNOS) has been shown to produce NO in sufficient quantities to inhibit respiration in isolated mitochondria, particularly upon the addition of exogenous L-arginine (Giulivi, 1998). This inhibition can be prevented by the inhibitor of NOS, N^{ω}-monomethyl-L-arginine (L-NMMA), or by oxyhaemoglobin which binds NO.

However, the exact identity of mtNOS remains unclear. Previous reports have shown that the isozyme exhibits Ca^{2+}-dependent activity, similar to that of the constitutive NOS enzymes (Ghafourifar and Richter, 1997). However, it has also been described to be similar

to iNOS, based on characteristics such as reactivity to monoclonal antibodies and molecular weight (Tatoyan and Giulivi, 1998).

Evidence demonstrating that mtNOS is either nNOS or eNOS has been provided by several studies. Cardiomyocytes obtained from nNOS knockout (KO) mice have been shown to lack mitochondrial NO production compared with the wild-type (WT; Kanai *et al.*, 2001). In addition, nNOS has been identified in only 13% of neuronal mitochondria obtained from nNOS KO mice, compared with 85% in the WT (Rothe *et al.*, 1999). These reports are consistent with the proposal that mtNOS is nNOS. In contrast, myocardial tissue obtained from eNOS KO mice does not demonstrate an inhibition of respiration upon stimulation with bradykinin (Loke *et al.*, 1999). This suggests that mtNOS may be eNOS.

Therefore it remains to be established whether or not a specific mtNOS exists or whether the NOS sited in the mitochondria is actually a previously-described isoform.

1.2.2 Targets of Nitric Oxide

Nitric oxide is able to bind to haem groups and to modulate the activities of the enzymes that contain them. The most established target for NO is sGC. In the last decade another biological target has risen to prominence. It too is a haemoprotein and is the terminal enzyme of the ETC, complex IV.

1.2.2.1 Soluble Guanylate Cyclase

Soluble guanylate cyclase is a heterodimeric protein that is composed of α and β subunits. It is the main target through which NO exerts its physiological actions. It contains a haem group to which NO binds and activates the enzyme (ED_{50} = ~20nM; Bellamy *et al.*, 2000). Once bound, NO induces a conformational change in the enzyme resulting in an increased activity of several hundred fold. The enzyme catalyses the conversion of guanosine 5'-triphosphate (GTP) into cyclic guanosine 3',5'-monophosphate (cGMP), which acts as a second messenger for signal transduction. Cyclic GMP has three principal targets: cGMP-dependent protein kinases (Lohmann *et al.*, 1997), cGMP-gated cation channels (Zagotta and Siegelbaum, 1996) and cGMP-regulated phosphodiesterases (Degerman *et al.*, 1997). Through these mechanisms NO exerts its physiological actions including the regulation of vascular tone (Warner *et al.*, 1994), the regulation of platelet aggregation (Buechler *et al.*, 1994) and neurotransmission (Jaffrey & Snyder, 1995).

1.2.2.2 The Electron Transport Chain

Complex IV of the ETC has also been shown to be a target of NO, which inhibits the enzyme in a manner that is reversible and competitive with O_2 (Brown and Cooper, 1994; Cleeter *et al.*, 1994; Schweizer and Richter, 1994). The site that NO and O_2 compete for is the binuclear centre of the enzyme, consisting of a haem (haem a_3) and copper (Cu_B). Both NO and O_2 have similar rates of association with the fully-reduced (Fe^{2+}-Cu_B^+) enzyme (k = 0.4-1.0x$10^8 M^{-1}s^{-1}$ and $10^8 M^{-1}s^{-1}$ respectively; Blackmore *et al.*, 1991; Babcock and Wikstrom, 1992). However, NO has been shown to inhibit complex IV rapidly (milliseconds to seconds) and potently, even when the concentration of O_2 is 40 to 500 times greater than that of NO.

It has been reported that in the range 125-165μM O_2, approximately the mean arterial blood concentration of O_2, the IC_{50} of NO is 270nM. Between 18-38μM O_2, approximately the tissue O_2 concentration, the IC_{50} is 60nM NO (Brown and Cooper, 1994). Others have reported similar IC_{50} values (364nM NO between 140-190μM O_2, 41nM at 50μM O_2) and have calculated the K_i for NO acting at complex IV to be 27nM (Koivisto *et al.*, 1997). The explanation for the greater potency of NO binding to complex IV compared to O_2 appears to be that NO can bind onto the enzyme when it is partially (Fe^{2+}-Cu_B^{2+} or Fe^{3+}-Cu_B^{+}) or fully-reduced (Fe^{2+}-Cu_B^{+}), whereas O_2 can only bind to the latter form (Babcock and Wikstrom, 1992).

The exact mechanism by which NO binds to complex IV remains to be clarified but two main hypotheses have been suggested. The first proposes that NO binds onto the Fe^{2+} of the partially-reduced enzyme and prevents the interaction of complex IV with O_2 (Giuffre *et al.*, 1996). The second hypothesis claims that NO initially binds onto Cu_B (Cu^+ or Cu^{2+}) of the partially-reduced enzyme, which then prevents O_2 from interacting with the haem iron (Torres *et al.*, 1995). This second hypothesis also suggests that the interaction of NO with the Cu_B leads to the hydration and metabolism of the gas to form nitrite (NO_2^-) within the binuclear centre. The NO_2^-, when formed, inhibits complex IV effectively because it diffuses out of the binuclear centre slowly (Torres *et al.*, 1998).

In addition to the reversible inhibition at complex IV, NO, or one of its related species, has been reported to inhibit the activity of the ETC persistently or irreversibly. Cells (Jurkat, L929 and J774) treated with (Z)-1-[2-aminoethyl-N-(2-ammonioethyl)amino] diazen-1-ium-

1,2-diolate (DETA), which releases a constant amount of NO over the course of the experiment, for prolonged periods resulted in the persistent inhibition of respiration that was attributed to S-nitrosylation of complex I (Clementi *et al.*, 1998; Beltran *et al.*, 2000; Orsi *et al.*, 2000). In these studies, respiration occurring through complexes I, III and IV was inhibited whereas respiration through complexes II, III and IV was unchanged after exposure to 1.5μM NO. Furthermore, nitration of complex I was discounted because the persistent inhibition was reversed by the addition of reduced glutathione ethyl-ester or by cold light, properties that are exhibited by S-nitrosylation. In addition, the ONOO- scavenger and the SOD mimetic MnTBAP did not prevent the inhibition of complex I, suggesting that ONOO- was not the nitrosylating species (Clementi *et al.*, 1998). It was also reported that S-nitrosylation of complex I only occurred after the intracellular concentrations of reduced glutathione had fallen. This was supported by the acceleration of the persistent inhibition upon treatment of the cells with the glutathione synthesis inhibitor L-buthionine (S, R)-sulphoximine. Thus, the persistent inhibition of NO on mitochondrial respiration may only occur when the redox state of the mitochondrion has been compromised.

In contrast, direct treatment of isolated mitochondria with peroxynitrite ($ONOO^-$), which can be produced intracellularly by the reaction between NO and O_2^- (Radi *et al.*, 1994; Murray *et al.*, 2003), resulted in the inhibition of complexes I, II and IV. This effect could not be reversed by the antioxidant dithiothreitol, ruling out inhibition by S-nitrosylation, and was suggested to be due to nitration of the enzyme complexes by $ONOO^-$ (Murray *et al.*, 2003). Previous work has also been carried out using NO donors that is in agreement with these observations (Riobo *et al.*, 2001). The inhibition of respiration by NO acting at complex IV was proposed to increase the formation of O_2^- from the mitochondria, which would then

react with NO to produce $ONOO^-$. This $ONOO^-$ could then react with and nitrate components of the ETC. An inhibition of complex I was observed that was prevented by SOD. This implicates $ONOO^-$ as the species involved in causing the effect. In contrast, however, no effects on complex II or III were observed in this study. Experiments using activated astrocytes have also provided evidence for $ONOO^-$ causing irreversible inhibition of the ETC (Bolanos *et al.*, 1994). Astrocytes activated with LPS and IFNγ exhibited an irreversible inhibition of complex IV that was prevented by SOD and catalase. No effects on complex I, however, were observed.

Recently, it has been reported that both S-nitrosylation and nitration of complex I may occur in concert in activated macrophages (Frost *et al.*, 2005). It was shown that an early NO-dependent inhibition of complex I activity could be prevented by addition of reduced glutathione ethyl-ester, indicating S-nitrosylation. The ability of glutathione ethyl-ester to prevent this inhibition diminished with time and was coupled with the appearance of nitrotyrosine residues.

A variety of cell types have been exposed to NO, whether endogenous or exogenous (Bolanos *et* al., 1996; Clementi *et al.*, 1998; Beltran *et al.*, 2000). Similar effects upon mitochondrial respiratory activity were observed and a decrease in intracellular GSH concentration was implicated as a contributor to loss of complex I activity. This phenomenon was exemplified by the increased susceptibility of astrocytes to $ONOO^-$ induced complex I damage after GSH depletion (Barker *et al.*, 1996). Astrocytes have a higher specific activity of γ-glutamylcysteine synthetase, the rate-limiting enzyme of GSH synthesis, than neurons. This may be responsible for the greater resistance of astrocytes

compared with neurons to $ONOO^-$ (Bolanos *et al.*, 1995). Furthermore, it has been shown that the ability of astrocytes to upregulate glycolysis in response to respiratory inhibition by NO protects it from apoptosis, as is observed in neurons, which cannot upregulate glycolysis (Almeida *et al.*, 2001). Thus, the effects of NO upon cells may be dictated by a variety of factors, such as the intracellular concentration of GSH and the ability to upregulate apoptosis, which have been described here.

1.3 Carbon Monoxide

Carbon monoxide (CO), like NO and O_2^-, is a gaseous diatomic molecule that is produced endogenously by cells. However, unlike NO and O_2^-, it is not a free radical and as a result is less reactive and more stable. In fact, the only clearly established targets for CO are haem-containing proteins. Nevertheless, CO has been implicated in a number of biological functions, such as neurotransmission, vasorelaxation and inhibition of smooth muscle proliferation (Ryter and Otterbein, 2004). It is produced enzymatically by a group of enzymes called haem oxygenases (HO; Tenhunen *et al.*, 1969; Maines, 1997), consuming the substrates haem, NADPH and oxygen.

1.3.1 Carbon Monoxide Biosynthesis

Haem oxygenase enzymes catalyse the rate-limiting step in haem catabolism, a mechanism that is responsible for over 85% of endogenously produced CO.

The HO enzymes are normally colocalised with another enzyme, NADPH:cytochrome P450 reductase, which provides the substrate NADPH as an electron donor. The electrons derived from NADPH have two functions: to activate O_2 and to reduce the haem iron to the ferrous state. Haem and O_2 are required for HO activity, which produces equimolar quantities of free divalent iron (Fe^{2+}), CO and linear tetrapyrrole biliverdin. Biliverdin is then rapidly converted into bilirubin by the enzyme biliverdin reductase (Tenhunen *et al.*, 1970).

The CO produced by HO is derived from the α-methene bridge carbon of the haem molecule. It is released by the oxidation of this atom by O_2 in an NADPH-dependent reaction.

Three isoforms of the HO enzyme have been identified: HO-1, HO-2 and HO-3.

1.3.1.1 Haem Oxygenase-1

Haem oxygenase-1 is the most widely studied isoform of HO. It is a 32kDa enzyme that can be induced by a wide range of stimuli including pro-inflammatory cytokines, heavy metals, heat shock, hypoxia and oxidative stress. As a result of its expression in response to stress, it was initially identified as heat shock protein 32 (HSP32) and proposed to possess cytoprotective properties (Keyse and Tyrrell, 1989).

The proposed cytoprotective effects of HO-1 have been attributed to its enzymatic products. Biliverdin and bilirubin have both been shown to be potent antioxidants (Stocker *et al.*, 1987; Marks *et al.*, 1991) whereas the free iron that is released induces the upregulation of ferritin, a protein which sequesters iron within a cell. The net effect of ferritin is to decrease intracellular free iron concentrations, which prevents the production of ROS via the Fenton reaction.

Carbon monoxide also has been proposed to possess cytoprotective actions. *In vitro* studies using cultured murine fibroblasts have shown that the overexpression of HO-1 is able to prevent TNFα-induced apoptosis. This effect can also be reproduced by the administration of exogenous CO gas (Petrache *et al.*, 2000). In studies of organ transplantation, pre-

treatment of the organ with CO prevents organ rejection (Sato *et al.*, 2001). Furthermore, in models of ischaemia/reperfusion (I/R) lung injury, HO-1 null mice displayed increased mortality compared with wild-type mice. This was partly attributed to the lack of CO production from HO-1 because inhalation of CO gas (1000ppm) compensated for the HO-1 deficiency and improved survival following I/R injury (Fujita *et al.*, 2001).

1.3.1.2 Haem Oxygenase-2 and -3

Haem oxygenase-2 is the isoform that is predominantly found in the brain (Sun *et al.*, 1990) and vasculature where it has been proposed to be involved in neurotransmission and regulation of vascular tone, respectively (Maines, 1997). Unlike HO-1, HO-2 is a constitutive isoform. They are products of different genes and share little similarity in primary structure, regulation and tissue distribution. However, they have similar mechanisms of haem catabolism, substrate specificity and coenzyme requirements. Haem oxygenase-2 is a slightly larger protein (36kDa in size) and has a lower affinity for haem (K_m = 0.24μM for HO-1, K_m = 0.67μM for HO-2; Maines *et al.*, 1986). In addition, the HO-2 gene does not contain consensus sequences for the binding of several regulatory factors, including heat shock factor, AP-1 and NF-κB. This explains why it is not induced by stimuli that induce HO-1 expression.

Little is known about HO-3. It is a homologue of HO-2 that shows little catalytic activity and its functions are not well understood (McCoubrey *et al.*, 1997).

1.3.2 Targets of Carbon Monoxide

Carbon monoxide, like NO, is able to bind to iron centres of haem groups (Maines, 1997). Unlike NO, however, CO only has affinity for ferrous iron whereas NO is capable of binding both the ferrous and ferric forms (Ryter *et al.*, 2004). It is through this interaction with haem groups that CO mediates most of its biological actions.

1.3.2.1 Soluble Guanylate Cyclase

Carbon monoxide activates sGC with a potency 30-100 times less than that of NO (Furchgott & Jothianandan, 1991). Nevertheless CO has been shown to be important in stimulating sGC, particularly in the olfactory neurones of the brain. These neurones do not express NOS but still produce cGMP, which can be inhibited by either haemoglobin (a scavenger of both NO and CO) or zinc protoporphyrin IX (ZnPPIX; an inhibitor of HO activity). Furthermore, cGMP production could be stimulated by treatment with exogenous CO (Ingi *et al.*, 1996b). These observations suggest a role for CO as an agonist for sGC in the brain. In contrast, however, CO has also been proposed to be an antagonist of sGC. This is because production of cGMP in the cerebellar granules of the brain can be decreased by inhibition of NOS, indicating that NO is activating sGC, and increased by inhibition of HO activity. This suggests that CO could be acting as an antagonist, preventing sGC activation by NO (Ingi *et al.*, 1996a).

Carbon monoxide also modulates vascular tone via activation of sGC (Zakhary *et al.*, 1996). In these studies, it was shown that tin protoporphyrin IX, another inhibitor of HO activity, reduced the vasorelaxation of porcine aortae in response to acetylcholine. Furthermore, in an isolated perfused rat liver model, inhibition of HO activity by ZnPPIX increased

perfusion pressure. This effect was reversed by the administration of either CO or cGMP analogues (Suematsu *et al.*, 1994).

1.3.2.2 Mitogen-Activated Protein Kinase Pathways

The mitogen-activated protein kinase (MAPK) pathways are involved in the signalling of stress and inflammatory events. The MAPKs are a family of Ser/Thr protein kinases that are activated by a variety of stimuli (Kyriakis & Avruch, 1996). There are three main MAPK pathways: extracellular signal-related protein kinase (ERK), JNK and p38 MAPK. Carbon monoxide activates the p38 MAPK pathway and increases the phosphorylation of p38. It is through this mechanism that CO has been proposed to exert its anti-inflammatory effects (Otterbein *et al.*, 2000).

1.3.2.3 The Electron Transport Chain

Carbon monoxide is also known to interact with the ETC by binding to complex IV. Unlike NO, which can bind to the cytochrome a_3 of complex IV in both the reduced and oxidised state, CO can only bind to it in the reduced state (Hansen & Nicholls, 1978; Haab, 1990; Piantadosi, 2002). By binding to complex IV CO inhibits the catalytic activity of the enzyme (K_i = 0.32μM; Petersen, 1977), preventing the reduction of O_2 to water, and therefore inhibiting the consumption of O_2 by mitochondria. This inhibition is competitive with O_2 (Hansen & Nicholls, 1978). Thus, when O_2 is plentiful, CO is unable to bind onto complex IV. In addition, when O_2 is abundant complex IV is maintained in the oxidised state, again preventing the binding of CO. In situations of hypoxia, where the O_2 concentration is low and complex IV is in a more reduced state (Kreisman *et al.*, 1981;

Palacios-Callender *et al.*, 2004), the binding of CO to complex IV is favoured (Wohlrab & Ogunmola, 1971; Piantadosi, 2002).

In studies of smokers, who experience a greater exposure to CO, it has been shown that complex IV activity in blood lymphocytes was inhibited by up to 76% compared with controls (Miro *et al.*, 1998). Other complex activities remained unaffected. Following cessation of smoking for 3 days, complex IV activity recovered to 48% of control levels.

1.4 Hypoxia

Hypoxia is generally defined as the lack of availability of O_2, whether it be for the alteration in cellular bioenergetics due to the changes in mitochondrial and glycolytic activity, or for the initiation of adaptive transcriptional events. In biological systems, adaptation to hypoxia has been associated with an alteration in gene transcription, specifically with the upregulation of genes that promote survival in low O_2 conditions, such as the haematopoietic growth factor erythropoietin, and those that alter the energy metabolism of a cell to a more anaerobic state, such as the enzymes that are involved in glycolysis (Semenza, 2003). Investigations into the mechanism by which these genes are induced led to the identification of a transcription factor that is responsive to low O_2 concentrations: hypoxia-inducible factor.

Hypoxia-inducible factor (HIF) is a heterodimeric transcription factor that is composed of two subunits: HIFα and HIFβ. Whereas HIFβ is a constitutive protein, HIFα is highly induced by hypoxia. In hypoxia HIFα and HIFβ dimerise to form the active HIF transcription factor. This is then able to recognise and bind onto specific DNA sequences (5'-G/ACGTG-3'; Semenza, 1996) in the promoter or enhancer regions of genes to initiate transcription. These DNA motifs are called hypoxia-response elements (HREs).

There are three isoforms of HIFα, each a product of separate genes: HIF1α, HIF2α and HIF3α. The isoform that displays the most robust response to hypoxia, and has subsequently been most studied and understood, is HIF1α. In addition, the active heterodimer of HIF1α, HIF1, has been proposed to be the most important transcription factor regulating gene expression in hypoxia (Sandau *et al.*, 2001).

1.4.1 Hypoxia-Inducible Factor 1α

Hypoxia-inducible factor 1α is a 120kDa protein that dimerises with HIF1β to form HIF1, which has been reported to control the expression of over 60 genes (Semenza, 2003). It is stabilised in hypoxia in most, if not all, cells.

Hypoxia-inducible factor 1α protein is induced exponentially by O_2 concentrations of less than 6% (Jiang *et al.*, 1996), but the mRNA levels are not upregulated (Wood *et al.*, 1996). This suggests that HIF1α protein is not regulated by hypoxia at the transcriptional level. When O_2 is abundant, the HIF1α protein is rapidly degraded and is subsequently unable to dimerise with HIF1β to form the active HIF1 transcription factor. In hypoxia the protein is no longer degraded and is stabilised. The mechanism by which the stability of HIF1α is regulated involves the prolyl hydroxylase enzymes (Figure 4).

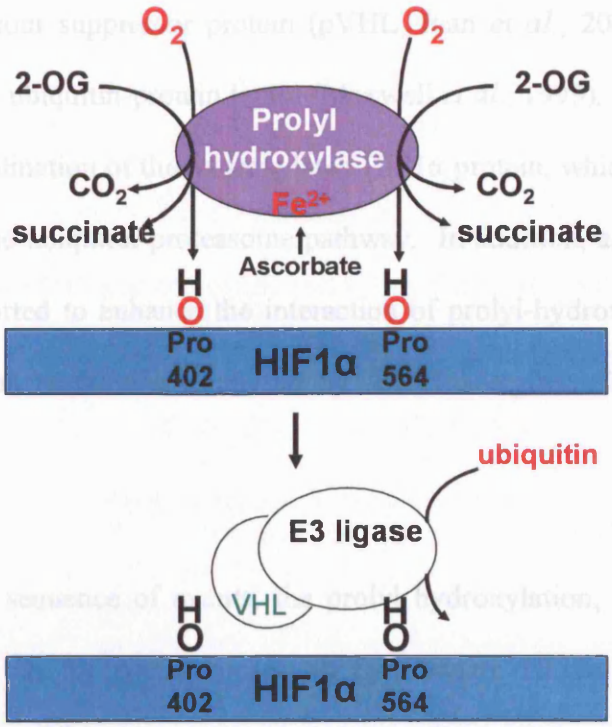

Figure 4. **Prolyl hydroxylation of HIF1α targets the protein for degradation.** The hydroxylation of two specific proline residues in HIF1α is catalysed by the enzyme prolyl hydroxylase. This enzyme requires the substrates O_2 and 2-oxoglutarate (2-OG) to function, as well the presence of iron and ascorbate. The prolyl hydroxylation of HIF1α results in the binding of the von Hippel Lindau tumour suppressor protein and E3 ligase, which catalyses the polyubiquitination of HIF1α. This targets the transcription factor for proteasomal degradation.

1.4.2 HIF1α Regulation by Prolyl Hydroxylases

When O_2 is abundant HIF1α is rapidly targeted for degradation via hydroxylation of specific proline residues (Pro^{402} and Pro^{564}; Bruick and McKnight, 2001) located in the carboxy- and amino-terminal oxygen-dependent degradation domains of the protein (CODDD and NODDD, respectively). These reactions are catalysed by the prolyl hydroxylase enzymes, which are members of the 2-oxoglutarate-dependent-oxygenase family (Schofield and Ratcliffe, 2004). Hydroxylation of the prolyl residues increases the affinity of HIF1α for the

von Hippel-Lindau tumour suppressor protein (pVHL; Ivan *et al.*, 2001; Jaakkola *et al.*, 2001) which recruits E3 ubiquitin-protein ligase (Maxwell *et al.*, 1999). This ligase enzyme catalyses the polyubiquitination of the hydroxylated HIF1α protein, which ultimately results in its degradation via the ubiquitin-proteasome pathway. In addition, acetylation of HIF1α at Lys^{532} has been reported to enhance the interaction of prolyl-hydroxylated HIF1α with pVHL. This acetylation reaction is catalysed by ARD1 acetyltransferase (Jeong *et al.*, 2002).

The initial step in this sequence of events, the prolyl hydroxylation, is an O_2-dependent reaction which utilises O_2 for two purposes: to hydroxylate HIF1α and to oxidatively decarboxylate 2-oxoglutarate into succinate and CO_2. This reaction is rapid and occurs when O_2 is abundant, preventing the formation of HIF1 and subsequent gene expression. When O_2 becomes limiting the enzyme activity is inhibited and HIF1α is no longer hydroxylated and is not, therefore, targeted for degradation. The K_m of the prolyl hydroxylases for O_2 has been reported to be in the region of 230-250μM (Hirsila *et al.*, 2003), explaining the lack of HIF1α prolyl hydroxylation at low (<3μM) O_2 concentrations. It has been proposed that such a high K_m renders the prolyl hydroxylases sensitive to changes in O_2 concentration and therefore makes them good cellular O_2 sensors.

Three isoforms of the HIF prolyl hydroxylase enzymes have been identified: PHD1, PHD2 and PHD3. Of these, it has been shown that inactivation of PHD2 is enough to upregulate HIF1α (Berra *et al.*, 2003). However, transfection studies have shown that each of the prolyl hydroxylases have the ability to regulate HIF1α protein levels in cells (Bruick & McKnight, 2001).

1.4.3 Regulation by Asparaginyl Hydroxylases

In addition to prolyl hydroxylation, HIF1α may also be subject to hydroxylation on Asn^{803}. This reaction is catalysed by an enzyme called factor inhibiting HIF (FIH). FIH requires the same co-substrates (O_2 and 2-oxoglutarate) as the prolyl hydroxylases but has a greater affinity for them (the K_m for O_2 is 90μM, 230-250μM for prolyl hydroxylases, and the K_m for 2-oxoglutarate is 25μM, 55-60μM for prolyl hydroxylases).

The hydroxylation of Asn^{803}, unlike that of the proline residues, does not initiate the degradation of HIF1α but prevents its association with transcriptional co-activators such as CBP/p300, resulting in a lower activity (Lando *et al.*, 2003). However, as a result of its high affinity for O_2, FIH has been reported to inhibit HIF1 transcriptional activity even at very low O_2 concentrations (Mahon *et al.*, 2001).

1.4.4 Regulation by ROS

Two main hypotheses have been proposed whereby ROS may modulate the stability of the HIF1α protein. The first involves the NADPH oxidase enzyme and the second proposes a role for ROS derived from the ETC.

NADPH oxidase produces O_2^- which is subsequently converted into H_2O_2 by SOD. It has been proposed that a fall in O_2 concentrations reduces the amount of ROS produced and that this signals for HIF1α stabilisation (Fandrey *et al.*, 1994). This is supported by the observation that H_2O_2 inhibited the hypoxic stabilisation of HIF1α (Huang *et al.*, 1996) and

that the administration of catalase or antioxidants to cells induced the non-hypoxic stabilisation of HIF1α (Salceda and Caro, 1997). However, experimental results from other laboratories are inconsistent with this hypothesis, for example diphenylene iodonium, which inhibits NADPH oxidase and therefore reduces ROS production, prevents the hypoxic stabilisation of HIF1α (Gleadle *et al.*, 1995).

Other studies investigating the role of mitochondria in the regulation of HIF1α have proposed that $O_2^{\cdot}$, and subsequently H_2O_2, generated from the ETC are required to stabilise HIF1α and to induce HIF1 activity (Chandel *et al.*, 2000a; Agani *et al.*, 2000). It has been shown that under hypoxic conditions the ETC is shifted to a more reduced state because of a decrease in activity of complex IV due to a lack of O_2 as its substrate (Palacios-Callender *et al.*, 2004). This change in redox state increases the 'leak' of electrons to O_2 from other complexes of the ETC, probably complex III, to form $O_2^{\cdot}$ which initiates the signal for HIF1α stabilisation (Chandel *et al.*, 2000a). In support of this hypothesis, a number of antioxidants (N-acetylcysteine and glutathione) have been shown to inhibit the hypoxic stabilisation of HIF1α. In contrast, however, the use of complex IV inhibitors (azide and cyanide), to mimic the decreased activity of the enzyme in hypoxia, have been reported not to inhibit HIF1 activity (Chandel *et al.*, 1998), and antimycin a, a complex III inhibitor that causes ROS production from this site, does not induce non-hypoxic stabilisation of HIF1α. These observations disagree with the hypothesis. In addition, the precise mechanism by which ROS may modulate HIF1α stabilisation has remained elusive.

1.4.5 Regulation by Nitric Oxide

Nitric oxide has been reported both to promote and inhibit the stabilisation of HIF1α (Huang *et al.*, 1999; Metzen *et al*; 2003). Upon hypoxic stabilisation of HIF1α, treatment of cells with NO, derived from NO donors, induces the destabilisation of the protein (Sogawa *et al.*, 1998; Agani *et al.*, 2002) and a reduction in DNA binding through a mechanism that does not involve sGC (Huang *et al.*, 1999). In contrast, however, HIF1α can be stabilised by NO under non-hypoxic conditions (Sandau *et al.*, 2001).

A variety of explanations have been proposed for the mechanism by which NO stabilises HIF1α protein. One hypothesis suggests that it may act through S-nitrosylation of the protein which subsequently prevents its degradation (Palmer *et al.*, 2000; Sumbayev *et al.*, 2003). However, this mechanism has been questioned because of a lack of a specific target for NO in the HIF1α protein. Alternatively, NO can inhibit prolyl hydroxylase activity, preventing the degradation of HIF1α protein. It has been proposed that NO can compete with O_2 for binding to the iron in the active site of the enzyme (Metzen *et al.*, 2003). However, other studies have reported that NO can increase the prolyl hydroxylation of HIF1α (Wang *et al.*, 2002). In addition, NO may stabilise HIF1α protein by increasing the production of ROS (Genius & Fandrey, 2000).

Recently, it has been shown that the contrasting effects of NO may be due to the concentration at which it is used, and that there is actually a biphasic effect of NO upon HIF1α stability, with low concentrations destabilising and high concentrations stabilising HIF1α. Furthermore, it has been proposed that the HIF1α destabilising effect of NO is

mitochondria-dependent whereas the stabilising effect is mitochondria-independent (Mateo *et al.*, 2003).

AIMS

A number of studies have demonstrated that exogenous NO can cause both a reversible and persistent inhibition of cellular respiration. Thus, the initial aims of my work were to confirm that:

- acute treatment with exogenous NO rapidly and reversibly inhibits cellular respiration, and
- prolonged exposure to NO can persistently inhibit cellular respiration, and that this phenomenon is associated with a decrease in complex I activity.

To verify that endogenous NO can also inhibit respiration, both reversibly and persistently, a macrophage-like cell line was activated to produce NO and the effects upon O_2 consumption were followed over time.

Having established the effects of NO, I wanted to determine whether CO can inhibit cellular respiration. CO has also been shown to inhibit complex IV of the ETC. However, little is known about the ability of CO to inhibit cellular respiration. Therefore, I wanted to:

- clarify whether exogenous CO could inhibit respiration,
- investigate if, by transfecting the HO-1 gene into HEK293 cells, endogenously-produced CO can inhibit respiration,
- investigate the time course of HO-1 expression in activated macrophages and to follow the effect that the CO has on the respiration of those cells,
- investigate the relative contribution of NO and CO to the mitochondrial defect that occurs in activated macrophages both under ambient and hypoxic conditions.

Although NO has been shown to potently inhibit cellular O_2 consumption, the significance of this phenomenon is currently not well understood. Recent evidence has indicated a link between mitochondrial respiratory activity and the stability of HIF1α protein. However, many of the reports have been contradictory and the precise mechanisms involved remain unclear. Therefore, to examine the consequences of respiratory inhibition, I aimed to:

- clarify the role of mitochondria in the modulation of hypoxia-induced stability of HIF1α protein,
- investigate the mechanism by which the inhibition of mitochondrial activity could be acting to affect this phenomenon.

2 MATERIALS AND METHODS

2.1 Growth and Maintenance of Cell Lines

Unless otherwise stated, cells were grown and maintained in appropriate culture media supplemented with 10% heat-inactivated (30mins at 56°C and then cooled on ice) New Zealand foetal bovine serum, 2mM L-glutamine, 100U/ml penicillin and 100μg/ml of streptomycin (Life Technologies, Paisley, U.K.). They were kept in an incubation chamber set at 37°C with 95% air and 5% CO_2.

2.1.1 Culture of $RAW_{264.7}$ Cells

$RAW_{264.7}$ (murine monocyte) cells were obtained from American Type Culture Collection (Manassas, VA). They were maintained as described above in RPMI 1640 medium (Life Technologies, Paisley, U.K.). Cells were grown as a suspension culture in biological stirrer bottles (Techne, Cambridge, UK) set with a continuous stirrer speed of 25rpm.

2.1.2 Culture of HEK293, Hep3B, 143B and HeLa Cells

HEK293 (human embryonic kidney), Hep3B (human hepatoma), 143B (human osteosarcoma) and HeLa (human cervix carcinoma) cells were cultured in DMEM (Life Technologies, Paisley, U.K.). They were grown in T175 tissue culture flasks (Becton Dickinson, Franklin Lanes, N.J.) and kept below 90% confluency.

2.2 Measurement of O_2 Consumption

Oxygen consumption was measured polarographically through the use of a Clark-type O_2 electrode (Figure 5; Rank Brothers, Bottisham, U.K.) which was equipped with a water

jacket thermostatically set at 37°C. Prior to use, the electrode was calibrated with air-saturated water, assuming an O_2 concentration of 200μM.

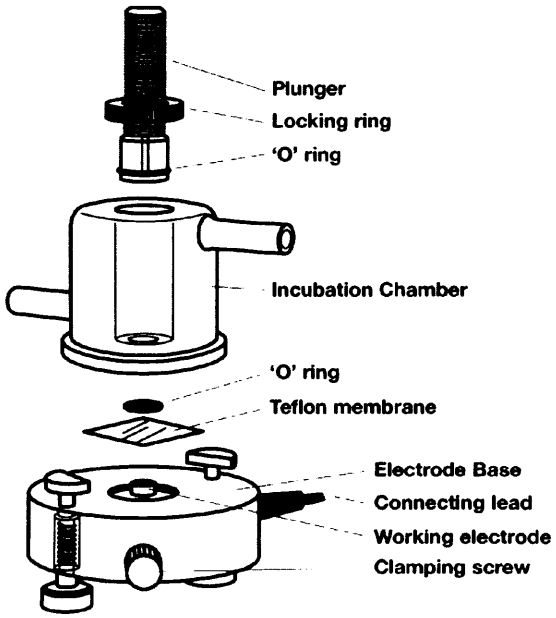

Figure 5. The Clark-type O_2 electrode. The illustration shows a Clark-type O_2 electrode that was used to measure cellular O_2 consumption. Cells (at a concentration of 10^7/ml) were sealed inside the incubation chamber and the decrease of O_2 was measured by the electrode and recorded by the DUO18 program.

Samples (1ml of cells suspended in incubation medium at a concentration of 10^7cells/ml) were placed into a gas-tight chamber which was sealed using a plastic plunger. A homogeneous sample was maintained by a magnetic stirrer (Rank Brothers, Bottisham, U.K.) situated at the bottom of the chamber, set at 600rpm. The decrease of O_2 (O_2 consumption) was recorded using the DUO18 (World Precision Instruments, Stevenage, U.K.) recording system.

Treatments were administered to the cells within the incubation chamber through the use of a microsyringe, which was inserted into the chamber via a port in the plunger.

2.3 Activation of $RAW_{264.7}$ Cells

$RAW_{264.7}$ cells were activated using LPS (100ng/ml; Sigma) and IFNγ (50U/ml). Both LPS and IFNγ were prepared in phosphate buffered saline solution and were added to the cells, which were in biological stirrer bottles.

2.4 Measurement of NO

Nitric oxide was measured either by an NO electrode or by the Griess assay, as described below.

2.4.1 Use of an NO Electrode

Nitric oxide was measured amperometrically through the use of an ISO-NOP NO electrode (Figure 6; World Precision Instruments, Stevenage, U.K.). Prior to use, the electrode was calibrated by generating known amounts of NO via a chemical reaction. The chemical reaction used was:

$$2KNO_2 + 2KI + 2H_2SO_4 \rightarrow 2NO + I_2 + H_2O + K_2SO_4$$

Since the amount of NO produced by the reaction is proportional to the amount of KNO_2 added, when KI and H_2SO_4 are in excess, then known amounts of NO can be generated by adding known quantities of KNO_2 (Figure 7).

The NO electrode was positioned in the incubation chamber of the O_2 electrode via a port in the plunger such that both NO and O_2 concentrations could be measured simultaneously during experiments.

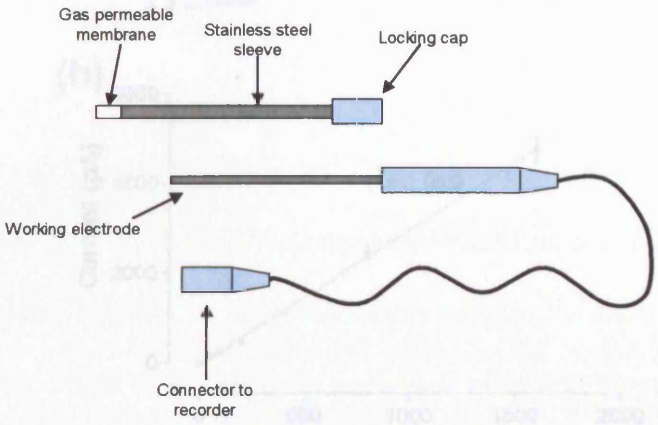

Figure 6. The structure of an NO electrode. Dynamic NO concentrations were measured using an NO electrode which was calibrated prior to use. The electrode was positioned into the incubation chamber of the Clark-type O_2 electrode through a port in the plunger such that both O_2 and NO concentrations were measured simultaneously.

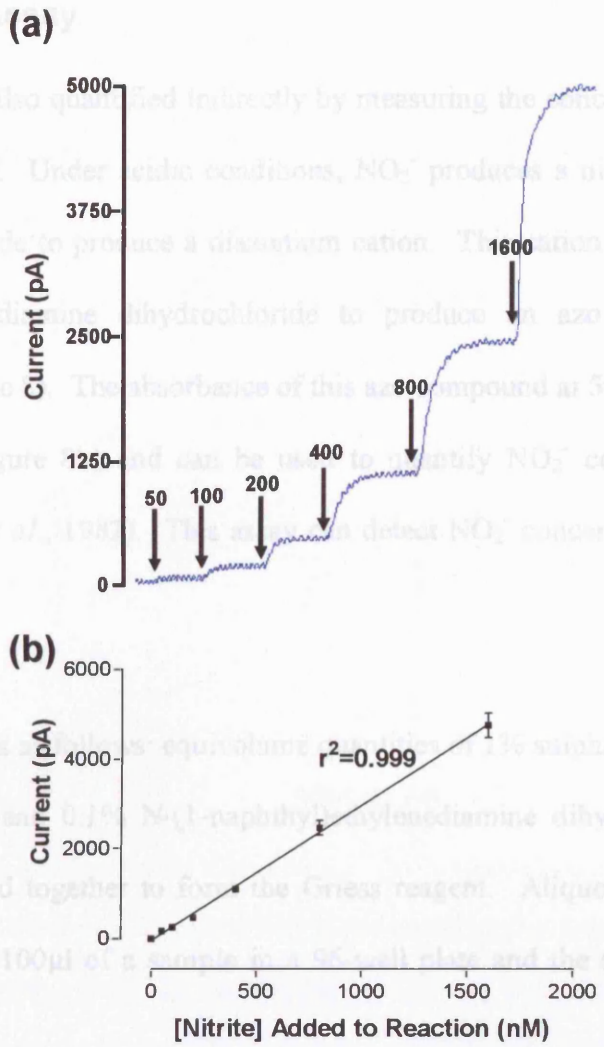

Figure 7. Calibration of the NO electrode. (a) A tracing obtained from the DUO18 recording program during the calibration of the NO electrode. Nanomolar concentrations of nitrite (indicated by arrows and numerical values) were added to the reaction mixture consisting of potassium iodide and sulphuric acid. The current detected from the reaction is proportional to the concentration of nitrite (and NO) present. (b) A calibration curve was plotted such that nitrite (and hence, NO) concentrations could be calculated from the currents measured. (n = 3).

2.4.2 Griess Assay

Nitric oxide was also quantified indirectly by measuring the concentration of NO_2^-, a stable metabolite of NO. Under acidic conditions, NO_2^- produces a nitrosating agent that reacts with sulphanilamide to produce a diazonium cation. This cation can then react with N-(1-naphthyl)ethylenediamine dihydrochloride to produce an azo compound of a purple colouration (Figure 8). The absorbance of this azo compound at 540nm is proportional to its concentration (Figure 8b) and can be used to quantify NO_2^- concentrations in unknown samples (Green *et al.*, 1982). This assay can detect NO_2^- concentrations as low as 100nM (Nagano, 1999).

The procedure was as follows: equivolume quantities of 1% sulphanilamide (acidified in 5% phosphoric acid) and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride (dissolved in water) were mixed together to form the Griess reagent. Aliquots (100µl) of this reagent were mixed with 100µl of a sample in a 96-well plate and the absorbance was measured immediately.

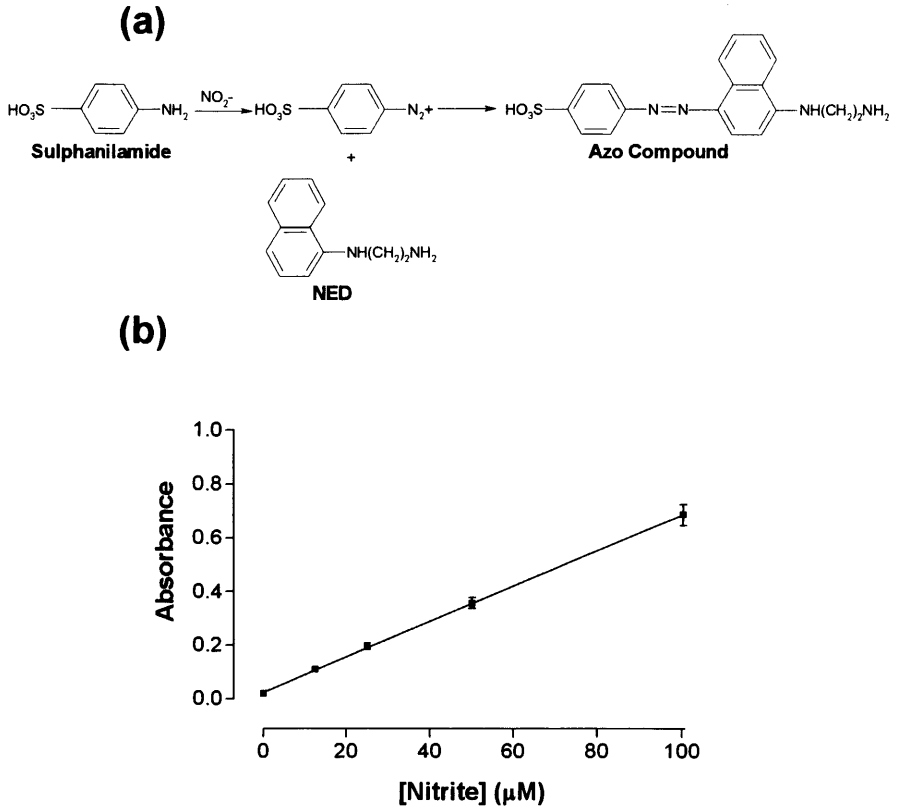

Figure 8. **The reactions of the Griess assay.** (a) Sulphanilamide reacts with the nitrite anion to produce a chemical species that reacts with N-(1-naphthyl)ethylenediamine dihydrochloride (NED) to produce an azo compound. This azo compound has a strong absorbance at 540nm. (b) A calibration curve obtained from the Griess assay using known concentrations of nitrite solutions. The absorbance measured was directly proportional to the concentration of nitrite. $n > 3$.

2.5 Measurement of Complex I Activity

Complex I activity was determined spectrophotometrically (Uvikon-XS, Biotek, Loughborough, U.K.) by the rate of rotenone-sensitive NADH oxidation (King, 1967). Cells were collected and centrifuged at 2500g for 5mins. The resultant pellet was then resuspended in 100μl of potassium phosphate buffer (10mM, pH8.0) and subjected to 3 freeze-thaw cycles (frozen in liquid nitrogen for 5mins and then warmed to 37°C in a water

bath). 20µl aliquots of the cell lysates were added to 955µl of phosphate buffer containing 50µM ubiquinone$_{10}$ (Sigma, Poole, U.K.). Freshly prepared NADH (Sigma, Poole, U.K.) was then added to a final concentration of 100µM and the formation of NAD^+ was measured at 340nm (ε = 6.81$mM^{-1}cm^{-1}$) for 2mins. To the same sample, 5µM rotenone (Aldrich Chemicals Company, Milw., WI) was added to distinguish the production of NAD^+ by complex I from other cellular processes. The difference in the rates of oxidation of NADH in the presence and absence of rotenone was due to the activity of complex I. The assayed sample was then collected and the protein content was measured using the bicinchoninic acid method (described below). Complex I activity was expressed as nanomoles of NAD^+ produced per minute per milligram of protein.

2.6 Measurement of Complex IV Activity

Complex IV activity was determined spectrophotometrically by the rate of oxidation of reduced cytochrome c (Wharton and Tzagoloff, 1967). Prior to analysis, samples were prepared as described for measurement of complex I activity. Bovine cytochrome c (Sigma, St. Louis, MO) was dissolved in phosphate buffered solution (100mM, pH7.0) at an initial concentration of 4g/100ml buffer and was reduced by the addition of a small amount of sodium dithionite. The solution was then passed through a sephadex G25 (Sigma, St. Louis, MO) column to purify the solution and the eluent was collected. The concentration of reduced cytochrome c was determined spectrophotometrically at 550nm (ε = 19.6$mM^{-1}cm^{-1}$). Cytochrome c (150µl) was added to cellular extracts (10µl; prepared by freeze-thaw lysis of cells described above) in 840µl buffer and the rate of oxidation was measured spectrophotometrically. This procedure was repeated in the presence of azide (5mM) to distinguish the oxidation of cytochrome c by complex IV from that by other processes. A

graph of time against log(absorbance) was then plotted and the first-order rate constant was determined. Protein concentrations were measured in the original samples and complex IV specific activity was calculated from the known cytochrome c concentration in the assay mixture and the first-order velocity constant. Specific activity was expressed as nanomoles of cytochrome c oxidised per minute per milligram of protein.

2.7 Measurement of Protein

Protein concentrations in samples were quantified using the bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Illinois, U.S.A.). This assay is based on the reduction of cupric (Cu^{2+}) cations into the cuprous (Cu^{+}) ion by proteins under alkaline conditions. The Cu^{+} ions then chelate with BCA resulting in the formation of a purple-coloured complex whose absorbance at 562nm is proportional to protein concentrations from 0.02-2mg/ml.

Albumin standards of known concentrations were prepared alongside unknown samples that were diluted appropriately. The assay reagent was prepared by mixing 50 parts of BCA reagent A with 1 part BCA reagent B. 200µl of the assay reagent was then mixed with 25µl of standard or unknown on a microplate and incubated for 30mins at 37°C. After incubation, the absorbance was measured using a microplate reader and the protein concentrations of the unknowns were obtained from the standard curve plotted.

2.8 Measurement of Bilirubin

In experiments where bilirubin was measured, the culture vessel was wrapped in foil to protect it from light. Treatments were performed and 1ml aliquots of culture medium were

collected at the allotted timepoints. The aliquots were then centrifuged for 5' at 13000rpm to remove any cells or debris. A 0.5ml sample was collected from the resultant supernatant and added to 250mg of $BaCl_2$. The mixture was then vortexed vigorously prior to the addition of 0.75ml benzene. The mixture was vortexed again and then centrifuged for 30' at 13000rpm. The upper benzene layer, containing the bilirubin, was then extracted and its absorbance was measured at 450nm with a reference at 600nm. The concentration of bilirubin in the sample was then calculated using the molar extinction coefficient of bilirubin dissolved in benzene, $\varepsilon_{450} = 27.3 mM^{-1}cm^{-1}$ (Foresti *et al.*, 1999). A separate sample of fresh culture medium was processed in the same way and used as a blank.

2.9 Western Blotting

Cells were lysed using an ice-cold lysis buffer containing 25mM Tris (pH7.5), 2.5mM EDTA, 2.5mM EGTA, 20mM NaF, 1mM sodium orthovanadate, 100mM NaCl, 20mM sodium β-glycerophosphate, 10mM sodium pyrophosphate, 0.5% triton, 0.1% β-mercaptoethanol and complete protease inhibitor cocktail (Roche, Lewes, U.K.). For detection of HIF1α protein, cells were lysed in an ice-cold buffer consisting of 10mM Hepes (pH7.5), 1.5mM $MgCl_2$, 10mM KCl, 0.2% NP-40, 0.2% 2-mercaptoethanol and complete protease inhibitor cocktail (Roche, Lewes, U.K). The lysates were vigorously vortexed and then centrifuged for 5mins at 5000rpm to remove cellular debris. The supernatant was collected and analysed for protein concentration. Samples were either used immediately or stored at -80°C for future use. Frozen samples were thawed gently on ice and then vortexed briefly to obtain a homogeneous lysate.

2.9.1 Protein Separation by SDS-PAGE

Equal amounts of protein (50μg) were prepared for electrophoresis under reducing conditions by mixing one part sample with three parts Laemmli sample buffer (Bio-rad) containing 5% 2-mercaptoethanol (Sigma). For example, a 5μl aliquot of a sample was mixed with 15μl of sample buffer. The mixture was then boiled at 100°C for 5mins and cooled on ice for a further 5mins.

Once cooled, the samples were loaded into separate wells of a sodium dodecyl sulphate-polyacrylamide gel (SDS-PAG) along with a well containing standard protein markers. The proteins then underwent electrophoresis (SDS-PAGE) at 125V for approximately 1.5hrs or until the solvent front reached the bottom of the gel. The stacking and resolving gels and the running buffer were prepared as follows:

Stacking gel: 5ml of a 5% stacking gel was prepared by mixing 3.4ml distilled water, 0.83ml 30% acrylamide mix, 0.63ml 1.0M Tris (pH6.8), 0.05ml 10% SDS, 0.05ml 10% ammonium persulphate and 0.005ml TEMED.

Resolving gel: 20ml of an 8% resolving gel was prepared by mixing 9.3ml distilled water, 5.3ml 30% acrylamide mix, 5.0ml 1.5M Tris (pH8.8), 0.2ml 10% SDS, 0.2ml 10% ammonium persulphate and 0.012ml TEMED.

20ml of a 12% resolving gel was prepared by mixing 6.6ml distilled water, 8.0ml 30% acrylamide mix, 5.0ml 1.5M Tris (pH8.8), 0.2ml 10% SDS, 0.2ml 10% ammonium persulphate and 0.008ml TEMED.

Running buffer: 3g Trizma base, 15 glycine and 1g lauryl sulphate were dissolved in distilled water and made up to a final volume of 1l.

2.9.2 Protein Transfer onto Nitrocellulose Membranes

Once the proteins had been separated, the gel was removed from the electrophoresis cassette and transferred onto a nitrocellulose membrane (Amersham Life Science, Bucks, U.K.) at 25V for 12h at 4°C. The gel was positioned in a cassette next to the nitrocellulose membrane and between fibre pads and filter paper (Whatman, U.K.) as illustrated (Figure 9). The entire apparatus was immersed in cooled (4°C) transfer buffer consisting of 3.1g Trizma base, 14.4g glycine, 200ml methanol and made up to 1l with distilled water.

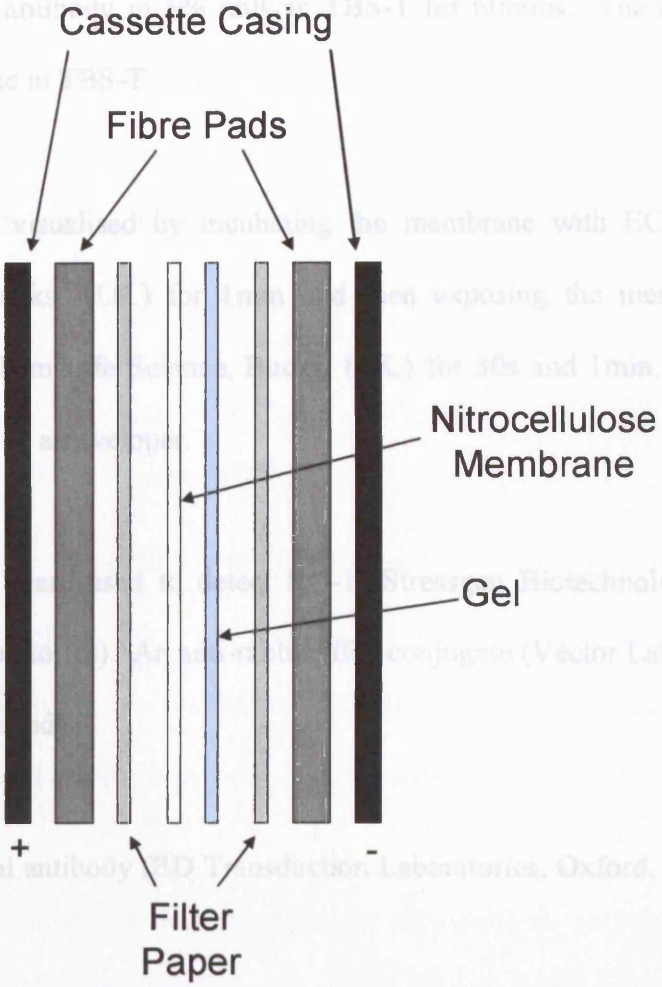

Figure 9. Protein transfer onto nitrocellulose membrane. The diagram illustrates the set up of the cassette for the transfer of proteins from a polyacrylamide gel onto a nitrocellulose membrane.

2.9.3 Immunoblotting of Proteins

Once transferred, the nitrocellulose membrane was initially blocked in 10% non-fat milk protein (Marvel) in TBS-T (6.6g NaCl, 25ml 1M Tris HCl (pH7.5), 0.05% Tween 20 and made up to 1l with distilled water) for 60mins. The membrane was then washed in TBS-T for 15mins and incubated with a primary antibody in TBS-T for 60mins. After incubation, the membrane was again washed for 15mins and incubated with a horseradish peroxidase

(HRP)-conjugated secondary antibody in 5% milk in TBS-T for 60mins. The membrane was then washed one final time in TBS-T.

Immunoreactive bands were visualised by incubating the membrane with ECL reagent (Amersham Life Science, Bucks, U.K.) for 1min and then exposing the membrane to autoradiography film (Amersham Life Science, Bucks, U.K.) for 30s and 1min. Exposed films were then developed using a developer.

Rabbit polyclonal antibodies were used to detect HO-1 (Stressgen Biotechnologies) and iNOS (BD Transduction Laboratories). An anti-rabbit HRP conjugate (Vector Laboratories) was used as the secondary antibody.

To detect HIF1α a monoclonal antibody (BD Transduction Laboratories, Oxford, U.K.) was used.

Measurement of p38 MAPK activity was determined by detection of phosphorylated p38 via Western blotting using a commercially available kit (PhosphoPlus p38 MAP Kinase Antibody Kit, Cell Signalling Technology).

2.10 Preparation of CO Solutions

Fresh stock solutions of CO gas were prepared each day by the method described by Naseem and Bruckdorfer, 1995. Briefly, double distilled water was boiled for 15min, and allowed to cool to 60°C whilst being pulled under vacuum into specially adapted gas sampling tubes. 10ml of the water was then flushed with nitrogen gas (BOC Gases,

Guildford, U.K.) for 45min and the container tubes were sealed with gas-impermeable rubber septa. CO gas (BOC Gases, Guildford, U.K.) was then bubbled into the flushed solutions for 1h. The concentration of CO in solution was determined spectrophotometrically by measuring the conversion of deoxymyoglobin to carbon monoxymyoglobin.

2.11 Measurement of CO Concentrations

The method used to measure the concentration of CO in water was similar to that described previously by Motterlini *et al.* (Motterlini *et al.*, 2002). The method utilised the binding of CO to deoxymyoglobin to form carbon monoxymyoglobin, whose concentration can be determined spectrophotometrically with an absorbance at 540nm (ε_{540} = 15.4$mM^{-1}cm^{-1}$).

Fresh deoxymyoglobin solutions (66μM) were prepared by dissolving the protein in 0.04M phosphate buffer (pH6.8). Prior to use, the reducing agent sodium dithionite (~5mg) was added to the deoxymyoglobin solution to completely reduce it. CO solutions were then mixed with the deoxymyoglobin and left for 1min before spectrophotometric measurement.

2.12 Determination of K_i Values

In order to calculate the K_i values of CO for complex IV, Lineweaver-Burk and secondary plots were constructed. The reaction velocity (rate of O_2 consumption) was measured in the presence of a fixed concentration of inhibitor (CO) and varying substrate (O_2) concentrations. The velocity was then plotted against the substrate concentration in a double reciprocal (Lineweaver-Burk) plot. The slopes obtained were then plotted against the

inhibitor concentrations (secondary plot). The K_i was calculated by interpolation to the x-axis.

2.13 Incubation under Hypoxia

Experiments that were performed under hypoxic (1% O_2) conditions were carried out within a humidified anaerobic chamber (Coy Laboratory Products; Grasslake, MI). The chamber was digitally set at the required O_2 concentration and was maintained at 37°C by a thermostatic controller.

2.14 Generation of HEK293-HO-1 Cells

Full length human HO-1 was amplified via PCR from human foetal brain cDNA. An XhoI restriction site and Kozak consensus sequence (GACGAG) was included in the PCR primers at the 5' end, and XbaI site at the 3' end. The following PCR primers were used: CACTCGAGGACGAGATGGAGCGTCCGCAACCCG (forward) and CATCTAGATTACATGGCATAAAGCCCTAC (reverse). The PCR product was then gel purified and, after addition of an A overhang, subcloned into pGEM-T easy. The sequence was confirmed by DNA sequencing, and the HO-1 gene was transferred to the pcDNA4/TO plasmid, using the XhoI and XbaI restriction sites. TREX293 cells (tet-on HEK293 cells) were then transfected with the HO-1-pcDNA4/TO expression plasmid, and stable clones were isolated using zeocin selection.

2.15 Measurement of HIF1 Gene Reporter Activity

The activity of the HIF1 transcription factor was assessed by an HRE-dependent luciferase reporter assay. The pGL3-HRE luciferase reporter construct that was used was a kind gift from Dr. Kaye Williams (The School of Pharmacy and Pharmaceutical Sciences, Manchester, U.K.). It contains three copies of the HRE from the phosphoglycerate kinase promoter, located upstream of a luciferase gene.

Cells were cultured in 12-well plates until approximately 50% confluency and then transfected with 0.2μg/ml of pGL3-HRE DNA for 24h. The cells were then incubated for 5h at 1% O_2 in the absence or presence of respiratory inhibitors. The resultant luciferase activity was then assayed using the Steady-Glo Luciferase Assay System (Promega, Southampton, U.K.). Luminescence was detected using a luminometer (Berthold Technologies, Bad Wildbad, Germany) and was expressed as relative fold-increase compared with non-hypoxic control cells.

2.16 Measurement of O_2-Dependent Renilla Luciferase Activity

2.16.1 Construction of a Mitochondrially-Targeted Renilla Luciferase Vector

The Renilla luciferase gene was obtained from the pRL-CMV vector (Promega, Southampton, U.K.). A V5 epitope tag was added and the sequence was amplified via polymerase chain reaction (PCR). The PCR product was then subcloned into the XhoI and XbaI sites of the pcDNA3 vector (Invitrogen, Paisley, U.K.). The mitochondrial-targeting

sequence of MnSOD (SOD-2), comprising residues 1-28 of the gene, was generated by PCR amplification and a Kozak consensus sequence was added at the 5' end. This targeting sequence was then inserted into the KpnI and XhoI sites of the pcDNA3 vector containing the Renilla luciferase gene to form the mtRLuc-pcDNA3 construct.

2.16.2 Transfection and Measurement of Renilla Luciferase Activity in HeLa Cells

HeLa cells were grown to 60-80% confluency on 10cm tissue culture plates. They were then transfected with 2μg of the mtRLuc-pcDNA3 plasmid using Fugene 6 (Roche, Lewes, U.K.) following the manufacturer's instructions. The medium was then changed following overnight incubation and the cells were then left for a further 24h at 37°C. They were then trypsinised, centrifuged and resuspended at a concentration of 3 x 10^6cells/ml at 1% O_2 using preconditioned medium that was left at 1% O_2 for 24h. The cells were then divided into 100μl aliquots and left to equilibrate for 10mins. DETA (0-100μM) was then added to the cell suspensions and left for a further 60mins. The luciferase substrate, coelenterazine-hcp (Sigma, Poole, U.K.), was then added at a final concentration of 5μg/ml and left for 5mins. Renilla luciferase luminescence in the cells was measured using a hand-held luminometer (Berthold Technologies, Bad Wildbad, Germany).

2.16.3 Measurement of Immunoprecipitated Renilla Luciferase Protein

HeLa cells were transfected with the mtRLuc-pcDNA3 plasmid and maintained as described above. Following trypsinisation, the cells were lysed using the HIF1α lysis buffer. The

lysates were then centrifuged for 5mins at 5000rpm and the supernatant was collected. The lysates were mixed with 2.5μg of monoclonal anti-V5 antibody coupled to protein G-sepharose and left for 2h on a shaking platform.

The pellets were washed gently 3 times in a buffer composed of 50mM Tris (pH7.5), 0.1mM EGTA and 0.5M NaCl. The pellets were washed a further 2 times in the same buffer but without NaCl. The protein was then resuspended in PBS and aliquots were incubated at ambient, 5% and 1% O_2 concentrations. To measure Renilla luciferase activity, the immunoprecipitated protein was treated as described for HeLa cells transfected with the mtRLuc-pcDNA3 plasmid.

2.17 Statistical Analysis

Data are expressed as the arithmetic mean ± SEM. Differences between pairs of variables were assessed with the Students's *t* test. Differences between multiple groups of variables were assessed by Analysis of Variance (ANOVA) followed by Tukey's Multiple Comparison post-test. $P < 0.05$ was considered to be statistically significant.

3 RESULTS

3.1 Inhibition of Cellular Oxygen Consumption

Mitochondrial respiration is the major component of total cellular O_2 consumption (Brown, 1999; Piantadosi, 2002). Manipulation of the mitochondrial ETC, through the use of pharmacological inhibitors, would be expected to prevent mitochondrial, and therefore cellular, respiration. In order to investigate this, $RAW_{264.7}$ cells were treated with rotenone (a complex I inhibitor; Lambert & Brand, 2004), myxothiazol (a complex III inhibitor; Raha *et al.*, 2000) or sodium azide (a complex IV inhibitor; Petersen, 1977). In addition, NO donors were administered to cells to examine the short- (minutes) and long-term (hours) effects of NO on cellular respiration.

3.1.1 Inhibition of Respiration by Pharmacological Inhibitors

Untreated control $RAW_{264.7}$ cells respired at a constant rate of $13.97 \pm 1.02\mu M$ O_2/min (n = 5), which was independent of the O_2 concentration in the range measured (Figure 10). Repeated additions of sodium azide (1mM) resulted in a dose-dependent inhibition of O_2 consumption (Figure 11); similar observations were obtained with rotenone and myxothiazol. A single bolus treatment with rotenone (500nM), myxothiazol (1μM) or azide (5mM) completely inhibited respiration (>90% inhibition; Figure 12), confirming that mitochondria are indeed responsible for most of cellular O_2 consumption. Since these inhibitors act at different complexes of the ETC, respiration can therefore be inhibited by blocking the ETC at various sites.

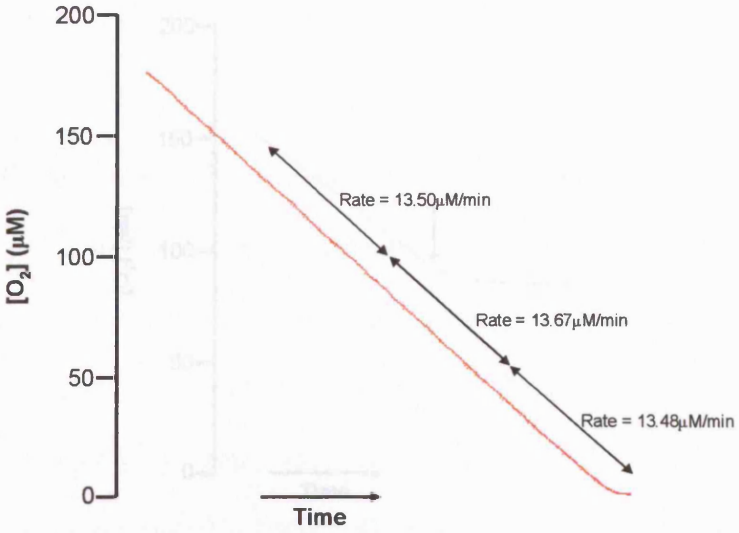

Figure 10. **The O_2 consumption of control $RAW_{264.7}$ cells.** The rate of O_2 consumption was independent of the O_2 concentration and was at a constant rate of 13.97μM/min (n = 5). The tracing was obtained from one of five independent experiments.

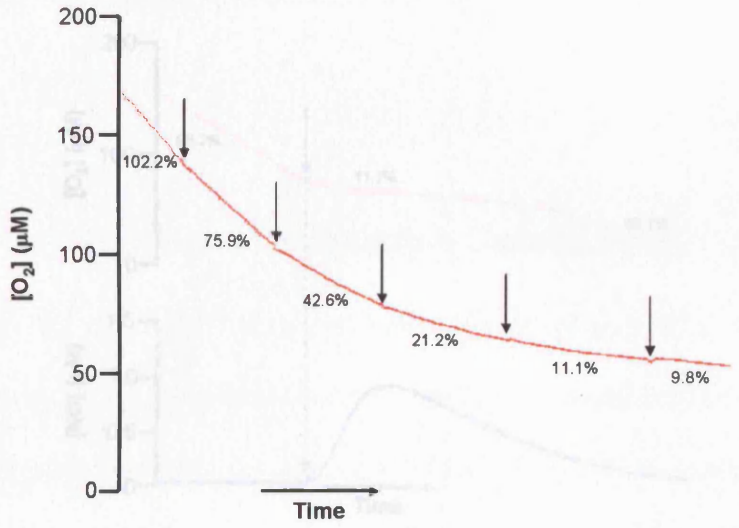

Figure 11. **The cumulative inhibition of O_2 consumption by repeated additions of sodium azide.** Cells were treated with sequential additions of sodium azide (1mM; indicated by the arrows) and the effects upon O_2 consumption were measured. Percentage values show the rate of respiration relative to the control tracing at the equivalent O_2 concentrations. The tracing was obtained from one of five independent experiments.

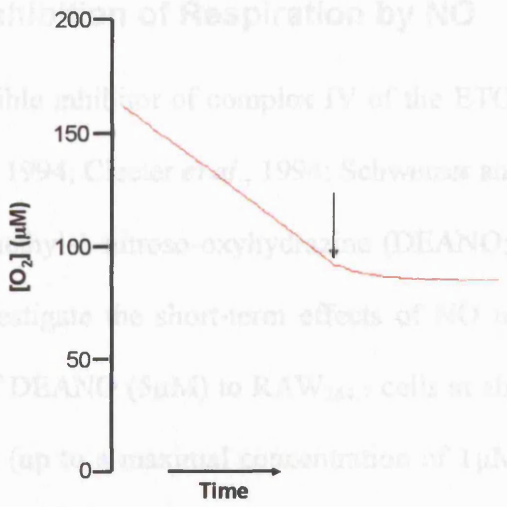

Figure 12. The inhibition of O_2 consumption by a bolus addition of a respiratory inhibitor. A single treatment with a respiratory inhibitor (indicated by the arrow) was made and the effects upon O_2 consumption were measured. Rotenone (500nM), myxothiazol (1μM) or sodium azide (5mM) completely inhibited (>90% inhibition) cellular O_2 consumption.

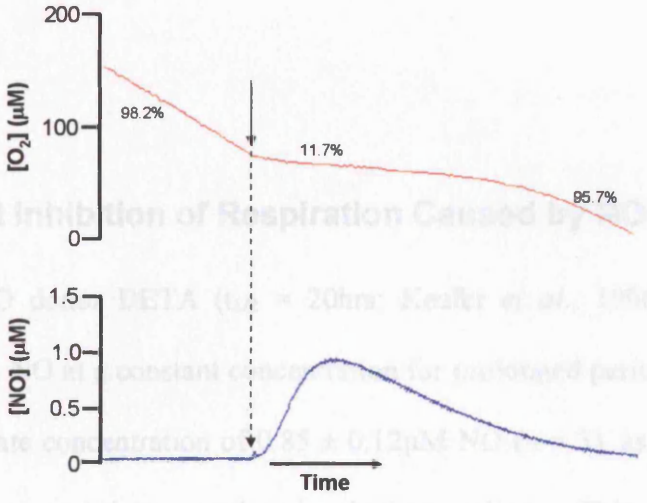

Figure 13. The inhibition of respiration by NO. DEANO (5μM) was added (indicated by arrow) and NO was released (up to a maximum concentration of 1μM; blue tracing). Cellular O_2 consumption (red tracing) was inhibited in the presence of NO but recovered after the NO was degraded. Numerical values represent the rates of respiration relative to the control at equivalent O_2 concentrations. The tracings were obtained from one of three independent experiments.

3.1.2 Reversible Inhibition of Respiration by NO

Nitric oxide is a reversible inhibitor of complex IV of the ETC, acting in competition with O_2 (Brown and Cooper, 1994; Cleeter *et al.*, 1994; Schweizer and Richter, 1994). The short-acting NO donor 2,2-diethyl-1-nitroso-oxyhydrazine (DEANO; $t_{1/2}$ = 2mins; Keefer *et al.*, 1996) was used to investigate the short-term effects of NO upon cellular respiration. A single bolus addition of DEANO (5μM) to $RAW_{264.7}$ cells at about 75μM O_2 resulted in the transient release of NO (up to a maximal concentration of 1μM, as measured using an NO electrode) with a concomitant inhibition of cellular respiration (Figure 13). This effect disappeared when the NO was completely degraded and respiration recovered to the control rate. The duration of the inhibitory effect of NO appeared to be greater at a lower O_2 concentration. However, the same concentration of DEANO produced greater maximal concentrations of NO at lower O_2 concentrations, as detected by an NO electrode (Figure 14).

3.1.3 Persistent Inhibition of Respiration Caused by NO

The long-acting NO donor DETA ($t_{1/2}$ = 20hrs; Keefer *et al.*, 1996) was used to treat $RAW_{264.7}$ cells with NO at a constant concentration for prolonged periods. DETA (2.5mM) released a steady-state concentration of 0.85 ± 0.12μM NO (n = 3), as measured by an NO electrode, to cells suspended in complete incubation medium. This concentration of NO inhibited cellular respiration (>90% inhibition compared with untreated controls) in a manner that was initially completely reversed by the addition of the NO scavenger oxyhaemoglobin (oxyHb; 20μM; Figure 15).

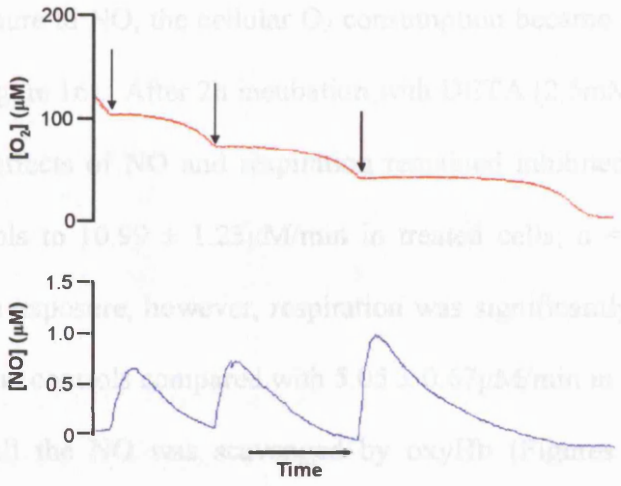

Figure 14. The inhibition of respiration by NO at different O_2 concentrations. DEANO (5μM) was added sequentially (indicated by arrows) and the NO that was released was detected by the NO electrode (blue tracing). The inhibition of respiration (red tracing) exerted by NO increased in duration at lower O_2 concentrations. However, larger quantities of NO were detected at lower O_2 concentrations, despite the addition of the same amount of DEANO. The tracings were obtained from one of at least three independent experiments.

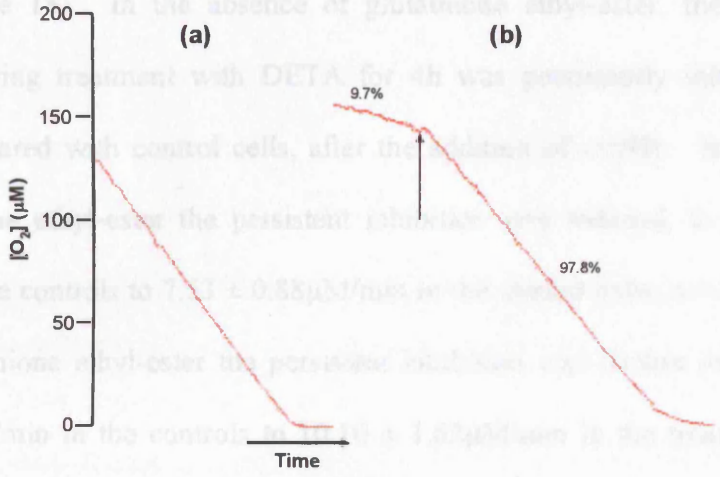

Figure 15. The reversible inhibition of respiration by NO released by DETA. (a) O_2 consumption of control cells. (b) O_2 consumption of cells incubated for 1h with DETA (2.5mM). The arrow indicates the addition of oxyhaemoglobin (20μM) which scavenged the NO. Percentage values indicate the rate of respiration relative to the control. The tracings were obtained from one of 3 independent experiments.

After prolonged exposure to NO, the cellular O_2 consumption became less reversible by the addition of oxyHb (Figure 16). After 2h incubation with DETA (2.5mM), oxyHb could only partially reverse the effects of NO and respiration remained inhibited by 20.2% (13.78 ± 0.71μM/min in controls to 10.99 ± 1.23μM/min in treated cells; n = 3) compared to the control rate. After 4h exposure, however, respiration was significantly inhibited by 63.8% (13.94 ± 0.95μM/min in controls compared with 5.05 ± 0.67μM/min in treated cells; n = 3; $P < 0.01$), even after all the NO was scavenged by oxyHb (Figures 16 and 17). Thus, prolonged treatment with the NO donor DETA caused an inhibition of respiration that persisted even after removal of NO by oxyHb.

It has been reported that this persistent inhibitory effect of NO can be prevented by the addition of exogenous antioxidants (Clementi *et al.*, 1998; Beltran *et al.*, 2000; Orsi *et al.*, 2000). Consistent with this, the addition of reduced glutathione ethyl-ester helped to prevent this effect (Figure 18). In the absence of glutathione ethyl-ester, the rate of cellular respiration following treatment with DETA for 4h was persistently inhibited by 63.8% (Figure 16) compared with control cells, after the addition of oxyHb. In the presence of 2.5mM glutathione ethyl-ester the persistent inhibition was reduced, to 46.0% (13.94 ± 0.95μM/min in the controls to 7.53 ± 0.88μM/min in the treated cells; n = 3; $P < 0.05$), and with 5mM glutathione ethyl-ester the persistent inhibition was further reduced, to 27.6% (13.94 ± 0.95μM/min in the controls to 10.10 ± 1.62μM/min in the treated cells; n = 3). Glutathione ethyl-ester itself did not affect the rate of cellular respiration. Thus, glutathione ethyl-ester significantly attenuated the persistent inhibition of respiration caused by prolonged treatment with NO.

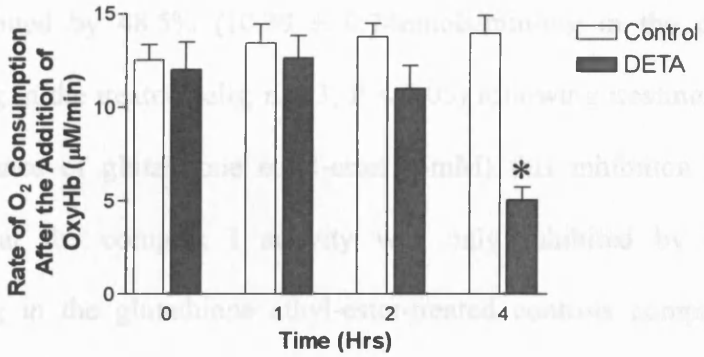

Figure 16. The persistent inhibition of respiration by NO. Treated cells were incubated with 2.5mM DETA. Oxyhaemoglobin (oxyHb; 20μM) was then added to scavenge the NO and the rate of respiration was measured. OxyHb became less able to reverse the inhibition of respiration indicating that NO had exerted a persistent effect upon respiration. * $P < 0.05$ compared with control. $n = 3$.

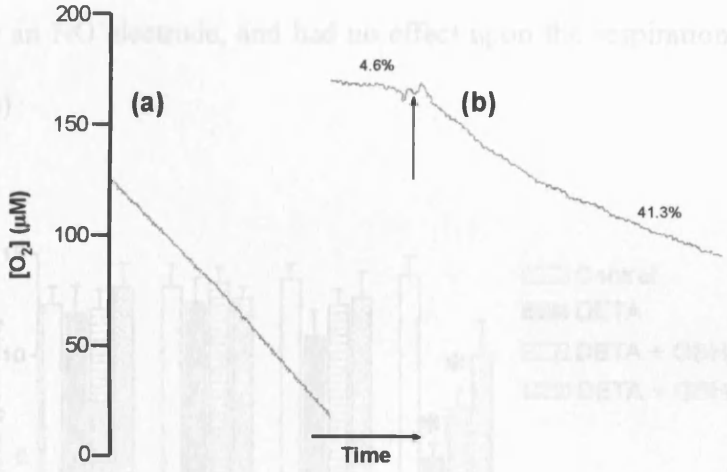

Figure 17. The persistent inhibition of respiration caused by prolonged exposure to NO. (a) O_2 consumption of control cells. (b) O_2 consumption of cells incubated with DETA (2.5mM) for 4h. Arrow indicates the addition of oxyhaemoglobin (20μM). Numerical values represent the O_2 consumption relative to the control. The tracings were obtained from one of at least 3 independent experiments.

Furthermore, the persistent inhibition of respiration has been attributed to the modification, and subsequent inhibition, of complex I as a result of its interactions with NO (Clementi *et al.*, 1998). In agreement with this, complex I activity in $RAW_{264.7}$ cells was found to be

significantly inhibited by 48.5% (10.39 ± 0.74nmols/min/mg in the control to 5.35 ± 0.71nmols/min/mg in the treated cells; n = 3; $P < 0.05$) following treatment with DETA for 4h. In the presence of glutathione ethyl-ester (5mM) this inhibition was significantly reduced, such that the complex I activity was only inhibited by 20.5% (11.15 ± 1.32nmols/min/mg in the glutathione ethyl-ester-treated controls compared with 8.86 ± 0.80nmols/min/mg in cells treated with DETA and glutathione ethyl-ester; n = 3; Figure 19). The onset of decrease in complex I activity was consistent with the appearance of the persistent inhibition of respiration caused by prolonged exposure to NO.

DETA that was completely decomposed, by leaving it for 24h at 37°C, did not release NO, as measured by an NO electrode, and had no effect upon the respiration of $RAW_{264.7}$ cells (data not shown).

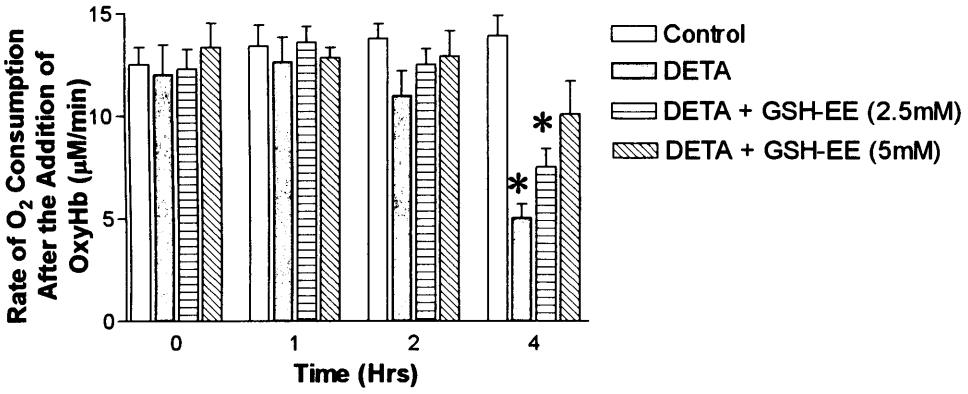

Figure 18. The effects of glutathione ethylester (GSH-EE) on the persistent inhibition of respiration caused by NO. Cells were incubated with DETA (2.5mM) in the presence or absence of GSH-EE (2.5 or 5mM) for the times indicated. Oxyhaemoglobin (20μM) was added prior to measurement of the rate of respiration. GSH-EE alone did not affect the rate of respiration compared with the controls. DETA caused a persistent inhibition of respiration after 4h of treatment, an effect that was dose-dependently prevented by GSH-EE. * $P < 0.05$ compared with control. n = 3.

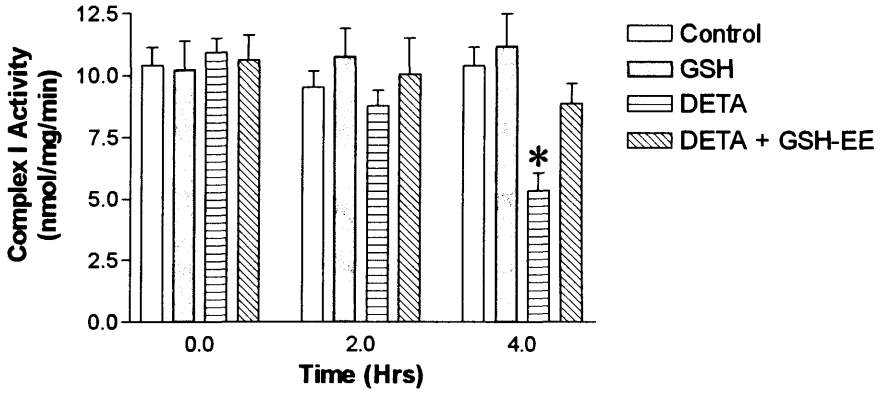

Figure 19. The effects of DETA on complex I activity in $RAW_{264.7}$ cells. Cells treated with DETA (2.5mM) for 4h showed a significant decrease in complex I activity. Co-treatment with GSH-EE (5mM) prevented this decrease. GSH-EE itself did not change complex I activities compared with controls. * $P < 0.05$ compared with control. $n = 3$.

3.2 Inhibition of Cellular Respiration in Activated $RAW_{264.7}$ Cells

In the previously described results, donor compounds were used to investigate the short- and long-term effects of NO on cellular O_2 consumption. However, the kinetics and quantities of NO released may not reflect endogenous production. Furthermore, certain NO donors release additional chemical species (Yamamoto and Bing, 2000), such as cyanide from sodium nitroprusside (Ruan *et al.*, 1999) and O_2^- from 3-morpholinosydnonimine hydrochloride (Lepore *et al.*, 1999), which may confound experiments. In order to investigate the effects of endogenously-produced NO on cellular respiration, $RAW_{264.7}$ cells were activated to express the iNOS protein.

3.2.1 Activation of $RAW_{264.7}$ Cells Induces iNOS Protein Expression and Nitric Oxide Synthesis

$RAW_{264.7}$ cells were activated with LPS (100ng/ml) and IFNγ (50U/ml). Activation of the cells resulted in the time-dependent expression of iNOS protein, as detected by Western blotting (Figure 20). The protein was not detected in control cells but was significantly elevated 4h after activation and increased throughout the experiment (up to 12h) as determined by densitometry (Figure 21).

The concentration of nitrite, a metabolite of NO, in the medium was measured to indicate iNOS protein activity and to quantify the amounts of NO that were produced. The nitrite concentration was significantly increased 6h after activation and continued to rise throughout the experiment, up to 178.02 ± 8.90μM (n = 3) at 24h post-treatment.

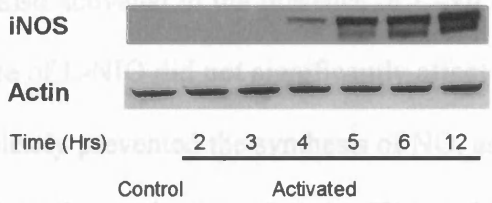

Figure 20. The time-dependent expression of iNOS in activated $RAW_{264.7}$ cells. $RAW_{264.7}$ cells were activated with LPS (100ng/ml) and IFNγ (50U/ml). The western blot shows the expression of iNOS protein, which increased throughout the experiment (up to 12h). iNOS was not detected in untreated control cells. Actin was used as a protein loading control. The Westerns blots shown were obtained from one of at three separate experiments.

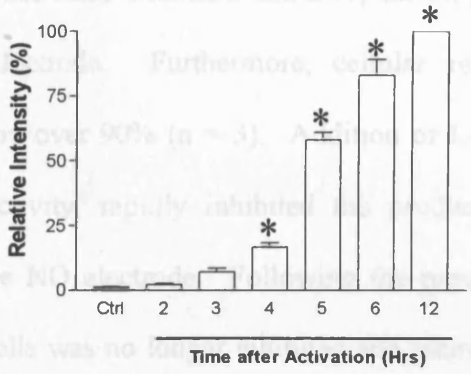

Figure 21. Relative band intensities obtained from densitometry measurements of iNOS Western blots from activated $RAW_{264.7}$ cells. The expression of the iNOS protein displayed a time-dependent increase, increasing throughout the experiment. A significant increase was initially detected at 4 hrs post-activation. * $P < 0.05$ compared with control. $n = 3$.

Between 18 and 24h, the rate of increase of nitrite concentration decreased compared with that between 6 and 18h (Figure 22). No nitrite was detected in the medium of control cells at any time.

$RAW_{264.7}$ cells were also activated in the presence of L-NIO (500μM), an inhibitor of NOS activity. The presence of L-NIO did not significantly affect the expression of iNOS protein (Figure 23) but completely prevented the synthesis of NO, as measured by an NO electrode, up to 18h and, therefore, the production of nitrite (Figure 24). L-NIO alone did not induce the expression of iNOS protein, neither did it alter nitrite concentrations in the medium.

3.2.2 Reversible Inhibition of Respiration by Nitric Oxide in Activated $RAW_{264.7}$ Cells

$RAW_{264.7}$ cells that were activated with LPS and IFNγ for 6h produced NO (over 2μM), as measured by an NO electrode. Furthermore, cellular respiration was found to be concomitantly inhibited by over 90% (n = 3). Addition of L-NMMA (1mM) to activated cells, to inhibit NOS activity, rapidly inhibited the production of NO, resulting in a decreased signal from the NO electrode. Following the prevention of NO synthesis, the respiration of activated cells was no longer inhibited and recovered to a rate similar to that of the controls. In comparison, the addition of myxothiazol (1μM) also completely inhibited O_2 consumption (Figure 25).

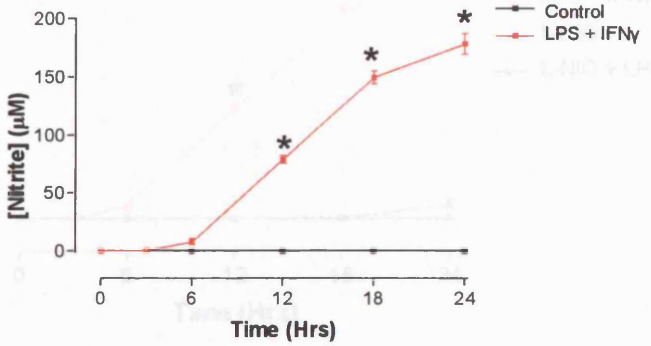

Figure 22. The increase in the concentration of nitrite in the medium of activated $RAW_{264.7}$ cells. Nitrite concentrations in the medium of control cells were unchanged. Nitrite in the medium of activated cells was significantly increased 12h after activation with LPS (100ng/ml) and IFNγ (50U/ml). * $P < 0.05$ compared with control. $n > 3$.

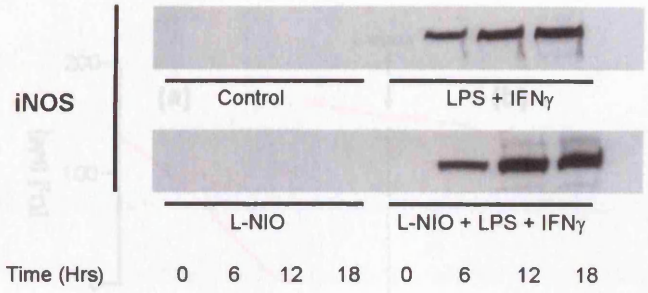

Figure 23. Activation of $RAW_{264.7}$ cells in the presence of L-NIO does not affect the expression of iNOS protein. Samples from control and treated cells (with LPS + IFNγ, L-NIO or a combination of both treatments) were collected at 0, 6, 12 and 18h. The cells were lysed and prepared for Western blot analysis. iNOS protein was detected using a polyclonal antibody. Blots are representative of at least 3 independent experiments.

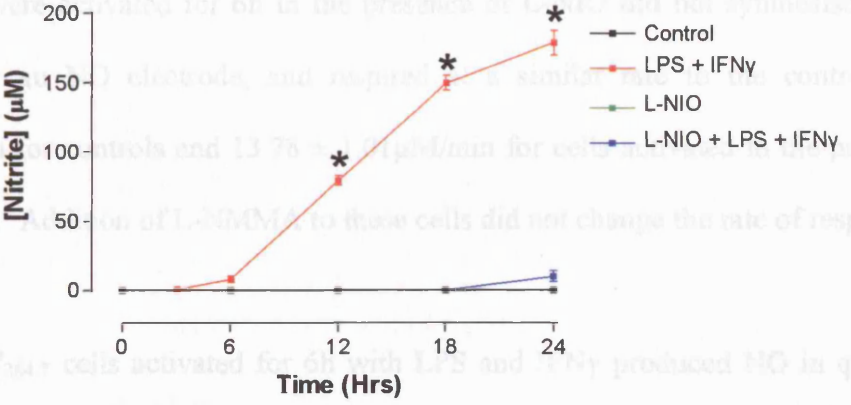

Figure 24. The increase of nitrite in the medium of activated $RAW_{264.7}$ cells can be prevented by L-NIO (500μM). Activated cells significantly increased nitrite concentrations 12 hrs after activation, and continued to rise throughout the experiment. Activation in the presence of L-NIO abolished this increase. L-NIO treatment alone did not alter the nitrite concentrations. * $P < 0.05$ compared with control. $n > 3$.

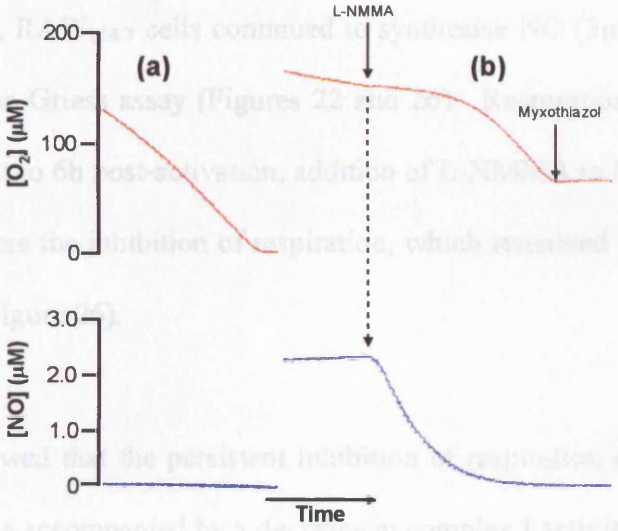

Figure 25. Activated $RAW_{264.7}$ cells produced NO which reversibly inhibited respiration. (a) Control cells did not produce NO. (b) Cells activated for 6h produced NO in sufficient quantities to inhibit respiration. Upon the addition of L-NMMA (500μM), NO synthesis was blocked and respiration recovered to control rates. Myxothiazol (1μM) completely inhibited O_2 consumption. The tracings were obtained from one of at least three independent experiments.

Cells that were activated for 6h in the presence of L-NIO did not synthesise any NO, as detected by an NO electrode, and respired at a similar rate to the controls (14.02 ± 1.19μM/min for controls and 13.78 ± 1.01μM/min for cells activated in the presence of L-NIO; n = 5). Addition of L-NMMA to these cells did not change the rate of respiration.

Thus, $RAW_{264.7}$ cells activated for 6h with LPS and IFNγ produced NO in quantities that were sufficient to inhibit cellular respiration by over 90%. This inhibition was sensitive to L-NMMA and was prevented when cells were activated in the presence of L-NIO.

3.2.3 Persistent Inhibition of Respiration Caused by Nitric Oxide in Activated $RAW_{264.7}$ Cells

After 12h activation, $RAW_{264.7}$ cells continued to synthesise NO (3μM), as measured by an NO electrode and the Griess assay (Figures 22 and 26). Respiration was also found to be inhibited. In contrast to 6h post-activation, addition of L-NMMA to block NO synthesis did not completely reverse the inhibition of respiration, which remained inhibited by two-thirds of the control rate (Figure 26).

Previous results showed that the persistent inhibition of respiration caused by NO, derived from NO donors, was accompanied by a decrease in complex I activity. Therefore, complex I activity in the activated $RAW_{264.7}$ cells was also measured.

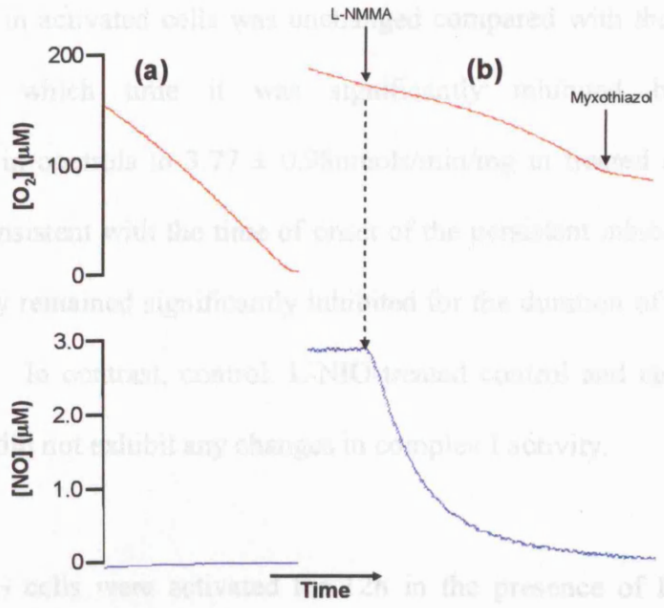

Figure 26. The persistent inhibition of respiration caused by NO in activated $RAW_{264.7}$ cells. (a) Control cells. (b) Cells activated for 12h produced NO and exhibited an inhibition of respiration. When L-NMMA (500μM) was added to block NO synthesis, cellular respiration did not return to control rates but remained inhibited. The tracings were obtained from one of at least three independent experiments.

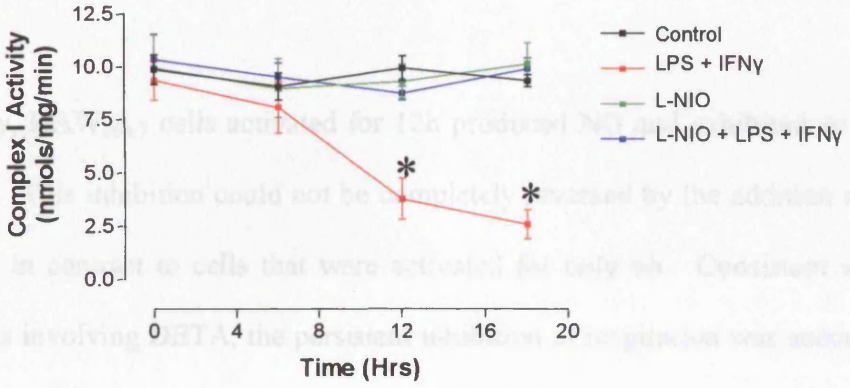

Figure 27. Complex I activities of control and activated $RAW_{264.7}$ cells in the presence and absence of L-NIO (500μM). The complex I activity of control and L-NIO cells remained unchanged throughout the experiment. Complex I activity of activated cells was inhibited by 62% at 12h post-treatment. This decrease in complex I activity was prevented by L-NIO. * $P < 0.05$ compared with control. $n > 3$.

Complex I activity in activated cells was unchanged compared with the controls until 12h post-treatment, at which time it was significantly inhibited by 62% (9.92 ± 0.57nmols/min/mg in controls to 3.77 ± 0.98nmols/min/mg in treated cells; n = 4-6; $P < 0.05$). This was consistent with the time of onset of the persistent inhibition of respiration. The enzyme activity remained significantly inhibited for the duration of the experiment (up to 18h; Figure 27). In contrast, control, L-NIO-treated control and cells activated in the presence of L-NIO did not exhibit any changes in complex I activity.

When the $RAW_{264.7}$ cells were activated for 12h in the presence of L-NIO, so that NO synthesis was completely prevented, the rate of respiration was found to be consistently inhibited by 20% (14.27 ± 0.57μM/min in controls to 11.57 ± 0.34μM/min; n = 3; $P < 0.05$) compared to the L-NIO-treated control rate (Figure 28). This small but significant inhibition of respiration was independent of NO, since NOS was completely inhibited and NO could not be detected by the NO electrode or by the Griess assay (Figures 24 and 28).

In summary, $RAW_{264.7}$ cells activated for 12h produced NO and exhibited an inhibition of respiration. This inhibition could not be completely reversed by the addition of L-NMMA, which was in contrast to cells that were activated for only 6h. Consistent with previous experiments involving DETA, the persistent inhibition of respiration was accompanied by a decrease in complex I activity. Furthermore, when cells were activated in the presence of L-NIO, which completely prevented NO synthesis, they

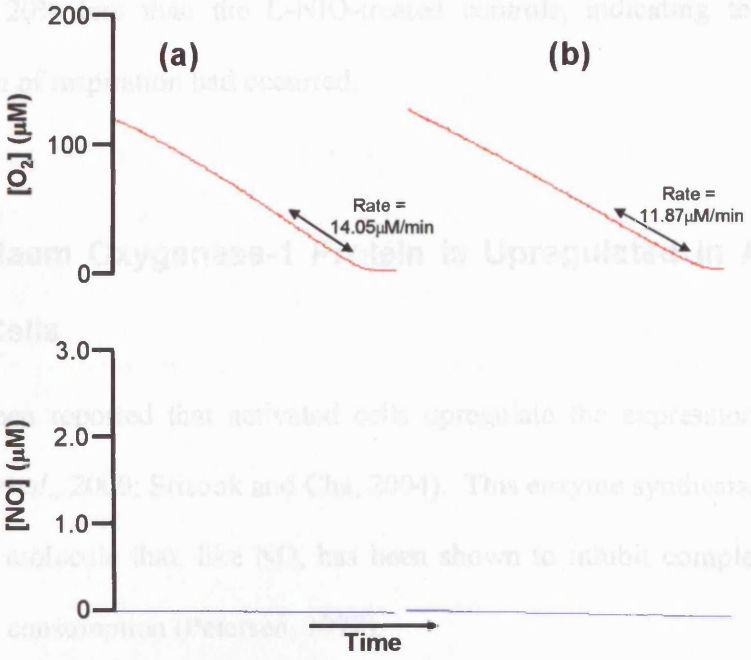

Figure 28. NO-independent inhibition of respiration in activated $RAW_{264.7}$ cells. (a) Cells treated with L-NIO (500μM) did not produce NO and respired at the same rate as controls. (b) Cells activated in the presence of L-NIO for 12h respired 20% less than the L-NIO-treated control. The tracings were obtained from one of at least three independent experiments.

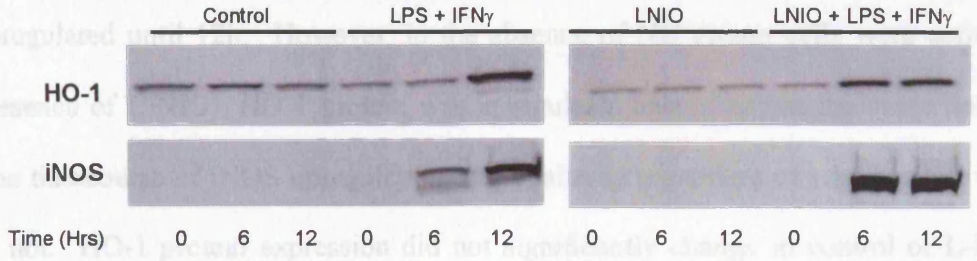

Figure 29. The expression of HO-1 and iNOS in activated $RAW_{264.7}$ cells. HO-1 was upregulated in cells activated in the presence and absence of L-NIO. In the absence of L-NIO, HO-1 was found to be upregulated 12h post-activation whereas it was upregulated after 6h in the presence of the inhibitor. L-NIO did not affect the timecourse of iNOS protein expression. The Western blots are representative of at least 3 independent experiments.

respired 20% less than the L-NIO-treated controls, indicating that an NO-independent inhibition of respiration had occurred.

3.2.4 Haem Oxygenase-1 Protein is Upregulated in Activated $RAW_{264.7}$ Cells

It has been reported that activated cells upregulate the expression of the HO-1 enzyme (Wiesel *et al.*, 2000; Srisook and Cha, 2004). This enzyme synthesises CO (Tenhunen *et al.*, 1969), a molecule that, like NO, has been shown to inhibit complex IV and subsequently block O_2 consumption (Petersen, 1977).

In agreement with published observations, activated $RAW_{264.7}$ cells did indeed strongly upregulate HO-1 protein as determined by Western blotting (Figure 29). The timecourse of HO-1 upregulation was delayed compared with that of iNOS in NO-producing cells. Whereas iNOS protein was upregulated within 6h of cell activation, HO-1 was not upregulated until 12h. However, in the absence of NO (when cells were activated in the presence of L-NIO), HO-1 protein was upregulated earlier and at the same time as iNOS. The timecourse of iNOS upregulation was unaltered regardless of whether NO was present or not. HO-1 protein expression did not significantly change in control or L-NIO-treated cells that were not activated (Figure 29).

Since CO is known to bind to and inhibit complex IV (Petersen, 1977), the activity of this enzyme was measured in activated cells in the presence and absence of L-NIO. Complex IV activity of cells activated for 12h was found to be significantly inhibited by 62% (157 ± 6.03nmols/min/mg in controls compared with 61.22 ± 16.56nmols/min/mg in activated cells;

n = 4-5; P < 0.05) compared with the control (Figure 30). Similarly, cells that were activated for 12h in the presence of L-NIO also displayed significantly reduced complex IV activity of a similar magnitude (68% inhibition compared with L-NIO-treated cells; 166.60 ± 12.56nmols/min/mg in L-NIO-treated controls and 53.09 ± 13.62nmols/min/mg in cells activated in the presence of L-NIO; n = 4-5; P < 0.05). Control and L-NIO-treated cells did not show significant changes in complex IV activity through the duration of the experiment.

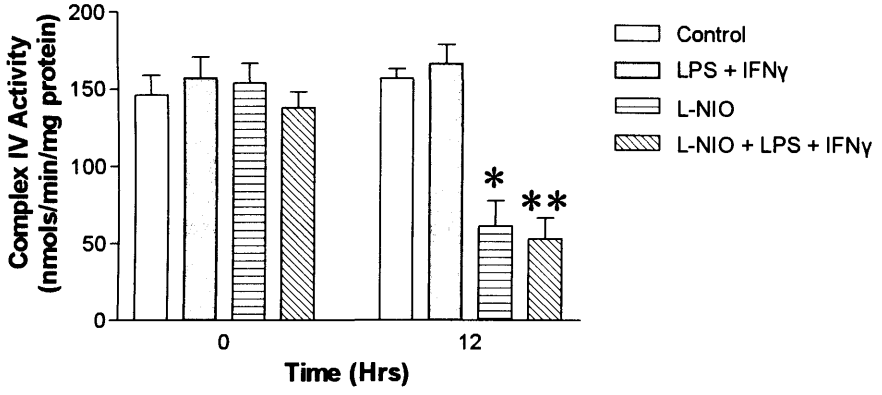

Figure 30. Complex IV activity of activated $RAW_{264.7}$ cells. Complex IV activity in activated cells was decreased in the presence or absence of L-NIO, suggesting an NO-independent cause (n = 4-5). * P < 0.05 compared with control. ** P < 0.05 compared with L-NIO-treated cells.

3.3 Inhibition of Respiration by Carbon Monoxide

Inhibition of respiration by exogenous CO has been reported to occur in mitochondria at complex IV of the ETC (Alonso *et al.*, 2003). However, previous work has mostly involved the use of the isolated cytochrome c oxidase enzyme (Petersen, 1977). Here, the effects of exogenous CO on the respiration of intact HEK293 cells were investigated. These cells were used because they could easily be transfected with the HO-1 gene under the control of a tetracycline-inducible promoter, and used to investigate whether endogenously-produced CO from HO-1 could also inhibit respiration.

3.3.1 Carbon Monoxide Gas Inhibits Cellular Respiration

Solutions of CO gas were administered to HEK293 cells at concentrations of 5-20μM. Cellular respiration was found to be inhibited in a dose-dependent manner. At a concentration of 20μM CO, respiration was inhibited by 40% (18.62 ± 0.74μM/min in controls to 11.63 ± 0.75μM/min, $n = 3$; $P < 0.05$) in the range 10-30μM O_2 on the respiration tracings (Figure 31A). Furthermore, the effects of CO were still evident 30mins after treatment (Figure 31B). Thus the effects of CO were persistent. From the data obtained, Lineweaver-Burk and the derived secondary plots were made and the K_i of CO for respiration in HEK293 cells was calculated to be 1.44μM.

The inhibition of respiration by CO that was observed was modest compared to that by NO (Figure 31A). However, CO is only able to bind to and inhibit complex IV when the enzyme is in the reduced state (Petersen, 1977; Piantadosi, 2002). The previous

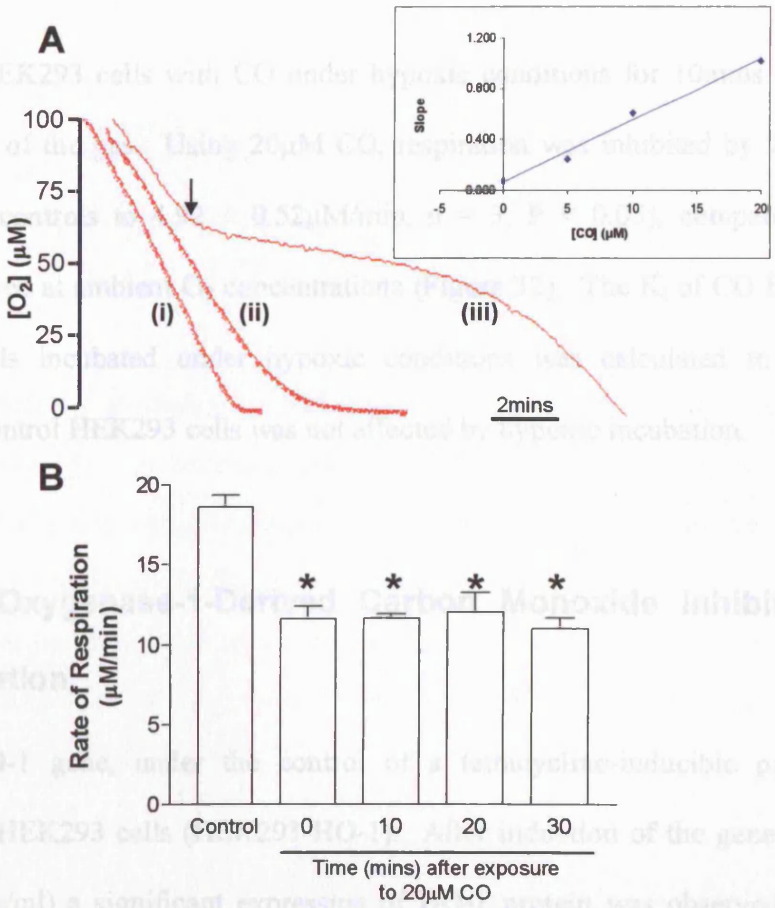

Figure 31. Inhibition of cellular respiration by exogenous CO. (A) Main figure: (i) A tracing of the O_2 consumption of control HEK293 cells. (ii) The O_2 consumption of cells treated with 20μM CO. (iii) A tracing showing the comparative effects of 5μM DEANO (addition indicated by arrow). The rate of respiration recovers after the NO is degraded. Insert: The slopes obtained from the Lineweaver-Burk plot were plotted against [CO] (secondary plot). The Ki of CO on respiration was calculated by interpolation to the x-axis and was 1.44μM. Tracings are representative of at least 3 independent experiments. (B) The rates of respiration of HEK293 cells were measured 0, 10, 20 and 30mins after the addition of 20μM CO (n = 3). *P<0.05 compared with controls.

experiments were carried out at ambient O_2 concentrations, a condition that maintains the ETC in an oxidised state. Thus, to shift complex IV into a more reduced state, experiments were also performed under hypoxic (1% O_2) conditions.

Incubation of HEK293 cells with CO under hypoxic conditions for 10mins increased the inhibitory effect of the gas. Using 20μM CO, respiration was inhibited by 75% (19.14 ± 0.82μM/min in controls to 4.92 ± 0.52μM/min, n = 3; $P < 0.05$), compared with 40% inhibition observed at ambient O_2 concentrations (Figure 32). The K_i of CO for respiration in HEK293 cells incubated under hypoxic conditions was calculated to be 0.35μM. Respiration in control HEK293 cells was not affected by hypoxic incubation.

3.3.2 Haem Oxygenase-1-Derived Carbon Monoxide Inhibits Cellular Respiration

The human HO-1 gene, under the control of a tetracycline-inducible promotor, was transfected into HEK293 cells (HEK293-HO-1). After induction of the gene for 24h with tetracycline (1μg/ml) a significant expression of HO-1 protein was observed (Figure 33). All following experiments were performed under these conditions.

Bilirubin is an indirect product of HO-1 activity and was used as an indicator of functional HO-1 enzyme. As shown in Figure 34, induced HEK293-HO-1 cells had 1.42μM of bilirubin in the medium compared with 0.97μM in the non-induced control. This indicated that the HO-1 protein induced was functional.

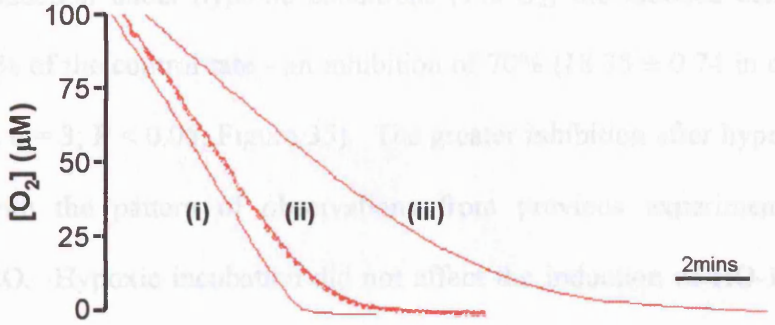

Figure 32. Inhibition of cellular respiration by exogenous CO in HEK293 cells incubated in hypoxia (1% O_2). (i) the O_2 consumption of control cells incubated in hypoxia. (ii) the O_2 consumption of cells treated with 20μM CO under ambient O_2 concentrations. (iii) the O_2 consumption of cells treated with 20μM CO in hypoxia. No difference was observed between untreated control cells kept under ambient O_2 concentrations and those incubated in hypoxia. The K_i of CO for cells in hypoxia was calculated to be 0.35μM. The tracings were obtained from one of at least 3 independent experiments.

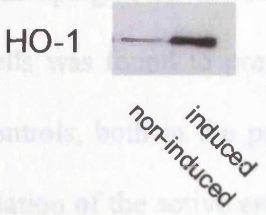

Figure 33. The expression of HO-1 following induction of HEK293-HO-1 cells. HEK293-HO-1 cells were treated with tetracycline (1μg/ml) for 24h and HO-1 protein expression was detected by Western blotting.

Following incubation under ambient O_2 conditions, the induced HEK293-HO-1 cells were found to respire less than the non-induced control cells. Within the range of 10-30μM O_2 on the respiration traces, the respiration of the induced HEK293-HO-1 cells was inhibited by 12% (18.57 ± 1.06μM/min in controls to 16.33 ± 0.87μM/min, n = 3; $P < 0.05$; Figure 35).

Following induction under hypoxic conditions (1% O_2) the induced cells were found to respire at 30% of the control rate - an inhibition of 70% (18.35 ± 0.74 in controls to 5.24 ± 0.67μM/min, n = 3; $P < 0.05$; Figure 35). The greater inhibition after hypoxic incubation is consistent with the pattern of observations from previous experiments that involved exogenous CO. Hypoxic incubation did not affect the induction of HO-1 protein in these cells, as determined by Western blotting, nor the activity of the enzyme, since the concentration of bilirubin in the medium of cells induced at 1% O_2 was not significantly different from that of the cells induced under ambient O_2 concentrations (Figure 36).

3.3.3 Haem Oxygenase-1-Derived Carbon Monoxide Inhibits Cellular Respiration in Activated $RAW_{264.7}$ Cells

$RAW_{264.7}$ cells activated for 12h upregulated the expression of HO-1 protein (Figure 29). This enzyme from activated cells was found to produce significantly greater quantities of bilirubin compared with the controls, both in the presence and absence of L-NIO (Figure 37), consistent with the upregulation of the active enzyme. In the absence of L-NIO 3.48 ± 0.19μM (n = 6) bilirubin was detected in the incubation medium of activated cells, compared with 1.34 ± 0.07μM (n = 3) in the untreated control. In the presence of L-NIO,

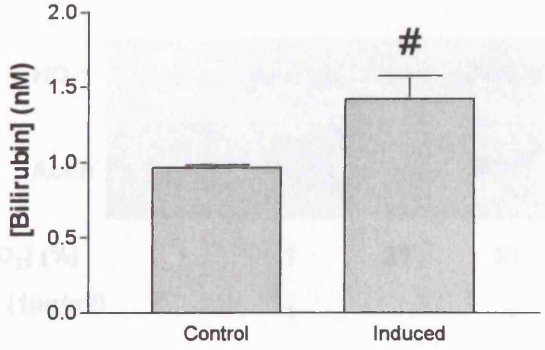

Figure 34. Induced HEK293-HO-1 cells produce more bilirubin. The concentration of bilirubin in the medium of induced HEK293-HO-1 cells was increased to 1.42μM compared with 0.97μM in the uninduced. This indicates that active HO-1 protein was expressed. n = 3. # $P < 0.05$ compared with control.

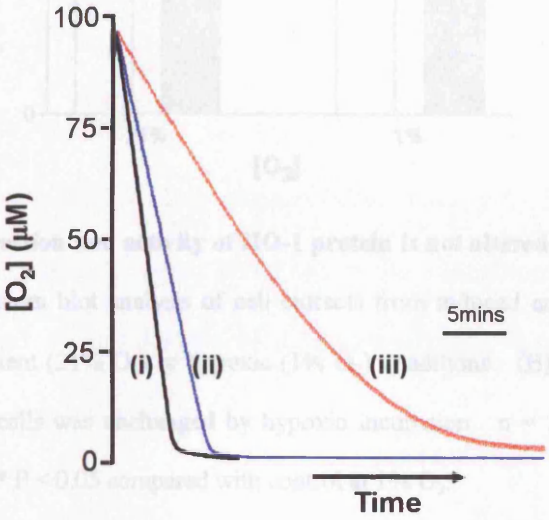

Figure 35. Induced HEK293-HO-1 cells exhibit a decreased rate of respiration. The respiration of induced HEK293-HO-1 cells incubated under ambient O_2 concentrations (ii) and hypoxic conditions (iii) was decreased compared with uninduced cells (i). The tracings were obtained from one of at least 3 independent experiments.

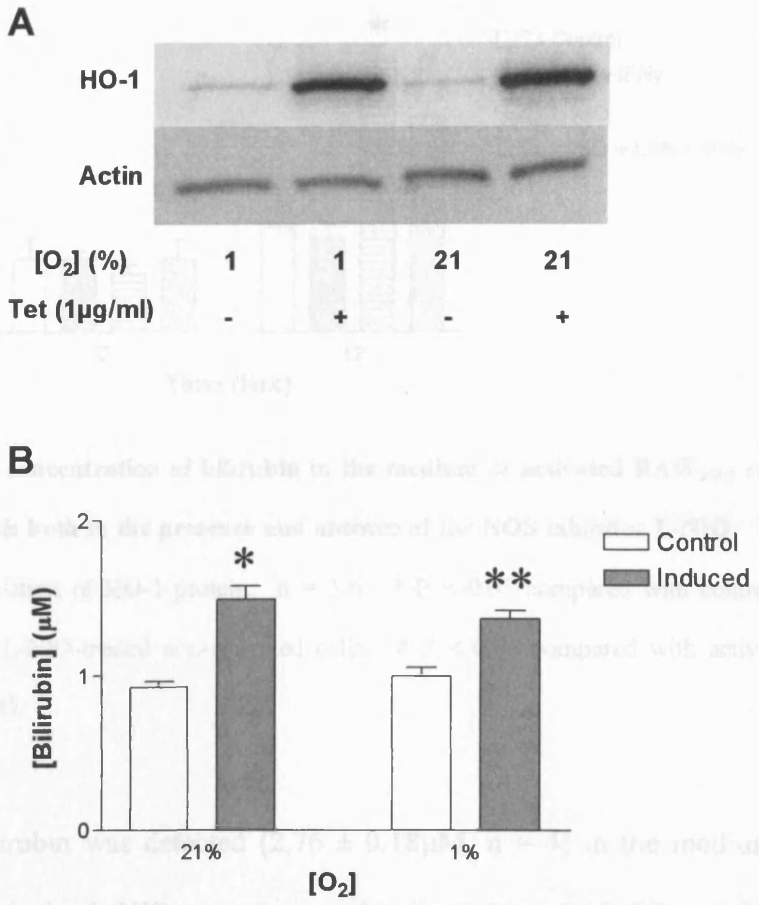

Figure 36. The expression and activity of HO-1 protein is not altered by incubation under hypoxic conditions. (A) Western blot analysis of cell extracts from induced and uninduced HEK-HO-1 cells incubated under ambient (21% O_2) or hypoxic (1% O_2) conditions. (B) Bilirubin concentration in the medium of induced cells was unchanged by hypoxic incubation. n = 3. * $P < 0.05$ compared with control at 21% O_2. ** $P < 0.05$ compared with control at 1% O_2.

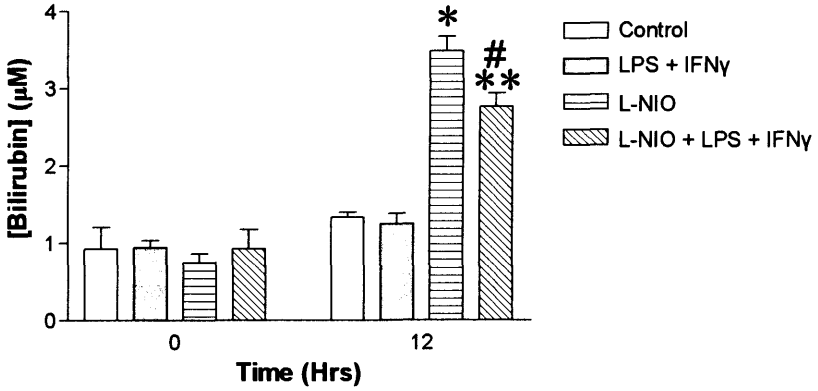

Figure 37. The concentration of bilirubin in the medium of activated $RAW_{264.7}$ cells was greater than the controls both in the presence and absence of the NOS inhibitor L-NIO. This is consistent with the upregulation of HO-1 protein. n = 3-6. * $P < 0.05$ compared with control. ** $P < 0.05$ compared with L-NIO-treated non-activated cells. # $P < 0.05$ compared with activated cells in the absence of L-NIO.

although more bilirubin was detected (2.76 ± 0.18μM, n = 4) in the medium of activated cells compared with the L-NIO-treated control cells (1.25 ± 0.13μM, n = 3), the increase caused by activation was less than that observed in the absence of L-NIO. Thus, activated cells that were allowed to synthesise NO produced significantly more bilirubin than activated cells that could not do so.

In cells activated for 12h cellular O_2 consumption was almost totally inhibited, but in the presence of L-NIO, which completely blocked NO synthesis, the inhibition of O_2 consumption was reduced to 20% (11.57 ± 0.34μM/min compared with 14.27 ± 0.57μM/min in controls, n = 3-4, $P < 0.05$). When the cells were activated at 1% O_2, this NO-independent inhibitory effect was increased to 60% (4.92 ± 0.40μM/min in activated cells compared with 11.33 ± 0.42μM/min in non-activated controls, n = 3-4, $P < 0.05$; Figure 38). In addition, complex IV activity was decreased to 53.09 ± 13.62nmol/min/mg from 166.60 ±

12.56 protein (n = 4, P < 0.05) following cell activation for 12h in the presence of L-NIO under ambient O_2 concentrations (Figure 30), and to 25.22 ± 7.74nmol/min/mg from 75.08 ± 19.21 protein (n = 4, P < 0.05) under hypoxic conditions.

Hypoxia has been reported to be a stimulus for the induction of HO-1 (Morita *et al.*, 1995). In agreement with this we observed an upregulation of HO-1 protein following hypoxic incubation in our control $RAW_{264.7}$ cells (Figure 39A). This was accompanied by a decrease in cellular respiration (Figure 39B). In the range 10-30μM O_2 the rate of respiration was initially 11.26 ± 0.91μM/min (n = 3). This fell to 6.68 ± 1.35μM/min (41% inhibition; n = 3, P < 0.05) after 12h in hypoxia and was reduced further to 4.41 ±

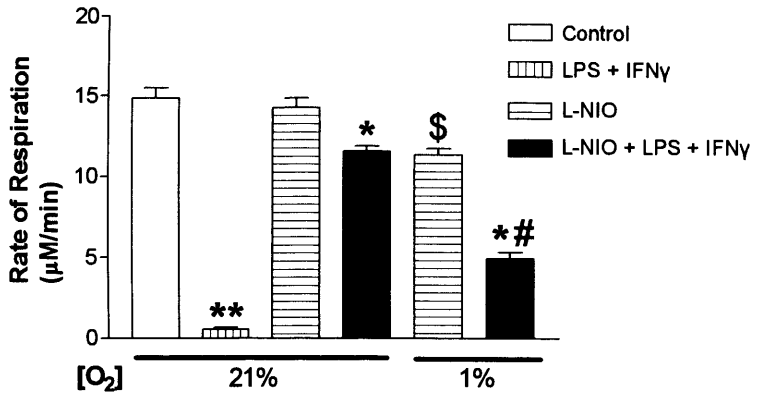

Figure 38. **The respiration of activated cells.** The respiration of cells activated for 12h was almost completely inhibited. Activated cells that did not synthesise NO still exhibited an inhibition of respiration that was increased by hypoxic incubation. n = 3-5. ** P < 0.05 compared with control. * P < 0.05 compared with respective L-NIO-treated controls. $ P < 0.05 compared with L-NIO-treated cells maintained at 21% O_2. # P < 0.05 compared with cells activated in the presence of L-NIO at 21% O_2.

1.85μM/min (61% inhibition; n = 3; P < 0.05) after 24h. Complex IV activity was also significantly decreased following hypoxic incubation for 12h from 157.15 ± 6.04 to 88.25 ±

4.31 nmol/min/mg protein (n = 3-4; $P < 0.05$; Figure 39C). This is consistent with CO binding to, and inhibiting, complex IV. The upregulation of HO-1 and the inhibition of respiration observed following hypoxic incubation was not observed in the non-induced control HEK293-HO-1 cells (Figures 32 and 36A).

Another interesting observation was that $RAW_{264.7}$ cells activated under hypoxic conditions produced less NO compared with cells activated at ambient O_2 concentrations (Figure 40). NO production, as determined by the Griess assay and an NO electrode, was significantly reduced at 1% (10μM) O_2. Nitrite, a metabolite of NO, concentration was reduced by 85% at 1% O_2 compared with ambient conditions after 24h (178.02 ± 8.90μM to 27.15 ± 2.60μM, $P < 0.05$). The concentration of NO in the incubation medium, measured using an NO electrode, was 0.3μM at 10μM O_2 compared with 2.3μM at 100μM. This decrease is in agreement with the observed 85% reduction in the amount of nitrite measured. In addition, the K_m of iNOS for O_2 was found to be between 20 and 30μM in our cells (Figure 40B).

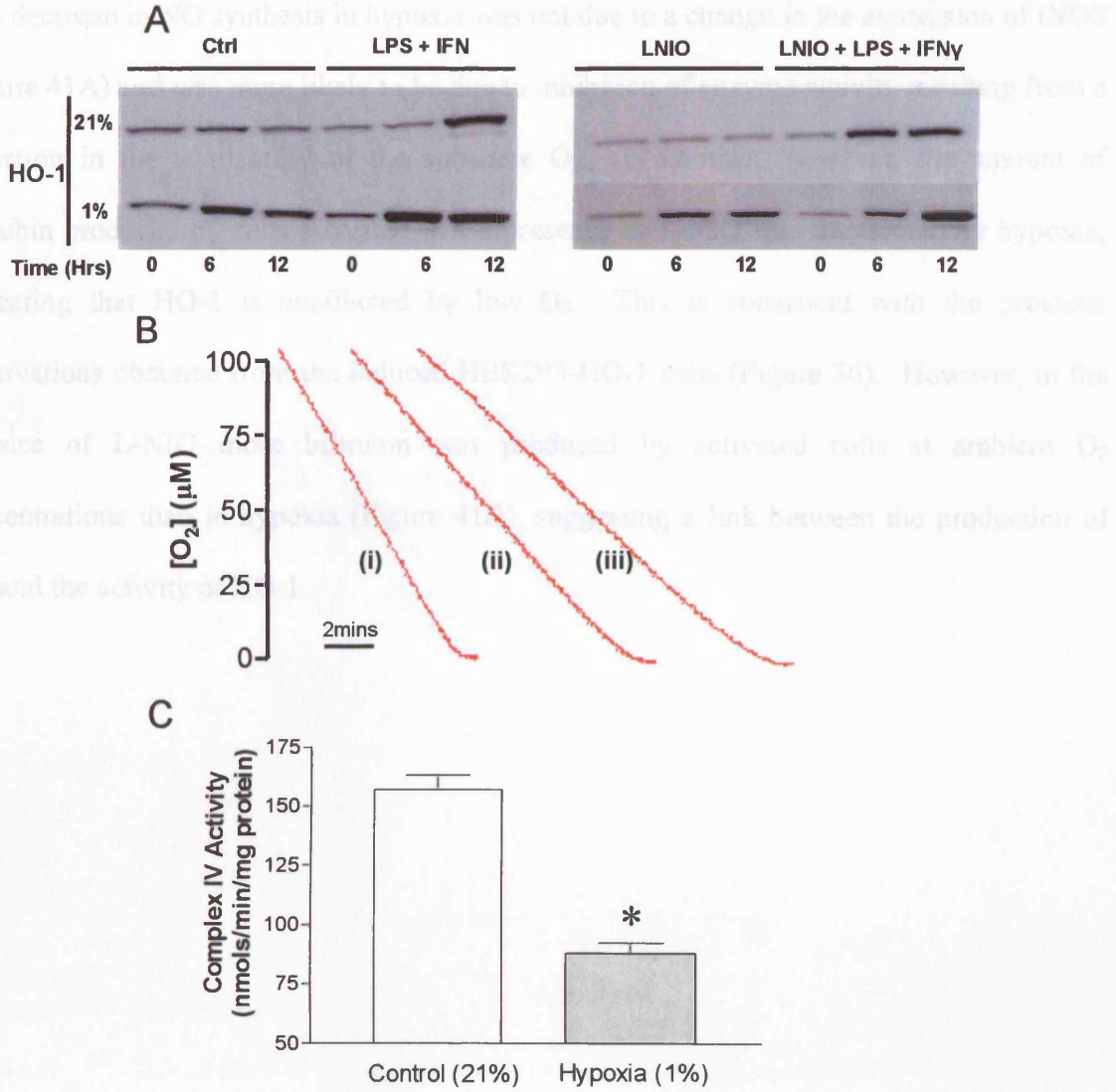

Figure 39. Hypoxic incubation of $RAW_{264.7}$ cells induced the expression of HO-1 protein and was accompanied by a decrease in the rate of cellular respiration and complex IV activity. (A) Hypoxia induced the expression of HO-1 protein both in controls and L-NIO treated cells. (B) Respiration in cells incubated at 1% O_2 for 12h (ii) or 24h (iii) was inhibited by 41% and 61% respectively. (C) Complex IV activity of cells incubated in hypoxia for 12h was decreased compared with those incubated under ambient O_2 concentrations. * $P < 0.05$ compared with control. (n = 3-4).

This decrease in NO synthesis in hypoxia was not due to a change in the expression of iNOS (Figure 41A) and was more likely to be due to inhibition of enzyme activity resulting from a reduction in the availability of the substrate O_2. In contrast, however, the amount of bilirubin produced by cells activated in the presence of L-NIO was unaffected by hypoxia, indicating that HO-1 is unaffected by low O_2. This is consistent with the previous observations obtained from the induced HEK293-HO-1 cells (Figure 36). However, in the absence of L-NIO more bilirubin was produced by activated cells at ambient O_2 concentrations than in hypoxia (Figure 41B), suggesting a link between the production of NO and the activity of HO-1.

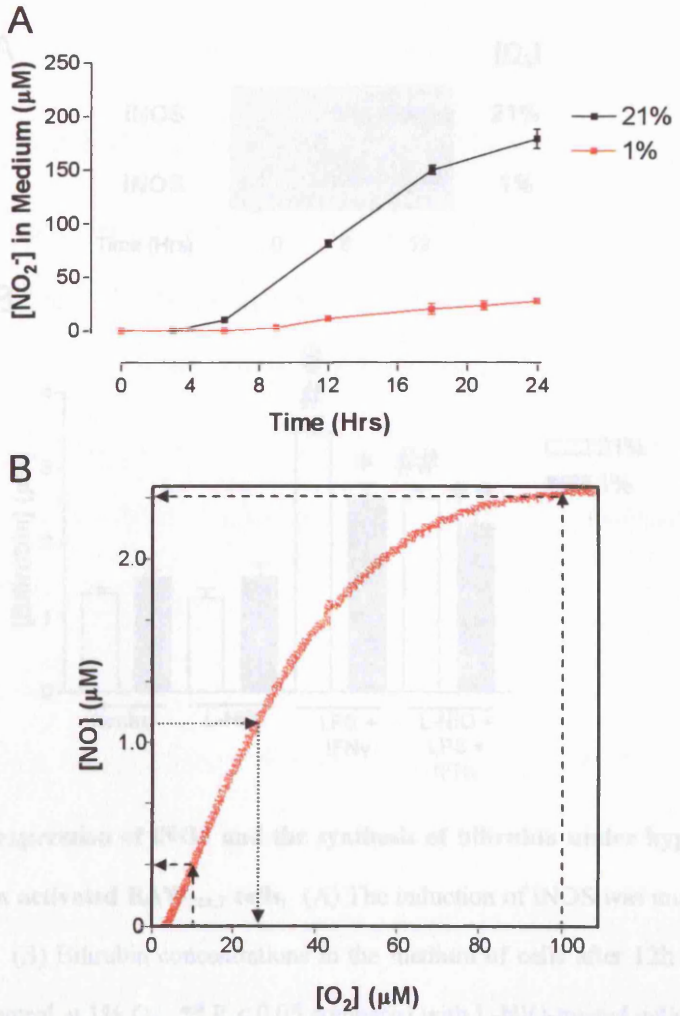

Figure 40. The synthesis of NO is inhibited by hypoxia. (A) Production of NO in activated $RAW_{264.7}$ cells was inhibited by 85% under hypoxia, as determined by the concentration of its metabolite nitrite. (B) NO decreased from a concentration of 2.3µM at 100µM O_2 to 0.3µM at 10µM O_2 (indicated by dashed arrows). The K_m for steady-state NO concentrations at different O_2 tensions in $RAW_{264.7}$ cells was between 20-30µM (indicated by the dotted line). n = 3.

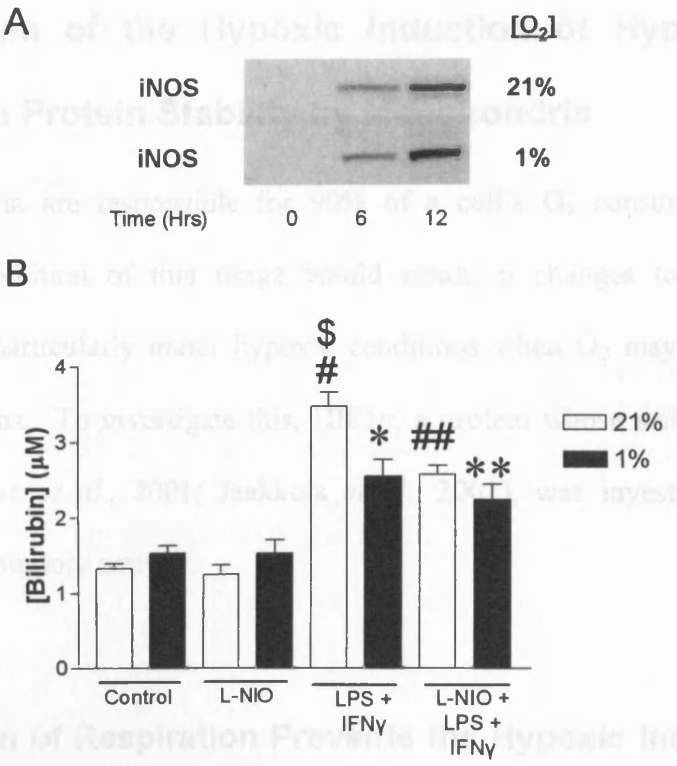

Figure 41. The expression of iNOS and the synthesis of bilirubin under hypoxic and ambient O_2 concentrations in activated $RAW_{264.7}$ cells. (A) The induction of iNOS was unaltered by activation of cells in hypoxia. (B) Bilirubin concentrations in the medium of cells after 12h (n = 3-6). * $P < 0.05$ compared with control at 1% O_2. ** $P < 0.05$ compared with L-NIO-treated cells at 1% O_2. # $P < 0.05$ compared with control at 21% O_2. ## $P < 0.05$ compared with L-NIO-treated cells at 21% O_2. $ $P <$ 0.05 compared with activated cells at 1% O_2.

3.4 Modulation of the Hypoxic Induction of Hypoxia Inducible Factor 1α Protein Stability by Mitochondria

Since mitochondria are responsible for 90% of a cell's O_2 consumption, it would be expected that inhibition of this usage would result in changes to the availability of intracellular O_2, particularly under hypoxic conditions when O_2 may be limiting for O_2-dependent reactions. To investigate this, HIF1α, a protein whose stability is known to be O_2-dependent (Ivan *et al.*, 2001; Jaakkola *et al.*, 2001), was investigated in relation to mitochondrial respiratory activity.

3.4.1 Inhibition of Respiration Prevents the Hypoxic Induction of HIF1α Protein and its Transcriptional Activity

HEK293 cells incubated in hypoxia (1% O_2) for 3h stabilised HIF1α protein. The presence of the respiratory inhibitors myxothiazol (1μM), sodium azide (5mM), antimycin A (1μg/ml) or rotenone (500nM) completely prevented this stabilisation (Figure 42A). Thus, inhibition of the ETC at various sites produces the same destabilising effect on hypoxia-induced HIF1α protein. A similar phenomenon was also observed in a variety of other cell lines, including Hep3B, 143B and HeLa cells, treated with myxothiazol (1μM; Figure 42B). Thus, this HIF1α destabilising effect is not a cell-specific phenomenon.

HIF1α-dependent reporter gene activity was also increased (11-fold) following hypoxic treatment for 5h. This too was abolished by the addition of the mitochondrial inhibitors,

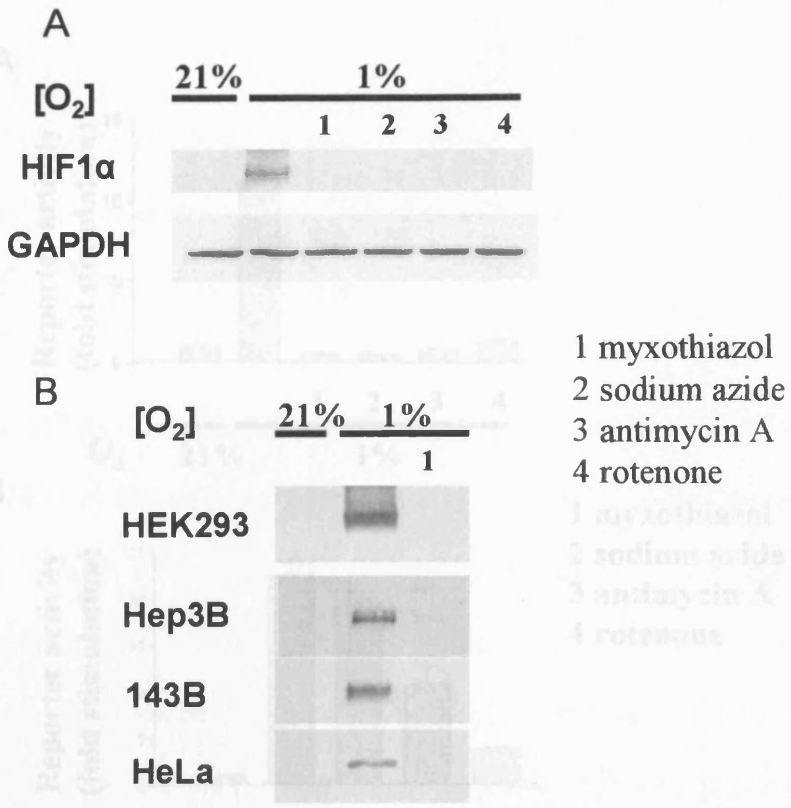

Figure 42. Inhibition of mitochondrial respiration prevents hypoxia-dependent HIF1α stabilisation. (A) HEK293 cells were incubated at 1% O_2 for 3h in the presence of the mitochondrial respiratory inhibitors myxothiazol (1μM), sodium azide (5mM), antimycin A (1μg/ml) and rotenone (0.5μM), as indicated. HIF1α was detected by Western blotting. (B) Four different human cell types (HEK293, Hep3B hepatocellular carcinoma cells, 143B osteosarcoma cells and HeLa cervix carcinoma cells) were incubated at 1% O_2 in the presence or absence of 1μM myxothiazol. HIF1α was detected by Western blotting.

indicating that the transcriptional effects of HIF1α are correlated with the stability of the protein and are similarly affected by respiratory inhibition at various sites of the ETC (Figure 43A). In addition, HIF1α protein stabilisation and HIF1α-dependent reporter gene activity were significantly inhibited by the addition of exogenous NO, released from DETA (10-100μM), in a dose-dependent manner (Figure 43B).

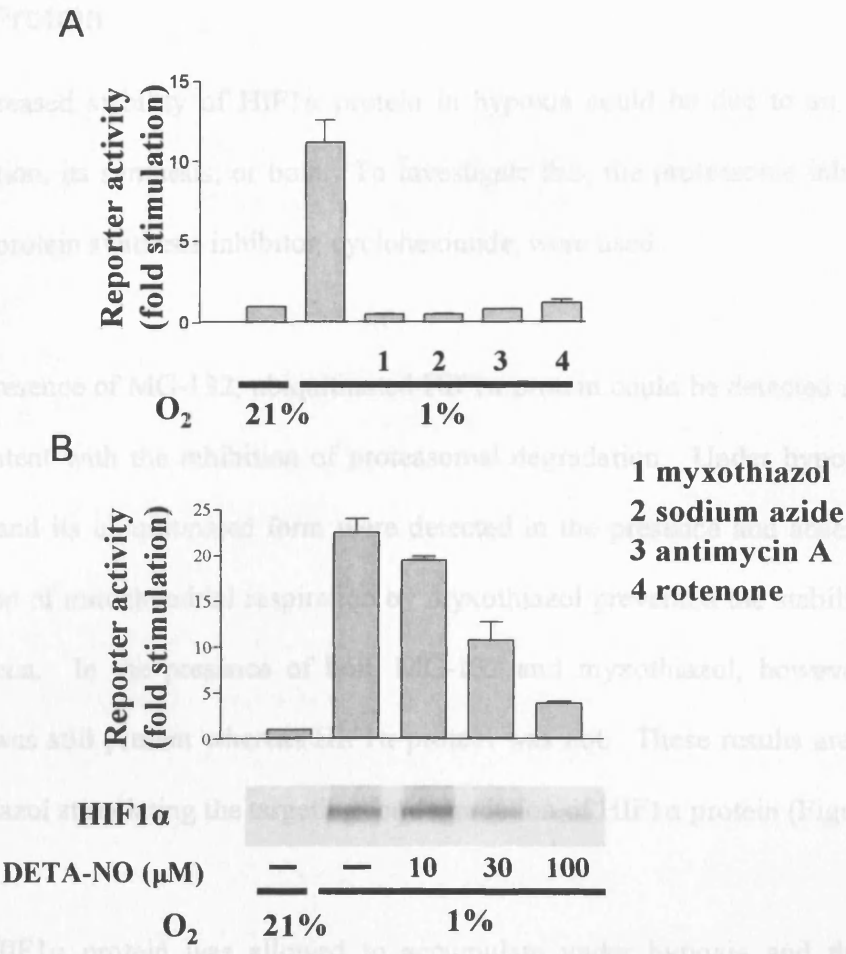

Figure 43. Inhibition of mitochondrial respiration prevents hypoxia-dependent HIF1α stabilisation. (A) HIF1α-dependent gene reporter activity was measured at 21% and 1% O_2 in the presence of inhibitors, as indicated. (B) HEK293 cells were incubated at 1% O_2 for 3h (HIF1α protein) or 5h (HIF1α reporter activity) in the absence or presence of the NO donor DETA.

3.4.2 Destabilisation of HIF1α is Due to Increased Degradation of the Protein

The increased stability of HIF1α protein in hypoxia could be due to an alteration in its degradation, its synthesis, or both. To investigate this, the proteasome inhibitor, MG-132, and the protein synthesis inhibitor, cycloheximide, were used.

In the presence of MG-132, ubiquitinated HIF1α protein could be detected at 21% O_2. This is consistent with the inhibition of proteasomal degradation. Under hypoxia, both HIF1α protein and its ubiquitinated form were detected in the presence and absence of MG-132. Inhibition of mitochondrial respiration by myxothiazol prevented the stabilisation of HIF1α in hypoxia. In the presence of both MG-132 and myxothiazol, however, ubiquitinated HIF1α was still present whereas HIF1α protein was not. These results are consistent with myxothiazol stimulating the targeting for degradation of HIF1α protein (Figure 44A).

When HIF1α protein was allowed to accumulate under hypoxia and then myxothiazol added, the protein was completely degraded within 45min. The addition of cycloheximide to prevent protein synthesis, however, resulted in a much slower rate of HIF1α disappearance, which was still visible after 90min (Figure 44B). The difference in the rates of HIF1α protein destabilisation indicates that myxothiazol does not act by inhibiting the synthesis of the transcription factor.

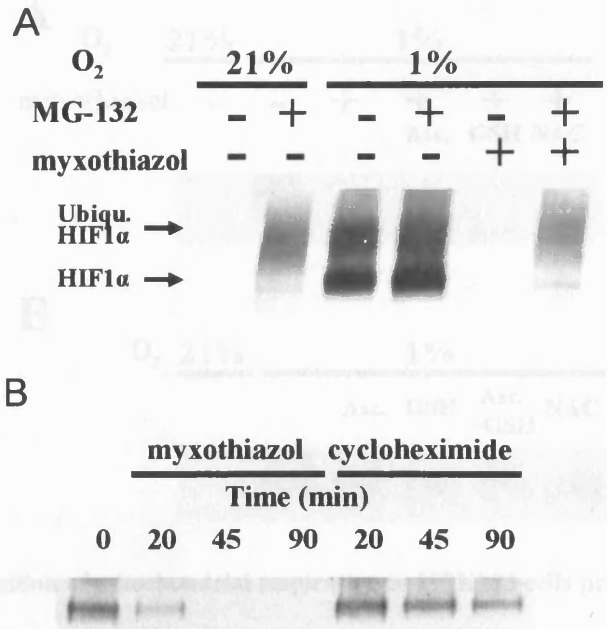

Figure 44. Inhibition of mitochondrial respiration in HEK293 cells prevents HIF1α stabilisation by promoting HIF1α degradation. (A) High-molecular-weight ubiquitinated HIF1α protein species were detected by Western blotting with a monoclonal antibody after incubation of cells at 21% or 1% O_2 for 3h in the presence or absence of 1μM myxothiazol and 25μM MG-132, as indicated. (B) HIF1α protein was allowed to accumulate to levels shown at time zero by incubating cells for 4h at 1% O_2. Cells were then treated with 1μM myxothiazol or 17.5μM cycloheximide. Cell lysates were obtained after 20, 45 and 90mins and analysed by Western blotting.

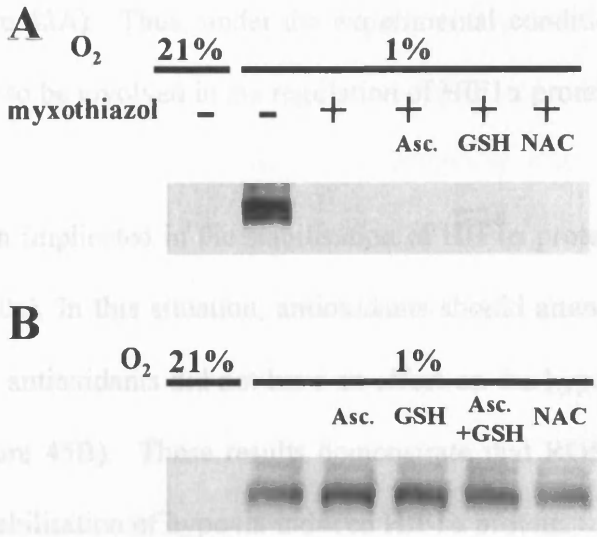

Figure 45. Inhibition of mitochondrial respiration in HEK293 cells prevents HIF1α stabilisation in a ROS-independent manner. Cells were incubated at 1% O_2 for 3 hours in the presence (A) or the absence (B) of 1μM myxothiazol. The antioxidants ascorbate (Asc., 2.5mM), glutathione ethyl ester (GSH, 5 mM), and N-acetylcysteine (NAC, 5mM) were included as indicated. HIF1α protein was determined by Western blotting.

3.4.3 Reactive O_2 Species are not Involved in the Regulation of HIF1α Protein Stability

Reactive O_2 species have been implicated in the regulation of hypoxia-induced HIF1α protein stability. If ROS destabilised HIF1α protein (Huang *et al.*, 1996; Salceda and Caro, 1997) then antioxidants should attenuate this effect. However, in the presence of ascorbate, glutathione ethyl-ester or N-acetylcysteine, myxothiazol still destabilised HIF1α in hypoxia (Figure 45A). In addition, the generation of ROS by mitochondria varies according to the site of the ETC which is inhibited (Waypa *et al.*, 2002; Chen *et al.*, 2003). However, all inhibitors of the ETC destabilised the hypoxia-induced HIF1α protein, regardless of the site

of inhibition (Figure 42A). Thus, under the experimental conditions and in the cells used ROS did not appear to be involved in the regulation of HIF1α protein stability.

ROS have also been implicated in the stabilisation of HIF1α protein (Chandel *et al.*, 1998; Chandel *et al.*, 2000a). In this situation, antioxidants should attenuate the accumulation of the protein. Again, antioxidants did not have an effect on the hypoxia-induced stabilisation of the protein (Figure 45B). These results demonstrate that ROS are not involved in the stabilisation or destabilisation of hypoxia-induced HIF1α protein.

3.4.4 Inhibition of Respiration in Hypoxia Increases Intracellular O_2 Availability

In order to investigate if the inhibition of mitochondrial respiration increases the intracellular O_2 concentration, Renilla luciferase, whose activity is dependent on its substrates coelenterazine (added in excess) and O_2 only, was targeted to the mitochondria of HeLa cells. The activity of this enzyme was found to be reduced by decreasing O_2 concentrations. At 5% O_2 the activity of the immunoprecipitated enzyme was significantly inhibited by 25% compared with control enzyme at 21% O_2. Further inhibition (80%) was observed at 1% O_2 (Figure 46A). In intact cells the activity was significantly inhibited by (55%) at 5% O_2 and was further inhibited (by 85%) at 1% O_2 (Figure 46B). Thus, the activity of the Renilla luciferase enzyme was correlated with the O_2 concentrations.

Cells containing Renilla luciferase and incubated at 1% O_2 were then treated with NO, from DETA (1-100μM), to inhibit respiration and the activity of the enzyme was measured. The activity of the luciferase was negligible in hypoxia in the absence of DETA. However, it

was found to be stimulated by the addition of increasing concentrations of DETA in hypoxia (Figure 47). In contrast, DETA had no significant effect on the activity of Renilla luciferase when the cells were incubated at ambient O_2 concentrations, eliminating the possibility that NO interacted with the enzyme. These observations are consistent with a lack of O_2 availability for luciferase activity under hypoxic conditions and an increase in intracellular O_2 availability upon inhibition of respiration by NO.

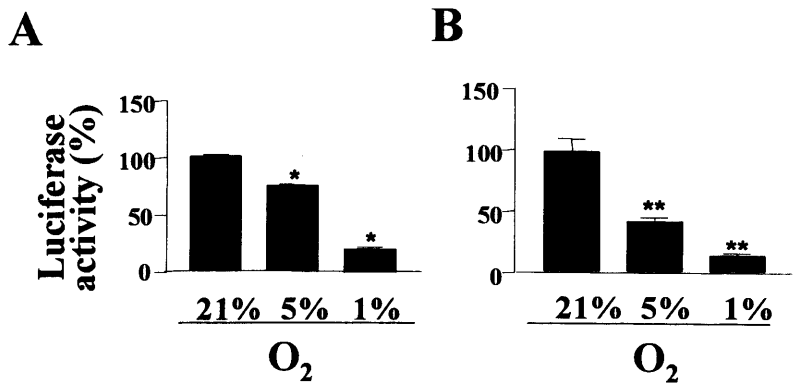

Figure 46. O_2-dependence of Renilla luciferase activity. (A) Immunoprecipitated mitochondrial Renilla luciferase activity is O_2-dependent in vitro. (B) Mitochondrial Renilla luciferase transfected into HeLa cells demonstrates O_2-dependence. (n = 3; single asterisk indicates $P < 0.01$; double asterisks, $P < 0.05$; means ± SEM).

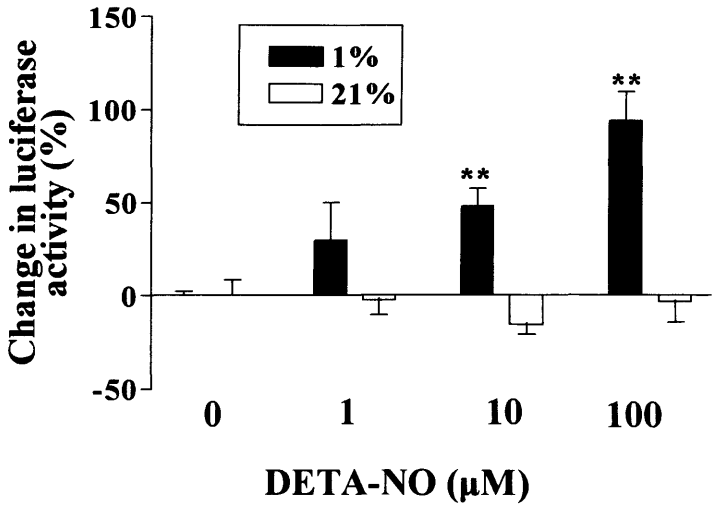

Figure 47. HeLa cells expressing mitochondrially targeted Renilla luciferase were incubated with increasing concentrations of DETA (0 to 100μM) in a hypoxia chamber at 1% O_2. After the addition of the substrate coelenterazine (5μg/ml), luminescence was determined in the hypoxia chamber. NO caused a concentration-dependent increase in luciferase activity. When re-equilibrated to 21% O_2, no effect of DETA-NO on luciferase activity was observed.

3.4.5 Inhibition of Mitochondrial Respiration Prevents Cell Density-Dependent HIF1α Stabilisation

HIF1α-dependent gene reporter activity was measured in HEK293 cells that were seeded onto cell culture plates at different densities in the presence or absence of myxothiazol (1μM) under ambient O_2 concentrations. As shown in Figure 48, increasing the density at which cells were maintained resulted in higher reporter activity, such that a 7-fold increase was observed at the highest density (1.40 x 10^6cells/cm^2) compared with that of the lowest (0.12 x 10^6cells/cm^2). However, in the presence of myxothiazol, the cell density-dependent gene reporter activity was abolished.

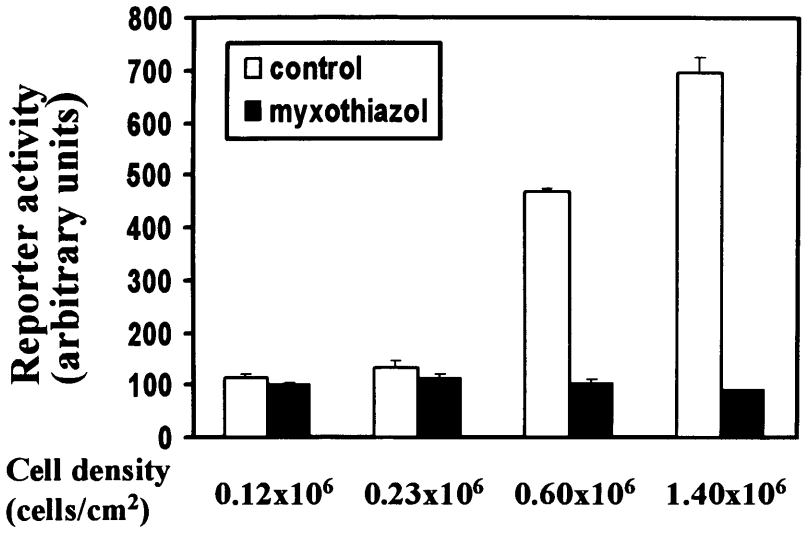

Figure 48. Inhibition of mitochondrial respiration prevents cell density-dependent HIF1α stabilisation. HIF1α-dependent gene reporter activity was measured in cells that were seeded onto culture plates at different densities in the presence or absence of myxothiazol (1μM) under ambient O_2 concentrations.

4 DISCUSSION

4.1 Pharmacological Inhibition of Cellular Oxygen Consumption

Mitochondria are responsible for approximately 90% of a cell's O_2 consumption (Brown, 1999; Piantadosi, 2002). Thus, inhibition of the ETC would be expected to significantly reduce cellular O_2 utilisation. In the present study, this was investigated by treatment of $RAW_{264.7}$ cells with rotenone (a complex I inhibitor; Lambert and Brand, 2004), myxothiazol (a complex III inhibitor; Raha *et al.*, 2000) or sodium azide (a complex IV inhibitor; Petersen, 1977).

Control $RAW_{264.7}$ cells respired at a constant of rate of 13.97μM O_2/min, between 200μM and ~10μM O_2, when suspended in incubation medium at a concentration of 10^7cells/ml. Respiration was completely inhibited (>90% reduction) by treatment with rotenone (500nM), myxothiazol (1μM) or sodium azide (5mM). This effect of the inhibitors is consistent with reports that mitochondria are responsible for the majority of cellular O_2 consumption (Brown, 1999; Piantadosi, 2002). Also, the inhibitors, which act at different complexes, illustrate that respiration can be inhibited by blockade of the ETC at various locations and not just at the site of O_2 metabolism (i.e. complex IV). These observations are in agreement with a number of studies that show similar inhibitions with the mitochondrial inhibitors used (Boveris and Chance, 1973; Chen *et al.*, 2003; Hollis *et al.*, 2003).

4.2 Inhibition of Respiration by Treatment with Nitric Oxide

In addition to the pharmacological inhibitors used, cells were also treated with exogenous NO, derived from NO donor compounds, for short (minutes) or long (hours) durations of time and their respiration was measured. NO is a potent inhibitor of complex IV, acting in a manner that is reversible and in competition with O_2 (Brown and Cooper, 1994; Cleeter *et al.*, 1994; Schweizer and Richter, 1994).

4.2.1 Reversible Inhibition of Respiration by Acute Treatment of Cells with Nitric Oxide

The NO donor DEANO has a half-life of 2mins (Keefer *et al.*, 1996) and was used to investigate the acute effects of NO on the respiration of $RAW_{264.7}$ cells. The concentration of DEANO that was used (5μM) produced a maximum of 1μM NO, which is similar to concentrations of NO that have been measured to be produced by activated macrophages (Brown *et al.*, 1998). This quantity of NO completely inhibited the O_2 consumption of the cells, an observation that has similarly been reported by others (Brown *et al.*, 1998). Furthermore, when the NO was broken down, respiration returned back to the control rate. Thus, the inhibition of respiration was reversible.

The mechanism by which NO was broken down may be due to a combination of different phenomena. It has been proposed that NO can be metabolised by complex IV of the ETC to produce NO_2^- (Torres *et al.*, 1995; Cooper *et al.*, 1997). However, it is not clear whether this is a major mechanism. NO also reacts rapidly with O_2^- at a near-diffusion-limited rate ($6.7 \times 10^9\ M^{-1}s^{-1}$; Huie and Padmaja, 1993). It has been shown that the addition of SOD, to

metabolise O_2^- into H_2O_2, noticeably increases the amount of NO (by approximately 100nM) detected in submitochondrial particles treated with DETA (Riobo *et al.*, 2001). This experiment shows that O_2^-, which is likely to be produced by the ETC, may be a significant mechanism by which NO is metabolised. In addition, NO can react directly with O_2. The rate at which this reaction occurs is thought to be slow at physiological concentrations of NO (10nM to 1μM) such that the half life of NO, due to the reaction with O_2, has been estimated to range from 9 to 900mins depending on its concentration (Beckman *et al.*, 1996). However, it has been reported that this reaction is increased by 300-fold in hydrophobic membranes (Liu *et al.*, 1998). This could make the reaction between NO and O_2 a significant pathway for the metabolism of NO and could explain why, at lower concentrations of O_2, more NO was found to be released from the same concentration of DEANO. Recently, it was reported that NO could be consumed by cells via a lipid peroxidation pathway (Keynes *et al.*, 2005). This mechanism was also O_2-dependent and required the presence of ascorbate.

Another interesting observation was that NO could inhibit respiration even when the O_2:NO ratio was high. For example, at 80μM O_2, 1μM NO was found to be sufficient to completely inhibit respiration. This is a phenomenon that has been commented on by others (Boveris *et al.*, 2000; Brown, 2001) and is interesting because both O_2 and NO have similar association rate constants for fully reduced complex IV (10^8 $M^{-1}s^{-1}$ and 0.4-1.0 x 10^8 $M^{-1}s^{-1}$ respectively; Babcock and Wikstrom, 1992; Blackmore *et al.*, 1991), yet NO is able to completely inhibit the enzyme even when there is a relative abundance of O_2. A mechanistic explanation for this has been proposed in which NO initially binds onto the Cu_B site of the binuclear centre giving it a kinetic advantage over O_2 for the binding to the haem

a_3 (Torres *et al.*, 1995). Alternatively, it has been suggested that NO is able to inhibit complex IV potently because it is capable of binding to the partially reduced enzyme, whereas O_2 can only bind onto the fully reduced enzyme (Antunes *et al.*, 2004). Thus, NO is able to bind to, and inhibit, complex IV before the enzyme becomes available for O_2. Nevertheless, the precise explanation for the high potency of NO as an inhibitor of complex IV, even at high O_2 concentrations, remains to be clearly defined.

4.2.2 Persistent Inhibition of Respiration Caused by Prolonged Treatment with NO

Although NO reversibly inhibited cellular respiration upon acute exposure, it also caused a persistent inhibition of respiration in cells following prolonged treatment. The NO donor DETA (2.5mM) has a half-life of 20hrs (Keefer *et al.*, 1996) and released NO at a steady concentration of 0.85μM, which was sufficient to inhibit cellular respiration completely. Under this treatment, $RAW_{264.7}$ cells were exposed to NO for up to 4hrs. Initially, the inhibition of respiration could be reversed back to the control rate upon the addition of oxyHb (20μM), which rapidly scavenged the NO released. After 2h of exposure, however, respiration could not be completely reversed by oxyHb and remained inhibited by 20.2%. After 4h of exposure to NO, respiration was persistently inhibited by 63.8% after the addition of oxyHb. Similar patterns of events have been reported by others in a variety of cells (Clementi *et al.*, 1998; Beltran *et al.*, 2000; Orsi *et al.*, 2000).

The explanation for these observations was proposed to be due to the S-nitrosylation of complex I of the ETC. In agreement with these studies, the addition of glutathione ethyl-ester reversed the persistent effects caused by NO, a characteristic indicative of S-

nitrosylation (Bauer *et al.*, 1995). In addition, complex I activity decreased concomitantly with the onset of the persistent inhibition of respiration, which could also be prevented by glutathione ethyl-ester. Recently, it was shown that there is a direct correlation between the rate of cellular O_2 consumption and complex I activity in activated macrophages (Frost *et al.*, 2005).

In contrast, others have reported an irreversible inhibition of respiration that was attributed to nitration by $ONOO^-$ (Bolanos *et al.*, 1994; Riobo *et al.*, 2001; Carreras *et al.*, 2004). In these studies the addition of SOD could prevent irreversible inhibition, by metabolising O_2^- and preventing the formation of $ONOO^-$, and they therefore concluded that the causative agent was $ONOO^-$. Although $ONOO^-$ is capable of both nitrosylation and nitration (Radi *et al.*, 2002), the work done by Clementi *et al* suggests that $ONOO^-$ is not involved. This is because scavengers of $ONOO^-$ did not prevent the persistent inhibition and no ROS were detected, arguing against the presence of $ONOO^-$. Furthermore, cold light and glutathione ethyl-ester could reverse this inhibition, a phenomenon that is consistent with S-nitrosylation (Bauer *et al.*, 1995). Interestingly, it has been reported that both S-nitrosylation and nitration of complex I may occur in concert in activated macrophages (Frost *et al.*, 2005). It was shown that an early NO-dependent inhibition of complex I activity could be prevented by the addition of reduced glutathione ethyl-ester, indicating S-nitrosylation. The ability of glutathione ethyl-ester to block this inhibition diminished with time and was coupled with the appearance of nitrotyrosine residues, indicating protein nitration. Thus, the manner by which complex I is inhibited by NO exhibits temporal changes. In the $RAW_{264.7}$ cells used in my experiments, it is possible that continued exposure to NO would result in the nitration of complex I, and the inability of glutathione ethyl-ester to prevent this inhibition.

The significance of the S-nitrosylation and inhibition of complex I remains to be clarified. It has been demonstrated that inhibition of complex I has detrimental effects upon cells and induces apoptosis (Seaton *et al.*, 1998; Barrientos and Moraes, 1999). In addition, S-nitrosylation may be involved in pathophysiological situations that occur when cells are under oxidative stress (Beltran *et al.*, 2000). In contrast, it has been suggested that nitrosylating reactions may be involved in cellular signalling events (Stamler, 1994). Furthermore, S-nitrosylation has been proposed to modulate the activity of GAPDH (Padgett and Whorton, 1995), creatine kinase (Wolosker *et al.* 1996) and the ryanodine receptor (Xu *et al.*, 1997). Thus, whether S-nitrosylation of complex I is a physiological or pathophysiological phenomenon remains to be determined.

4.3 Inhibition of Cellular Respiration in Activated $RAW_{264.7}$ Cells

In previous experiments, exogenous NO, through the use of NO donors, was used to investigate the effects of NO on cellular respiration. However, these donors may not release NO in the quantities, nor with the kinetics, that reflects endogenous production. In addition, some NO donors may release other chemical species which may confound experiments (Yamamoto and Bing, 2000). Sodium nitroprusside, for example, releases the cyanide anion (Ruan *et al.*, 1999), which is also a known inhibitor of complex IV. As a result, exogenous NO donors may not be suitable for experiments on cellular respiration. Cells can be stimulated, however, to synthesise NO for prolonged periods, such that the acute and long-term effects of endogenous NO on cellular respiration can be investigated.

Although murine macrophage cells can be stimulated to express iNOS with relative ease, stimulation of human macrophages is comparatively difficult. The poor reproducibility or minimal response of human macrophages, even following treatment with a variety of cytokines, has led to the suggestion that iNOS induction in these cells may not be important in humans. Nevertheless, iNOS has been shown to be present in other human phagocytic cells, such as neutrophils, and an increase in nitrite and nitrate is observed during infections (Harvey, 2000).

4.3.1 Reversible Inhibition of Respiration in Activated $RAW_{264.7}$ Cells

$RAW_{264.7}$ cells activated with LPS and IFNγ expressed iNOS and produced NO in micromolar quantities (see also Brown *et al.*, 1998 and Connelly *et al.*, 2003). 6h post-activation, the cells produced NO in sufficient quantities to completely inhibit respiration.

Reports by others (Brown *et al.*, 1998) have also observed similar inhibitions in activated cells. This inhibition was completely abolished upon addition of the NOS inhibitor L-NMMA and the cells respired at a similar rate to the non-activated controls. Thus, the inhibition of respiration was attributed to NO synthesised from iNOS and was reversible at this 6h timepoint. In addition, cells activated in the presence of L-NIO, which inhibited NO synthesis throughout the experiment, respired at a similar rate to the control. This further supports the observation that NO was responsible for the inhibition of respiration.

The upregulation of iNOS was found to be significant 4h after activation of the $RAW_{264.7}$ cells. However, nitrite concentrations were not significantly elevated until 12h post-activation. This discrepancy can be attributed to the relative insensitivity of the Griess assay that was used to measure nitrite concentrations. It has been reported that the Griess assay is able to detect nitrite concentrations with a sensitivity limit of 0.1-1.0μM (Nagano, 1999). At 4h post-activation, it is possible that the nitrite, produced from the metabolism of NO, was below this detection limit. At 6h post-activation, however, enough nitrite had accumulated to allow the detection of a significant elevation in the medium. In contrast, the NO electrode has a detection limit of 1nM (Zhang and Broderick, 2000) and is, therefore, more sensitive in the detection of NO.

Although the nitrite concentrations were measured, nitrate, the other stable metabolite of NO, was not. The total nitrate and nitrite concentrations would have provided a better indication of NO synthesis and possibly have resolved the discrepancy between the expression of iNOS and the detected increase in NO synthesis indicated by the Griess assay.

The rate of increase in nitrite concentrations was linear between 6 and 18h post cell activation. Between 18 and 24h, however, the rate decreased. This could be due to a decrease in the availability of L-arginine in the medium. L-arginine is a substrate for NO synthesis and is metabolised as NO is produced. As the concentration of L-arginine decreases it is reasonable to expect that the rate of NO, and therefore nitrite, synthesis would similarly decrease. In addition, the enzyme arginase is upregulated following cell activation (Chicoine *et al.*, 2004). This enzyme has a higher activity in metabolising L-arginine than does NOS and it has been shown that inhibition of arginase can significantly increase the synthesis of NO by NOS (Chang *et al.*, 1998). Furthermore, it has been reported that 90% of L-arginine is consumed by arginase, whereas the remaining 10% is consumed by NOS (Hrabak *et al.*, 1994). This would help to explain why extracellular L-arginine is required for iNOS activity in activated cells.

Nevertheless, reduction in the availability of other necessary cofactors or cosubstrates for NOS activity can also not be discounted. Alternatively, the activity of iNOS could have become inhibited by NO. It has been shown that the activity of iNOS is decreased in the presence of NO (Assreuy *et al.*, 1993). The mechanism by which this occurs was described as being due to the formation of a haem-NO complex within the enzyme. This would have the subsequent effect of increasing the K_m of the enzyme for O_2 and inhibiting the consumption of NADPH and the production of citrulline, all indicative of an inhibition of the enzyme's activity.

Cells that were activated in the presence of the NOS inhibitor L-NIO did not synthesise NO, as determined by the Griess assay, up to the 18h timepoint. Between 18 and 24h, however,

an increase in the concentration of nitrite in the medium of the cells was detected, suggesting iNOS activity. For this reason, experiments measuring respiration in cells activated in the presence of L-NIO were measured at the 12h timepoint when no NO was detected by either the Griess assay or by the NO electrode.

4.3.2 Persistent Inhibition of Respiration in Activated $RAW_{24.7}$ Cells

At 12h after cell activation, the inhibition of respiration could not be completely reversed upon blockade of NO synthesis by the addition of L-NMMA, indicating that cellular O_2 consumption had been inhibited persistently. Since the onset of persistent inhibition was accompanied by a decrease in complex I activity in cells treated with exogenous NO, complex I activity was also measured in the activated $RAW_{264.7}$ cells. In agreement with previous experiments using exogenous NO, complex I activity of activated cells was significantly decreased (by 62%). Since the activity of complex I in cells activated in the presence of L-NIO did not change, this effect can be attributed to NO or one of its derived species.

As discussed previously, the mechanism by which the complex was inhibited is likely to involve S-nitrosylation. This is supported by the effects of glutathione ethyl-ester, which prevented the inhibition of complex I. However, it remains possible that complex I may also be nitrated at a later stage, as has been proposed (Frost *et al.*, 2005)

It was also observed that cells activated for 12h in the presence of L-NIO respired less than L-NIO-treated control cells by 20%. This inhibition was not due to NO because no NO synthesis was detected by the NO electrode or by the Griess assay. Also, this inhibition was

not due to an inhibition of complex I, since the activity of this enzyme was not altered. However, measurement of complex IV activity showed that it was significantly inhibited suggesting an alternative mechanism.

4.3.3 Expression of Haem Oxygenase-1 in Activated $RAW_{264.7}$ Cells

Complex IV activity of $RAW_{264.7}$ cells activated in the presence of L-NIO for 12h was inhibited by 68%. It has been reported that activated cells are induced to express the HO-1 protein (Wiesel *et al.*, 2000; Srisook and Cha, 2004) and that exogenously-added CO can bind to and inhibit complex IV as the isolated enzyme (Petersen, 1977) or in intact cells (Alonso *et al.*, 2003). Therefore, HO-1 protein expression was investigated in my activated $RAW_{264.7}$ cells and, in agreement with other reports (Wiesel *et al.*, 2000; Srisook and Cha, 2004), was found to be elevated. Thus, it was hypothesised that HO-1-derived CO was responsible for the NO-independent inhibition of complex IV and respiration in the activated cells. However, it has not previously been thoroughly investigated whether or not exogenous or endogenous CO, produced by HO enzymes, is able to inhibit cellular respiration.

Although both complex I and IV activities were measured, it would have been beneficial to have measured the activities of complex II and III. This would have provided a more complete picture of the changes in the mitochondrial ETC and would have shown if the changes observed were specific for the complexes investigated. This is particularly important since contrasting effects upon the different complexes have been reported (Bolanos *et al.*, 1994; Beltran *et al.*, 2000).

4.4 Inhibition of Cellular Respiration by Carbon Monoxide

4.4.1 Carbon Monoxide Gas Inhibits Cellular Respiration

When HEK293 cells were treated with solutions of CO gas, the rate of O_2 consumption was found to be reduced in a dose-dependent manner. Similar observations have been reported by others (Alonso *et al.*, 2003). Furthermore, this inhibition was greater when cells were incubated under hypoxic conditions. From my data I have calculated that the K_i of CO for complex IV was 1.44μM following incubation under ambient O_2 concentrations. This was decreased to 0.35μM at 1% O_2, indicating that complex IV has a greater affinity for CO under hypoxic conditions. These values are similar to those previously reported for purified complex IV and for complex IV contained in proteoliposomes (Petersen, 1977; Hansen and Nicholls, 1978). They indicate that CO is a much less potent inhibitor of complex IV than is NO, which has a reported IC_{50} of 60nM at 30μM O_2 (Brown and Cooper, 1994) and a K_i of 27nM (Koivisto *et al.*, 1997). In addition to the different affinities of complex IV for NO and CO, the redox state of cytochrome a3 could also determine the potency of CO to inhibit the enzyme. CO is only able to bind to complex IV when cytochrome a_3 is reduced (in the ferrous form), whereas NO is capable of binding to complex IV when cytochrome a_3 is in the reduced or oxidised form. Under low O_2 concentrations the ETC is in a more reduced state (Duranteau *et al.*, 1998; Palacios-Callender *et al.*, 2004), which may favour the inhibition of complex IV by CO. This may explain, at least in part, the increased potency of CO as an inhibitor of complex IV in hypoxia.

The inhibition of respiration that was observed by treating cells with 20μM CO lasted for at least 30mins. However, the rate of dissociation ('k_{off}') of CO from complex IV has been reported to be 2 x $10^{-2}s^{-1}$ (Erecinska and Wilson, 1980), a value that is similar to that of NO (1-13 x $10^{-2}s^{-1}$; Antunes *et al.*, 2004), and the inhibition due to NO is rapidly reversible. Thus, there is a discrepancy between the duration of the observed respiratory inhibition by CO, and its reported kinetics with complex IV. Nevertheless, others have shown that the inhibition of complex IV activity by CO persists for several days (Miro *et al.*, 1998). Similarly, the binding of CO to haemoglobin, another haem protein, has been reported to last for over 6 hrs. In addition, the rate of dissociation of CO from myoglobin has been reported to be significantly slower than that of O_2 and NO (Gibson *et al.*, 1986).

In contrast, however, the rates of association ('k_{on}') of both O_2 and NO have been reported to be greater than that of CO (1 x $10^8M^{-1}s^{-1}$ for O_2, 0.4-1.0 x $10^8M^{-1}s^{-1}$ for NO and 8 x $10^4M^{-1}s^{-1}$ for CO; Babcock and Wikstrom, 1992; Blackmore *et al.*, 1991; Erecinska and Wilson, 1980). Thus, the association of O_2 or NO to complex IV is approximately 3-4 orders of magnitude greater than that for CO, suggesting that O_2 and NO are more dynamic than CO for the binding to the enzyme.

4.4.2 Haem Oxygenase-1-Derived Carbon Monoxide Inhibits Cellular Respiration

HEK293 cells were transfected with the human HO-1 gene and expression was controlled by a tetracycline-dependent promoter. Induction of the protein following treatment with tetracycline was verified by Western blotting and the enzyme activity was indicated by an increase in bilirubin concentration in the cellular medium.

Induced HEK293-HO-1 cells were able to produce CO in sufficient quantities to inhibit cellular respiration. This was accompanied by an inhibition of complex IV activity which is consistent with CO binding to, and inhibiting, the enzyme. As with exogenous CO, the inhibition of respiration was also increased by incubation at 1% O_2. Since HO-1 is an O_2-dependent enzyme (Tenhunen *et al.*, 1969), the effect of lowering the O_2 tension to 1% on the enzyme's activity was investigated. We discovered that there was no change in HO-1 activity, as assessed by the accumulation of bilirubin. The K_m of HO-1 for O_2 has been reported to be 12μM (Piantadosi, 2002) and in our system we observed no significant change in enzyme activity by lowering the O_2 concentration to 1% (10μM). In addition, the expression of the enzyme in these cells was unchanged by incubation of the cells at different O_2 concentrations. Thus, the increased inhibition of respiration observed under hypoxic conditions was likely to be due to an increased binding of CO to complex IV and not due to a change in the expression or activity of HO-1.

4.4.3 Activated $RAW_{264.7}$ Cells Synthesise Bilirubin and Exhibit an Inhibition of Respiration

$RAW_{264.7}$ cells activated in both the presence and absence of L-NIO upregulated the expression of HO-1 protein and produced significantly greater quantities of bilirubin compared with the controls, as measured by the accumulation of bilirubin in the cellular medium. This indicates that the HO-1 protein was active and producing CO, since the enzyme synthesises bilirubin and CO in stoichiometric quantities (Tenhunen *et al.*, 1969). In addition, activated cells that were allowed to produce NO (i.e. cells that were activated in the absence of L-NIO) synthesised significantly more bilirubin than cells that could not

produce NO. This was unexpected because NO has been reported to be capable of inhibiting HO enzymes (Ding *et al.*, 1999). Since NO is a known inducer of HO-1 protein (Foresti *et al.*, 1997; Bouton and Demple, 2000; Srisook and Cha, 2004) it is possible that NO induced a higher expression of the enzyme resulting in a greater total activity. However, the upregulation of HO-1 was found to be delayed (till 12h) in the presence compared with the absence of NO, when the protein was upregulated 6h post-activation. The explanation for an apparent greater HO-1 activity in the presence of NO is as yet unclear and remains to be investigated.

As discussed previously, the induction of HO-1 was hypothesised to be responsible for the NO-independent inhibition of respiration in activated $RAW_{264.7}$ cells. Consistent with previous experiments involving exogenous or endogenous CO, the hypoxic incubation of cells significantly increased (to 60%) this inhibition, providing further evidence that CO may be involved in the inhibition of cellular O_2 consumption. In addition, complex IV activity was inhibited by 66%, and this was likely to have contributed directly to the decreased O_2 consumption. Again, these observations are in agreement with the hypothesis that CO is responsible for the inhibition of respiration observed.

4.4.4 Reduction of NO, but not CO, Synthesis by Hypoxia in Activated $RAW_{264.7}$ Cells

When $RAW_{264.7}$ cells were activated under ambient conditions in the absence of L-NIO, nitrite, a metabolite of NO, accumulated in the culture medium. The concentration of NO, measured by an NO electrode, produced by the cells was approximately 3μM at 12h post-activation. Under hypoxic conditions the nitrite produced was 6.5-fold less, suggesting that

O_2 was limiting for NO synthesis. These results are consistent with previous reports which show that iNOS activity is affected by the availability of O_2. The K_m of iNOS in cells for O_2 has been shown to be 137μM (McCormick *et al.*, 2000). In contrast, it has been reported that the K_m of the purified iNOS protein for O_2 is much lower, at 6μM (Rengasamy and Johns, 1996). In our cells, however, we obtain a value of between 20-30μM for the K_m of iNOS for O_2. This difference from the value reported by McCormick *et al.* may be due to the maintenance of our cells in biological stirrer bottles, which could provide a more homogeneous O_2 concentration throughout the medium than in culture plates. It is also greater than the value reported by Rengasamy and Johns. However, their system involved the use of the purified enzyme with the addition of exogenous substrates and cofactors. This may not reflect the availability of the constituents for NO synthesis within a cell.

In addition, HO-1 activity was found to be unaffected by hypoxic incubation in the HEK293-HO-1 cells. A similar observation was seen in the $RAW_{264.7}$ cells activated in the presence of L-NIO. This would suggest that at low O_2 tensions CO, produced by HO-1, might be more capable of inhibiting mitochondrial respiration whereas the effects of NO, due to the inactivation of iNOS, might be less significant.

However, cells that were activated in the absence of L-NIO did exhibit a difference in HO-1 activity depending on the O_2 concentration, as indicated by the accumulation of bilirubin in the medium of cells. Under ambient O_2 concentrations, 35% more bilirubin was produced by activated cells compared with cells activated at 1% O_2. Since HO-1 activity is independent of the O_2 concentration, as previously shown, the difference in the bilirubin concentrations is possibly due to the presence of NO. As described previously, a greater

quantity of bilirubin accumulated in the medium of cells in the presence of NO than in the absence. Similarly, under hypoxic conditions, where less NO is being produced, less bilirubin is synthesised.

4.4.5 Hypoxia Induces Haem Oxygenase-1 which is accompanied by an Inhibition of Respiration

Hypoxic incubation alone induced the expression of HO-1 protein in $RAW_{264.7}$ cells, an observation that has also been reported by others (Morita *et al.*, 1995). The induction of the enzyme was accompanied by a decrease in the rate of respiration. This phenomenon was not observed in the non-induced HEK293-HO-1 cells, which did not upregulate HO-1 in response to hypoxia. In addition, complex IV activity was measured in $RAW_{264.7}$ cells incubated in hypoxia for 12h and was found to be decreased by 44% compared with non-hypoxic controls. This is consistent with our hypothesis that CO inhibits respiration, particularly under low O_2 concentrations, through inhibition of complex IV. Previous reports have similarly described a decrease in complex IV activity caused by hypoxia (Chandel *et al.*, 1996). It was hypothesised that this may be due to an allosteric alteration to the conformation of complex IV caused by the absence of O_2 (Chandel *et al.*, 1996), such that the enzyme was modified. However, this does not explain the unchanged rate of respiration in the non-induced HEK293-HO-1 cells following hypoxic incubation.

4.5 Modulation of the Hypoxic Induction of Hypoxia Inducible Factor 1α Protein Stability by Mitochondria

Previous observations have shown that the inhibition of the mitochondrial ETC results in the inhibition of cellular respiration, demonstrating that mitochondria are responsible for the vast majority of a cell's O_2 consumption. However, there are other O_2-dependent processes within a cell, which may compete with mitochondria for the common substrate. When O_2 is plentiful, enough is available for these other processes to occur. When O_2 becomes limiting, however, non-mitochondrial O_2-requiring processes may become inhibited because complex IV preferentially consumes the available O_2 due to its low K_m (high affinity; Gnaiger and Kuznetsov, 2002; Cooper *et al.*, 2003) for O_2. If respiration is inhibited, however, then the O_2 would be expected to become available for other processes. The effect of the inhibition of respiration under hypoxic conditions was therefore investigated with respect to the stability of HIF1α, a protein whose degradation is known to be an O_2-dependent process (Ivan *et al.*, 2001; Jaakkola *et al.*, 2001).

4.5.1 Inhibition of Respiration Prevents the Hypoxic Induction of Hypoxia Inducible Factor 1α Protein and its Transcriptional Activity

HEK293 cells incubated in hypoxia (1% O_2) for 3h stabilised the HIF1α protein, as determined by Western blotting. This was similar to the observations reported by other groups (Berra *et al.*, 2003; Mateo *et al.*, 2003; Appelhoff *et al.*, 2004). This stabilisation was prevented when the cells were incubated in the presence of the respiratory inhibitors rotenone, myxothiazol, antimycin A, sodium azide or NO. Therefore, inhibition of the ETC

at complex I, III or IV prevented the hypoxic stabilisation of HIF1α. These observations have also been previously reported by others (Mateo *et al.*, 2003). Furthermore, the transcriptional activity of HIF1α was similarly prevented by respiratory inhibition, which is consistent with the destabilisation of HIF1α protein. Thus, active (O_2-consuming) mitochondria are required for HIF1α protein stabilisation and its subsequent transcriptional activity, whereas inactivated mitochondria prevents the transcription factor's stabilisation and activity. In contrast, it has been reported that active mitochondria are not required for HIF1α stabilisation (Srinivas *et al.*, 2001). However, in these experiments, 0.5% O_2 was used for the hypoxia treatment. This low O_2 concentration may have been too low for even the O_2-dependent prolyl hydroxylases to function, resulting in the inhibition of degradation and subsequent stabilisation of HIF1α.

Contrasting effects of NO on the stabilisation of HIF1α have been reported. In fact both stabilisation (Sandau *et al.*, 2000; Sumbayev *et al.*, 2003) and destabilisation (Sogawa *et al.*, 1998; Huang *et al.*, 1999) of the protein has been described. These studies often use a variety of NO donors which release NO at a range of concentrations (which were rarely measured), and which may release other interfering species. Thus, it is difficult to reconcile the reported effects of NO with the effects upon HIF1α protein stability. Recently, however, it has been shown that NO has a biphasic effect upon HIF1α stability. Using cells that produced controlled concentrations of NO it was demonstrated that at low concentrations (<400nM) NO inhibited the hypoxia-induced stabilisation of HIF1α, whereas at high concentrations (>1μM) NO stabilised HIF1α even under non-hypoxic conditions (Mateo *et al.*, 2003). It was concluded that the destabilising effect of NO on HIF1α protein stability

was due to its inhibitory effect upon the ETC, whereas the stabilising effect was due to an as yet undetermined non-mitochondrial phenomenon.

4.5.2 Destabilisation of Hypoxia Inducible Factor 1α is due to Increased Degradation of the Protein

The destabilisation of HIF1α protein could be due to an increase in its degradation, a decrease in synthesis, or a combination of both. The results obtained indicate that the inhibition of mitochondrial respiration stimulates the degradation of HIF1α protein, since myxothiazol increased the rate at which HIF1α disappeared compared with cycloheximide, which inhibited protein synthesis (Figure 44). The mechanism by which HIF1α is degraded involves the prolyl hydroxylation of the protein, interaction with pVHL, ubiquitination and then subsequent degradation by the 26S proteasomes (Giaccia *et al.*, 2003; Semenza, 2003). It has been reported that this pathway is regulated by the initial prolyl hydroxylation of the protein, a reaction that is regulated by the availability of O_2. These prolyl hydroxylase enzymes have been shown to have a high K_m (low affinity) for O_2, making them good O_2 sensors (Hirsila *et al.*, 2003). Therefore, at low O_2 concentrations they are likely to be inactivated as a result of their low affinity for the substrate, resulting in decreased degradation and subsequent stabilisation of HIF1α. If O_2 was made available again, the prolyl hydroxylases could be reactivated, initiating the targeting for degradation of HIF1α. Thus, the stimulation of degradation of HIF1α protein by myxothiazol is consistent with the idea that the inhibition of respiration increases the availability of intracellular O_2.

4.5.3 Inhibition of Mitochondrial Respiration in Hypoxia Increases the Intracellular O_2 Availability

The activity of the enzyme Renilla luciferase requires only O_2 and coelenterazine. When coelenterazine was available in excess, the enzyme's activity was found to be directly related to the concentration of O_2 both in the immunoprecipitated enzyme and when transfected into HeLa cells. Thus, the activity of the luciferase could be used as a monitor of intracellular O_2 concentrations. When respiration was inhibited by NO in cells containing the luciferase, the enzyme's activity was significantly increased, as determined by an increase in luminescence. This indicates that there was an increase in the availability of intracellular O_2. This increase in O_2 could reactivate the prolyl hydroxylase enzymes, which were previously inhibited, explaining how the inhibition of respiration destabilises HIF1α protein by stimulating its degradation. A similar mechanism of O_2 diversion has been reported in firefly flashing experiments whereby NO increased the bioluminescence of fireflies by inhibiting respiration and diverting O_2 towards the peroxisomes (Trimmer *et al.*, 2001). When cells were treated with NO under ambient O_2 concentrations, however, no change in luciferase activity was detected. This shows that NO does not interact directly with the luciferase enzyme and that under ambient conditions, O_2 was not limited.

In support of this mechanism, HEK293 cells incubated at higher densities exhibited greater HIF1α-dependent gene reporter activity. Cells at a higher density would consume more O_2 from their environment causing local hypoxia and stabilisation of HIF1α. The presence of myxothiazol, however, inhibited mitochondrial respiration, preventing O_2 consumption and hypoxia. This was mirrored by the abolition of the cell density-dependent activation of the

gene reporter activity. In addition, cells lacking a functional mitochondrial ETC only stabilise HIF1α at lower O_2 concentrations (Mateo *et al.*, 2003).

4.5.4 The Destabilisation of HIF1α does not Involve Reactive O_2 Species

Reactive O_2 species have been proposed to be responsible for the stabilisation of HIF1α (Chandel *et al.*, 2000a; Agani *et al.*, 2000). In this model, it was suggested that the increase in mitochondrial ROS production occurred under hypoxia as a result of decreased complex IV activity (Duranteau *et al.*, 1998) and this signalled for HIF1α protein stabilisation. However, in the HEK293 cells used, a variety of antioxidants (ascorbate, glutathione ethyl-ester and N-acetylcysteine) did not have an effect upon hypoxia-induced HIF1α protein stabilisation. In contrast, ROS, produced by NADPH oxidase, have been claimed to be the signal for the stabilisation of HIF1α. Under hypoxic conditions, the activity of this enzyme was proposed to decrease (due to a fall in available O_2), resulting in the decreased production of ROS and providing the signal for HIF1α protein stabilisation. Again, antioxidants did not have an effect on either the stabilisation or destabilisation of HIF1α protein, suggesting that ROS were unlikely to be involved in the regulation of HIF1α in the HEK293 cells used.

Recently evidence has been presented whereby inactivation of the Rieske iron-sulphur protein in complex III of the ETC prevented the formation of mitochondrial ROS in hypoxia (Brunelle *et al.*, 2005). This disruption blocked the stabilisation of HIF1α by hypoxia, indicating that mitochondrially-derived ROS are involved in signalling for the stabilisation of the protein. Furthermore, cells from $p38\alpha^{-/-}$, $MKK3^{-/-}$ or $MKK6^{-/-}$ mice failed to stabilise HIF1α in response to hypoxia (Emerling *et al.*, 2005). Reintroduction of p38α restored this

ability. In addition, the overexpression of glutathione peroxidase I, an enzyme that metabolises H_2O_2 into water, abolished the hypoxic activation of the p38 MAPK pathway indicating that H_2O_2 was responsible for conveying the stabilisation of HIF1α via this mechanism. Thus, these reports provide evidence that support the role of ROS in the modulation of HIF1α protein stability.

5 GENERAL CONCLUSION

NO is known to inhibit complex IV of the mitochondrial ETC rapidly and reversibly, preventing O_2 consumption by mitochondria. After prolonged exposure, however, mitochondrial respiration can become persistently inhibited. Although much is known about the effects of exogenous NO on the ETC, relatively little work has been done to demonstrate whether endogenous NO can exert similar effects. Thus, the initial objective of my work was to investigate the role of NO in the respiratory changes that occur in macrophages during inflammatory activation by LPS and IFNγ.

Following cell activation, it was found that the expression of iNOS was upregulated, resulting in the synthesis of micromolar quantities of NO. This NO initially inhibited cellular respiration in a reversible manner, with the rate of O_2 consumption reverting to that of the control when NO synthesis was blocked with L-NMMA. The reversible inhibition was a consequence of NO acting at complex IV of the mitochondrial ETC. Following prolonged exposure to endogenous NO, a phase of persistent respiratory inhibition was observed. This could not be reversed by blocking the synthesis of NO and was associated with a decrease in the activity of complex I. However, this phenomenon could be prevented by the addition of glutathione ethyl-ester, indicating that it was probably due to S-nitrosylation of the enzyme.

In addition, exogenous CO is also known to inhibit complex IV, and HO-1, an enzyme that synthesises this gas, was found to be upregulated in activated cells. Therefore, the possibility that CO contributes to the respiratory inhibition was investigated.

It was discovered that endogenous CO, produced by HEK293 cells transfected with an inducible HO-1 gene, inhibited respiration, albeit mildly. However, this effect was found to be greatly amplified at low O_2 concentrations. In addition, it was observed that activated macrophages exhibited a significant mitochondrial defect under hypoxic conditions, even when the generation of NO is inhibited. The results obtained suggest that this is due to CO, released by HO-1, acting at complex IV. Furthermore, it was observed that HO-1 retained its activity at low O_2 concentrations, whereas iNOS activity was greatly reduced. Thus, mitochondrial dysfunction, which occurs in the hypoxic environment often found at inflammatory sites, may be a consequence of CO rather than NO.

The consequence of inhibition of mitochondrial activity under these low O_2 concentrations was investigated. It was found that the inhibition of cellular respiration in hypoxia resulted in the redistribution of intracellular O_2 from mitochondrial consumption, as measured by an increase in activity of the Renilla luciferase enzyme. The subsequent increase in the availability of O_2 reactivated the prolyl hydroxylase enzymes, resulting in the decrease in stability of hypoxia-induced HIF1α protein, creating a situation in which the cells might fail to register hypoxia. The consequences of this effect are unclear at present, and merit further investigation.

6 FUTURE WORK

Although the inhibition of cellular respiration by NO has been abundantly studied, the inhibition by CO has been given less attention. It is well known that CO can bind to, and inhibit the activity of, isolated complex IV, but only recently has work been reported on the effects upon cellular respiration (Alonso *et al.*, 2003). However, in these experiments, cells were incubated in a CO gas/air mixture where the concentration of CO was in the region of several hundred ppm and it is unclear whether such quantities of CO are produced endogenously to affect O_2 consumption. The results presented in this thesis provide evidence that endogenous CO may indeed inhibit cellular respiration. However, definitive demonstration that this occurs through the action of CO on complex IV remains lacking. Thus, further studies investigating this phenomenon should address this issue, possibly through the use of spectroscopic tools to show shifts in the absorption spectra of the complex IV cytochromes due to the binding of CO.

If endogenous CO does indeed inhibit respiration through complex IV, it would be interesting to investigate the consequences of this effect under hypoxic conditions. As shown in this thesis, inhibition of respiration in hypoxia results in the redistribution of intracellular O_2. Evidence is provided suggesting that endogenous CO rather, rather than endogenous NO, may perform this role:

- the inhibitory effect of CO is enhanced at lower O_2 concentrations,
- CO synthesis, as indicated by HO-1 expression and bilirubin synthesis, is increased in hypoxia, and
- HO-1 activity is not affected at 1% O_2, whereas iNOS activity is greatly reduced.

In addition to the effects upon prolyl hydroxylase activity and HIF1α protein stability, it would be interesting to investigate the manner in which other O_2-dependent enzymes are affected by the redistribution of O_2. One such enzyme is iNOS. The concept that HO-1-derived CO could stimulate the synthesis of NO by increasing intracellular O_2 concentrations is an intriguing possibility.

REFERENCES

Abu-Soud, H.M., Yoho, L.L., Stuehr, D.J. (1994). Calmodulin controls neuronal nitric-oxide synthase by a dual mechanism. Activation of intra- and interdomain electron transfer. *J.Biol.Chem.* **269**: 32047-32050.

Agani, F.H., Pichiule, P., Chavez, J.C., Lamanna, J.C. (2000). The role of mitochondria in the regulation of hypoxia-inducible factor 1 expression during hypoxia. *J.Biol.Chem.* **275**: 35863-35867.

Agani, F.H., Puchowicz, M., Chavez, J.C., Pichiule, P., LaManna, J. (2002). Role of nitric oxide in the regulation of HIF-1alpha expression during hypoxia. *Am.J.Physiol Cell Physiol* **283**: C178-C186.

Alderton, W.K., Cooper, C.E., Knowles, R.G. (2001). Nitric oxide synthases: structure, function and inhibition. *Biochem.J.* **357**: 593-615.

Almeida, A., Almeida, J., Bolanos, J.P., Moncada, S. (2001). Different responses of astrocytes and neurons to nitric oxide: the role of glycolytically generated ATP in astrocyte protection. *Proc.Natl.Acad.Sci.U.S.A* **98**: 15294-15299.

Alonso, J.R., Cardellach, F., Lopez, S., Casademont, J., Miro, O. (2003). Carbon monoxide specifically inhibits cytochrome c oxidase of human mitochondrial respiratory chain. *Pharmacol.Toxicol.* **93**: 142-146.

Antunes, F., Boveris, A., Cadenas, E. (2004). On the mechanism and biology of cytochrome oxidase inhibition by nitric oxide. *Proc.Natl.Acad.Sci.U.S.A* **101**: 16774-16779.

Appelhoff, R.J., Tian, Y.M., Raval, R.R., Turley, H., Harris, A.L., Pugh, C.W., Ratcliffe, P.J., Gleadle, J.M. (2004). Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor. *J.Biol.Chem.* **279**: 38458-38465.

Assreuy, J., Cunha, F.Q., Liew, F.Y., Moncada, S. (1993). Feedback inhibition of nitric oxide synthase activity by nitric oxide. *Br.J.Pharmacol.* **108**: 833-837.

Babcock, G.T. and Wikstrom, M. (1992). Oxygen activation and the conservation of energy in cell respiration. *Nature* **356**: 301-309.

Barker, J.E., Bolanos, J.P., Land, J.M., Clark, J.B., Heales, S.J. (1996). Glutathione protects astrocytes from peroxynitrite-mediated mitochondrial damage: implications for neuronal/astrocytic trafficking and neurodegeneration. *Dev.Neurosci.* **18**: 391-396.

Barrientos, A. and Moraes, C.T. (1999). Titrating the effects of mitochondrial complex I impairment in the cell physiology. *J.Biol.Chem.* **274**: 16188-16197.

Bauer, J.A., Booth, B.P., Fung, H.L. (1995). Nitric oxide donors: biochemical pharmacology and therapeutics. *Adv.Pharmacol.* **34**: 361-381.

Beckman, J.S., Ye, Y.Z., Chen, J., Conger, K.A. (1996). The interactions of nitric oxide with oxygen radicals and scavengers in cerebral ischemic injury. *Adv.Neurol.* **71**: 339-350.

Bellamy, T.C., Wood, J., Goodwin, D.A., Garthwaite, J. (2000). Rapid desensitization of the nitric oxide receptor, soluble guanylyl cyclase, underlies diversity of cellular cGMP responses. *Proc.Natl.Acad.Sci.U.S.A* **97**: 2928-2933.

Bellamy, T.C. and Garthwaite, J. (2002). The receptor-like properties of nitric oxide-activated soluble guanylyl cyclase in intact cells. *Mol.Cell Biochem.* **230**: 165-176.

Beltran, B., Orsi, A., Clementi, E., Moncada, S. (2000). Oxidative stress and S-nitrosylation of proteins in cells. *Br.J.Pharmacol.* **129**: 953-960.

Berra, E., Benizri, E., Ginouves, A., Volmat, V., Roux, D., Pouyssegur, J. (2003). HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia. *EMBO J.* **22**: 4082-4090.

Blackmore, R.S., Greenwood, C., Gibson, Q.H. (1991). Studies of the primary oxygen intermediate in the reaction of fully reduced cytochrome oxidase. *J.Biol.Chem.* **266**: 19245-19249.

Bolanos, J.P., Peuchen, S., Heales, S.J., Land, J.M., Clark, J.B. (1994). Nitric oxide-mediated inhibition of the mitochondrial respiratory chain in cultured astrocytes. *J.Neurochem.* **63**: 910-916.

Bolanos, J.P., Heales, S.J., Land, J.M., Clark, J.B. (1995). Effect of peroxynitrite on the mitochondrial respiratory chain: differential susceptibility of neurones and astrocytes in primary culture. *J.Neurochem.* **64**: 1965-1972.

Bolanos, J.P., Heales, S.J., Peuchen, S., Barker, J.E., Land, J.M., Clark, J.B. (1996). Nitric oxide-mediated mitochondrial damage: a potential neuroprotective role for glutathione. *Free Radic.Biol.Med.* **21**: 995-1001.

Bossy-Wetzel, E., Barsoum, M.J., Godzik, A., Schwarzenbacher, R., Lipton, S.A. (2003). Mitochondrial fission in apoptosis, neurodegeneration and aging. *Curr.Opin.Cell Biol.* **15**: 706-716.

Bouton, C. and Demple, B. (2000). Nitric oxide-inducible expression of heme oxygenase-1 in human cells. Translation-independent stabilization of the mRNA and evidence for direct action of nitric oxide. *J.Biol.Chem.* **275**: 32688-32693.

Boveris, A. and Chance, B. (1973). The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochem.J.* **134**: 707-716.

Boveris, A., Costa, L.E., Poderoso, J.J., Carreras, M.C., Cadenas, E. (2000). Regulation of mitochondrial respiration by oxygen and nitric oxide. *Ann.N.Y.Acad.Sci.* **899**: 121-135.

Brand, M.D., Affourtit, C., Esteves, T.C., Green, K., Lambert, A.J., Miwa, S., Pakay, J.L., Parker, N. (2004). Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins. *Free Radic.Biol.Med.* **37**: 755-767.

Bredt, D.S. and Snyder, S.H. (1994). Nitric oxide: a physiologic messenger molecule. *Annu.Rev.Biochem.* **63**: 175-195.

Brown, G.C. and Cooper, C.E. (1994). Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase. *FEBS Lett.* **356**: 295-298.

Brown, G.C., Foxwell, N., Moncada, S. (1998). Transcellular regulation of cell respiration by nitric oxide generated by activated macrophages. *FEBS Lett.* **439**: 321-324.

Brown, G.C. (1999). Nitric oxide and mitochondrial respiration. *Biochim.Biophys.Acta* **1411**: 351-369.

Brown, G.C. (2001). Regulation of mitochondrial respiration by nitric oxide inhibition of cytochrome c oxidase. *Biochim.Biophys.Acta* **1504**: 46-57.

Bruick, R.K. and McKnight, S.L. (2001). A conserved family of prolyl-4-hydroxylases that modify HIF. *Science* **294**: 1337-1340.

Buechler, W.A., Ivanova, K., Wolfram, G., Drummer, C., Heim, J.M., Gerzer, R. (1994). Soluble guanylyl cyclase and platelet function. *Ann.N.Y.Acad.Sci.* **714**: 151-157.

Bush, P.A., Gonzalez, N.E., Griscavage, J.M., Ignarro, L.J. (1992). Nitric oxide synthase from cerebellum catalyzes the formation of equimolar quantities of nitric oxide and citrulline from L-arginine. *Biochem.Biophys.Res.Commun.* **185**: 960-966.

Carreras, M.C., Franco, M.C., Peralta, J.G., Poderoso, J.J. (2004). Nitric oxide, complex I, and the modulation of mitochondrial reactive species in biology and disease. *Mol.Aspects Med.* **25**: 125-139.

Chandel, N.S., Budinger, G.R., Schumacker, P.T. (1996). Molecular oxygen modulates cytochrome c oxidase function. *J.Biol.Chem.* **271**: 18672-18677.

Chandel, N.S., Maltepe, E., Goldwasser, E., Mathieu, C.E., Simon, M.C., Schumacker, P.T. (1998). Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. *Proc.Natl.Acad.Sci.U.S.A* **95**: 11715-11720.

Chandel, N.S., Trzyna, W.C., McClintock, D.S., Schumacker, P.T. (2000). Role of oxidants in NF-kappa B activation and TNF-alpha gene transcription induced by hypoxia and endotoxin. *J.Immunol.* **165**: 1013-1021.

Chandel, N.S., McClintock, D.S., Feliciano, C.E., Wood, T.M., Melendez, J.A., Rodriguez, A.M., Schumacker, P.T. (2000). Reactive oxygen species generated at mitochondrial

complex III stabilize hypoxia-inducible factor-1alpha during hypoxia: a mechanism of O2 sensing. *J.Biol.Chem.* **275**: 25130-25138.

Chang, C.I., Liao, J.C., Kuo, L. (1998). Arginase modulates nitric oxide production in activated macrophages. *Am.J.Physiol* **274**: H342-H348.

Chen, Q., Vazquez, E.J., Moghaddas, S., Hoppel, C.L., Lesnefsky, E.J. (2003). Production of reactive oxygen species by mitochondria: central role of complex III. *J.Biol.Chem.* **278** : 36027-36031.

Chicoine, L.G., Paffett, M.L., Young, T.L., Nelin, L.D. (2004). Arginase inhibition increases nitric oxide production in bovine pulmonary arterial endothelial cells. *Am.J.Physiol Lung Cell Mol.Physiol* **287**: L60-L68.

Cleeter, M.W., Cooper, J.M., Darley-Usmar, V.M., Moncada, S., Schapira, A.H. (1994). Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide. Implications for neurodegenerative diseases. *FEBS Lett.* **345**: 50-54.

Clementi, E., Brown, G.C., Feelisch, M., Moncada, S. (1998). Persistent inhibition of cell respiration by nitric oxide: crucial role of S-nitrosylation of mitochondrial complex I and protective action of glutathione. *Proc.Natl.Acad.Sci.U.S.A* **95**: 7631-7636.

Clementi, E., Brown, G.C., Foxwell, N., Moncada, S. (1999). On the mechanism by which vascular endothelial cells regulate their oxygen consumption. *Proc.Natl.Acad.Sci.U.S.A* **96**: 1559-1562.

Connelly, L., Jacobs, A.T., Palacios-Callender, M., Moncada, S., Hobbs, A.J. (2003). Macrophage endothelial nitric-oxide synthase autoregulates cellular activation and pro-inflammatory protein expression. *J.Biol.Chem.* **278**: 26480-26487.

Cooper, C.E., Torres, J., Sharpe, M.A., Wilson, M.T. (1997). Nitric oxide ejects electrons from the binuclear centre of cytochrome c oxidase by reacting with oxidised copper: a general mechanism for the interaction of copper proteins with nitric oxide? *FEBS Lett.* **414**: 281-284.

Cooper, C.E., Davies, N.A., Psychoulis, M., Canevari, L., Bates, T.E., Dobbie, M.S., Casley, C.S., Sharpe, M.A. (2003). Nitric oxide and peroxynitrite cause irreversible increases in the K(m) for oxygen of mitochondrial cytochrome oxidase: in vitro and in vivo studies. *Biochim.Biophys.Acta* **1607**: 27-34.

Degerman, E., Belfrage, P., Manganiello, V.C. (1997). Structure, localization, and regulation of cGMP-inhibited phosphodiesterase (PDE3). *J.Biol.Chem.* **272**: 6823-6826.

Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R., Zeiher, A.M. (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* **399**: 601-605.

Dinerman, J.L., Dawson, T.M., Schell, M.J., Snowman, A., Snyder, S.H. (1994). Endothelial nitric oxide synthase localized to hippocampal pyramidal cells: implications for synaptic plasticity. *Proc.Natl.Acad.Sci.U.S.A* **91**: 4214-4218.

Ding, Y., McCoubrey, W.K., Jr., Maines, M.D. (1999). Interaction of heme oxygenase-2 with nitric oxide donors. Is the oxygenase an intracellular 'sink' for NO? *Eur.J.Biochem.* **264** : 854-861.

Duranteau, J., Chandel, N.S., Kulisz, A., Shao, Z., Schumacker, P.T. (1998). Intracellular signaling by reactive oxygen species during hypoxia in cardiomyocytes. *J.Biol.Chem.* **273** : 11619-11624.

Emerling, B.M., Platanias, L.C., Black, E., Nebreda, A.R., Davis, R.J., Chandel, N.S. (2005). Mitochondrial reactive oxygen species activation of p38 mitogen-activated protein kinase is required for hypoxia signaling. *Mol.Cell Biol.* **25**: 4853-4862.

Fandrey, J., Frede, S., Jelkmann, W. (1994). Role of hydrogen peroxide in hypoxia-induced erythropoietin production. *Biochem.J.* **303**: 507-510.

Foresti, R., Clark, J.E., Green, C.J., Motterlini, R. (1997). Thiol compounds interact with nitric oxide in regulating heme oxygenase-1 induction in endothelial cells. Involvement of superoxide and peroxynitrite anions. *J.Biol.Chem.* **272**: 18411-18417.

Foresti, R., Sarathchandra, P., Clark, J.E., Green, C.J., Motterlini, R. (1999). Peroxynitrite induces haem oxygenase-1 in vascular endothelial cells: a link to apoptosis. *Biochem.J.* **339**: 729-736.

Frost, M.T., Wang, Q., Moncada, S., Singer, M. (2005). Hypoxia accelerates nitric oxide-dependent inhibition of mitochondrial complex I in activated macrophages. *Am.J.Physiol Regul.Integr.Comp Physiol* **288**: R394-R400.

Fujita, T., Toda, K., Karimova, A., Yan, S.F., Naka, Y., Yet, S.F., Pinsky, D.J. (2001). Paradoxical rescue from ischemic lung injury by inhaled carbon monoxide driven by derepression of fibrinolysis. *Nat.Med.* **7**: 598-604.

Furchgott, R.F. and Jothianandan, D. (1991). Endothelium-dependent and -independent vasodilation involving cyclic GMP: relaxation induced by nitric oxide, carbon monoxide and light. *Blood Vessels* **28**: 52-61.

Gao, S., Chen, J., Brodsky, S.V., Huang, H., Adler, S., Lee, J.H., Dhadwal, N., Cohen-Gould, L., Gross, S.S., Goligorsky, M.S. (2004). Docking of endothelial nitric oxide synthase (eNOS) to the mitochondrial outer membrane: a pentabasic amino acid sequence in the autoinhibitory domain of eNOS targets a proteinase K-cleavable peptide on the cytoplasmic face of mitochondria. *J.Biol.Chem.* **279**: 15968-15974.

Genius, J. and Fandrey, J. (2000). Nitric oxide affects the production of reactive oxygen species in hepatoma cells: implications for the process of oxygen sensing. *Free Radic.Biol.Med.* **29**: 515-521.

Ghafourifar, P. and Richter, C. (1997). Nitric oxide synthase activity in mitochondria. *FEBS Lett.* **418**: 291-296.

Ghosh, D.K., Wu, C., Pitters, E., Moloney, M., Werner, E.R., Mayer, B., Stuehr, D.J. (1997). Characterization of the inducible nitric oxide synthase oxygenase domain identifies a 49 amino acid segment required for subunit dimerization and tetrahydrobiopterin interaction. *Biochemistry* **36**: 10609-10619.

Giaccia, A., Siim, B.G., Johnson, R.S. (2003). HIF-1 as a target for drug development. *Nat.Rev.Drug Discov.* **2**: 803-811.

Gibson, Q.H., Olson, J.S., McKinnie, R.E., Rohlfs, R.J. (1986). A kinetic description of ligand binding to sperm whale myoglobin. *J.Biol.Chem.* **261**: 10228-10239.

Giuffre, A., Sarti, P., D'Itri, E., Buse, G., Soulimane, T., Brunori, M. (1996). On the mechanism of inhibition of cytochrome c oxidase by nitric oxide. *J.Biol.Chem.* **271**: 33404-33408.

Giulivi, C. (1998). Functional implications of nitric oxide produced by mitochondria in mitochondrial metabolism. *Biochem.J.* **332**: 673-679.

Gleadle, J.M., Ebert, B.L., Ratcliffe, P.J. (1995). Diphenylene iodonium inhibits the induction of erythropoietin and other mammalian genes by hypoxia. Implications for the mechanism of oxygen sensing. *Eur.J.Biochem.* **234**: 92-99.

Gnaiger, E. and Kuznetsov, A.V. (2002). Mitochondrial respiration at low levels of oxygen and cytochrome c. *Biochem.Soc.Trans.* **30**: 252-258.

Green, D. and Kroemer, G. (1998). The central executioners of apoptosis: caspases or mitochondria? *Trends Cell Biol.* **8**: 267-271.

Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R. (1982). Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal.Biochem.* **126**: 131-138.

Grigorieff, N. (1999). Structure of the respiratory NADH:ubiquinone oxidoreductase (complex I). *Curr.Opin.Struct.Biol.* **9**: 476-483.

Haab, P. (1990). The effect of carbon monoxide on respiration. *Experientia* **46**: 1202-1206.

Hansen, F.B. and Nicholls, P. (1978). Control of respiration in proteoliposomes containing cytochrome aa3. II. Inhibition by carbon monoxide and azide. *Biochim.Biophys.Acta* **502**: 400-408.

Harvey, B.H. (2000). Acid-dependent dismutation of nitrogen oxides may be a critical source of nitric oxide in human macrophages. *Med Hypotheses* **54**: 829-831.

Hayashi, Y., Nishio, M., Naito, Y., Yokokura, H., Nimura, Y., Hidaka, H., Watanabe, Y. (1999). Regulation of neuronal nitric-oxide synthase by calmodulin kinases. *J.Biol.Chem.* **274**: 20597-20602.

Hinkle, P.C. and McCarty, R.E. (1978). How cells make ATP. *Sci.Am.* **238**: 104-3.

Hinz, B., Brune, K., Pahl, A. (2000). Nitric oxide inhibits inducible nitric oxide synthase mRNA expression in RAW 264.7 macrophages. *Biochem.Biophys.Res.Commun.* **271**: 353-357.

Hirsila, M., Koivunen, P., Gunzler, V., Kivirikko, K.I., Myllyharju, J. (2003). Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor. *J.Biol.Chem.* **278**: 30772-30780.

Hollis, V.S., Palacios-Callender, M., Springett, R.J., Delpy, D.T., Moncada, S. (2003). Monitoring cytochrome redox changes in the mitochondria of intact cells using multi-wavelength visible light spectroscopy. *Biochim.Biophys.Acta* **1607**: 191-202.

Hrabak, A., Idei, M., Temesi, A. (1994). Arginine supply for nitric oxide synthesis and arginase is mainly exogenous in elicited murine and rat macrophages. *Life Sci.* **55**: 797-805.

Huang, L.E., Arany, Z., Livingston, D.M., Bunn, H.F. (1996). Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. *J.Biol.Chem.* **271**: 32253-32259.

Huang, L.E., Willmore, W.G., Gu, J., Goldberg, M.A., Bunn, H.F. (1999). Inhibition of hypoxia-inducible factor 1 activation by carbon monoxide and nitric oxide. Implications for oxygen sensing and signaling. *J.Biol.Chem.* **274**: 9038-9044.

Huie, R.E. and Padmaja, S. (1993). The reaction of NO with superoxide. *Free Radic.Res.Commun.* **18**: 195-199.

Ingi, T., Chiang, G., Ronnett, G.V. (1996). The regulation of heme turnover and carbon monoxide biosynthesis in cultured primary rat olfactory receptor neurons. *J.Neurosci.* **16**: 5621-5628.

Ingi, T., Cheng, J., Ronnett, G.V. (1996). Carbon monoxide: an endogenous modulator of the nitric oxide-cyclic GMP signaling system. *Neuron* **16**: 835-842.

Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J.M., Lane, W.S., Kaelin, W.G., Jr. (2001). HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science* **292**: 464-468.

Jaakkola, P., Mole, D.R., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., Kriegsheim, A., Hebestreit, H.F., Mukherji, M., Schofield, C.J., Maxwell, P.H., Pugh, C.W., Ratcliffe, P.J. (2001). Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science* **292**: 468-472.

Jaffrey, S.R. and Snyder, S.H. (1995). Nitric oxide: a neural messenger. *Annu.Rev.Cell Dev.Biol.* **11**: 417-440.

Jeong, J.W., Bae, M.K., Ahn, M.Y., Kim, S.H., Sohn, T.K., Bae, M.H., Yoo, M.A., Song, E.J., Lee, K.J., Kim, K.W. (2002). Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation. *Cell* **111**: 709-720.

Jiang, B.H., Semenza, G.L., Bauer, C., Marti, H.H. (1996). Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O2 tension. *Am.J.Physiol* **271**: C1172-C1180.

Kanai, A.J., Pearce, L.L., Clemens, P.R., Birder, L.A., VanBibber, M.M., Choi, S.Y., de Groat, W.C., Peterson, J. (2001). Identification of a neuronal nitric oxide synthase in isolated cardiac mitochondria using electrochemical detection. *Proc.Natl.Acad.Sci.U.S.A* **98**: 14126-14131.

Keefer, L.K., Nims, R.W., Davies, K.M., Wink, D.A. (1996). "NONOates" (1-substituted diazen-1-ium-1,2-diolates) as nitric oxide donors: convenient nitric oxide dosage forms. *Methods Enzymol.* **268**: 281-293.

Keynes, R.G., Griffiths, C.H., Hall, C., Garthwaite, J. (2005). Nitric oxide consumption through lipid peroxidation in brain cell suspensions and homogenates. *Biochem.J.* **387**: 685-694.

Keyse, S.M. and Tyrrell, R.M. (1989). Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. *Proc.Natl.Acad.Sci.U.S.A* **86**: 99-103.

King, T.E. (1967). Preparation of succinate cytochrome c reductase and the b-c1 particle and reconstruction of succinate cytochrome c reductase. *Methods Enzymol.* **10**: 216-225.

Koivisto, A., Matthias, A., Bronnikov, G., Nedergaard, J. (1997). Kinetics of the inhibition of mitochondrial respiration by NO. *FEBS Lett.* **417**: 75-80.

Kreisman, N.R., Sick, T.J., Lamanna, J.C., Rosenthal, M. (1981). Local tissue oxygen tension-cytochrome a,a3 redox relationships in rat cerebral cortex in vivo. *Brain Res.* **218**: 161-174.

Kyriakis, J.M. and Avruch, J. (1996). Sounding the alarm: protein kinase cascades activated by stress and inflammation. *J.Biol.Chem.* **271**: 24313-24316.

Lambert, A.J. and Brand, M.D. (2004). Superoxide production by NADH:ubiquinone oxidoreductase (complex I) depends on the pH gradient across the mitochondrial inner membrane. *Biochem.J.* **382**: 511-517.

Lando, D., Gorman, J.J., Whitelaw, M.L., Peet, D.J. (2003). Oxygen-dependent regulation of hypoxia-inducible factors by prolyl and asparaginyl hydroxylation. *Eur.J.Biochem.* **270**: 781-790.

Lepore, D.A., Stewart, A.G., Tomasi, A., Anderson, R.L., Hurley, J.V., Morrison, W.A. (1999). The survival of skeletal muscle myoblasts in vitro is sensitive to a donor of nitric oxide and superoxide, SIN-1, but not to nitric oxide or peroxynitrite alone. *Nitric.Oxide.* **3**: 273-280.

Liu, X., Miller, M.J., Joshi, M.S., Thomas, D.D., Lancaster, J.R., Jr. (1998). Accelerated reaction of nitric oxide with O2 within the hydrophobic interior of biological membranes. *Proc.Natl.Acad.Sci.U.S.A* **95**: 2175-2179.

Lohmann, S.M., Vaandrager, A.B., Smolenski, A., Walter, U., De Jonge, H.R. (1997). Distinct and specific functions of cGMP-dependent protein kinases. *Trends Biochem.Sci.* **22**: 307-312.

Loke, K.E., Laycock, S.K., Mital, S., Wolin, M.S., Bernstein, R., Oz, M., Addonizio, L., Kaley, G., Hintze, T.H. (1999). Nitric oxide modulates mitochondrial respiration in failing human heart. *Circulation* **100**: 1291-1297.

Mahon, P.C., Hirota, K., Semenza, G.L. (2001). FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. *Genes Dev.* **15**: 2675-2686.

Maines, M.D., Trakshel, G.M., Kutty, R.K. (1986). Characterization of two constitutive forms of rat liver microsomal heme oxygenase. Only one molecular species of the enzyme is inducible. *J.Biol.Chem.* **261**: 411-419.

Maines, M.D. (1997). The heme oxygenase system: a regulator of second messenger gases. *Annu.Rev.Pharmacol.Toxicol.* **37**: 517-554.

Marks, G.S., Brien, J.F., Nakatsu, K., McLaughlin, B.E. (1991). Does carbon monoxide have a physiological function? *Trends Pharmacol.Sci.* **12**: 185-188.

Mateo, J., Garcia-Lecea, M., Cadenas, S., Hernandez, C., Moncada, S. (2003). Regulation of hypoxia-inducible factor-1alpha by nitric oxide through mitochondria-dependent and -independent pathways. *Biochem.J.* **376**: 537-544.

Maxwell, P.H., Wiesener, M.S., Chang, G.W., Clifford, S.C., Vaux, E.C., Cockman, M.E., Wykoff, C.C., Pugh, C.W., Maher, E.R., Ratcliffe, P.J. (1999). The tumour suppressor

protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* **399**: 271-275.

McCall, T.B., Palmer, R.M., Moncada, S. (1992). Interleukin-8 inhibits the induction of nitric oxide synthase in rat peritoneal neutrophils. *Biochem.Biophys.Res.Commun.* **186**: 680-685.

McCormick, C.C., Li, W.P., Calero, M. (2000). Oxygen tension limits nitric oxide synthesis by activated macrophages. *Biochem.J.* **350**: 709-716.

McCoubrey, W.K., Jr., Huang, T.J., Maines, M.D. (1997). Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3. *Eur.J.Biochem.* **247**: 725-732.

Metzen, E., Zhou, J., Jelkmann, W., Fandrey, J., Brune, B. (2003). Nitric oxide impairs normoxic degradation of HIF-1alpha by inhibition of prolyl hydroxylases. *Mol.Biol.Cell* **14**: 3470-3481.

Michel, J.B., Feron, O., Sase, K., Prabhakar, P., Michel, T. (1997). Caveolin versus calmodulin. Counterbalancing allosteric modulators of endothelial nitric oxide synthase. *J.Biol.Chem.* **272**: 25907-25912.

Miro, O., Casademont, J., Barrientos, A., Urbano-Marquez, A., Cardellach, F. (1998). Mitochondrial cytochrome c oxidase inhibition during acute carbon monoxide poisoning. *Pharmacol.Toxicol.* **82**: 199-202.

Mitchell, P. (1961). Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature* **191**: 144-148.

Moncada, S., Palmer, R.M., Higgs, E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol.Rev.* **43**: 109-142.

Mori, M. and Gotoh, T. (2000). Regulation of nitric oxide production by arginine metabolic enzymes. *Biochem.Biophys.Res.Commun.* **275**: 715-719.

Morita, T., Perrella, M.A., Lee, M.E., Kourembanas, S. (1995). Smooth muscle cell-derived carbon monoxide is a regulator of vascular cGMP. *Proc.Natl.Acad.Sci.U.S.A* **92**: 1475-1479.

Motterlini, R., Clark, J.E., Foresti, R., Sarathchandra, P., Mann, B.E., Green, C.J. (2002). Carbon monoxide-releasing molecules: characterization of biochemical and vascular activities. *Circ.Res.* **90**: E17-E24.

Murray, J., Taylor, S.W., Zhang, B., Ghosh, S.S., Capaldi, R.A. (2003). Oxidative damage to mitochondrial complex I due to peroxynitrite: identification of reactive tyrosines by mass spectrometry. *J.Biol.Chem.* **278**: 37223-37230.

Nagano, T. (1999). Practical methods for detection of nitric oxide. *Luminescence.* **14**: 283-290.

Naseem, K.M. and Bruckdorfer, K.R. (1995). Hydrogen peroxide at low concentrations strongly enhances the inhibitory effect of nitric oxide on platelets. *Biochem.J.* **310**: 149-153.

Nishida, C.R. and Ortiz de Montellano, P.R. (1998). Electron transfer and catalytic activity of nitric oxide synthases. Chimeric constructs of the neuronal, inducible, and endothelial isoforms. *J.Biol.Chem.* **273**: 5566-5571.

Okun, J.G., Lummen, P., Brandt, U. (1999). Three classes of inhibitors share a common binding domain in mitochondrial complex I (NADH:ubiquinone oxidoreductase). *J.Biol.Chem.* **274** : 2625-2630.

Orsi, A., Beltran, B., Clementi, E., Hallen, K., Feelisch, M., Moncada, S. (2000). Continuous exposure to high concentrations of nitric oxide leads to persistent inhibition of oxygen consumption by J774 cells as well as extraction of oxygen by the extracellular medium. *Biochem.J.* **346**: 407-412.

Otterbein, L.E., Bach, F.H., Alam, J., Soares, M., Tao, L.H., Wysk, M., Davis, R.J., Flavell, R.A., Choi, A.M. (2000). Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nat.Med.* **6**: 422-428.

Padgett, C.M. and Whorton, A.R. (1995). S-nitrosoglutathione reversibly inhibits GAPDH by S-nitrosylation. *Am.J.Physiol* **269**: C739-C749.

Palacios-Callender, M., Quintero, M., Hollis, V.S., Springett, R.J., Moncada, S. (2004). Endogenous NO regulates superoxide production at low oxygen concentrations by modifying the redox state of cytochrome c oxidase. *Proc.Natl.Acad.Sci.U.S.A* **101**: 7630-7635.

Palmer, L.A., Gaston, B., Johns, R.A. (2000). Normoxic stabilization of hypoxia-inducible factor-1 expression and activity: redox-dependent effect of nitrogen oxides. *Mol.Pharmacol.* **58**: 1197-1203.

Palmer, R.M., Ferrige, A.G., Moncada, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* **327**: 524-526.

Palmer, R.M., Ashton, D.S., Moncada, S. (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* **333**: 664-666.

Parone, P.A., James, D., Martinou, J.C. (2002). Mitochondria: regulating the inevitable. *Biochimie* **84**: 105-111.

Petersen, L.C. (1977). The effect of inhibitors on the oxygen kinetics of cytochrome c oxidase. *Biochim.Biophys.Acta* **460**: 299-307.

Petrache, I., Otterbein, L.E., Alam, J., Wiegand, G.W., Choi, A.M. (2000). Heme oxygenase-1 inhibits TNF-alpha-induced apoptosis in cultured fibroblasts. *Am.J.Physiol Lung Cell Mol.Physiol* **278**: L312-L319.

Piantadosi, C.A. (2002). Biological chemistry of carbon monoxide. *Antioxid.Redox.Signal.* **4**: 259-270.

Radi, R., Turrens, J.F., Chang, L.Y., Bush, K.M., Crapo, J.D., Freeman, B.A. (1991). Detection of catalase in rat heart mitochondria. *J.Biol.Chem.* **266**: 22028-22034.

Radi, R., Rodriguez, M., Castro, L., Telleri, R. (1994). Inhibition of mitochondrial electron transport by peroxynitrite. *Arch.Biochem.Biophys.* **308**: 89-95.

Radi, R., Cassina, A., Hodara, R., Quijano, C., Castro, L. (2002). Peroxynitrite reactions and formation in mitochondria. *Free Radic.Biol.Med.* **33**: 1451-1464.

Radomski, M.W., Palmer, R.M., Moncada, S. (1990). An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. *Proc.Natl.Acad.Sci.U.S.A* **87**: 5193-5197.

Raha, S., McEachern, G.E., Myint, A.T., Robinson, B.H. (2000). Superoxides from mitochondrial complex III: the role of manganese superoxide dismutase. *Free Radic.Biol.Med.* **29**: 170-180.

Rengasamy, A. and Johns, R.A. (1996). Determination of Km for oxygen of nitric oxide synthase isoforms. *J.Pharmacol.Exp.Ther.* **276**: 30-33.

Ricquier, D., Mory, G., Nechad, M., Combes-George, M., Thibault, J. (1983). Development and activation of brown fat in rats with pheochromocytoma PC 12 tumors. *Am.J.Physiol* **245**: C172-C177.

Riobo, N.A., Clementi, E., Melani, M., Boveris, A., Cadenas, E., Moncada, S., Poderoso, J.J. (2001). Nitric oxide inhibits mitochondrial NADH:ubiquinone reductase activity through peroxynitrite formation. *Biochem.J.* **359**: 139-145.

Rothe, F., Huang, P.L., Wolf, G. (1999). Ultrastructural localization of neuronal nitric oxide synthase in the laterodorsal tegmental nucleus of wild-type and knockout mice. *Neuroscience* **94**: 193-201.

Ruan, R.S., Leong, S.K., Yeoh, K.H. (1999). Ototoxicity of sodium nitroprusside is not due to nitric oxide. *Exp.Neurol.* **158**: 192-201.

Ryter, S.W. and Otterbein, L.E. (2004). Carbon monoxide in biology and medicine. *Bioessays* **26**: 270-280.

Ryter, S.W., Morse, D., Choi, A.M. (2004). Carbon monoxide: to boldly go where NO has gone before. *Sci.STKE.* **2004**: RE6.

Salceda, S. and Caro, J. (1997). Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. *J.Biol.Chem.* **272**: 22642-22647.

Sandau, K.B., Fandrey, J., Brune, B. (2001). Accumulation of HIF-1alpha under the influence of nitric oxide. *Blood* **97**: 1009-1015.

Sato, K., Balla, J., Otterbein, L., Smith, R.N., Brouard, S., Lin, Y., Csizmadia, E., Sevigny, J., Robson, S.C., Vercellotti, G., Choi, A.M., Bach, F.H., Soares, M.P. (2001). Carbon monoxide generated by heme oxygenase-1 suppresses the rejection of mouse-to-rat cardiac transplants. *J.Immunol.* **166**: 4185-4194.

Schofield, C.J. and Ratcliffe, P.J. (2004). Oxygen sensing by HIF hydroxylases. *Nat.Rev.Mol.Cell Biol.* **5**: 343-354.

Schweizer, M. and Richter, C. (1994). Nitric oxide potently and reversibly deenergizes mitochondria at low oxygen tension. *Biochem.Biophys.Res.Commun.* **204**: 169-175.

Seaton, T.A., Cooper, J.M., Schapira, A.H. (1998). Cyclosporin inhibition of apoptosis induced by mitochondrial complex I toxins. *Brain Res.* **809**: 12-17.

Sell, H., Deshaies, Y., Richard, D. (2004). The brown adipocyte: update on its metabolic role. *Int.J.Biochem.Cell Biol.* **36**: 2098-2104.

Semenza, G.L., Jiang, B.H., Leung, S.W., Passantino, R., Concordet, J.P., Maire, P., Giallongo, A. (1996). Hypoxia response elements in the aldolase A, enolase 1, and lactate

dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. *J.Biol.Chem.* **271**: 32529-32537.

Semenza, G.L. (2003). Targeting HIF-1 for cancer therapy. *Nat.Rev.Cancer* **3**: 721-732.

Shaul, P.W., Smart, E.J., Robinson, L.J., German, Z., Yuhanna, I.S., Ying, Y., Anderson, R.G., Michel, T. (1996). Acylation targets emdothelial nitric-oxide synthase to plasmalemmal caveolae. *J.Biol.Chem.* **271**: 6518-6522.

Shiva, S., Brookes, P.S., Patel, R.P., Anderson, P.G., Darley-Usmar, V.M. (2001). Nitric oxide partitioning into mitochondrial membranes and the control of respiration at cytochrome c oxidase. *Proc.Natl.Acad.Sci.U.S.A* **98**: 7212-7217.

Sogawa, K., Numayama-Tsuruta, K., Ema, M., Abe, M., Abe, H., Fujii-Kuriyama, Y. (1998). Inhibition of hypoxia-inducible factor 1 activity by nitric oxide donors in hypoxia. *Proc.Natl.Acad.Sci.U.S.A* **95**: 7368-7373.

Srinivas, V., Leshchinsky, I., Sang, N., King, M.P., Minchenko, A., Caro, J. (2001). Oxygen sensing and HIF-1 activation does not require an active mitochondrial respiratory chain electron-transfer pathway. *J.Biol.Chem.* **276**: 21995-21998.

Srisook, K. and Cha, Y.N. (2004). Biphasic induction of heme oxygenase-1 expression in macrophages stimulated with lipopolysaccharide. *Biochem.Pharmacol.* **68**: 1709-1720.

St-Pierre, J., Buckingham, J.A., Roebuck, S.J., Brand, M.D. (2002). Topology of superoxide production from different sites in the mitochondrial electron transport chain. *J.Biol.Chem.* **277**: 44784-44790.

Stamler, J.S. (1994). Redox signaling: nitrosylation and related target interactions of nitric oxide. *Cell* **78**: 931-936.

Stocker, R., Yamamoto, Y., McDonagh, A.F., Glazer, A.N., Ames, B.N. (1987). Bilirubin is an antioxidant of possible physiological importance. *Science* **235**: 1043-1046.

Stuehr, D.J., Kwon, N.S., Nathan, C.F., Griffith, O.W., Feldman, P.L., Wiseman, J. (1991). N omega-hydroxy-L-arginine is an intermediate in the biosynthesis of nitric oxide from L-arginine. *J.Biol.Chem.* **266**: 6259-6263.

Suematsu, M., Kashiwagi, S., Sano, T., Goda, N., Shinoda, Y., Ishimura, Y. (1994). Carbon monoxide as an endogenous modulator of hepatic vascular perfusion. *Biochem.Biophys.Res.Commun.* **205**: 1333-1337.

Sumbayev, V.V., Budde, A., Zhou, J., Brune, B. (2003). HIF-1 alpha protein as a target for S-nitrosation. *FEBS Lett.* **535**: 106-112.

Sun, Y., Rotenberg, M.O., Maines, M.D. (1990). Developmental expression of heme oxygenase isozymes in rat brain. Two HO-2 mRNAs are detected. *J.Biol.Chem.* **265**: 8212-8217.

Tatoyan, A. and Giulivi, C. (1998). Purification and characterization of a nitric-oxide synthase from rat liver mitochondria. *J.Biol.Chem.* **273**: 11044-11048.

Tenhunen, R., Marver, H.S., Schmid, R. (1969). Microsomal heme oxygenase. Characterization of the enzyme. *J.Biol.Chem.* **244**: 6388-6394.

Tenhunen, R., Ross, M.E., Marver, H.S., Schmid, R. (1970). Reduced nicotinamide-adenine dinucleotide phosphate dependent biliverdin reductase: partial purification and characterization. *Biochemistry* **9**: 298-303.

Torres, J., Darley-Usmar, V., Wilson, M.T. (1995). Inhibition of cytochrome c oxidase in turnover by nitric oxide: mechanism and implications for control of respiration. *Biochem.J.* **312**: 169-173.

Torres, J., Cooper, C.E., Wilson, M.T. (1998). A common mechanism for the interaction of nitric oxide with the oxidized binuclear centre and oxygen intermediates of cytochrome c oxidase. *J.Biol.Chem.* **273**: 8756-8766.

Trimmer, B.A., Aprille, J.R., Dudzinski, D.M., Lagace, C.J., Lewis, S.M., Michel, T., Qazi, S., Zayas, R.M. (2001). Nitric oxide and the control of firefly flashing. *Science* **292**: 2486-2488.

Turrens, J.F. and Boveris, A. (1980). Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. *Biochem.J.* **191**: 421-427.

Vodovotz, Y. and Bogdan, C. (1994). Control of nitric oxide synthase expression by transforming growth factor-beta: implications for homeostasis. *Prog.Growth Factor Res.* **5**: 341-351.

Wang, F., Sekine, H., Kikuchi, Y., Takasaki, C., Miura, C., Heiwa, O., Shuin, T., Fujii-Kuriyama, Y., Sogawa, K. (2002). HIF-1alpha-prolyl hydroxylase: molecular target of nitric oxide in the hypoxic signal transduction pathway. *Biochem.Biophys.Res.Commun.* **295**: 657-662.

Wang, M., Roberts, D.L., Paschke, R., Shea, T.M., Masters, B.S., Kim, J.J. (1997). Three-dimensional structure of NADPH-cytochrome P450 reductase: prototype for FMN- and FAD-containing enzymes. *Proc.Natl.Acad.Sci.U.S.A* **94**: 8411-8416.

Warner, T.D., Mitchell, J.A., Sheng, H., Murad, F. (1994). Effects of cyclic GMP on smooth muscle relaxation. *Adv.Pharmacol.* **26**: 171-194.

Waypa, G.B., Marks, J.D., Mack, M.M., Boriboun, C., Mungai, P.T., Schumacker, P.T. (2002). Mitochondrial reactive oxygen species trigger calcium increases during hypoxia in pulmonary arterial myocytes. *Circ.Res.* **91**: 719-726.

Wharton, D.C. and Tzagoloff, A. (1967). Cytochrome oxidase from beef heart mitochondria. *Methods Enzymol.* **10**: 245-250.

Wiesel, P., Foster, L.C., Pellacani, A., Layne, M.D., Hsieh, C.M., Huggins, G.S., Strauss, P., Yet, S.F., Perrella, M.A. (2000). Thioredoxin facilitates the induction of heme oxygenase-1 in response to inflammatory mediators. *J.Biol.Chem.* **275**: 24840-24846.

Winyard, P.G., Moody, C.J., Jacob, C. (2005). Oxidative activation of antioxidant defence. *Trends Biochem.Sci.* **30**: 453-461.

Wohlrab, H. and Ogunmola, G.B. (1971). Carbon monoxide binding studies of cytochrome a3 hemes in intact rat liver mitochondria. *Biochemistry* **10**: 1103-1106.

Wolosker, H., Panizzutti, R., Engelender, S. (1996). Inhibition of creatine kinase by S-nitrosoglutathione. *FEBS Lett.* **392**: 274-276.

Wood, S.M., Gleadle, J.M., Pugh, C.W., Hankinson, O., Ratcliffe, P.J. (1996). The role of the aryl hydrocarbon receptor nuclear translocator (ARNT) in hypoxic induction of gene expression. Studies in ARNT-deficient cells. *J.Biol.Chem.* **271**: 15117-15123.

Yamamoto, T. and Bing, R.J. (2000). Nitric oxide donors. *Proc.Soc.Exp.Biol.Med.* **225**: 200-206.

Zagotta, W.N. and Siegelbaum, S.A. (1996). Structure and function of cyclic nucleotide-gated channels. *Annu.Rev.Neurosci.* **19**: 235-263.

Zakhary, R., Gaine, S.P., Dinerman, J.L., Ruat, M., Flavahan, N.A., Snyder, S.H. (1996). Heme oxygenase 2: endothelial and neuronal localization and role in endothelium-dependent relaxation. *Proc.Natl.Acad.Sci.U.S.A* **93**: 795-798.

Zhang, Y. and Chen, F. (2004). Reactive oxygen species (ROS), troublemakers between nuclear factor-kappaB (NF-kappaB) and c-Jun NH(2)-terminal kinase (JNK). *Cancer Res.* **64**: 1902-1905.

PUBLICATIONS

Hagen, T., Taylor, C.T., Lam, F., Moncada, S. (2003). Redistribution of intracellular oxygen in hypoxia by nitric oxide: effect on HIF1alpha. *Science* **302**: 1975-1978.

Hagen, T., D'Amico, G., Quintero, M., Palacios-Callender, M., Hollis, V., Lam, F., Moncada, S. (2004). Inhibition of mitochondrial respiration by the anticancer agent 2-methoxyestradiol. *Biochem.Biophys.Res.Commun.* **322**: 923-929.

D'Amico, G., Lam, F., Hagen, T., Moncada, S. (2005) Inhibition of cellular respiration by endogenously-produced carbon monoxide. Submitted.