PHYSIOLOGICAL REGULATION AND PATHOLOGICAL INHIBITION OF TISSUE RESPIRATION BY NITRIC OXIDE

Submitted by

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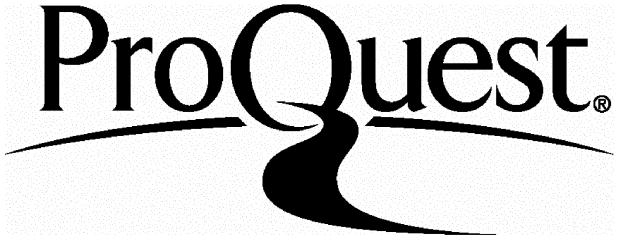

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ABSTRACT

PHYSIOLOGICAL REGULATION AND PATHOLOGICAL INHIBITION OF TISSUE RESPIRATION BY NITRIC OXIDE

Antonia Orsi

Long-term exposure of Jurkat, L929 and J774 cells to exogenous NO inhibits cellular respiration at the level of complex IV that is reversed by oxyHb and at the level of complex I that is not reversed by oxyHb. Long-term exposure of NO to the cells causes a decrease in reduced glutathione concentration that precedes the inhibition at the level of complex I. This inhibition is reversed by addition of reduced glutathione or cold light, suggesting that S-nitrosylation of thiols is necessary for the activity of the enzyme. Furthermore, long-term exposure of the cells to NO decreases oxygen utilisation due to two distinct actions, one being the inhibition of cellular oxygen consumption and the other, the generation of a hypoxic microenvironment due to the oxygen consumption by the extracellular medium.

Inhibition of basal NO, with a NO synthase inhibitor, increases cellular respiration in iNOS mutant, iNOS and eNOS wild type, but not in eNOS mutant mice. Administration of a low dose of endotoxin to the mice leads to a short-term generation of NO associated with a short-term inhibition of mitochondrial respiration resulting in non-lethal shock. Whereas administration of a high dose of endotoxin leads to a sustained generation of NO accompanied by a sustained inhibition of mitochondrial respiration, at the level (at least in part) of complex I, resulting in lethal shock.

These results suggest that NO can be considered as a physiological regulator and a pathological inhibitor of tissue respiration. The basal release of small amounts of NO acts as a regulating mechanism, whereby cells respond to changes in their environment and regulate ATP production. By contrast the generation of high concentrations of NO over a sustained period decreases reduced glutathione concentration and pathologically inhibits cellular respiration leading to multiple organ failure and ultimately death.

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Chapters 3, 4 and 5 were performed in collaboration with Dr B. Beltran. In particular Dr B. Beltran performed a large part of the complex I and GSH measurements and are included for completeness. The measurement of blood pressure in chapters 7 and 8 were performed with the help of Miss D. Morren and Dr D. D. Rees.

Abbreviations

ACh	Acetylcholine
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BH_4	Tetrahydrobiopterin
Bk	Bradykinin
CoQ	Coenzyme Q or ubiquinone
$CoQH_2$	Ubiquinol
COX II	Cyclooxygenase II
DEA-NO	Diethylamine nonoates
DETA-NO	Diethylenetriamine nonoates or (z)-1-[2-(2-aminoethyl)-N-(2-ammmoniothyl)amino]diazen-1-ium-1,2diolate
DMEM	Dulbecco's Modified Eagle's Medium
D-NMMA	D-N^G (mono)methylarginine HCl
eNOS	Endothelial nitric oxide synthase
EDTA	N,N'-1,2-ethanediybis[N-(carboxymethyl)glycine] tetra sodium salt
FAD	Flavine adenine dinucleotide
FCCP	Carbonyl cyanide-p- (trifluoro-methoxy) phenylhydrazone
FeS	Ferrous sulphide
FMN	Flavin mononucleotide
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSH	Reduced glutathione
5-HT	5-Hydroxytryptamine
HEPES	4-(2-Hydroxyethyl)-1-piperazineethane-sulphonic acid
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon-γ
IL-1,4,6,8,10	Interleukin-1,4,6,8,10
iNOS	Inducible nitric oxide synthase
L-NAME	L-N^G-nitro-arginine-methyl ester
LNMMA	L-N^G-(mono)methylarginine HCl
LPS	Lipopolysaccharide
NAD	Nicotinamide adenine dinucleotide

NADH	Nicotinamide adenine dinucleotide reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate reduced form
NFκB	Nuclear transcriptor factor kappa B
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO^-	Nitroxyl anion
NO^+	Nitrosonium ion
$ONOO^-$	Peroxynitrite
OxyHb	Oxyhaemoglobin
PHP	Pyridoxylated haemoglobin polyoxyethylene conjugate
PMNs	Polymorphonuclear leukocytes
PTP	Permeability transition pore
PVC	Polyvinyl chloride
RSH	Mercaptans
SPER-NO	Spermine nonoates
TGF-β	Transforming growth factor β
TMPD	N,N,N',N'-tetramethyl-p-phenylenediamine
TNF-α	Tumour necrosis factor-α
VCAM	Vascular cell adhesion molecule
1400W	N-(3-(aminomethyl)benzyl)acetamide

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CHAPTER 1

GENERAL INTRODUCTION

1 GENERAL INTRODUCTION

1.1 NITRIC OXIDE

Nitric oxide (NO) is a free-radical gaseous molecule, which has been shown over the last decade to play a wide range of roles in biological systems. It is soluble in both lipid and water and diffuses freely within and between cells and, as such, can transmit signals between cells or from one part of a cell to another (Rees, 1999a). It has a half-life of only a few seconds and readily reacts with oxygen free radicals (Moncada *et al.*, 1991). Nitric oxide forms complexes with haem-containing proteins particularly soluble guanylyl cyclase, which accounts for many of its physiological actions (Ignarro *et al.*, 1987a). In the blood, haemoglobin inactivates NO by binding it to form nitrosohaemoglobin and by catalysing the degradation of NO to nitrite and nitrate, resulting in the formation of methaemoglobin (Wennmalm *et al.*, 1992). Nitric oxide will also bind to other plasma constituents including thiols, albumin and a variety of other proteins (Stamler & Loscalzo, 1992). Several studies have shown that NO is involved in the regulation of diverse processes including smooth muscle relaxation, inhibition of platelet activation, central and peripheral neurotransmission and the cytotoxic actions of immune cells. More recently, it has been suggested that NO plays an important role in the regulation of the mitochondrial respiration in physiological and pathological conditions (Rees, 1999a).

1.1.1 BIOSYNTHESIS OF NITRIC OXIDE

Nitric oxide is synthesised from the guanidino nitrogen of L-arginine by the enzyme, nitric oxide (NO) synthase. Nitric oxide synthases are haem-containing enzymes, named according to the cell type or conditions under which they were first identified - endothelial NO synthase (eNOS or type III NOS), neuronal NO synthase (nNOS or type I NOS) and inducible NO synthase (iNOS or type II NOS). In humans, the genes encoding for these enzymes are located on chromosome 12 (eNOS), 17 (nNOS) and 7 (iNOS). Distinct genes encode for each isoform of NOS and consist of either 26 exons (iNOS and eNOS) or 29 exons (nNOS Knowles & Moncada, 1994) (Nathan, 1992). The isoforms of NO synthase are large (125-155 kDa), dimeric enzymes, containing both oxidative and reductive domains. The oxygenase of each subunit interacts to form the dimer and the reductase domains are attached as independent extensions. The oxygenase domain contains binding sites for L-arginine, tetrahydrobiopterin (BH_4) and iron protoporphyrin IX (haem). The reductase domain binds flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and nicotinamide adenine dinucleotide phosphate, reduced form (NADPH). The reductase domain of NO synthase is similar to cytochrome P450 reductase and provides an electron shuttling transport chain from NADPH, FAD and FMN to the oxygenase domain, where oxidation of L-arginine occurs (Bredt *et al.*, 1991; White & Marletta, 1992; Stuehr, 1997). Molecular oxygen is also a substrate for this reaction, which proceeds via the formation of L-N^G-hydroxyarginine and results in the formation of NO, with L-citrulline as the co-product. The isoform eNOS and nNOS are constitutive and regulated by

calmodulin and require an elevation of intracellular calcium for activation. By contrast, the iNOS isoform is induced, by inflammatory mediators and bacterial products, and binds calmodulin tightly so that its activation appears functionally independent of intracellular calcium concentration.

All of the isoforms may be inhibited either by natural analogues of arginine such as L-N^G-methylarginine (L-NMMA, Hibbs, *et al.*, 1987) and N^G-dimethylarginine (asymmetric dimethylarginine, (de Belder *et al.*, 1994) or by synthetic analogues such as L-N^G-nitroarginine methylester (L-NAME; (Rees *et al.*, 1990a) and N-(3-(aminomethyl)benzyl) acetamidine (1400W; (Garvey *et al.*, 1997). Although there is close homology between the isoforms, inhibitors have been developed with selectivity for iNOS over eNOS conferred by substituting the guanidino function of arginine with an amidine group (Garvey *et al.*, 1997). L-NMMA and other inhibitors of NO synthase have been used extensively as probes to characterise the physiological and pathophysiological roles of NO.

1.1.1.1 Endothelial and neuronal NOS

Endothelial NOS is found in the endothelium (Figure 1.1) and in other cell types including platelets and the endocardium. Neuronal NOS is found in the brain and in nitrergic nerves throughout the peripheral nervous system.

The isoforms eNOS and nNOS are constitutive and regulated by calmodulin and require an elevation of calcium for activation. Recent studies have shown that a local increase in calcium near the plasma membrane may be sufficient for the activation of eNOS (Lin *et al.*, 2000) whereas the opening of

calcium channels in peripheral nerves involved in nitrergic transmission has been shown to activate nNOS (Rand & Li, 1995).

In addition the activities of eNOS and nNOS may also be affected by phosphorylation or various post-translational modifications that regulate localisation within the cell. Indeed the N-terminal thiopalmitoylation of eNOS is required for targeting the enzyme to the specialised signal-transducing membrane domain termed caveolae (Liu *et al.*, 1996; Michel *et al.*, 1997). Caveolin, the principal structural protein in caveolae, interacts with eNOS leading to a calmodulin-reversible regulation (Michel & Feron, 1997).

1.1.1.2 Inducible NOS

The inducible calcium-independent NO synthase (iNOS) is expressed in macrophages, neutrophils, lymphocytes, hepatocytes and other cells. The combination of cytokines and microbial products required to activate iNOS and the subsequent time course of expression varies according to the type of cell and the species (Rees *et al.*, 1990b; Salter *et al.*, 1991; Rees, 1995a; Figure 1.1).

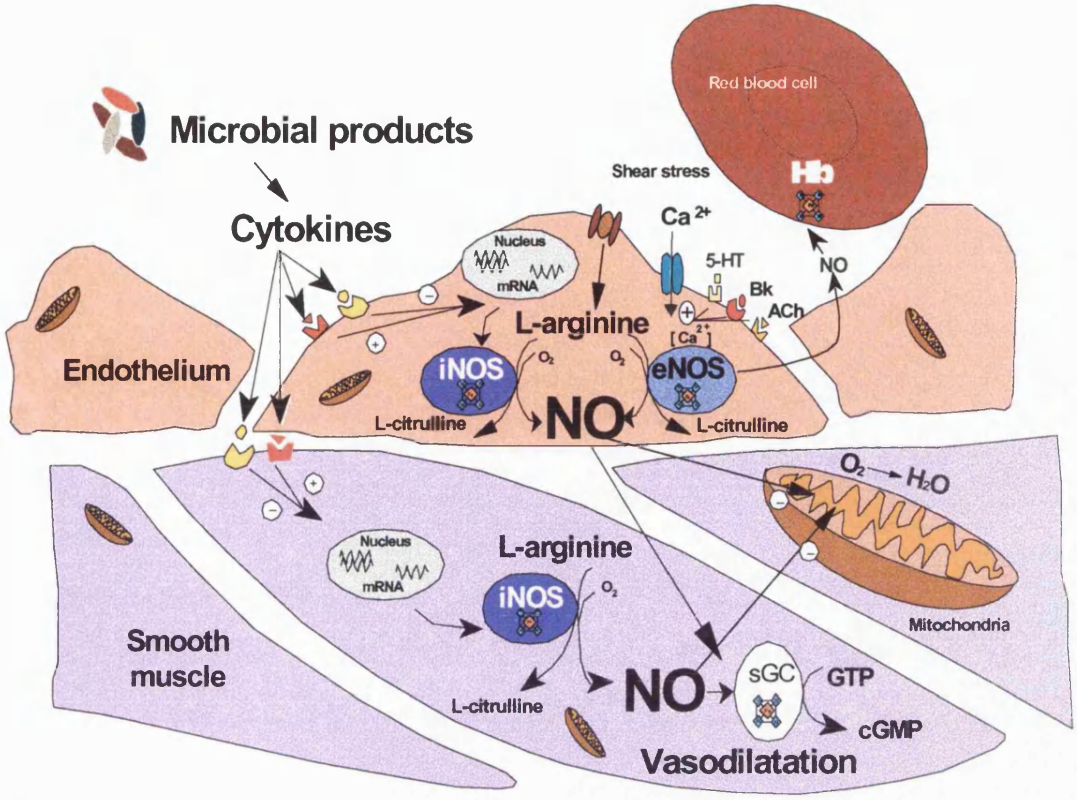

Figure 1.1 Mechanisms by which NO is regulated in the cells and potential targets of NO. Shear stress of circulating blood, chemical action of vasoactive mediators such as serotonin (5-HT), acetylcholine (ACh) and bradykinin (BK) elevate intracellular calcium activating eNOS. Microbial products and/or cytokines activate the expression of iNOS. The expression of this enzyme requires *de novo* protein synthesis over several hours and large quantities of NO are produced.

1.1.1.2.1 Transcriptional regulation

Cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-4 in human epithelial cells, IL-2, and interferon-γ (IFN-γ) and certain microbial products activate iNOS (Rees *et al.*, 1990b; Radomski *et al.*, 1990a) whereas IL-4, IL-8 and IL-10, in several cell types, inhibit this process (McCall *et al.*, 1992; Schini *et al.*, 1992). Nuclear factor kappa B (NFκB) is one of the key transcriptional factors regulating iNOS expression and exists as a silent dimeric protein formed by two subunits, p65 (Rel-A) and p50 (NF-

κB1), present in the cytoplasm bound to an inhibitory protein inhibitory κB (IκB). As a consequence of cellular activation by various immunostimulants, viruses, cytokines, reactive oxygen intermediates and protein kinase C activators, the IκB protein is phosphorylated by IκB kinase. This results in the liberation of NFκB, which translocates into the nucleus, where it binds to the promoter regions of various inflammatory genes such as iNOS as well as other inducible products such as cyclooxygenase II (COX-II), 5'-lipoxygenase, cytosolic phospholipase A2, genes encoding for proinflammatory cytokines, adhesion molecules and receptors such as IL-2 (Barnes PJ, 1997).

1.1.1.2.2 Post-transcriptional regulation

Transforming growth factor β (TGF-β) post-transcriptionally regulates iNOS expression, destabilising iNOS mRNA; thus the synthesis of iNOS protein is suppressed and its degradation is accelerated (Vodovotz & Bogdan, 1994). TGF-β also attenuates induction of iNOS in macrophages. Whereas serum or tetradecanoylphorbolacetate augments induction of iNOS. The stability of mRNA also appears to be reduced by IL-4, IL-8 and IL-10 as well as macrophage deactivating factor.

1.1.1.2.3 Post-translational regulation

At a post-translational level, iNOS is regulated by both substrate and cofactor availability. L-arginine is the physiological nitrogen donor for NO formation so its availability represents a key controlling point. NADPH is an essential cofactor in NO synthesis, functioning as an electron donor and is upregulated by the same inflammatory mediators that induce iNOS

expression (Garcia-Nogales *et al.*, 1999). Tetrahydrobiopterin (BH_4), another essential cofactor in NO synthesis is involved in the binding of two inactive iNOS isoforms into the active dimeric form (Wong & Billiar, 1995).
In addition, high concentrations of NO can 'feed back' to the enzyme and inhibit its activity, providing an additional regulatory step (Assreuy *et al.*, 1993).

1.1.2 CELLULAR TARGETS

Nitric oxide moves freely within and between cells and is also transported from the cell by putative intracellular carriers such as dinitrosyl-iron(II) complexes (Mulsch *et al.*, 1991), N^G-hydroxyl-L-arginine-NO adduct (Hecker *et al.*, 1995) and glutathione-NO complex (Hogg *et al.*, 1996). Nitric oxide interacts at the surface or within the plasma membrane of the target cell. After diffusion into the target cell, NO can S-nitrosylate enzymes such as glyceraldehyde-3-phosphate dehydrogenase (Padgett & Whorton, 1995), protein kinase C (Gopalakrishna *et al.*, 1993), phosphotyrosine protein phosphatase (Caselli *et al.*, 1994), glutathione peroxidase (Asahi *et al.*, 1995), glutathione reductase (Becker *et al.*, 1995), methionine synthase (Nicolaou *et al.*, 1996) and creatine kinase (Gross *et al.*, 1996). Nitric oxide also mediates Fe^{2+} release from target cells, destroying Fe-S clusters in enzymes, including the citric acid cycle enzyme aconitase (Hibbs, *et al.*, 1988). Other intracellular targets include proteins containing zinc-fingers such as protein kinase C (Gopalakrishna *et al.*, 1993) and alcohol dehydrogenase (Gergel & Cederbaum, 1996).

Other targets of NO are at the level of the nucleus: NO can cause guanidine/cytosine to adenine/thymidine transition (Wink *et al.*, 1991), mediates DNA strand breaks (Nguyen *et al.*, 1992), activates poly (ADPribose) polymerase (PARP, (Zhang *et al.*, 1994a) and inhibits DNA repair enzymes (Stuehr *et al.*, 1991; Lepoivre *et al.*, 1991). Nitric oxide can directly regulate inflammatory genes such as COX-II (Hughes *et al.*, 1999), heat shock protein 70 (Malyshev *et al.*, 1996), manganese superoxide dismutase (Lewis-Molock *et al.*, 1994) and activator protein-1 (Peunova & Enikolopov, 1993). The respiratory chain is also an important intracellular target of NO.

1.2 RESPIRATORY CHAIN

Mitochondria produce most of the energy in animal cells through oxidative phosphorylation, a process in which electrons are passed along a series of carrier molecules called the respiratory chain (Figure 1.2). These electrons are generated from NADH and $FADH_2$ and are ultimately transferred to oxygen. The respiratory chain comprises four complexes (complex I-IV) situated in the inner membrane of the mitochondria. The passage of electrons between these complexes releases free energy that generates a proton gradient across the membrane and is then used by adenosine triphosphate (ATP) synthase to make ATP from adenosine diphosphate (ADP, Figure 1.2).

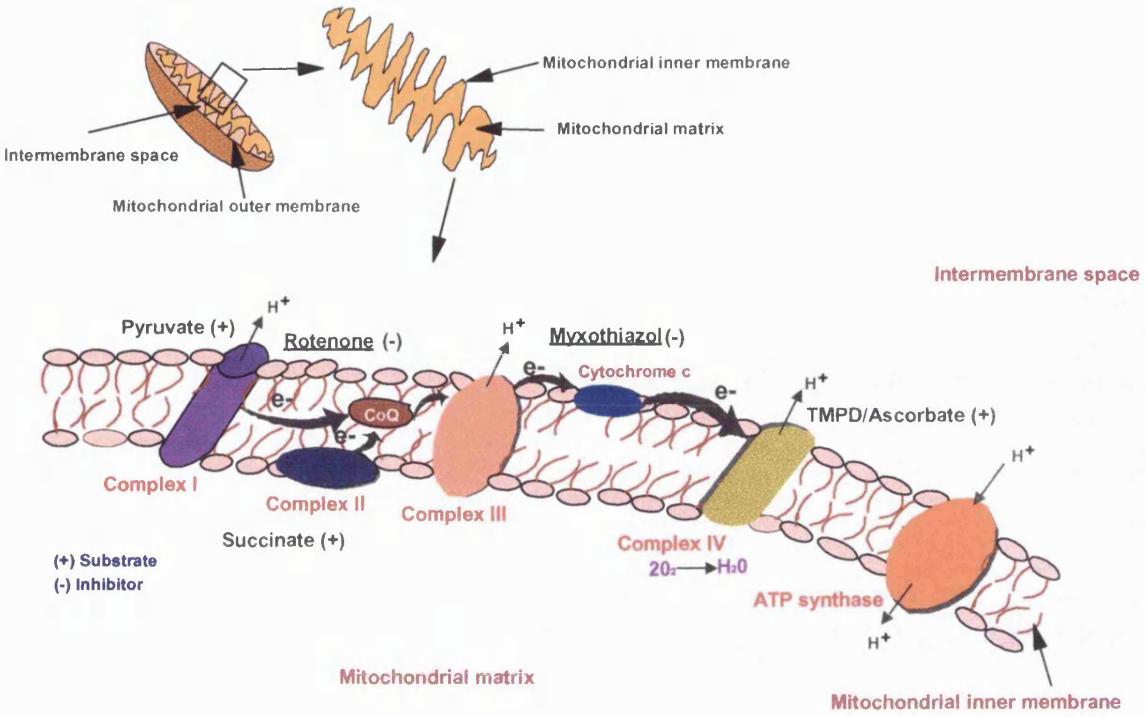

Figure 1.2 The respiratory chain situated in the inner membrane of the mitochondria includes 4 complexes (I-IV) and two smaller electron carriers, coenzyme Q (CoQ) and cytochrome c that act as shuttles between the complexes. The passages of electrons (e^-) through complexes I to III and IV result in pumping of protons (H^+) from the matrix to the intermembrane compartment.

1.2.1 COMPLEX I

Complex I (also known as NADH dehydrogenase or NADH-ubiquinone-reductase) is a large protein, tightly bound to the respiratory chain. It consists of 43 different subunits of unknown stoichiometry, one flavin mononucleotide (FMN), different FeS clusters in iron-sulphur proteins, and at least three bound quinol molecules. Electrons from NADH are transferred to the FMN group to produce $FMNH_2$ and then passed to the iron atoms of the FeS clusters. Once the iron atoms accept the electrons they are reduced from ferric (Fe^{3+}) to ferrous status (Fe^{2+}). Electrons are then passed to

ubiquinone (coenzyme Q or CoQ), converting it to ubiquinol ($CoQH_2$), and then to complex III (Lehninger, 1979; Wolfe & Dasta, 1995; Figure1.3).

1.2.2 COMPLEX II

Complex II (also known as succinate-dehydrogenase or succinate-ubiquinone reductase) participates in the respiratory chain by transferring electrons from succinate to the ubiquinone pool. It contains FAD (flavinin-adenine dinucleotide) and several FeS centres and is anchored to the membrane by a b-type cytochrome. Cytochromes are proteins with a bound haem group that contains an iron atom. Different cytochromes have different haem groups but all cytochromes have the ability to act as an electron carrier. Electrons from $FADH_2$ pass to FeS clusters and are then passed to ubiquinone. The electrons then enter the main electron transport chain and cause protons to be pumped out of the mitochondria. However complex II itself is not a proton pump because the energy change of the overall reaction is too small (Lehninger, 1979; Wolfe & Dasta, 1995; Figure 1.3).

1.2.3 COMPLEX III

Complex III (also known as ubiquinol cytochrome reductase and cytochrome bc_1) contains two b-type cytochromes, b_l and b_h, a cytochrome c_1, an FeS protein and between four and six additional subunits. Complex III delivers the electrons from ubiquinol ($CoQH_2$) to cytochrome c. Ubiquinol, a lipid-soluble compound moving within the membrane, is a two-electron carrier whereas cytochromes are one-electron carriers. For this reason the pathway of electron transfer within cytochrome bc_1 is complicated and involves ubiquinol releasing first one electron and a proton to become

ubisemiquinone and then releasing the second electron and a second proton to become ubiquinone. The electrons pass from ubiquinol through the cytochrome b, FeS cluster, cytochrome c_1 to the next electron carrier cytochrome c. Cytochrome c is a peripheral membrane protein that is loosely bound to the outer surface of the inner mitochondrial membrane. It binds to the cytochrome bc_1 complex and accepts an electron via a Fe^{3+} to Fe^{2+} transition. Then it binds to complex IV and donates the electron, reverting to the ferric state ($Fe^{3+;}$ Lehninger, 1979; Wolfe & Dasta, 1995; Figure1.3).

1.2.4 COMPLEX IV

Complex IV (also known as cytochrome c oxidase and cytochrome aa_3) is the terminal complex of the mitochondrial respiratory chain, contains two cytochromes, a and a_3 and two copper centres (CuA and CuB). During electron transfer, the iron atoms of the cytochromes cycle between the Fe^{3+} and Fe^{2+} states whilst the copper atoms cycle between the Cu^{2+} and Cu^{+}. Complex IV transfers four electrons from four cytochrome c molecules and four protons to molecular oxygen to form two molecules of water (Lehninger, 1979; Wolfe & Dasta, 1995; Figure 1.3).

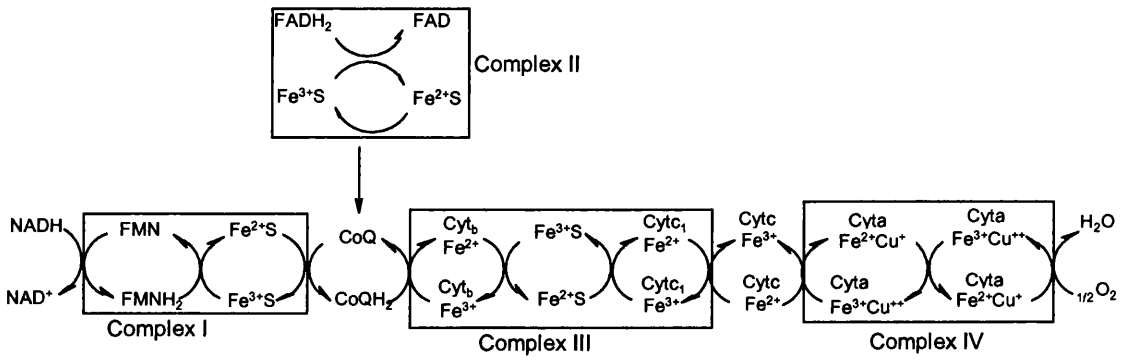

Figure 1.3 Electron transport of the respiratory chain (see the text for further details).

1.3 PHYSIOLOGICAL ROLE OF NITRIC OXIDE

Constitutive NOS is involved in a variety of physiological processes including regulation of cellular respiration in addition to that of the cardiovascular system.

1.3.1 CELLULAR RESPIRATION

In 1955, Wainio and co-workers reported that addition of NO to the reduced form of isolated cytochrome c induces a shift in the optical spectrum of complex IV (Wainio, 1955). Nitric oxide binds to the reduced form of haem a_3 (complex IV) as does oxygen (Blokzijl-Homan & Gelder, 1971; Stevens *et al.*, 1979; Brudvig & Chan, 1979; Boelens *et al.*, 1983). The addition of exogenous NO to rat skeletal muscle mitochondria reversibly inhibits oxygen consumption at the level of complex IV (Cleeter *et al.*, 1994). Furthermore the addition of NO to isolated cytochrome oxidase causes an immediate inhibition of oxygen consumption that is reversed upon removal of NO, suggesting that NO may be a physiological regulator of respiration (Brown *& Cooper*, 1994). In addition, NO causes rapid and oxygen-dependent inhibition of respiration at complex IV in synaptosomes (Brown *& Cooper*, 1994), astrocytes (Brown *et al.*, 1995) and pancreatic β-cells (Laffranchi & Spinas, 1996).

The concept that endogenous NO might act as a physiological modulator of respiration has emerged from studies both in isolated cells and in tissues *in vitro* and *in vivo.*

1.3.1.1 Studies in cells

Inhibition of NO synthase in lung alveolar type II cells increases cellular oxygen consumption, while an NO donor produces an opposing effect, suggesting that constitutive NO production inhibits respiration (Miles *et al.*, 1996). In addition, the basal release of NO, in primary cultures of endothelial cells, is sufficient to partially inhibit endothelial cell respiration, to the extent that inhibition of NOS leads to a stimulation of respiration. Furthermore, following stimulation with bradykinin, a brief pulse of NO produces a reversible inhibition of cellular respiration (Clementi *et al.*, 1999).

1.3.1.2 Studies In tissues *in vitro* and in the whole body

Addition of bradykinin or carbachol to slices of skeletal and heart muscle *in vitro* causes an NO-dependent inhibition of tissue respiration, principally due to a direct action of NO on the mitochondrial respiratory chain (Shen *et al.*, 1995; Xie *et al.*, 1996). Furthermore, NO donors and bradykinin decrease the oxygen consumption in kidney slices (Laycock *et al.*, 1998). In addition bradykinin added to myocardial tissue *in vitro* induces a significant decrease in oxygen consumption measured by a Clark-type oxygen electrode (Loke *et al.*, 1999).

In conscious dogs, L-N-nitroarginine causes a rapid and sustained increase in whole body oxygen consumption *in vivo* (Shen *et al.*, 1994). Furthermore, in exercising dogs, addition of L-N-nitroarginine causes an increase in myocardial oxygen consumption (Ishibashi *et al.*, 1998). All these results are consistent with the interpretation that basal NO regulates tissue respiration.

1.3.2 CARDIOVASCULAR SYSTEM

In 1980, Furchgott and Zawadzki demonstrated that the relaxation of rabbit aorta in response to acetylcholine (ACh) is entirely dependent on the presence of an intact endothelial cell layer. Nitric oxide was later shown to account for this endothelium-dependent relaxation and the release of NO from the endothelium has since been demonstrated in arteries, arterioles, veins and venules from a wide range of species, including humans, both in *vitro* and *in vivo* (Palmer *et al.*, 1988; Furchgott & Vanhoutte, 1989; Rees *et al.*, 1989a; Vallance *et al.*, 1989; Ignarro *et al.*, 1987b). Furthermore, the vasorelaxant properties of many hormones and autocoids including bradykinin, substance P and serotonin have been shown to be endothelium-dependent (Furchgott, 1983; Moncada *et al.*, 1991). Pharmacological inhibition of NOS with substrate analogues such as L-NMMA not only impairs the response to 'endothelium-dependent' dilators, particularly in conduit vessels, but also causes an endothelium-dependent vasoconstriction of isolated arteries, arterioles and, to a lesser extent, veins in many species including humans (Rees *et al.*, 1989a; Vallance *et al.*, 1989; Calver *et al.*, 1993). Similarly, administration of L-NMMA *in vivo* causes widespread vasoconstriction and elevation of blood pressure (Rees *et al.*, 1989b). In humans, infusion of L-NMMA into the brachial artery of healthy volunteers reduces resting blood flow by about 40-50% (Vallance *et al.*, 1989) and its administration intravenously increases systemic vascular resistance and blood pressure (Haynes *et al.*, 1993). This indicates that there is a continuous generation of NO that maintains resistance vessels in a dilated state and that the cardiovascular system as a whole is in a state of active

vasodilatation in both animals and humans (Rees *et al.*, 1989b; Moncada *et al.*, 1991; Calver *et al.*, 1993).

In addition to its effects on smooth muscle within the blood vessel, NO also affects blood cells. Nitric oxide prevents the adhesion of platelets and white cells to the endothelium, inhibits the aggregation of platelets and induces disagregation of aggregated platelets (Radomski *et al.*, 1996). Platelets themselves produce NO by a constitutive NOS, which may act as a negative feedback mechanism to inhibit platelet aggregation and adhesion (Radomski *et al.*, 1990b). The antiaggregatory/antiadhesive effects of NO are mediated by activation of the soluble guanylyl cyclase and an elevation in cGMP concentrations, which lead to a decrease in intracellular calcium.

1.4 PATHOLOGICAL ROLE OF NITRIC OXIDE

The inducible Ca^{2+}-independent NO synthase (iNOS) is expressed in phagocytes, macrophages, neutrophils, lymphocytes, hepatocytes and other cells following activation by live organisms, microbial products and/or certain cytokines. The activation of iNOS produces much larger quantities of NO than the constitutive enzyme.

Although overproduction of NO plays a significant role in inflammatory, ischaemic and neurogenerative pathologies, as well as in host defence against pathogens, it is hard to interpret its actions in terms of specific mechanisms. In addition to NO, other agents can be involved or NO may give rise to a variety of chemical products such as peroxynitrite ($ONOO^-$). Peroxynitrite can react with a wide variety of biomolecules, including proteins via nitration of tyrosine (Ischiropoulos *et al.*, 1992; Grune *et al.*, 1998) and tryptophan residues (Alvarez *et al.*, 1996; Padmaja *et al.*, 1996) or oxidation

of methionine (Pryor *et al.*, 1994). Peroxynitrite also reacts with DNA (Beckman & Koppenol, 1996), lipids and antioxidants (Radi *et al.*, 1991).

1.4.1 CELLULAR RESPIRATION

In the last decade many studies have sought to clarify the role of NO on the inhibition of respiration in pathological conditions. For many years it has been known that activated macrophages produce substances that are cytotoxic to other cells by irreversibly inhibiting their respiration (Hibbs, *et al.*, 1987, Granger *et al.*, 1980; Drapier & Hibbs, 1986). More recently several studies have shown that macrophages (Hibbs, *et al.*, 1988) and several other cells including astrocytes (Bolanos *et al.*, 1994), hepatocytes (Stadler *et al.*, 1991), smooth muscle cells (Geng *et al.*, 1992) and myocytes (Oddis & Finkel, 1995) activated with cytokines and endotoxin also produce cytotoxic substances and inhibit cellular respiration. Although these activated cells produce a variety of potentially toxic substances many of their toxic properties have been associated with the expression of iNOS and a sustained overproduction of NO. Indeed inhibitors of NOS or scavengers of NO prevent this cytotoxic activity (Hibbs, *et al.*, 1988; Drapier *et al.*, 1988; Stuehr & Nathan, 1989). However, it is difficult to define the specific mechanism underlying the inhibition of the respiration because, as already mentioned, NO may give rise to a variety of chemical products such as peroxynitrite. Peroxynitrite causes inhibition of ATP synthase and complex I-II (Radi *et al.*, 1994a). Furthermore addition of peroxynitrite to mitochondria causes opening of the permeability transition pore (PTP), a pore in the outer and inner mitochondrial membrane, causing the exit of cytochrome c (Liu *et al.*, 1996; Skulachev, 1998). Despite the clear importance of iNOS

expression in the cytotoxicity of activated cells, the role of NO itself on the respiratory enzymes requires further clarification. Short-term exposure, of the rat skeletal muscle mitochondria to exogenous NO, inhibits mitochondrial oxygen consumption in a reversible manner, by competing with oxygen at the level of complex IV (Cleeter *et al.*, 1994). Furthermore incubation of neurones for 24 h in the presence of exogenous NO decreases glutathione concentrations and inhibits complex I, II-III and IV (Bolanos *et al.*, 1996). In addition, non-activated J774 macrophages exposed to a constant concentration of exogenous NO induces a progressive inhibition of respiration occurring at the level of complex I (Clementi *et al.*, 1998).

1.4.2 INFLAMMATION

Inflammation is a defensive reaction to injuries such as mechanical trauma (crushing), radiation, direct chemical damage and invading microorganisms. In the acute phase of inflammation, which usually spans 2-6 hours, vascular and cellular events take place. The vascular events start immediately with dilatation of blood vessels, an increase in blood flow and subsequently an increase in vascular permeability with exudation of the plasma. The plasma contains numerous systems such as coagulation, complement and the kinin system. These systems act as mediators of the reaction against the initiating inflammatory triggers. The cellular events involve the activation and recruitment of mast cells, polymorphonuclear cells (neutrophils, eosinophils and basophils) and mononuclear cells (monocytes and lymphocytes, Dale & Foreman, 1984). During the acute phase, the chemotatic stimulation and the phagocytosis of neutrophils activates the respiratory burst oxidase of neutrophils. This membrane-bound enzyme generates the respiratory burst,

transferring electrons from cytosolic NADPH to oxygen producing superoxide. The products of this burst are essential for killing microorganisms, but are also a cause of tissue damage (Baggiolini & Wymann, 1990).

As a temporary reaction to trauma or pathogenic insult, constitutive NOS is increased (Salvemini *et al.*, 1989; Bhagat *et al.*, 1999) in the "acute phase" of inflammation and alters the expression of adhesion molecules on the surface of the endothelium and circulating cells (Radomski *et al.*, 1996). Nitric oxide reduces P-selectin expression from alpha granules of platelets (Michelson *et al.*, 1996), reduces intercellular adhesion molecule -1 (ICAM-1) expression by endothelial cells (Biffl *et al.*, 1996) and ICAM-1 expression induced by IL-1 in mesangial cells *in vitro* (Ikeda *et al.*, 1996). Indeed, the basal release of NO by the vascular endothelium can decrease the adhesion of polymorphonuclear leukocytes (PMNs) due, at least in part, to inhibition of endothelial expression of the neutrophil ligand (CD11a/CD18), adhesion molecules including ICAM-1 and vascular cell adhesion molecule (VCAM; Hickey & Kubes, 1999). Consequently, rolling of leukocytes along the endothelium is inhibited and migration from the vasculature is impeded (Kanwar & Kubes, 1995; Kubes *et al.*, 1991). In addition, inhibition of eNOS, with the NO synthase inhibitor L-NMMA, causes a superoxide dependent leukocyte adhesion (Kubes *et al.*, 1993; Gaboury *et al.*, 1993); indeed superoxide dismutase and other anti-oxidants reduce some of the L-NMMA-induced effects (mast cell degranulation, leukocyte adhesion and respiratory burst) and reduce the mast cell-dependent increase in leukocyte adhesion *in vitro* (Niu *et al.*, 1994) and *in vivo* (Kubes *et al.*, 1993). These data suggest

an interaction between constitutive NO and superoxide produced either constitutively in small amounts or by activated neutrophils (Warren *et al.*, 1987). Thus the generation of NO by the constitutive NOS maintains blood vessels in a state of active vasodilatation in the systemic and pulmonary circulation, inhibits activation and adhesion of white cells and platelets, inhibits increases in vascular permeability and thus plays a predominantly anti-inflammatory or at least "counterbalancing" role to these inflammatory processes. Associated with the progression of inflammation is the induction of iNOS, which produces much higher amounts of NO. Nitric oxide from iNOS may replace some of the essential functions of eNOS, whose increased expression decreases, with the progression of inflammation (Hickey & Kubes, 1999). Therefore it is possible that the increase in iNOS expression can play an important role in inflammation depending on amounts of NO generated. Indeed overproduction of NO may lead to inhibition of respiration, excessive vasodilatation, hypotension, vascular leakage and metabolic disruption (Section 1.4.5).

1.4.3 VASCULAR PERMEABILITY

Although inflammatory mediators and microbial products including endotoxin acutely increase microvascular permeability, the expression of iNOS in a variety of tissues following administration of endotoxin *in vivo* is associated with much greater increases in vascular leakage in those tissues (Rees, 1999a; Kubes, 1997). The processes by which pathological concentrations of NO produce this increase in microvascular permeability are still unclear. Cytoskeletal derangement may be a common factor and formation of peroxynitrite from NO and superoxide has been implicated in cellular injury

(Hibbs, *et al.*, 1987; Whittle, 1995; Salzman *et al.*, 1995). Indeed, the stimuli that lead to induction of iNOS often lead to increased generation of superoxide and thus peroxynitrite is formed. The increased generation of NO and peroxynitrite suppress cell respiration and oxygen consumption resulting in a reduction of ATP and excessive lactate formation (Rees, 1999a). This inability to utilise oxygen following excessive NO production, if long-lasting, could induce cell damage, trigger apoptosis and lead to cell death. This may explain, at least in part, the increased microvascular permeability and other cellular and tissue defects observed in conditions such as septic shock (Section 1.4.5; Rees, 1999a).

1.4.4 INFECTION

Nitric oxide produced by activated rodent macrophages has been implicated in the killing of intracellular parasites such as *Leishmania major* and *Toxoplasma gondii* and enteric bacteria such as *Escherichia coli* and *Salmonella typhimurium* (Hibbs, & Bastian, 1999). Whereas iNOS expression occurs readily in rodent cells or tissues exposed to endotoxin or cytokines, the induction of functionally active iNOS in human cells or tissues in experimental studies requires a more complex mixture of cytokines or other stimuli. There is good evidence for overproduction of NO in several clinical conditions, but whether this overproduction plays a role in the killing of microorganisms remains to be clarified (Rees, 1999b).

Inducible NOS also plays a crucial role in the control of *Mycobacterium tuberculosis*. Indeed iNOS mutant mice succumb quickly to infection with virulent *Mycobacterium tuberculosis* with 100 to 1000 fold greater pulmonary bacterial burdens than those in control mice (MacMicking *et al.*, 1997).

Furthermore, NO appears to play an important role in the protective immune response during all stages of *Plasmodium falciparum* infection. The association between NO production and protection from the infection shown in African children may be mediated by the ability of NO to downregulate TNFα, or to inhibit endothelial expression of receptors used by parasitised red cells to adhere to vascular endothelium (Anstey *et al.*, 1999).

1.4.5 SEPTIC SHOCK

Septic shock is the result of a pathogenic cascade of inflammatory mediators, which are initiated by the presence of infection and amplified by the action of the body's defence mechanism. The body is constantly subjected to attack from a wide variety of microorganisms and as such provides an effective first line of defence by a variety of mechanisms. However when the first line defence fails to halt the proliferation of microorganisms, sepsis develops whereby systemic inflammation, is driven by a variety of cytokines. Sepsis is associated with vascular dilatation resulting in lowering of systemic vascular resistance. In addition, plasma leaks from the circulation into the extravascular space. Both these factors produce hypovolaemia (reduction in blood volume). Sepsis develops into septic shock when hypotension persists, despite adequate fluid resuscitation. Indeed septic shock is defined as "severe sepsis with hypotension (a reduction of > 40 mmHg from baseline) in the absence of other causes for hypotension despite adequate fluid resuscitation" (Hinds & Watson, 1996). Septic shock in man is characterised by a decrease in systemic vascular resistance, an increase in cardiac output (volume of blood expelled per unit of time) and heart rate. Although cardiac output and heart

rate is increased the ventricular function is abnormal, ejecting less blood than in normal conditions. Although these abnormalities may in part account for the reduced oxygen delivery seen in patients with septic shock, there also appears to be a primary defect in cellular oxygen utilisation (Hinds & Watson, 1996). It has been suggested that in septic shock patients oxygen consumption often increases if oxygen delivery is actively increased (e.g. by fluid resuscitation, catecholamines or blood transfusion, Danek *et al.*, 1980; Haupt *et al.*, 1985; Gilbert *et al.*, 1986; Kruse *et al.*, 1990). This pathological supply dependency is due largely to a consequence of maldistribution of flow in the microcirculation, predominantly related to inappropriate vasodilatation, which is exacerbated by microemboli, interstitial oedema, localised vasoconstriction and arteriovenuos shunting (Hinds & Watson, 1996). However several studies suggest that the concept of pathological supply dependency should be interpreted cautiously (Hinds & Watson, 1996). Indeed oxygen delivery is calculated as the product of cardiac output and arterial oxygen content and oxygen consumption has been derived using an equation that share two variables (cardiac output and arterial oxygen content). The presence of shared variables may give rise to mathematical coupling resulting in an incorrect interpretation of supply dependency (Ronco *et al.*, 1991; Phang *et al.*, 1994). There is growing evidence that patients with septic shock show a primary defect in the ability of tissues to consume oxygen (Manthous *et al.*, 1993; Hayes *et al.*, 1993; Pilas *et al.*, 1995). This inability to consume oxygen has been suggested to be due to mitochondrial dysfunction (Schumer *et al.*, 1970; Mela *et al.*, 1971; Nicholas *et al.*, 1972; Poderoso *et al.*, 1978). Overproduction of NO has been implicated as a key

mediator in causing this cytopathic dysoxia. Thus, the overproduction of NO may be a common mechanism by which microbial invasion, bacterial products and cytokines lead to the excessive vasodilatation, myocardial depression, increased microvascular permeability, reduced oxygen consumption and multiple organ failure observed in septic shock.

1.4.5.1 Endotoxin shock

A common cause of septic shock is gram-negative bacterial infection. The administration of gram negative bacterial endotoxin, (lipopolysaccharide or LPS) a component of the outer membrane, produces a shock-like syndrome in animals and man (Parratt, 1973; Suffredini *et al.*, 1989). Endotoxin activates a variety of host mediators, which have been implicated in the pathogenesis of shock, in particular cytokines such as tumour necrosis factor-α, IL-1, IL-6 and IFN-γ. Indeed administration of TNF-α and IL-1 in animals and man produces the systemic and pathological features of endotoxin-induced shock (Rees, 1995a). Several studies have shown that endotoxin generates a time-dependent expression of iNOS over several hours, which begins after a lag period of approximately 2h, both in isolated blood vessels *in vitro* (Rees *et al.*, 1990b) and in mice (Rees *et al.*, 1998) and other species *in vivo* (Thiemermann & Vane, 1990; Wright *et al.*, 1992) including humans (Ochoa *et al.*, 1991; Evans *et al.*, 1993). The induction of iNOS is accompanied by an increase in the plasma concentrations of nitrite/nitrate over a similar time course. These responses are closely associated with a progressive fall in blood pressure and a significantly reduced vasopressor response to noradrenaline in mice (Rees *et al.*, 1998). This has been further confirmed by observations using iNOS mutant mice in

which endotoxin treatment results in a greatly reduced fall in blood pressure compared with wild type animals (MacMicking *et al.*, 1995; Rees *et al.*, 1998). Bacterial gram-positive wall fragments, including lipoteichoic acid and peptidoglycan appear to induce a similar profile (Zembowicz & Vane, 1992; Cunha *et al.*, 1993).

1.5 NITRIC OXIDE DONORS

As the number of studies on NO has increased, there has been a substantial interest in using compounds that are capable of generating NO *in situ* (Table 1.1). A wide variety of NO donors generate NO when applied in biological systems and thus mimic endogenous NO responses. However, the pathways leading to NO formation differ significantly among individual compound classes, as do their physical and chemical properties. While some compounds require enzymatic catalysis, others produce NO non-enzymatically. Some NO donors require interaction with thiols to release NO, while others spontaneously release NO. In addition, the kinetics of NO release from a given compound is an additional factor in the choice of NO-donor in order to best mimic the biological response (Feelisch & Stamler, 1996).

Table 1.1 Four classes of NO donors.

Compound class	N-Oxide species generated		
	$NO^{\cdot}$	NO^{-}	NO^{+}
Organic nitrates	+	-	-
Organic nitrites	+	-	+
S-Nitrosothiols	+	+	+
NONOates	+	(+)	(+)

($NO^{\cdot}$ = nitric oxide, NO^{-} = nitroxyl anion and NO^{+} = nitrosonium ion, (Feelisch & Stamler, 1996))

1.5.1 NONOATES

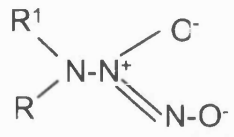

Figure 1.4 Chemical structure of NONOates.

NONOates (Figure 1.4) relax vascular tissue in an endothelium-independent manner, increase tissue cGMP and inhibit platelet aggregation and cell proliferation *in vitro* (Morley *et al.*, 1993; Maragos *et al.*, 1993; Mooradian *et al.*, 1995). NONOates generate NO spontaneously without being affected by biological reactants. This important property, taken with predictable rates of

NO release over minutes to several hours, makes NONOates a valuable tool in NO research (Keefer *et al.*, 1996). Despite these strengths it is relevant to emphasise that high concentrations of thiols may decrease NO release considerably, and enzymatic metabolism *in vivo* remains a distinct possibility (Feelisch & Stamler, 1996). The nucleophile backbone and the temperature of the incubation medium influences the release of NO. The decomposition of NONOates is also pH-dependent, proceeding at an extremely slow rate at values of pH > 9, with moderate rates at neutral pH and almost instantaneous release at pH < 5, suggesting that protonation is a pre-requisite for NO release (Fitzhugh & Keffer, 2000). The reaction follows first order kinetics, producing 2 moles of NO per mole of NONOate (Keefer *et al.*, 1996). Members of this group of donors release NO within minutes to several hours (Table 1.2, Figure 1.5; Feelisch & Stamler, 1996).

Table 1.2 Half-lives of NONOates.

NONOates	**$T_{1/2}$**	**pH**	**Temp. [°C]**
DEA/NO	2.1 min	7.4	37
SPER/NO	39 min	7.4	37
DETA/NO	20 h	7.4	37

(DEA/NO = diethylamine nonoates, SPER/NO = spermine nonoates and DETA/NO = diethylenetriamine nonoates)

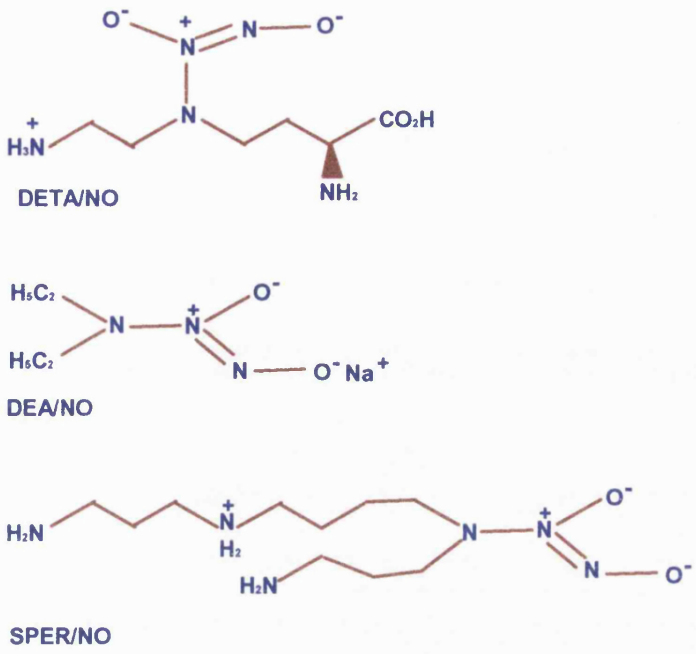

Figure 1.5 Chemical structures of selected NONOates (DETA/NO, DEA/NO and SPER/NO).

In addition to DETA/NO, DEA/NO and SPER/NO that release NO, a number of NONOates, e.g. SULFI/NO and PAPA/NO, can also release nitrous oxide (N_2O) and nitroxyl anion (NO^-), respectively (Maragos *et al.*, 1993).

The spontaneous first order of decomposition of NONOates may be disadvantageous in some therapeutic settings. Therefore, prodrugs have been designed that cannot release NO unless enzymatically converted to the corresponding NONOates in the target tissue (Feelisch & Stamler, 1996). O-alkylation (Figure 1.6) of secondary amines of NONOates leads to derivatives with considerably enhanced stability (Saavedra *et al.*, 1997).

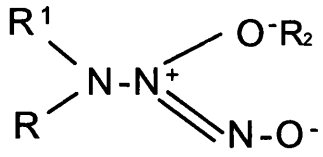

Figure 1.6 Chemical structure of O-alkylated NONOates.

The O-alkylated NONOates are resistant to hydrolysis; indeed DEA/NO has a short half life (2.1 min) while its ethylated derivative is considerably resistant to the hydrolysis under the same conditions (37 °C at pH 7.4). The enzymatic bioactivation of O-alkylated NONOates occurs *in vivo*, but the decomposition products are less predictable than those of the non-alkylated species (Feelisch & Stamler, 1996, Saavedra *et al.*, 1997).

1.5.2 ORGANIC NITRATES

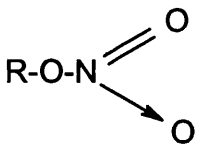

Figure 1.7 Chemical structure of organic nitrates.

Glyceryl trinitrate, one of the more widely used organic nitrates was first introduced as a treatment for angina pectoris more than a century ago. Organic nitrates (Figure 1.7) require either enzymatic or non enzymatic bioactivation to release NO. Non-enzymatic NO formation requires interaction with thiol groups (RSH; Feelisch *et al.*, 1988). The main drawback of the clinical use of organic nitrates is the impairment of bioactivity on long-term administration *in vivo* which may be due to depletion

of intracellular thiol concentration or down regulation of some enzymes involved (Feelisch, 1993).

1.5.3 ORGANIC NITRITES (R-O-N=O)

This class of NO donors displays a rapid onset and short duration of action. Organic nitrites are more susceptible to alkaline hydrolysis than their respective nitrates. However, in aqueous buffers at pH 7.4 hydrolysis is relatively slow, yielding inorganic nitrite and the corresponding alcohol (Feelisch & Stamler, 1996). Under these conditions, release of NO requires reaction with sulphydryl groups (Ignarro *et al.*, 1981). S-nitrosothiols are active intermediates in this process and rates of NO release are a function of the rate of formation and metabolism of the S-nitrosothiols involved. The vasodilator and antianginal effect of inhaled amyl nitrite was discovered more than a century ago (Brunton, 1867).

1.5.4 S-NITROSOTHIOLS (R-S-N=O)

S-nitrosothiols (RSNOs) are endogenous intermediates of NO (Myers *et al.*, 1990), as such they may be important in transport and targeting of the NO group to specific, thiolregulatory effector sites, including enzymes and signalling proteins (Stamler, 1995). S-nitrosothiol intermediates are also implicated in the smooth muscle relaxant effects of nitrovasodilators (Ignarro *et al.*, 1981) and might be beneficial in a variety of cardiovascular disorders (Al-Sa'doni & Ferro, 2000). S-nitrososthiols act as donors of NO, nitroxyl anion (NO^-) and nitrosonium ion (NO^+) depending on the reaction conditions (Feelisch & Stamler, 1996).

In addition to the groups detailed above, many other groups of nitric oxide donors are available; these include N-nitroso compounds, C-nitroso compounds, oximes, sydnonimines and related compounds, oxadiazoles, oxatriazoles, nitroxyl-generating compounds, hydroxylamine, N-hydroxy-guanidines, and inorganic NO donors.

1.6 NITRIC OXIDE INHIBITORS

The wide range of physiological and pathological processes controlled by NO has stimulated the development of many approaches and agents useful in experimental or therapeutic control of NO formation.

1.6.1 L-ARGININE ANALOGUES

Interest in NOS inhibitors predates the discovery of NOS itself. Indeed L-N^G-methylarginine (L-NMMA, Figure 1.8) was identified as an inhibitor of the arginine-dependent cytotoxic response of murine macrophages before it was determined that the cytotoxic factor was related to NO or that the arginine-dependent enzyme was iNOS (Hibbs, *et al.*, 1987). L-N^G-methylarginine inhibits eNOS under basal and stimulated conditions demonstrating that NO plays an important role in the regulation of blood flow and the control of blood pressure (Rees *et al.*, 1989a). Several studies have analysed the different structure activity relationships of L-arginine analogues both *in vitro* and *in vivo,* demonstrating that substitution of the guanidino moiety of L-arginine alters the potency of NOS inhibitors (Figure 1.8; Rees *et al.*, 1990b).

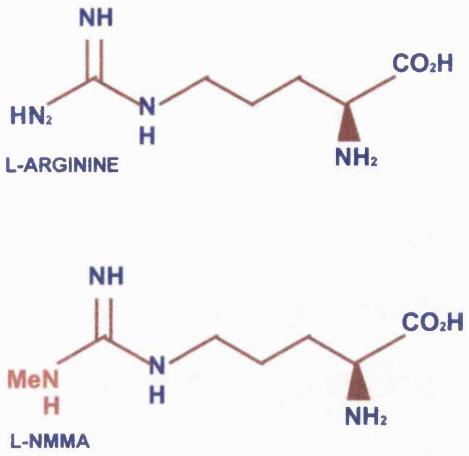

Figure 1.8 Chemical structures of L-arginine and L-N^G-methylarginine (L-NMMA).

1.6.2 ALTERNATIVE APPROACHES TO REDUCING NOS ACTIVITY OR ITS EFFECTS

Although the use of NOS inhibitors is the most common means of reducing NOS activity, other approaches are possible and potentially useful experimentally and clinically. Because iNOS-mediated overproduction of NO accounts for several disorders, substantial efforts have been directed at reducing or preventing expression of active iNOS.

Glucocorticoids inhibit the induction of iNOS by suppressing transcription of iNOS mRNA, but have no effect on iNOS activity once the enzyme is expressed (Radomski *et al.*, 1990c; Rees *et al.*, 1990b). Accordingly clinical data indicate that steroids are more effective if administered before or at the onset of conditions that involve overproduction of NO by iNOS such as septic shock (Rees, 1999a).

Pyridoxylated haemoglobin polyoxyethylene conjugate (PHP), a chemically modified haemoglobin, is used to scavenge NO extracellularly within the

vascular space to block the vasodilator effects of NO. Clinical studies show that PHP, by scavenging NO, may be used as an agent able to reduce selectively only the NO-induced vasodilated beds. Indeed it differs from the current therapies, since vasoconstrictors affect all vascular beds. In this respect vascular areas which have greater NO production would be expected to be vasopressor-insensitive while other vascular beds which have less NO production would have a greater vasoconstrictor response, thus further worsening the imbalance of perfusion (Kilbourn & Deangelo J, 1999). The clinical utilitiy of PHP in conditions such as septic shock remains to be investigated.

Inhibitors of tetrahydrobiopterin (BH_4) a cofactor for all NOS isoforms is a potential target for reducing NOS activity. Indeed agents, which selectively inhibit *de novo* BH_4 synthesis, prevent iNOS activation in these cells. (Griffith & Kilbourn, 1996).

1.7 AIMS OF THE THESIS

Recent evidence has demonstrated that NO inhibits tissue respiration both in physiological and pathological conditions. However, in spite of much research, many fundamental questions remain in relation to the sequence of events that lead from physiological regulation to pathological inhibition and the biochemical conditions necessary for this process to take place.

The specific aims of the thesis are as follows:

- To observe, following long term exposure to exogenous NO, whether the reversible inhibition of complex IV and the persistent inhibition of complex I is a general mechanism observed in different cells.

- To examine whether the same mechanism occurs following long term exposure to high concentrations of exogenous NO, mimicking pathological concentrations generated in conditions such as septic shock.
- To study the physiological role of NO on the respiratory chain *ex vivo* using eNOS and iNOS mutant and wild type mice.
- To investigate *ex vivo,* the pathological role of NO on the respiratory chain using iNOS mutant and wild type mice following sub-lethal endotoxin administration.
- To investigate *ex vivo,* the pathological role of NO on the respiratory chain using iNOS mutant and wild type mice following lethal endotoxin administration.

CHAPTER 2

METHODS AND MATERIALS

2 METHODS AND MATERIALS

2.1 OXYGEN CONSUMPTION MEASUREMENT

Oxygen consumption was measured by a Clark type oxygen electrode (Rank brothers, Bottisham, UK). The electrode is located at the base of a stirred, sealed chamber (1 ml) thermostatically set at 37°C and connected to a chart recorder. Prior to each experiment, the oxygen electrode immersed in incubation medium in the chamber was calibrated to atmospheric oxygen (200 μM) at 37°C (Clementi *et al.*, 1998). The cells or the chopped tissues were suspended in incubation medium and 1 ml samples were added to the chamber. The oxygen consumption of the cells or the tissues was monitored for at least 3 min and subtracted from the value of the oxygen consumption of the incubation medium (Figure 2.1).

2.2 NITRIC OXIDE MEASUREMENT

Nitric oxide was measured by a nitric oxide meter and sensor (Iso-NO World Precision Instruments, Stevenage, U.K) inserted in the chamber described above (Figure 2.1). The Iso-NO meter and sensor (2 mm diameter stainless steel shielded sensor) provides measurements of NO over a range of concentrations in both aqueous solutions and in gas mixtures. The sensor is amperometric: NO diffuses through the selective membrane and is oxidised at the electrode, resulting in an electrical current. This current is proportional to the concentration of NO in the sample. The NO electrode was calibrated following the titration method described previously (Tsukahara *et al.*, 1993). This method of calibration is based on the following reaction:

Equation 2.1:

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

A known amount of KNO_2 (50 µM) is added into a stirred chamber, kept at 37°C, containing KI (0.1M) and H_2SO_4 (0.1M), to produce a stoichiometric amount of NO.

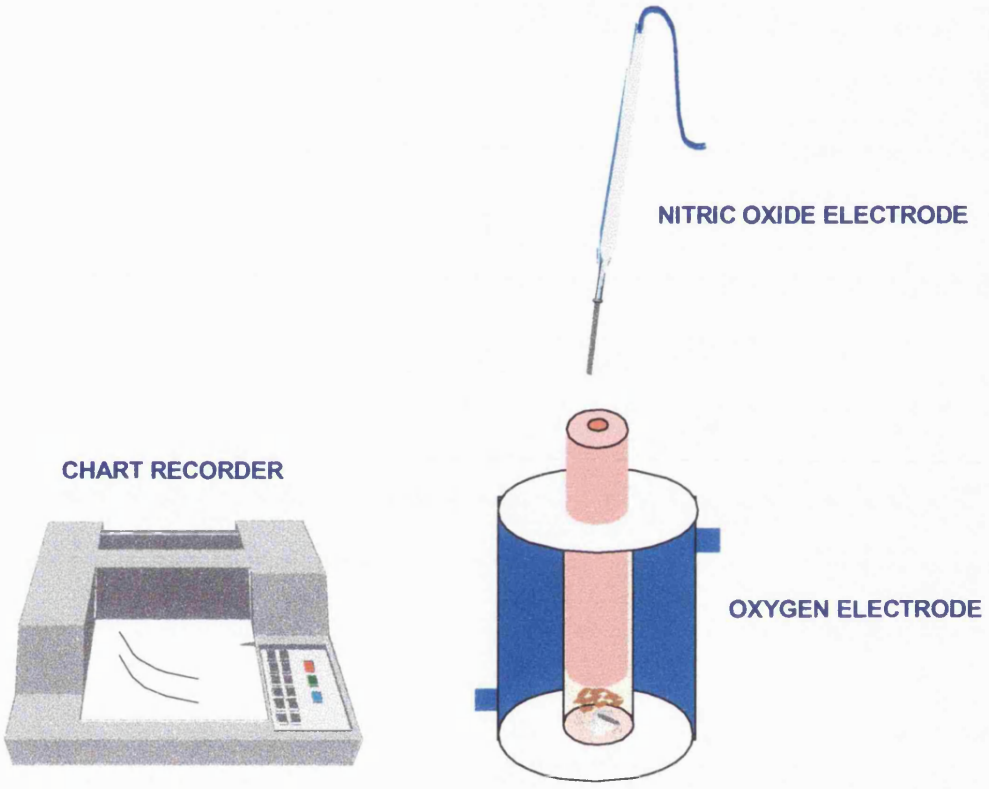

Figure 2.1 Clark type oxygen electrode and nitric oxide electrode connected to a chart recorder.

2.3 STUDIES IN VITRO

To study the effect of NO on cellular respiration *in vitro* three different cells (Jurkat, L929 and J774) were exposed to exogenous NO.

2.3.1 CULTURE AND MAINTENANCE OF CELLS

2.3.1.1 Jurkat cells

Jurkat, human adult T derived-lymphocyte, cells were obtained from the American Tissue and Cell Culture (ATCC TIB 152). These cells were cultured in suspension with a biological stirrer bottle (Techne Laboratories) using RPMI medium supplemented with heat-inactivated foetal bovine serum (10 %), L-glutamine (4 mM), penicillin (100 U ml^{-1}) and streptomycin (100 µg ml^{-1}) at 37°C in a humidified atmosphere containing 5 % CO_2.

2.3.1.2 L929 cells

L929, human derived-fibroblast L929 cells were obtained from Glaxo-Wellcome. These cells were cultured in flasks using RPMI medium supplemented as above and passaged with trypsin (0.05 % w/v) and EDTA (0.02 % w/v) every two days upon confluence.

2.3.1.3 J774 cells

J774, murine derived-macrophage cells, J774, were obtained from the European Collection of Animal Cell Cultures (Porton Down, Wilts). These cells were cultured in suspension using a biological stirrer bottle, in Dulbecco's Modified Eagle's Medium (DMEM) supplemented as above.

2.3.1.4 Preparation of cells

Cells were used in the logarithmic growth phase. At the time of the experiments, the cells (Jurkat, L929 and J774) were centrifuged at 400 g for 5 min at 4°C. The cells were resuspended at a density of 10^7cells ml^{-1} in

Krebs medium (in mM: NaCl 118, KCl 4.8, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1, glucose 20 and HEPES 25).

In experiments aimed at investigating the effect of immunostimulation of cells on oxygen consumption, J774 macrophages were grown in suspension in DMEM, in the absence or presence of L-arginine (0.5 mM). Cells were activated by addition of endotoxin (lipopolysaccharide; LPS, *W.S. Typhosa* 0901, 10 μg ml^{-1}) and IFN-γ (50 U ml^{-1}) to the culture medium and incubated for 18 h.

2.3.1.5 Cell viability

Cell viability was assessed using trypan blue (0.4 %) stain and a haemocytometer to determine total cell counts and viable cell number. The cell viability was calculated by the following equation:

Equation 2.2

$$\text{Cell viability (\%)} = \frac{\text{Total viable cells (unstained)}}{\text{Total cells (stained and unstained)}} \times 100\ (\%)$$

Trypan blue is one of several dyes used in dye exclusion procedures for viable cell counting, based on the principle that "dead" but not "living" cells take up certain dyes (Molecular Biology, Sigma, 1998 Trypan Blue, 1844 - 1845).

2.3.2 MEASUREMENT OF OXYGEN CONSUMPTION AND NITRIC OXIDE GENERATION

Cells suspended in the incubation medium at a density of 10^7 cells ml^{-1} were incubated in the presence or absence of (z)-1-[2-(2-aminoethyl)-N-(2-ammoniothyl)amino]diazen-1-ium-1,2diolate (DETA-NO; 0.5-10 mM). The cells were maintained during incubation in Falcon tubes, open to the atmosphere, at 37°C and gently agitated. Samples (1 ml) were analysed at the time points indicated in the various experiments in a gas-tight chamber thermostatically set at 37°C, equipped with both a Clark type oxygen electrode and a NO electrode connected to a chart recorder, as described above (Sections 1.1 and 1.2). The oxygen consumption of the cells treated with DETA-NO were expressed as a percentage of that measured in cells subjected to the same treatment in the absence of the NO donor (control).

In experiments examining the effect of higher concentration of DETA-NO (0.5-10 mM), cellular and extracellular oxygen consumption were investigated separately (Chapter 5). At indicated time-points cells were separated from the extracellular medium by two consecutive centrifugations (2,500 × g for 2 min), resuspended in incubation medium with DETA-NO (at the same concentration present during the incubation) and the oxygen consumption of the cells and of the extracellular medium were analysed immediately (Chapter 5).

In some experiments, the extracellular medium was subjected to a further treatment i.e. ultrafiltration or temperature change or illumination with a visible cold light source (~8 Mlx; KL 1500; Schott, Mainz, Germany, Chapter

5). Extracellular medium from endotoxin-activated cells was obtained by centrifugation as described above, except that the supernatant was obtained from the original culture medium (Chapter 5).

2.3.2.1 Pharmacological studies on the respiratory chain

The different metabolic steps related to oxidative phosphorylation were dissected pharmacologically, using specific inhibitors and appropriate substrates added to the cells at indicated time-points (Chapter 3 and 5). The addition of the protonophore carbonyl cyanide-p-(trifluoro-methoxy) phenylhydrazone (FCCP, 1 μM) to control cells uncouples the flow of electrons from the synthesis of ATP and generates an increase in the oxygen consumption. The addition of FCCP was used in some experiments to determine whether NO was acting at a step downstream to ATP synthase (complex V). To study whether impairment of the citric acid cycle was involved, experiments were performed with 3-nitropropionic acid (0.6 mM), an inhibitor of succinate dehydrogenase, in the presence of β-hydroxybutyrate (6 mM), which supplies the mitochondria with NADH via its conversion to acetoacetate. In another series of experiments the oxygen consumption sustained by the activity of complex IV was tested in the presence of the complex III inhibitor, myxothiazol (0.5 μM) and the supply of electrons to complex IV was maintained using N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD, 80 μM) plus ascorbic acid (4 mM). Finally, the oxygen consumption via complex II was examined using the respiratory substrate succinate (6 mM) in the presence of the complex I inhibitor rotenone (2 μM). The concentrations of the compounds were selected from preliminary experiments as described previously (Clementi *et al.*, 1998): the

various inhibitors (3 nitropropionic acid, myxothiazol, rotenone) were added at increasing concentrations until the oxygen consumption was reduced to less than 10 % of control. The corresponding respiratory substrates (β-hydroxybutyrate, TMPD plus ascorbate, succinate) were then added at increasing concentrations until the oxygen consumption was restored to at least 90% of the level of untreated controls. The maintenance of the respiration rate, thus obtained, was then measured and compared with that of untreated cells (control).

In some experiments, cells were incubated with DETA-NO (0.5-10 mM) together with GSH-ethyl ester (2 mM, Chapters 4 and 5). In further experiments, a persistent inhibition of respiration was established by incubation with DETA-NO for 5 h. The cells were then divided into two groups: one group received GSH-ethyl ester (2 mM) and the other group was subjected to illumination with high intensity (~ 8 Mlx) light from a cold light source (KL1500, Schott, Mainz, F.R.G.), for a further incubation period of 1.5 h in both cases (Chapters 4 and 5).

2.3.3 ASSAY OF COMPLEX I ACTIVITY

The activity of complex I was measured as described previously (Ragan & Heron, 1978). Complex I catalyses NADH oxidation, electrons are transferred from NADH through complex I to ubiquinone, which is reduced to ubiquinol. Briefly Jurkat, L929 and J774 cells were centrifuged (2,500 x g for 2 min) and the pellet freeze-thawed three times. Cellular homogenate (20 µl) was added to potassium phosphate buffer (1 ml, 10 mM), pH 8.0 containing NADH (100 µM) in a cuvette (~1 ml) at 37°C. The rate of NADH oxidation

was followed at 340 nm in a UV spectrophotometer. After recording the absorbance for 1 min, 5 µl of ubiquinone (10 mM) was added and the rate of NADH oxidation, (complex I activity), was followed for a further 2 min. The NADH oxidation rate was calculated from the slope of absorbance decrease over time using an extinction coefficient for NAD of 6.81 mM^{-1} cm^{-3} at 340 nm (Ragan & Heron, 1978). Rotenone (2 µM), a specific inhibitor of complex I, was added to calculate the non-specific NADH oxidation rate, which was 2.75 ± 0.5, 2.91 ± 0.3 and 4.2 ± 0.08 nmol min^{-1} $protein^{-1}$ in Jurkat, L929 and J774 cells respectively.

2.3.4 MEASUREMENT OF ATP

Measurements of ATP were performed using the ATP bioluminescence assay kit, CLS II (sensitivity range 10^{-6} - 10^{-13} M, Boehringer Mannheim).
A standard curve was prepared for every experiment using the stabilised ATP standard stock provided in the kit. At the time point indicated, cell suspensions were diluted 1:1 with Krebs solution. After addition of perchloric acid (0.09 M) cells were centrifuged (2,500 x g for 2 min) and KOH (0.08 M) was added to the supernatant. After centrifugation (2,500 x g for 2 min) the samples were diluted 1/5 with Krebs solution. Luciferase reagent was added and the absorbance was measured by a luminometer (Wallac -LKB 1250, ATP Bioluminescence assay kit protocol, Boehringer Mannheim).

2.3.5 MEASUREMENT OF GLUTATHIONE

The concentration of glutathione (GSH) in the three cells was studied during the time course experiments. At the time indicated, cells were centrifuged at 2,500 x g for 2 min; the pellet was resuspended in cold metaphosphoric acid

solution and the GSH content analysed using the Bioxytech GSH-400 kit (Oxis Int., Portland, Oregon, USA). The GSH-400 method is based on a chemical reaction, which proceeds in two steps. The first step leads to the formation of substitution products (thioethers) between a patented reagent, R_1 (4-chloro-1-methyl-7-trifluoromethyl-quinolinium methylsulfate) and all mercaptans (RSH) which are present in the sample (Figure 2.2; Anderson, 1989).

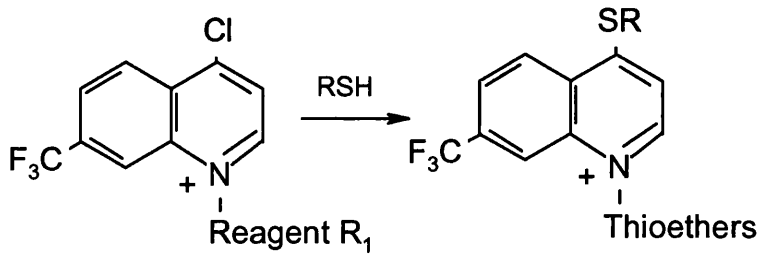

Figure 2.2 First step for the detection of GSH by GSH-400 kit.

The second step is a β-elimination reaction which takes place under alkaline conditions (Figure 2.3). This reaction is mediated by reagent R_2 (NaOH 30 %) which specifically transforms the substitution product (thioether) obtained with GSH into a chromophoric thione which has a maximal absorbance wavelength at 400 nm (Anderson, 1989).

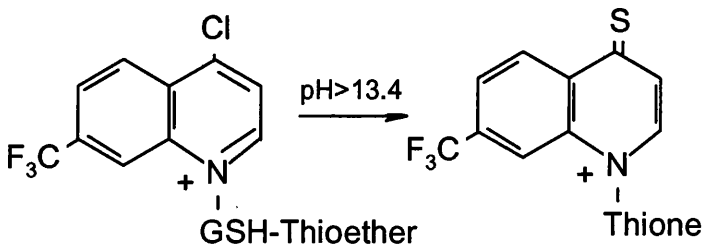

Figure 2.3 Second step for the detection of GSH by GSH-400 kit.

2.3.6 GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE ACTIVITY

The activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was determined by following the reduction of NAD^+ to NADH at 340 nm.

Equation 2.3

$$\text{Glyceraldehyde-3-phosphate} + NAD^+ + HPO_4^- \overset{\text{GAPDH}}{\leftrightarrow} \text{Glycerate-1,3-P}_2 + NADH$$

The assay mixture, in a total volume of 1 ml, contained sodium pyrophosphate (10mM, pH 8.5), sodium arsenate (25 mM, added to buffer the sytem), NAD^+ (0.25 mM), and glyceraldehyde-3-phosphate (1 mM). At the indicated time points, an aliquot of cells (0.7 ml) was centrifuged at 2,500 x g for 2 min and the pellet was freeze-thawed three times. Then, 4 µl of the cellular homogenate was added to the assay mixture to start the reaction and the activity was followed spectrophotometrically (340 nm, 25°C; Padgett & Whorton, 1995).

2.3.7 MEASUREMENT OF TOTAL PROTEINS

The protein content in the cells and extracellular medium samples was measured using a bicinchoninic acid (BCA) protein assay kit. The Pierce BCA protein assay (Pierce, Rockford, IL, USA) is a detergent formulation for the colourimetric detection and quantitation of total protein. This method combines the reduction of cupric ion (Cu^{2+}) to cuprous ion (Cu^{1+}) by protein in an alkaline medium with highly sensitive and selective colourimetric detection of cuprous ion using bicinchoninic acid (Smith *et al.*, 1985). The purple-coloured reaction product of this assay is formed by the chelation of

two molecules of BCA with one cuprous ion. This complex exhibits a strong absorbance at 562 nm that is linear with the increasing protein concentrations over a broad range (20-2,000 μg ml^{-1}; Smith *et al.*, 1985).

2.4 STUDIES *IN VIVO*

2.4.1 ANIMAL HUSBANDRY

Female eNOS mutant (Huang *et al.*, 1995) and wild type mice (C57BL/6x129, 18-25g; P. Huang, USA) and iNOS mutant (Laubach *et al.*, 1995) and wild type mice (C57BL/6, 18-25 g; Jackson laboratories) were housed in a temperature-controlled room with water and food *ad libitum*. In some experiments endotoxin was administered (0-24 h, *E. coli* 026:B6 i.v.) and the plasma concentration of nitrite/nitrate and oxygen consumption measured in iNOS mutant and wild type mice (Figure 2.4). In a separate series of experiments the survival profile following the administration of two different doses of endotoxin (6 mg kg^{-1} and 12.5 mg kg^{-1}) were examined. A further series of experiments examined the cardiovascular profile of the mice following endotoxin administration. All the studies were in accordance with the United Kingdom Home Office regulations for the care and the use of animals (Scientific Procedures Act. 1986).

2.4.2 EXTRACTION OF THE BLOOD AND PREPARATION OF THE TISSUES

At different time points the mice were anaesthetised briefly (2 min) with isofluorane (2 %). Blood samples were taken by cardiac puncture into heparin/saline filled syringes (25 U ml^{-1}). This was followed by cervical

dislocation. The heart and liver were immediately excised. Following removal of connective tissue and fat, the heart and liver were washed three times at room temperature in Krebs solution, containing (in mM) NaCl 118, KCl 4.8, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1, glucose 5, HEPES 25, pH 7.2. Tissues were then incubated for 5-10 min in the Krebs solution, briefly dried and then weighed. The tissues were cut into small pieces and resuspended in Krebs solution (1 ml).

2.4.3 MEASUREMENT OF OXYGEN CONSUMPTION

The samples (1 ml) were immediately analysed at 37°C in a gas-tight vessel, equipped with a Clark-type oxygen electrode connected to a chart recorder (Figure 2.4).

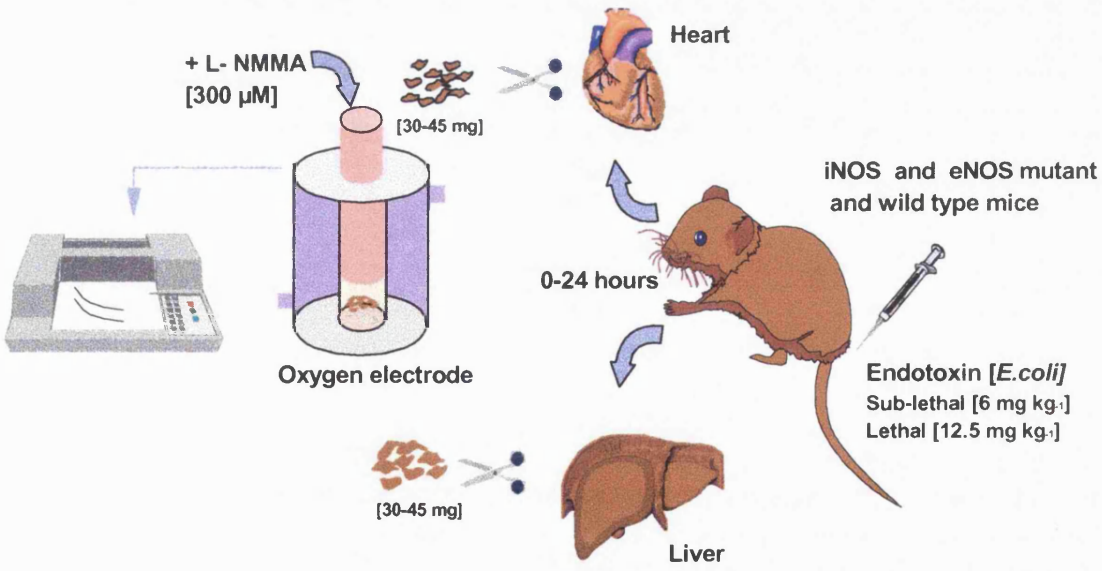

Figure 2.4 Schematic representation of the measurement of myocardial and hepatic oxygen consumption of eNOS and iNOS mutant and wild type mice in physiological conditions and following endotoxin administration.

The oxygen consumption by tissues was followed at least twice over the range from atmospheric oxygen (200 μM) to the complete absence of oxygen (0 μM). The oxygen consumption of each sample was only measured over the physiological range (~5-40 mmHg = ~7.5-60 μM). L-N^G methylarginine HCl (L-NMMA; 300 μM), D-N^G methylarginine HCl (D-NMMA; 300 μM) or vehicle was added to the gas-tight chamber. The effect of myxothiazol (1-100 μM), an inhibitor of complex III, was examined to establish that the oxygen consumption measured was due to tissue respiration (Chapter 6, Figure 6.2). Succinic acid (100 μM), a substrate of complex II, was used to study whether complex I activity was inhibited following endotoxin. In all the studies there was a linear relationship between tissue weight and oxygen consumption (Chapter 6, Figure 6.1).

2.4.4 ASSAY FOR PLASMA CONCENTRATIONS OF NITRITE/NITRATE

Blood samples (0.5 ml) were withdrawn by cardiac puncture and the plasma obtained following centrifugation (2,500 × g, for 5 min). The plasma nitrate/nitrite concentrations were determined using the Griess reaction (Thomsen *et al.*, 1990) incorporating some modifications (Verdon *et al.*, 1995). Briefly, the nitrate (NO_3^-) was enzymatically reduced using nitrate reductase from Aspergillus species. The conversion of nitrate to nitrite results in the formation of $NADP^+$ which interferes with the Griess reaction. To reduce the interference a low concentration of NADPH (1 μM) was added. However this concentration was inadequate to drive the nitrate reductase reaction quantitatively to completion. To avoid this limitation the nitrate reductase reaction was coupled to the oxidation of glucose-6-phosphate by glucose-6-phosphate dehydrogenase, which serves to

replenish NADPH from its oxide form. $NADP^+$ was recycled by the dehydrogenation of glucose-6-phosphate (G-6-P) to 6-phosphoglucolactone (6-PGL), a reaction catalysed by glucose-6-phosphate dehydrogenase (Figure 2.5).

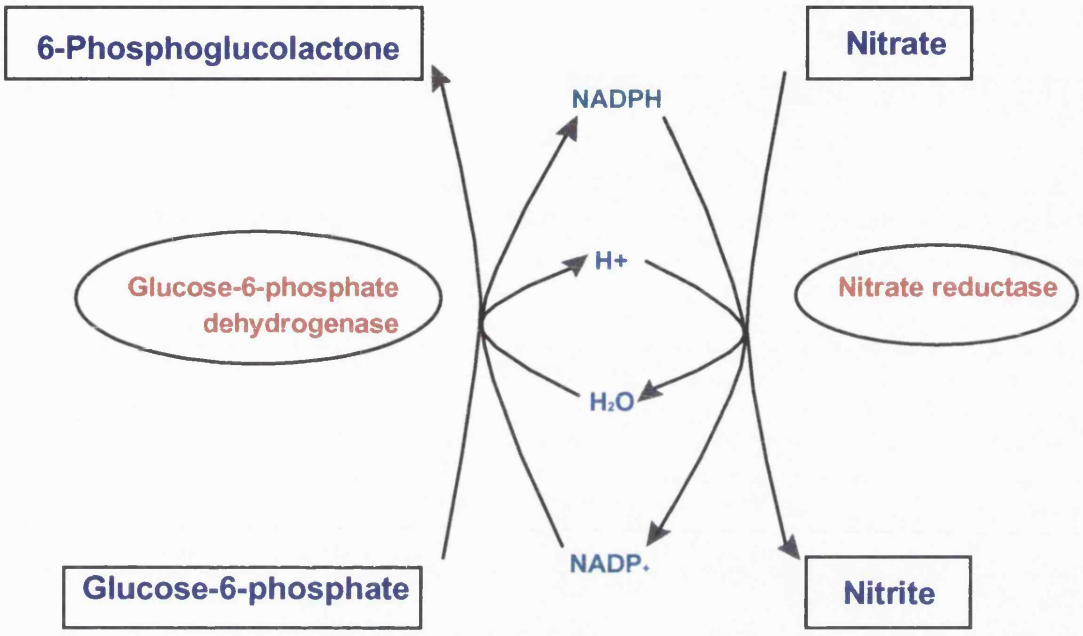

Figure 2.5 Conversion of nitrate to nitrite by nitrate reductase.

All the assays were conducted in 96-well microassay plates (Falcon 3912 Microtest III Flexible). The multi-well and all the pipette tips were washed with Milli-Q water three times before use to remove nitrite/nitrate contamination. NADPH (1 µM) and the reaction buffer (glucose-6-phosphate dehydrogenase 160 mU ml^{-1}, glucose-6-phosphate 500 µM, nitrate reductase 100 mU ml^{-1}) were added to the multi-well plate and incubated for 1 h at 37°C. Naphthalethylenediamine HCl (0.1 %; Griess reagent A) was added to each well, followed by the addition of sulphanilamide (1 %; Griess

reagent B). After an incubation of 15 minutes the absorbance was read at 540 nm with a filter at 620 nm.

2.4.5 TETHERED CONSCIOUS MICE

The femoral vein and artery in mice were cannulated to enable fluid and drug administration, monitoring of heart rate and blood pressure (Rees *et al.*, 1995; Rees *et al.*, 1998). Mice were attached to the monitoring system via a cantilever system involving a swivel mechanism, which allows the mouse free movement and access to food and water (Figure 2.6).

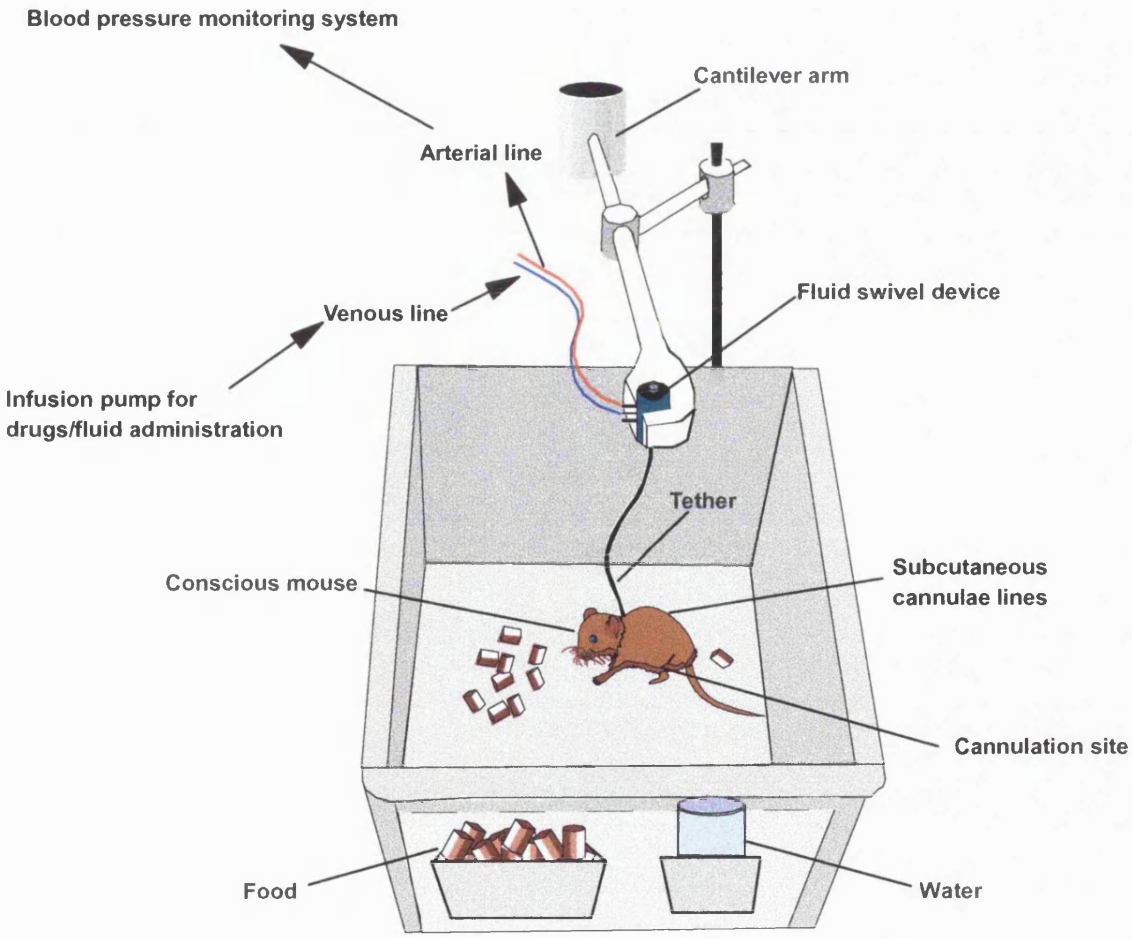

Figure 2.6 Tethered conscious mouse set up.

2.4.5.1 Arterial line

A 2 ml syringe on an infusion pump (Linton Instrumentation) was attached to a cannula line (10 cm, Vygon and 60 cm, SIMS Portex 06-0060N). This was connected to a flow-flush device (Ohmeda TA4005 Critiflo flush) attached on one side to a Nipro stopcock (3 way luer slip) and on the other side to a pressure transducer (Ohmeda P23XL-1). The second side of the Nipro stopcock was attached to a 1 ml syringe and the third side linked to a monoject needle connected to a single line PVC tubing (0.58 x 0.96 mm, 75 cm length) which was attached to the entry port of the arterial line of the swivel system (Instech; Dual Channel 375 series). At the exit port of the swivel, PVC tubing (0.58 x 0.96 mm, 5 cm length and 0.28 x 0.61 mm, 45 cm length) extended into the femoral artery as described below (Section 2.4.5.3.2), protected externally by a silicone rubber tube and sewn into the base of the neck-region.

The transducer was linked to a computerised data capture system (Modular Instruments, MI^2 BioReport system), with data additionally recorded on a chart recorder.

2.4.5.2 Venous line

A Discofix 3-way tap was attached to a 2 ml syringe on an infusion pump, and linked to the appropriate monoject needle connected with PVC tubing (0.58x0.96 mm, 75 cm length) directly attached to the venous entry port of the swivel system. The exit port was attached to PVC tubing (0.58 x 0.96 mm, 5 cm length and 0.28 x 0.61mm, 45 cm length) that enters the femoral

vein, as described below (Section 2.4.5.3.2), protected by a silicone rubber tube sewn into the base of the neck region.

2.4.5.3 Surgical procedure

2.4.5.3.1 Anaesthesia

The mouse was anaesthetised with isofluorane (5 %) for 3 min in the anaesthetic box. During cannulation, the anaesthesia was maintained with isofluorane (2.5 %) via a nose mask. After the required position for the button was marked (Figure 2.7), the mouse was laid on its ventral side and the two lower limbs were extended and kept in place by surgical tape.

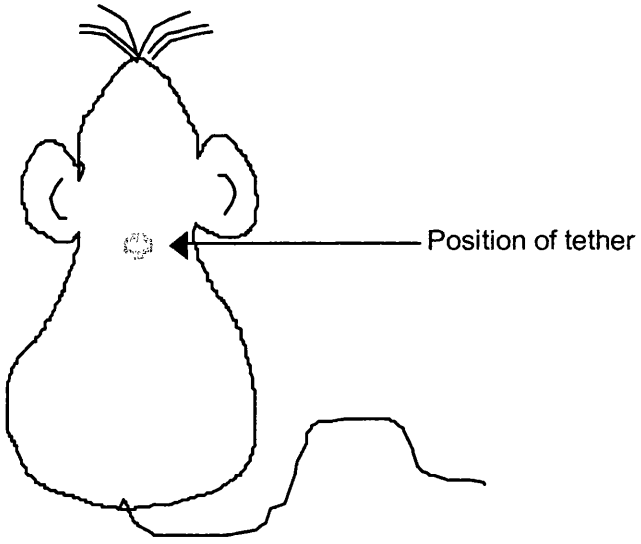

Figure 2.7 Marked position of tether.

2.4.5.3.2 Preparation of femoral site

A small incision was made in the left groin, to expose the femoral vein and artery (Figure 2.8).

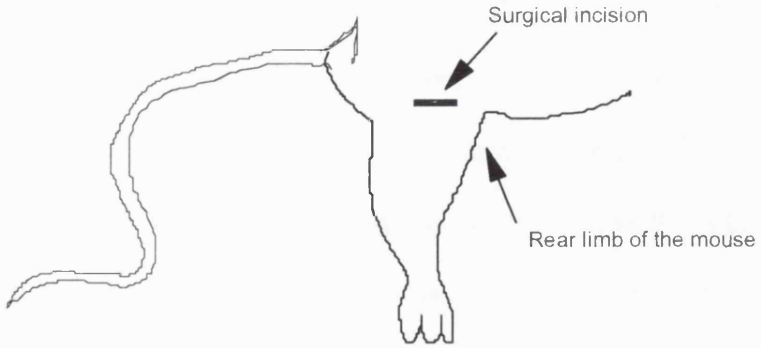

Figure 2.8 Small incision on femoral site.

A trocar was gently inserted at the incision point and positioned to emerge through the skin to the back of the neck. The cannula lines were threaded into the trocar, which was then removed so that the cannula lines emerge at the site of the groin incision. The mouse was put on its ventral side and the femoral vessels exposed.

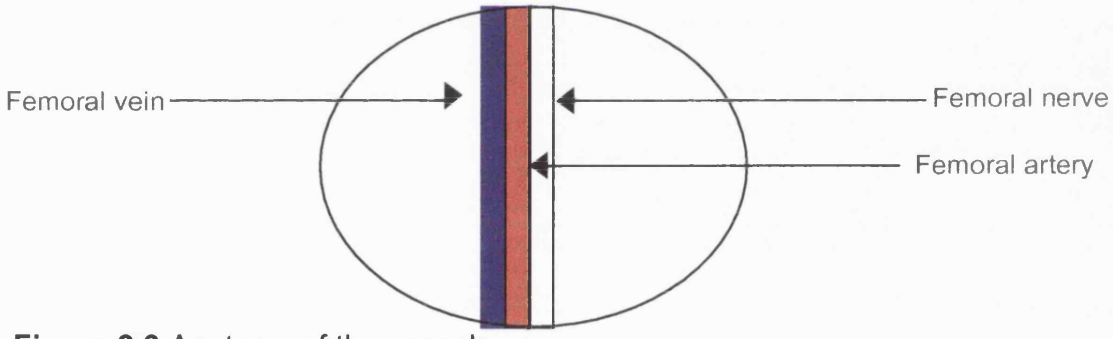

Figure 2.9 Anatomy of the vessels.

The nerve was gently teased away from the blood vessels (Figure 2.9) and a vessel support was put at the lower end of the incision (Figure 2.10).

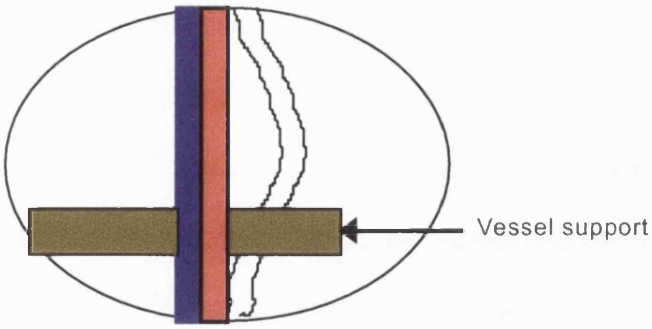

Figure 2.10 Vessel support at the lower end of the incision.

The vein and the artery were gently separated and then cannulated separately. If the blood flow in the two cannulae lines were not restricted the vessel support was removed. The incision was sewn up using a mattress stitch, (5-0 Ticron suture). The mouse was then turned over so that it lay on its ventral side. Gently the incision at the base of the neck was enlarged to accommodate the button (approx 1 cm) and a purse-string suture was put around the incision (5-0 Ticron suture, Figure 2.11).

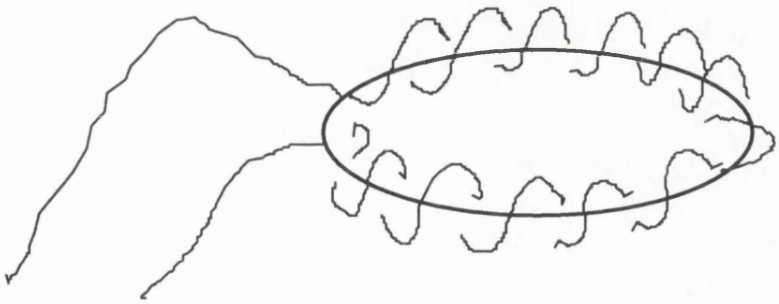

Figure 2.11 A purse-string suture around the incision.

The button was carefully inserted into the incision at the desired position, then the suture was pulled gently but firmly and knotted several times to secure the suture. The mouse and tether were gently transferred back to the cage. The whole procedure usually lasts approximately 40 min, with the mouse fully conscious in the cage approximately 1 hour after the start of the surgical procedure. A solution of heparinised saline (25 U ml^{-1}) was flushed

through the arterial and venous lines to eliminate air bubbles. Patency of the lines was maintained over the time course of the experiments by infusion of heparinised saline (50 µl h^{-1}) at a constant rate. All experiments began the day after the surgical procedure. Following administration of endotoxin the infusion of heparinised saline was increased to 100 µl h^{-1} through both the arterial and venous lines.

2.4.5.4 Monitoring of blood pressure

Blood pressure was monitored and recorded on the MI^2 computer system with additional data capture on a chart recorder. Once all the mice were in place, the system was calibrated using a manometer. Blood pressure was continuously monitored and mice were removed from the study if the blood pressure fell below 90 mmHg after 18-24 h following surgery, since this may indicate blood loss or other complications.

2.5 MATERIALS

The following materials were obtained as indicated: culture media, foetal bovine serum and trypan blue were obtained from (GIBCO, Paisley, UK). Endotoxin (Lipopolysaccharide, LPS) from *W.S. typhosa* 0901 (Insight Biotechnology, Germany, for studies *in vitro*), endotoxin (Lipopolysaccharide, LPS) from *E.Coli,* 026: B6 (Difco, UK for studies *in vivo*) and interferon-γ (IFN-γ, Insight Biotechnology, Germany). (z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl) amino]diazen-1-ium-1,2diolate (DETA-NO), a compound known to release NO with a half-life of 20 h at pH 7.4 (Hausladen & Fridovich, 1996). Oxyhaemoglobin (oxyHb) was prepared from crystallised haemoglobin (Sigma-Aldrich, Poole, UK), as described by

Feelisch (Feelish *et al.*, 1996). Myxothiazol, rotenone, succinic acid, FCCP, TMPD, β-hydroxybutyrate, 3-nitropropionic acid, NAD+, NADH, NADPH, glyceraldehyde-3-phosphate (GAP), GSSG, GSH-ethyl ester, ubiquinone, luminol naphthalethylenediamine HCl, sulphanilimide (1%), sodium nitrate and nitrite, sodium chloride solution (0.9 %), glucose-6-phosphate and glucose-6-phosphate dehydrogenase and all other reagents (Sigma-Aldrich, Poole, UK). Stock solutions (20 mM) were kept on ice in the dark and diluted in incubation buffer immediately before use. Ultrafiltration membranes (centrifugal filter devices, Microcon YC-3, -10 and -30, Millipore, Watford, UK). Nitrate reductase (Boehringer Mannheim, Germany) and heparin (Leo Laboratories Ltd, Buck U.K). L-N^G methylarginine HCl and D-NMMA were synthesised by Dr H. Hodson, as described previously (Scannell *et al.*, 1972; Patthy *et al.*, 1977).

2.6 STATISTICAL ANALYSIS

The results are expressed as mean ± standard error; *n* represents the number of individual experiments. Statistical analyses for the *in vitro* studies were performed by Student's t test for unpaired variables (Chapters 3, 4 and 5). Statistical analyses for the *in vivo* studies were performed by 2 tail Student's t test for paired variables in chapter 6. One way analysis of variance (ANOVA) was used for *in vivo* studies (Chapters 7 and 8), followed by Bonferroni Multiple Comparison test. Values were considered significant when $p < 0.05$. Survival data were represented as a Kaplan-Meier plot.

CHAPTER 3

EXOGENOUS NITRIC OXIDE INHIBITS CELLULAR RESPIRATION

3 EXOGENOUS NITRIC OXIDE INHIBITS CELLULAR RESPIRATION

3.1 SUMMARY

This study investigates the effect of prolonged exposure of exogenous NO on cellular respiration in Jurkat (human T lymphocyte-derived) and L929 (human fibroblast-derived) cells.

Exogenous NO, generated by DETA-NO (0.5 mM), inhibited, over 5 h, cellular respiration, which was reversed by oxyhaemoglobin (oxyHb). However, the ability of oxyHb to reverse the effect of NO lessened with time and was completely ineffective after 5 h in both cells.

The use of different substrates and inhibitors of the respiratory chain demonstrated that the site of inhibition by NO was downstream the citric acid cycle and upstream of ATP synthesis. The study further demonstrated that NO caused an oxyHb reversible inhibition of complex IV and an irreversible inhibition of complex I activity. Direct measurement of complex I activity and ATP concentrations showed a decrease in both over 5 h following incubation of cells with DETA-NO (0.5 mM).

This study indicates that prolonged exposure of exogenous NO to different cells causes an oxyHb reversible inhibition of complex IV and an irreversible inhibition of complex I suggesting that overproduction of NO in pathological conditions can compromise cellular energy metabolism.

3.2 INTRODUCTION

For many years it has been known that activated macrophages produce substances that are cytotoxic to other cells by irreversibly inhibiting their respiration (Hibbs, *et al.*, 1987; Granger *et al.*, 1980; Drapier & Hibbs, Jr., 1986). More recently several studies have shown that macrophages (Hibbs, Jr. *et al.*, 1988) and several other cells including astrocytes (Bolanos *et al.*, 1994), hepatocytes (Stadler *et al.*, 1991), smooth muscle cells (Geng *et al.*, 1992) and myocytes (Oddis & Finkel, 1995) activated with cytokines and endotoxin also produce cytotoxic substances that inhibit cellular respiration. Although these activated cells produce a variety of potentially toxic substances their toxic properties have been associated with the expression of iNOS leading to a sustained NO production. Indeed inhibitors of NOS or scavengers of NO prevent this cytotoxic activity (Hibbs, *et al.*, 1988; Drapier *et al.*, 1988; Stuehr & Nathan, 1989). However, it is difficult to define the specific mechanism underlying the inhibition of the respiration because NO may give rise to a variety of chemical products such as peroxynitrite. Peroxynitrite can react with a wide variety of biomolecules including proteins, via nitration of tyrosine (Ischiropoulos *et al.*, 1992); (Grune *et al.*, 1998) and tryptophan residues (Alvarez *et al.*, 1996); (Padmaja *et al.*, 1996), lipids and antioxidants (Radi *et al.*, 1991) and can oxidise methionine (Pryor *et al.*, 1994) and react with DNA (Beckman & Koppenol, 1996). Peroxynitrite can cause inhibition of ATP synthase and complex I-II (Radi *et al.*, 1994a). Despite the clear importance of iNOS expression in the cytotoxicity of activated cells, the role of NO itself on the respiratory enzymes requires further clarification. Short-term exposure of exogenous NO, to the rat

skeletal muscle mitochondria, inhibits mitochondrial oxygen consumption in a reversible manner, by competing with oxygen at the level of complex IV (Cleeter *et al.*, 1994). Furthermore, incubation of neurones with exogenous NO for 24 h inhibits complex I, II-III and IV (Bolanos *et al.*, 1996). In addition, non-activated J774 macrophages exposed to a constant concentration of exogenous NO induces a progressive inhibition of respiration occurring at the level of complex I (Clementi *et al.*, 1998).

This study investigates the effect of prolonged exposure of exogenous NO on cellular respiration and the potential target(s).

3.3 RESULTS

The NO donor, (z)-1-[2-(2-ami-noethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2 diolate, (DETA-N0, 0.5 mM), dissolved in the incubation medium, generated increasing concentrations of NO, which reached a plateau after about 20 min and remained constant throughout the time course of the experiments. In the incubation medium the concentration of NO generated at the plateau by DETA-NO (0.5 mM) was 1.2 ± 0.3 μM, (n = 4) as detected by the NO electrode.

3.3.1 EFFECT OF EXOGENOUS NITRIC OXIDE ON CELLULAR OXYGEN CONSUMPTION

The oxygen consumption in Jurkat (34.1 ± 2.2 nmol min^{-1} 10^7 $cells^{-1}$, at time 0 h, n = 6) and L929 cells (33.5 ± 1.2 nmol min^{-1} 10^7 $cells^{-1}$, at time 0 h, n = 6) did not alter significantly over the 5 h experiment (33.6 ± 1.2 and 31.1 ± 2.5 nmol min^{-1} 10^7 $cells^{-1}$ after 5 h in Jurkat and L929 cells, respectively, n = 6).

Addition of DETA-NO (0.5 mM) to the cells generated an immediate inhibition of respiration (76 ± 1% and 87 ± 1% inhibition in Jurkat and L929 cells respectively, *n* = 6), which was reversed by oxyHb (8 μM, Figure 3.1 A, B). However, the ability of oxyHb (8 μM) to reverse the effect of NO lessened with time and was ineffective after 5 h of incubation with DETA-NO (0.5 mM), in both cells (Fig. 3.1 A, B).

The irreversible inhibition of respiration by DETA-NO was not due to a non-specific toxic effect of NO since the cells were viable at the end of the experiment (5 h), as assessed by trypan blue exclusion (*n* = 10, Table 3.1 A, B).

No changes in the respiration, of both cells, were observed following incubation with decomposed DETA-NO (0.5 mM, *n* = 3, Table 3.1 A, B).

Table 3.1 Viability assessed by trypan blue exclusion of (A) Jurkat and (B) L929 cells during 5 hours of incubation with and without DETA-NO (0.5 mM, n = 3 -10).

A

Time [h]	Jurkat cells	Jurkat cells + decomposed DETA-NO [0.5 mM]	Jurkat cells + DETA-NO [0.5 mM]
0	95 ± 3 %	94 ± 2 %	96 ± 2 %
1	93 ± 5 %	93 ± 4 %	92 ± 4 %
2	91 ± 2 %	92 ± 4 %	89 ± 3 %
3	87 ± 4 %	88 ± 3 %	87 ± 5 %
4	84 ± 3 %	85 ± 4 %	86 ± 4 %
5	82 ± 5 %	81 ± 5 %	85 ± 3 %

(100 % = all the cells were viable)

B

Time [h]	L929 cells	L929 cells + decomposed DETA-NO [0.5 mM]	L929 cells +DETA-NO [0.5 mM]
0	83 ± 5 %	81 ± 3 %	82 ± 5 %
1	80 ± 3 %	80 ± 5 %	81 ± 5 %
2	78 ± 4 %	79 ± 5 %	77 ± 3 %
3	75 ± 2 %	74 ± 2 %	74± 2 %
4	73 ± 4 %	73 ± 4 %	72 ± 4 %
5	71 ± 3 %	72 ± 2 %	70 ± 5 %

(100 % = all cells were viable)

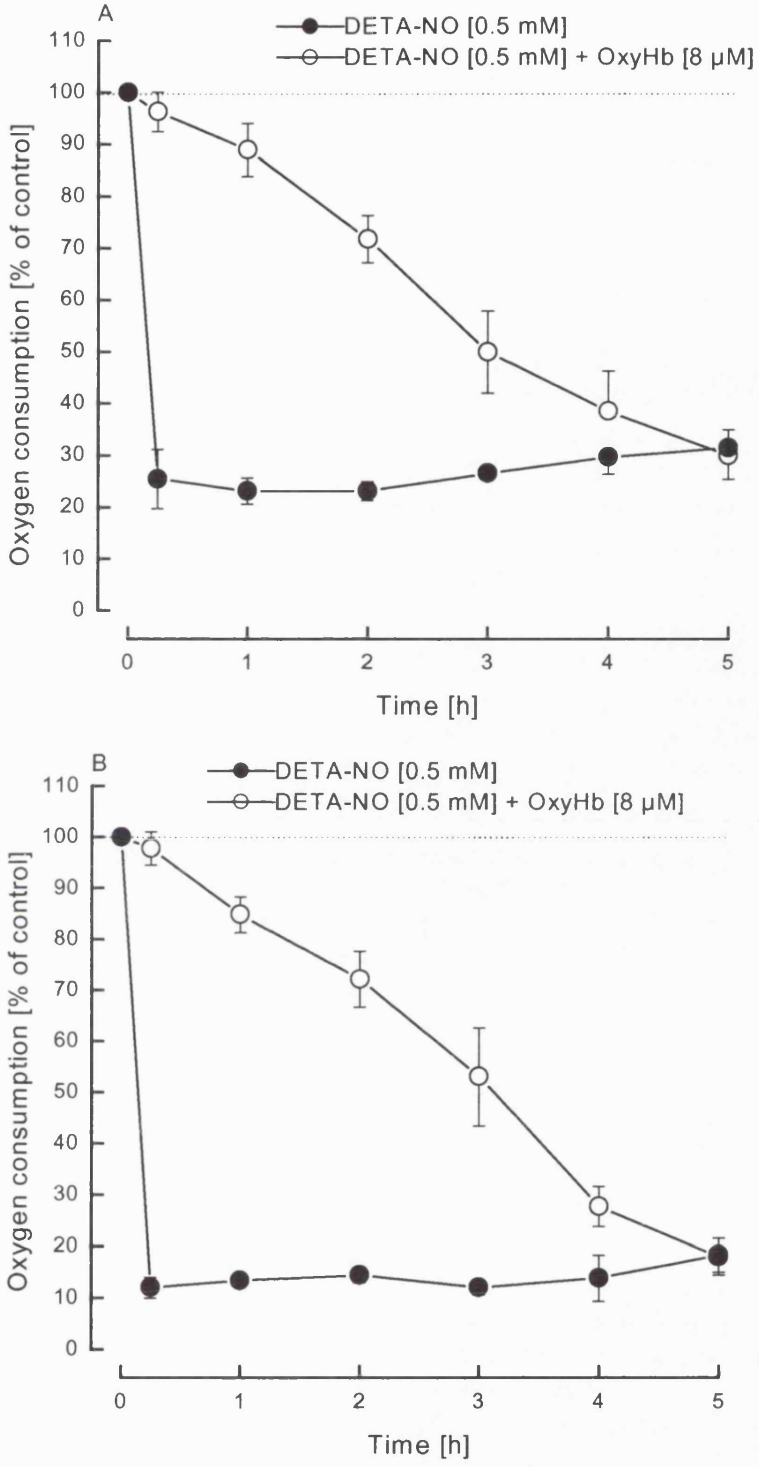

Figure 3.1 The reversal by oxyhaemoglobin (8 µM) of the inhibitory effect of DETA-NO (0.5 mM) on oxygen consumption decreases with time in (A) Jurkat and (B) L929 cells (n = 6).

3.3.2 IRREVERSIBLE INHIBITION OF CELLULAR RESPIRATION BY NITRIC OXIDE

Jurkat and L929 cells, resuspended in incubation medium in the presence of DETA-NO (0.5 mM), were treated with various inhibitors of mitochondrial respiratory complexes (Figure 3.2) in the presence of specific substrates as indicated ($n \geq 4$, Table 3.2 A, B).

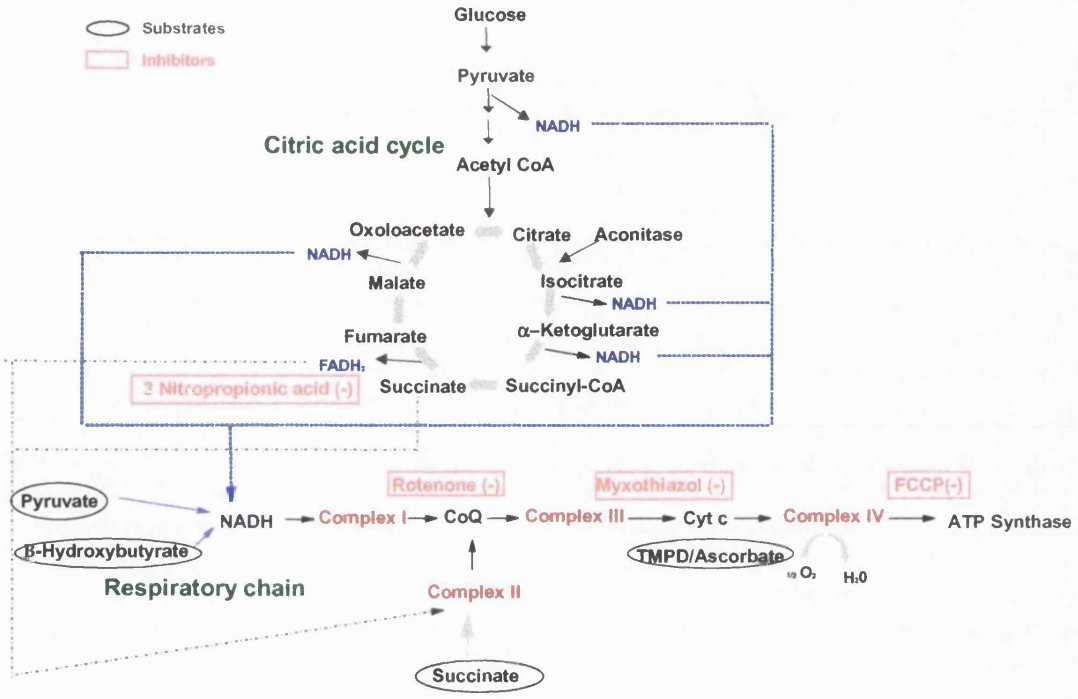

Figure 3.2 Substrates and inhibitors of the citric acid cycle and respiratory chain.

Oxygen consumption was measured in the presence of inhibitors and substrates of the respiratory chain to determine the site of inhibition by DETA-NO (0.5 mM).

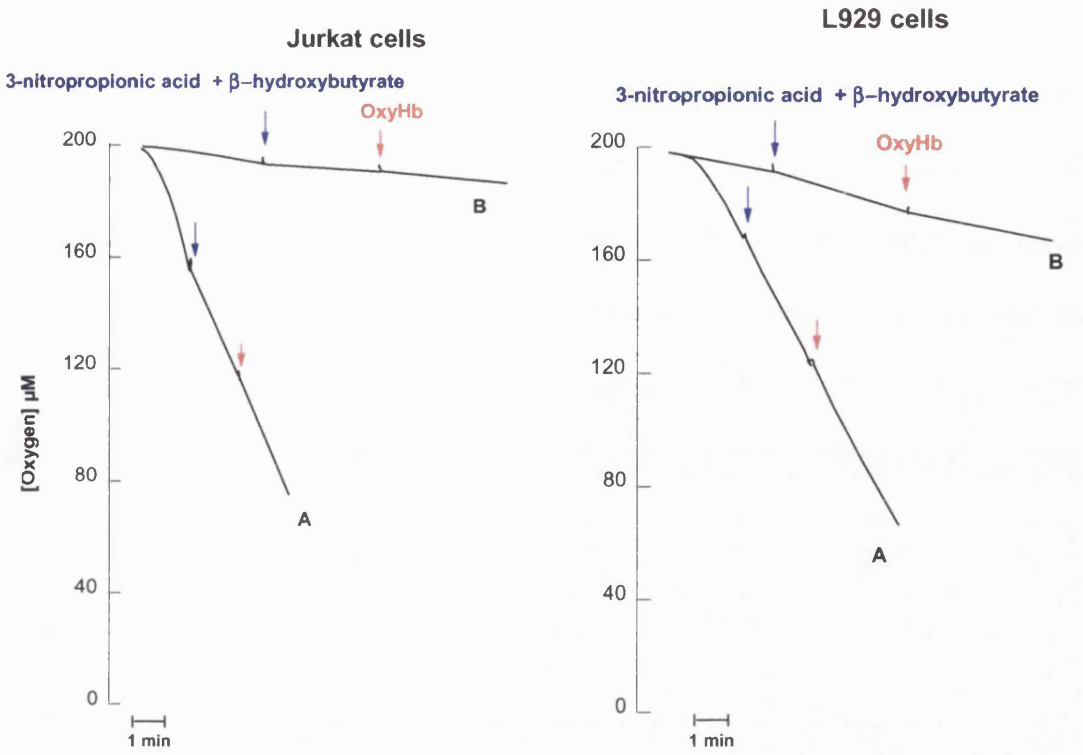

Figure 3.3 Example of oxygen consumption of Jurkat (left panel) and L929 (right panel) cells incubated with and without DETA-NO for 5 h following addition of 3-nitropropionic acid (0.6 mM) /β-hydroxybutyrate (6 mM) and oxyHb (8 µM).

Addition of 3-nitropropionic acid (0.6 mM) and β-hydroxybutyrate (6 mM) to Jurkat and L929 cells did not significantly alter the oxygen consumption over the time course of the experiment (at 5 h, before addition of 3-nitropropionic acid (0.6 mM) and β-hydroxybutyrate (6 mM) 33.6 ± 1.2 and 31.1 ± 2.5 nmol min^{-1} 10^7 $cells^{-1}$ in Jurkat and L929 cells respectively; after addition of 3-nitropropionic acid (0.6 mM) and β-hydroxybutyrate (6 mM) 32.9 ± 0.9 and 30.4 ± 1.9 nmol min^{-1} 10^7 $cells^{-1}$ in Jurkat and L929 cells respectively, n = 4-6, Figure 3.3). Addition of DETA-NO (0.5 mM) to Jurkat and L929 cells inhibited oxygen consumption. Addition of 3-nitropropionic acid (0.6 mM) and β-hydroxybutyrate (6 mM) did not significantly alter the cellular oxygen consumption of Jurkat and L929 cells exposed to DETA-NO (0.5 mM, n = 4-6, Figure 3.3). Furthermore addition of oxyHb (8 µM), to scavenge NO, did

not reverse the inhibition of cellular oxygen consumption at 5 h (Figure 3.3, Table 3.2, *n* = 4-6).

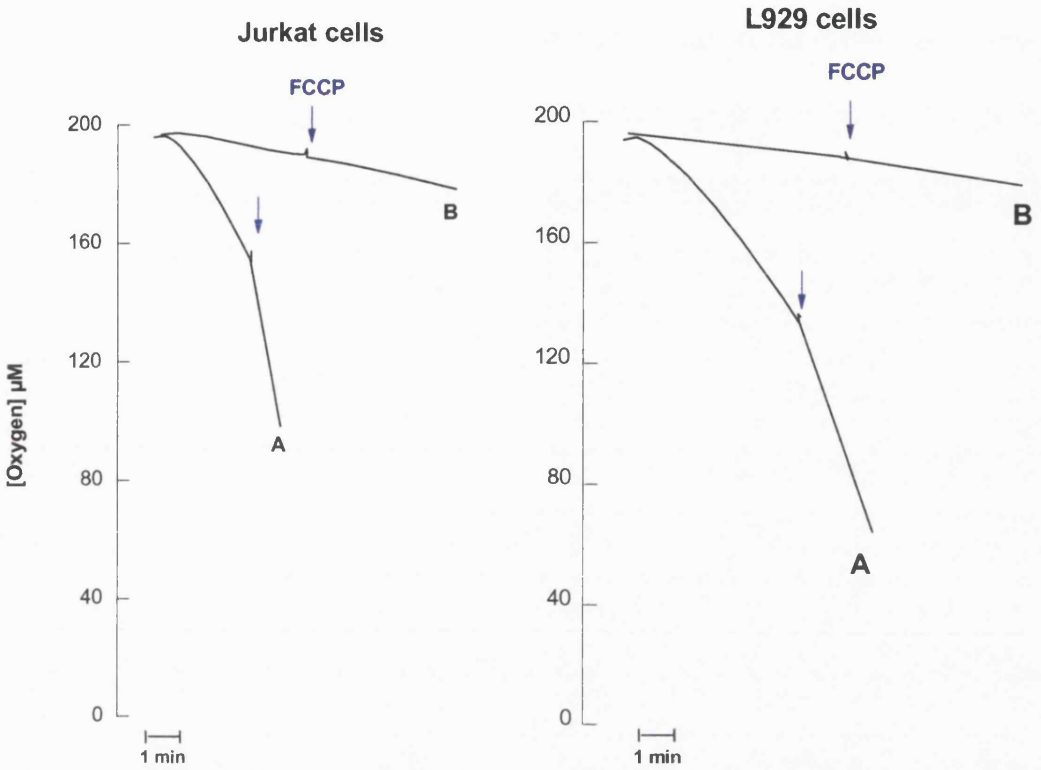

Figure 3.4 Example of oxygen consumption of Jurkat (left panel) and L929 (right panel) cells incubated with and without DETA-NO for 5 h following addition of FCCP (1 µM).

After 5 h incubation addition of the protonophore, carbonyl cyanide p-(trifluoro-methoxy) phenylhydrazone (FCCP, 1 µM, an uncoupler of the flow of electrons from the synthesis of ATP) increased oxygen consumption in Jurkat and L929 cells (from 33.5 ± 1.2 to 46.6 ± 2.2 nmol min^{-1} 10^7 $cells^{-1}$ in Jurkat cells and from 31.1 ± 2.5 to 45.6 ± 1.9 nmol min^{-1} 10^7 $cells^{-1}$ in L929 cells, *n* = 4-6, Figure 3.4).

Addition of DETA-NO (0.5 mM) to Jurkat and L929 cells inhibited oxygen consumption. Afetr 5 h incubation in the presence of DETA-NO, addition of FCCP (1 µM) had no effect on the oxygen consumption of the cells (from

10.55 ± 1.1 to 10.9 ± 1.2 nmol min $^{-1}$ 10^7 cells^{-1} in Jurkat cells and from 6.2 ± 0.09 to 6.5 ± 0.1 nmol min $^{-1}$ 10^7 cells^{-1} in L929 cells, n = 4-6 for each group, Figure 3.4).

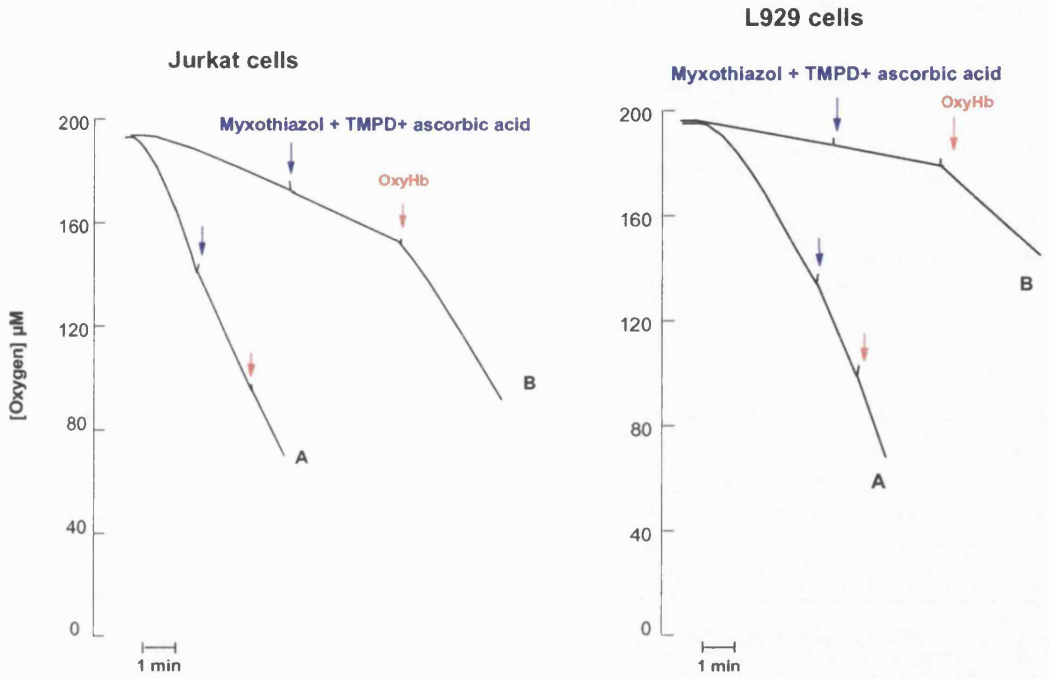

Figure 3.5 Example of oxygen consumption of Jurkat (left panel) and L929 (right panel) cells incubated with and without DETA-NO for 5 h following addition of myxothiazol (0.5 µM) and TMPD (80 µM)/ ascorbic acid (4 mM).

Addition of myxothiazol (0.5 µM), *N,N,N*^{1}*N*^{1}-tetramethyl-*p*-phenylene diamine (TMPD, 80 µM) and ascorbic acid (4 mM), to Jurkat and L929 cells did not significantly inhibit the cellular oxygen (Figure 3.5). Addition of DETA-NO (0.5 mM) to Jurkat and L929 cells inhibited oxygen consumption. After 5 h incubation addition of myxothiazol (0.5 µM) and TMPD (80 µM) / ascorbic acid (4 mM, n = 3) did not significantly alter the oxygen consumption (Figure 3.5). Whereas addition of oxyHb (8 µM), to scavenge NO, reversed the inhibition of respiration in both cells (Figure 3.5, Table 3.2, n = 4-6).

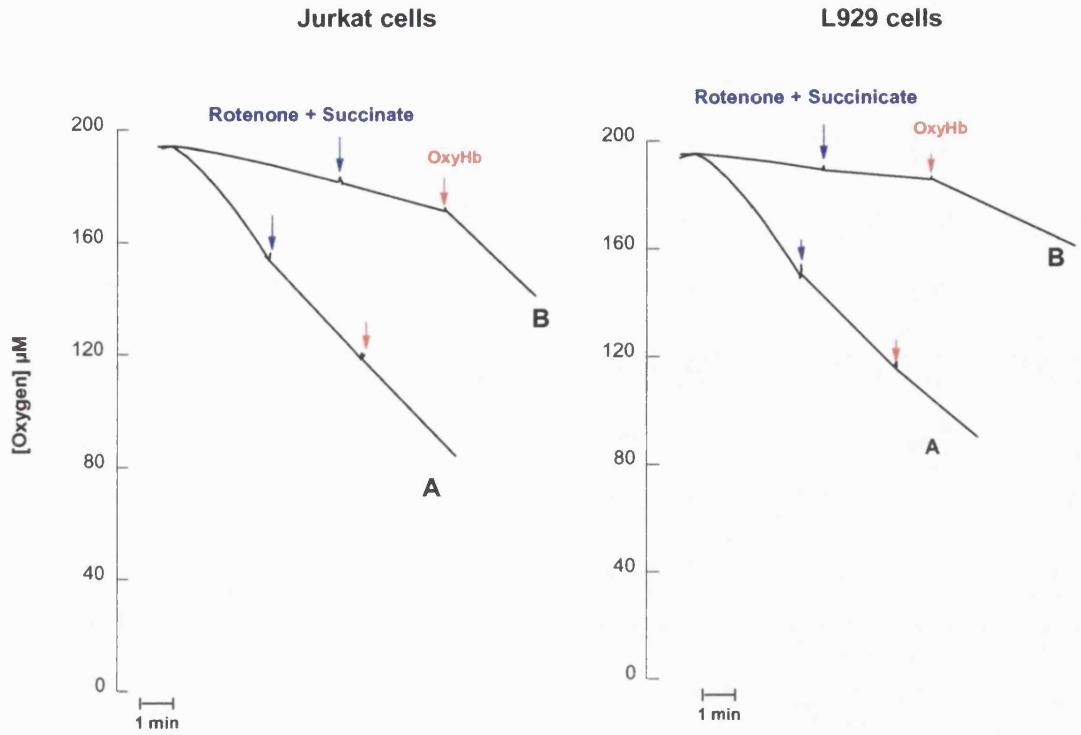

Figure 3.6 Example of oxygen consumption of Jurkat (left panel) and L929 (right panel) cells incubated with and without DETA-NO for 5 h following addition of rotenone (2 µM) and succinate (6 mM).

Addition of rotenone (2 µM) and succinate (6 mM), to Jurkat and L929 cells did not significantly alter the oxygen consumption (Figure 3.6, Table 3.2, *n* = 4).

Addition of DETA-NO (0.5 mM) to Jurkat and L929 cells inhibited oxygen consumption. Addition of rotenone (2 µM) and succinate (6 mM) did not alter the oxygen consumption of Jurkat and L929 cells exposed to DETA-NO (0.5 mM) for 5h (Figure 3.6). Whereas addition of oxyHb (8 µM), reversed the inhibition of respiration (Figure 3.6, Table 3.2, *n* = 4-6).

Table 3.2 Effects of DETA-NO (0.5 mM) on oxygen consumption in (A) Jurkat and (B) L-929 cells using different inhibitors and substrates of the respiratory chain. Cellular oxygen consumption was measured before and after the addition of inhibitor, substrates and oxyHb. The values are expressed as a percentage of the oxygen consumption observed in control cells treated with the same inhibitors and substrates but without DETA-NO (n = 4).

(A)

Treatment	Pathways	20 min	20 min + oxyHb [8 µM]	5 h	5 h + oxyHb [8 µM]
DETA-NO [0.5 mM]		26.0 ± 5.6	97.2 ± 3.8	31.5 ± 3.4	30.0 ± 4.5
3-Nitropropionic acid [0.6 mM, (-)]+ β-Hydroxybutyrate [6 mM, (+)]	Complexes I-III-IV	29.3 ± 2.6	104.6 ± 4.6	42.1 ± 4.7	36.6 ± 11.7
Myxothiazol [0.5 µM, (-)]+ Ascorbate [4mM, (+)] / TMPD [80 µM, (+)]	Complex IV	28.0 ± 1.6	101.7 ± 0.8	45.0 ± 5.0	107.0 ± 3.0
Rotenone [2 µM, (-)] + Succinate [6 mM, (+)]	Complexes II-III-IV	27.6 ± 10.8	98.6 ± 9.4	35.7 ± 7.2	97.0 ± 3.1

(B)

Treatment	Pathways	20 min	20 min + oxyHb [8 µM]	5 h	5 h + oxyHb [8 µM]
DETA-NO [0.5 mM]		10.6 ± 2.6	103.3 ± 3.3	18.3 ± 3.3	18.0 ± 3.6
3-Nitropropionic acid [0.6 mM, (-)]+ β-Hydroxybutyrate [6 mM, (+)]	Complexes I-III-IV	19.0 ± 9.0	96.6 ± 15.0	31.6 ± 2.0	26.0 ± 2.8
Myxothiazol [0.5 µM, (-)]+ Ascorbate [4mM, (+)] / TMPD [80 µM, (+)]	Complex IV	20.2 ± 3.1	107.0 ± 2.6	29.7 ± 3.0	101.0 ± 4.3
Rotenone [2 µM, (-)] + Succinate [6 mM, (+)]	Complexes II-III-IV	17.0 ± 4.9	108.0 ± 2.5	36.3 ± 3.2	99.6 ± 5.2

3.3.3 EFFECT OF NITRIC OXIDE ON COMPLEX I ACTIVITY

Complex I activity, measured as the rate of NADH oxidation, was analysed in Jurkat and L929 cell homogenates incubated with and without DETA-NO (0.5 mM) for 5 h.

The activity of complex I in both cells was constant over the time course of the experiment (5.8 ± 0.5 and 7.4 ± 0.6 nmol min^{-1} mg $protein^{-1}$ at 20 min and 6.1 ± 0.5 and 6.5 ± 0.4 nmol min^{-1} mg $protein^{-1}$ at 5 h in Jurkat and L929 cells, respectively, *n* = 4 for each, Figure 3.7 A, B).

The activity of complex I was inhibited by rotenone (2 μM; 2.7 ± 0.2 and 3.8 ± 0.6 nmol min^{-1} mg $protein^{-1}$ at 20 min and 2.8 ± 0.5 and 2.8 ± 0.4 nmol min^{-1} mg $protein^{-1}$ at 5 h in Jurkat and L929 cells, respectively, *n* = 4 for each, Figure 3.7 A, B).

Incubation with DETA-NO (0.5 mM) for 20 min did not inhibit the activity of complex I (5.4 ± 0.4 and 7.4 ± 1.2 nmol min^{-1} mg $protein^{-1}$ in Jurkat and L929 cells, respectively, *n* = 4 for each, Figure 3.7 A, B).

Whereas, incubation with DETA-NO (0.5mM) for 5 h inhibited the activity of complex I (3.1 ± 0.2 and 2.5 ± 0.6 nmol min^{-1} mg $protein^{-1}$ in Jurkat and L929 cells, respectively, *n* = 4 for each, Figure 3.7 A, B).

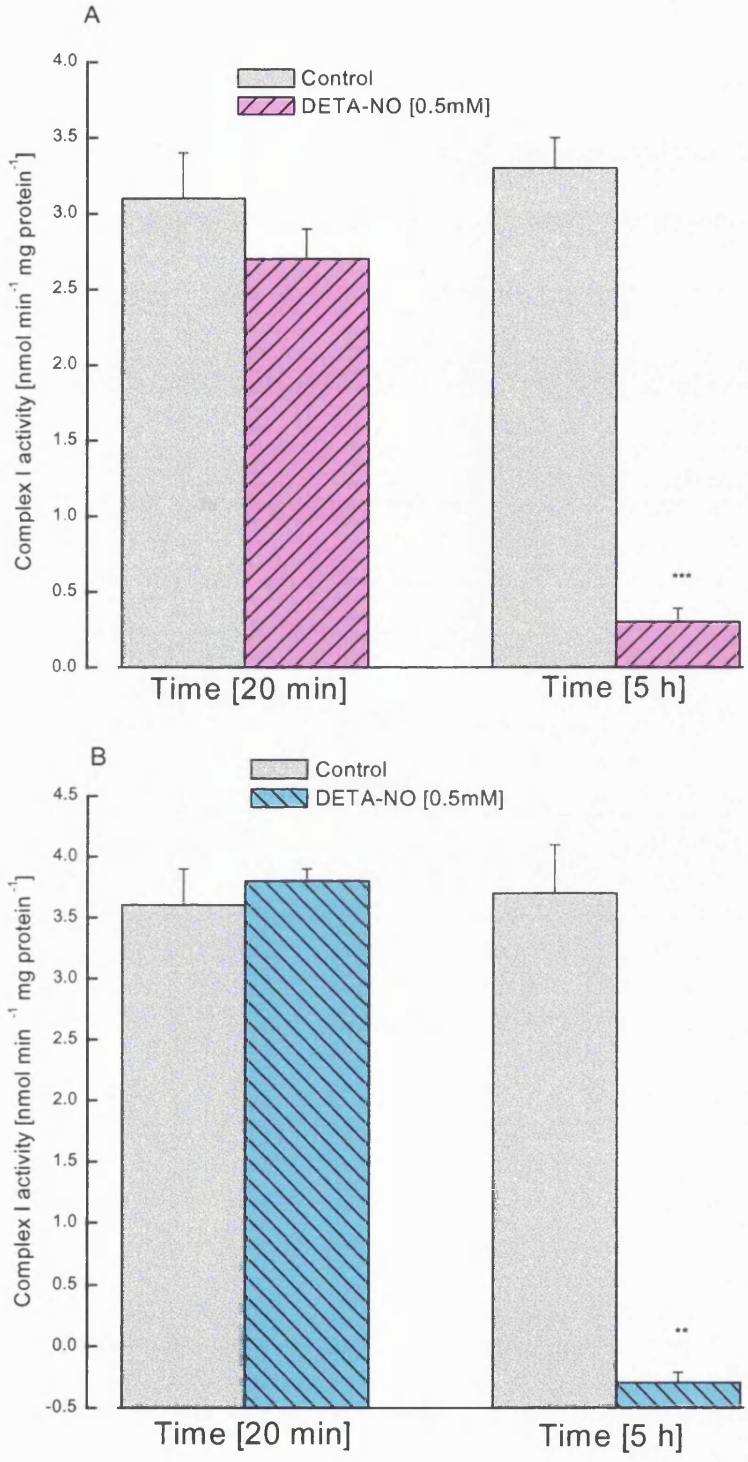

Figure 3.7 Effect of DETA-NO (0.5 mM) on complex I activity in (A) Jurkat and (B) L929 cells. Statistical analysis: * cells treated versus the control cells at the same time point. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$ ($n = 4$).

3.3.4 EFFECT OF NITRIC OXIDE ON ATP CONCENTRATION

The ATP concentration decreased in Jurkat and L929 cells during 5 h of incubation (Figure 3.8 A, B).

In Jurkat and L929 cells with DETA-NO (0.5 mM) for 5h, the concentrations of ATP were further reduced compared to the control (Figure 3.8 A, B).

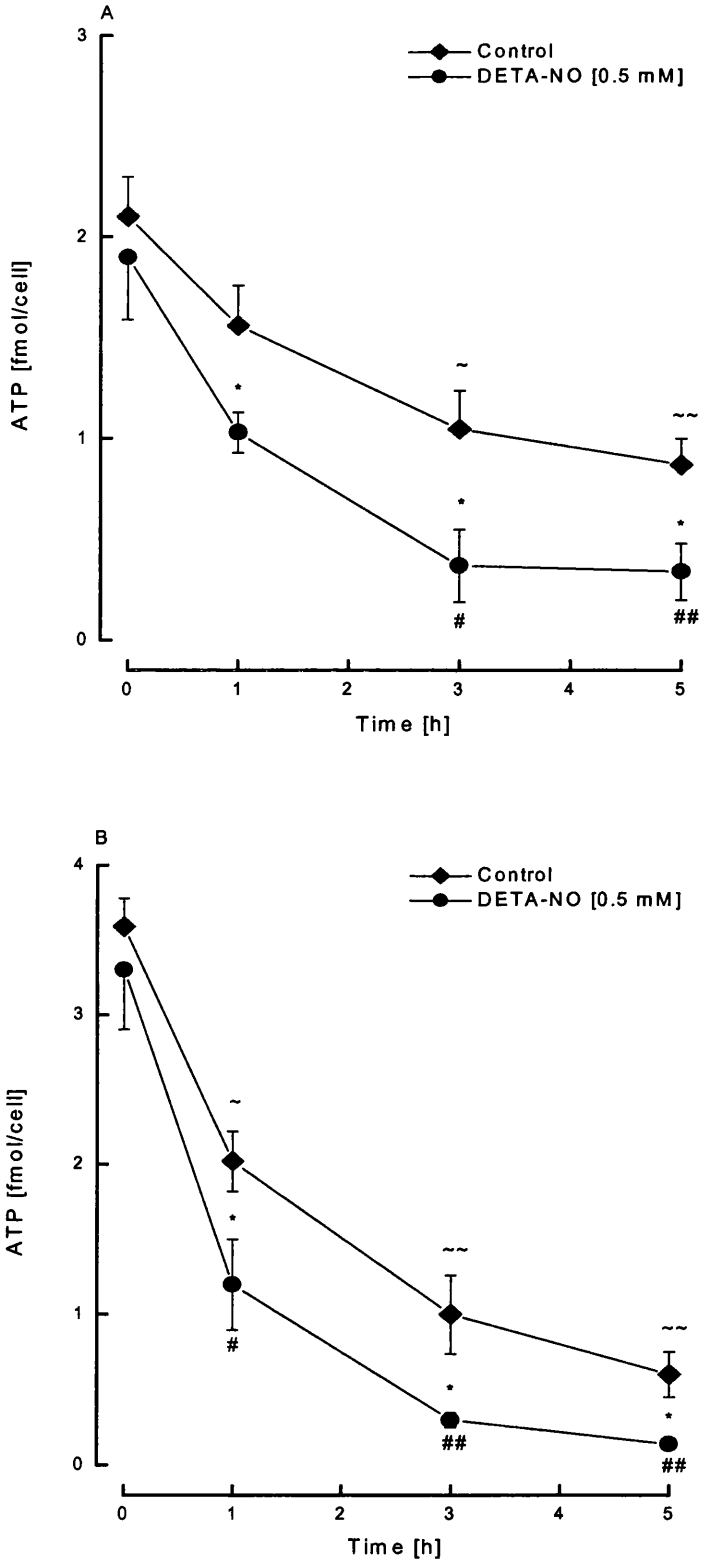

Figure 3.8 Effect of DETA-NO (0.5 mM) on ATP concentrations in (A) Jurkat and (B) L929 cells over 5 h. Statistical analysis: #, ~ within same group, * treated cells versus control cells at the same time points. *, ~, # = $p < 0.05$;**, ~~, ## = $p < 0.01$, ($n = 4$).

3.4 DISCUSSION

These results show that prolonged exposure of NO to different cells (Jurkat and L929) inhibits cell respiration. The ability of oxyHb to reverse the inhibition decreased over the time course of the experiment, and became inefficient at 5 h. Experiments carried out using substrates or compounds that inhibit specific enzymes of the citric acid cycle or the respiratory chain indicated that a reversible inhibition of oxygen consumption occurred at the level of complex IV and an irreversible inhibition by oxyHb occurred downstream of the citric acid cycle and upstream of complex II-III, suggesting that NO inhibits the activity of complex I. The inhibition of complex I activity was then confirmed by direct measurement of the rate of NADH oxidation in homogenates from cells treated with DETA-NO. These results are in accordance with previous studies in which long-term exposure of NO to J774 cells leads to the irreversible inhibition of complex I (Clementi *et al.*, 1998). Whether the action of NO on complex I is due to NO itself or to some related species is not yet clear. The mitochondria are a source of superoxide anion, which can react with NO in a rapid reaction to form peroxynitrite (Beckman *et al.*, 1990; Radi *et al.*, 1991). However, Clementi and co-workers did not show any detectable reactive oxygen species using the same experimental conditions with J774 cells and J774 C3C mutant clone, which does not release superoxide. Furthermore, the addition of peroxynitrite scavengers in the incubation medium did not affect the time course of onset of the irreversible inhibition of respiration (Clementi *et al.*, 1998). However, published results show that peroxynitrite is formed in close proximity of complex I due to the generation of superoxide anion in

complex I itself (Radi, 1996). In light of this, most of the inhibition of complex I could be related to the formation of peroxynitrite. Irreversible inhibition of complex I could represent a turning point in the cell leading from physiological regulation of complex IV (Brown & Cooper, 1994) to a pathophysiological inhibition of complex I. Indeed it has been shown that inhibition of complex I has toxic effects and leads to the triggering of an apoptotic programme (Seaton *et al.*, 1998; Barrientos & Moraes, 1999).

These results also show that over the time course of the experiment there is a gradual decrease in ATP concentration in the cells. This is probably associated with the change in the medium (from the culture medium to incubation medium) and the small decrease in viability of the cells as determined by trypan blue. Interestingly the ATP concentration is further decreased in cells incubated in the presence of DETA-NO. This is probably due to an upstream effect on the respiratory chain. Although the inhibition of ATP synthesis may also play a part in the inhibitory effect of NO (Brookes *et al.*, 1999).

These results show that long-term exposure of exogenous NO inhibits selectively complexes IV and I in two different cells. Thus it seems likely that cellular energy metabolism may be compromised in pathological conditions associated with overproduction of NO.

CHAPTER 4

INHIBITION OF RESPIRATION BY EXOGENOUS NITRIC OXIDE IS PRECEDED BY A DECREASE IN GLUTATHIONE CONCENTRATIONS

4 INHIBITION OF RESPIRATION BY EXOGENOUS NITRIC OXIDE IS PRECEDED BY A DECREASE IN GLUTATHIONE CONCENTRATIONS

4.1 SUMMARY

This study investigates whether glutathione depletion following long term exposure of exogenous nitric oxide plays a role in the inhibition of cellular respiration at the level of complex I. Exposure of exogenous NO (DETA-NO, 0.5 mM) to Jurkat (human T lymphocyte-derived) and L929 (human fibroblast) cells decreased the concentration of the intracellular reduced glutathione. This decrease in glutathione preceded the inhibition of complex I activity. In addition, the inhibition of complex I activity was reversed by addition of reduced glutathione or by exposure to cold light.

Exposure of these cells to exogenous NO (DETA-NO 0.5 mM) also inhibited the activities of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The activity of GAPDH progressively decreased over a time course similar to that observed for inhibition of complex I activity. Furthermore, inhibition of GAPDH was reversed and prevented by increasing the intracellular concentrations of reduced glutathione.

This study suggests that following long term exposure of NO to the cells, depletion of glutathione plays a role in the inhibition of respiration at the level of complex I.

Thus in the presence of overproduction of NO, glutathione is a key factor in determining the pathological and physiological status of cells.

4.2 INTRODUCTION

Glutathione (GSH) plays a critical role as a scavenger of free radicals and as a reducing cofactor essential for the detoxification of lipid and organic peroxides, including peroxynitrite, and lipid decomposition products (Radi *et al.*, 1991); (Lizasoain *et al.*, 1996). Incubation of neurones with a GSH synthesis inhibitor, L-buthione-[S,R]-sulfoximine (L-BSO), leads to a depletion of neuronal GSH accompanied by a marked decrease of complex II-III and IV activity, with only a small decrease in complex I activity (Bolanos *et al.*, 1996). In addition, in purified mitochondria and in the whole brain of pre-weanling rats, complex IV activity is significantly reduced, following depletion of GSH by L- BSO administration for up 10 days (Heales *et al.*, 1995). Furthermore, long term exposure of J774 murine macrophages to exogenous NO decreases the concentration of intracellular glutathione (GSH), resulting in a progressive inhibition of respiration. This inhibition occurs earlier following addition of L-BSO, whereas addition of GSH or exposure to cold light reverses these effects. These results suggest a possible S-nitrosylation of thiols necessary for the activity of the enzyme (Clementi *et al.*, 1998). S-nitrosylation can inhibit many enzymes including glutathione reductase (GR; Butzer *et al.*, 1999) and the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Padgett & Whorton, 1995). The inhibition of the latter can result in cytotoxicity for endothelial cells relying on glycolysis as their primary source of ATP generation (Padgett & Whorton, 1995). S-nitrosylation can activate the protooncogene $p21^{ras}$ (Lander *et al.*, 1997), L-type calcium channels (Broillet, 1999), cyclic nucleotide-gated channels (Broillet, 1999; Broillet & Firestein, 1997), or

modulate the DNA binding activity of transcription factors such as nuclear transcription factor B (NFκB; delaTorre *et al.*, 1998). Furthermore, S-nitrosylation has been proposed to be an important pathway of transducing NO signals in the cell (Stamler, 1994). In view of this, it is important to understand the conditions under which S-nitrosylation occurs in the whole cell.

This study investigates whether depletion of glutathione plays a role in the inhibition of complex I and GAPDH activity over a 5 h exposure to exogenous NO.

4.3 RESULTS

Jurkat (human T lymphocyte-derived) and L929 (human fibroblast) cells were exposed continuously to exogenous NO (1.2 ± 0.3 μM) over 5 h by addition of DETA-NO (0.5 mM) in the following studies.

4.3.1 MEASUREMENT OF GLUTATHIONE

Glutathione (GSH) concentration was not significantly different in both cell types over the time course of the experiments (13.9 ± 1.4 and 14.1 ± 1.1 nmol mg protein^{-1} at 0 h and 10.8 ± 1.5 and 11.6 ± 0.4 nmol mg protein^{-1} at 5 h in Jurkat and L929 cells, respectively (NS, n = 5-7 for each). Incubation with DETA-NO (0.5 mM) resulted in progressive decrease in the intracellular GSH concentration in Jurkat and L929 cells over 5 h (Figure 4.1 A, B).

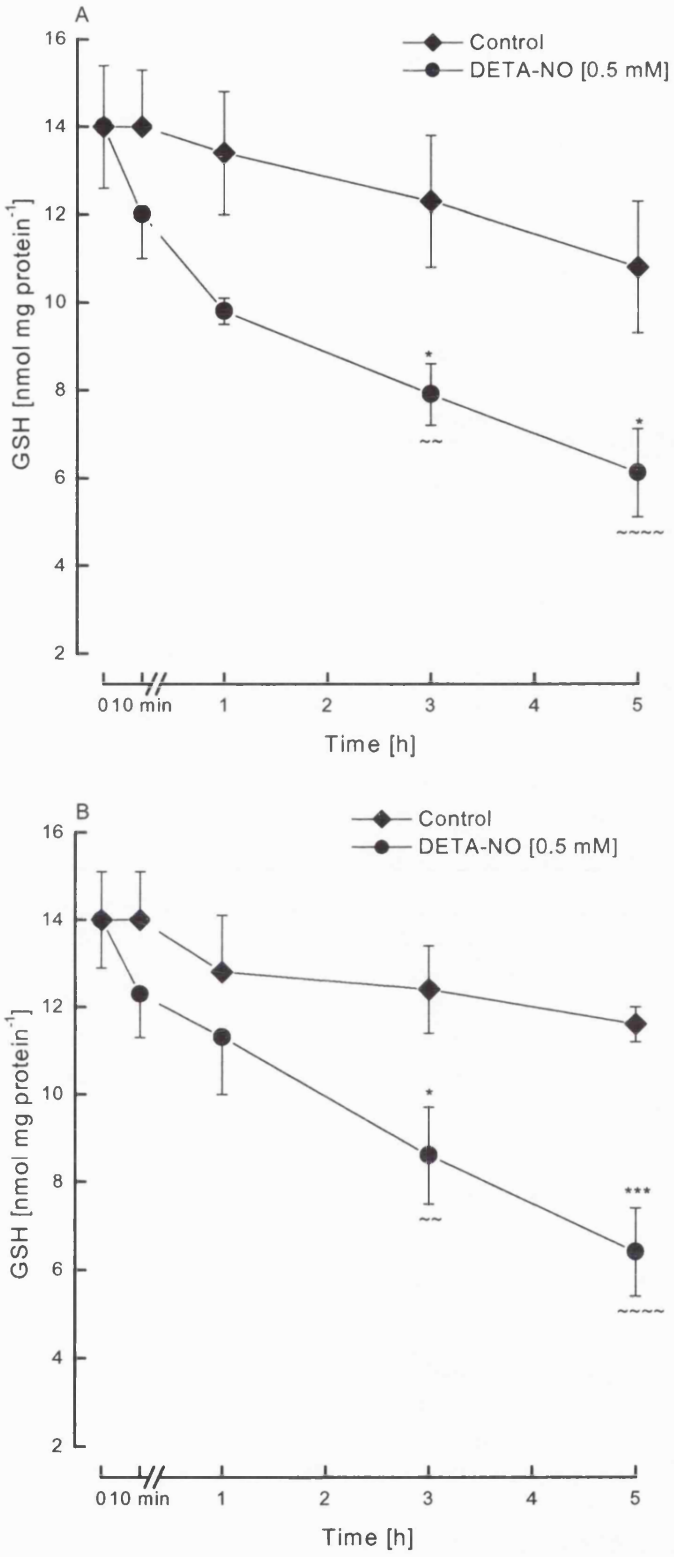

Figure 4.1 Intracellular GSH concentration in (A) Jurkat and (B) L929 cells incubated with and without DETA-NO (0.5 mM). Statistical analysis: * cells treated versus the control cells at the same time point. ~ within the same group. * = $p < 0.05$; **, ~~ = $p < 0.01$; ***, ~~~ = $p < 0.005$; ~~~~ = $p < 0.001$ (n = 5-7).

4.3.2 EFFECTS OF GSH ON CELLULAR OXYGEN CONSUMPTION, COMPLEX I AND GAPDH ACTIVITY

4.3.2.1 Oxygen consumption

DETA-NO (0.5 mM) inhibited the mitochondrial respiration at the level of complex I (Chapter 3), which was irreversible following addition of oxyHb at 5 h (Chapter 3). Following incubation of the cells with DETA-NO (0.5 mM) for 5 h, the GSH analogue, GSH-ethyl ester (2 mM), was added to the cells for a further 1.5 hours. GSH-ethyl ester (2 mM) restored oxygen consumption (by 63 ± 8 %, and 61 ± 1 % in Jurkat and L929 cells, *n* = 4 and 3, respectively) towards that of control cells. The addition of GSH-ethyl ester (2 mM) to the cells incubated without DETA-NO did not significantly change the oxygen consumption (by 1.5. ± 0. 5 %, and 1.1 ± 0.2 % in Jurkat and L929 cells, *n* = 4 and 3, respectively).

Coincubation of GSH-ethyl ester (2 mM) with DETA-NO (0.5 mM) showed no inhibition of oxygen consumption (*n* = 4 for each, Table 4.1).

The irreversible inhibition observed with oxyHb, after incubation with DETA-NO (5 h), was also reduced by doubling the concentration of cells in the medium (i.e. 20 x 10^6 cells ml^{-1}, *n* = 4, Table 4.1).

Exposure to cold light (8 Mlx) for a further 1.5 hours also reversed the inhibition of oxygen consumption (by 73 ± 18 % and 94 ± 3 % in Jurkat and L929 cells respectively, *n* = 3 for each group).

Table 4.1 Effect of DETA-NO and GSH on the oxygen consumption in (A) Jurkat and (B) L929 cells, (n = 4). The oxygen consumption of the cells was analysed before and after the addition of oxyHb (8 μM) at the time points indicated. Values are expressed as a percentage of the cells treated without DETA-NO.

(A)

Treatment	Oxygen consumption			
	20 min	**20 min + oxyHb [8 μM]**	**5 h**	**5 h + oxyHb [8 μM]**
n°cells [1x10^7]+ DETA-NO [0.5 mM]	26 ± 2%	97.5 ± 2.5%	33 ± 3.3%	33.5 ± 2.5%
n°cells [2x10^7]+ DETA-NO [0.5 mM] + GSH-ethyl ester [2 mM]	26.5 ± 1.5%	106 ± 1%	29.5 ± 0.5%	98 ± 6%
n°cells [2x10^7]+ DETA-NO [0.5 mM]	33.3 ± 8.1%	107 ± 6.3%	24.2 ± 2%	66.7 ± 12%

(B)

Treatment	Oxygen consumption			
	20 min	**20min + oxyHb [8 μM]**	**5 h**	**5 h + oxyHb [8 μM]**
n°cells [2x10^7]+ DETA-NO [0.5 mM]	11.7 ± 3 %	100 ± 0.5%	14 ± 3.3%	13.4 ± 4.1%
n°cells × [2x10^7]+ DETA-NO [0.5 mM] + GSH-ethyl ester [2 mM]	10.6 ± 1.4%	103.3 ± 2.3%	15 ± 2%	77.1 ± 1.4%

4.3.2.2 Complex I activity

The activity of complex I, measured by the oxidation of NADH, (6.1 ± 0.5 and 6.3 ± 0.4 nmol min^{-1} mg $protein^{-1}$, Jurkat and L929 cells respectively, at 20 min, *n* = 4 for each) was inhibited by incubation with DETA-NO (0.5 mM) at 5 h (3.1 ± 0.2 and 2.5 ± 0.6 nmol min^{-1} mg $protein^{-1}$, Jurkat and L929, respectively, *n* = 4 for each, $p < 0.05$ in both the cells, Figure 4.2 A, B). GSH-ethyl ester (2 mM) alone did not alter the activity of complex I at 5 h (6.5 ± 0.4 and 6.1 ± 0.9 nmol min^{-1} mg $protein^{-1}$, Jurkat and L929, respectively, *n* = 4 for each). Coincubation of GSH-ethyl ester (2 mM) with DETA-NO (0.5 mM) prevented the inhibition of the activity of complex I at 5 h (6.0 ± 0.5 and 5.1 ± 1.0 nmol min^{-1} mg $protein^{-1}$, Jurkat and L929 cells, respectively, *n* = 4 for each, Figure 4.2 A, B).

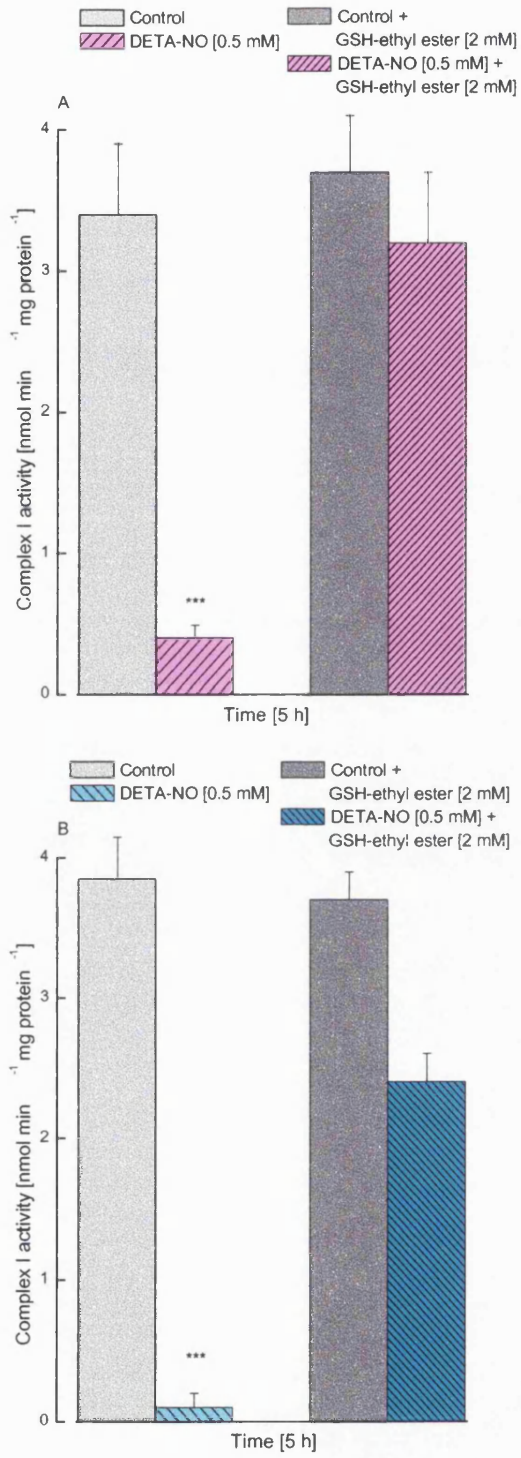

Figure 4.2 DETA-NO (0.5 mM) inhibited the activity of complex I at 5 h in (A) Jurkat and (B) L929 cells. GSH-ethyl ester (2 mM) coincubated with DETA-NO (0.5 mM) for 5 h prevented the inhibition of complex I. Statistical analysis: * cells treated with DETA-NO versus the control. *** = $p < 0.005$ ($n = 4$).

4.3.2.3 Glyceraldehyde-3-phosphate dehydrogenase activity

Glyceraldehyde-3-phosphate dehydrogenase activity, measured by the reduction of NAD^+, was not significantly different throughout the time course of the experiments (0.33 ± 0.01 and 0.37 ± 0.03 µmol min^{-1} mg $protein^{-1}$ at 20 min and 0.31 ± 0.01 and 0.34 ± 0.01 µmol min^{-1} mg $protein^{-1}$ at 5 h in Jurkat and L929 cells, respectively, *n* = 4 for each).

Incubation of Jurkat and L929 cells with DETA-NO (0.5 mM) inhibited GAPDH activity (by 6 % and 5% at 20 min; by 20 % and 18 % at 3 h and by 48 and 66 % at 5 h in Jurkat and L929 cells, respectively, *n* = 4 for each, Figure 4.3 A, B).

In Jurkat cells, GSH-ethyl ester (2 mM) alone did not alter the activity of GAPDH (0.1 ± 0.002 µmol min^{-1} mg $protein^{-1}$, NS, *n* = 4). Coincubation of GSH-ethyl ester (2 mM) with DETA-NO (0.5 mM) resulted in a slower rate of inhibition of the enzyme ($p > 0.05$ at 3 h and $p < 0.01$ at 5 h in Jurkat cells, *n* = 4, Figure 4.4).

In Jurkat cells inhibition of GAPDH by DETA-NO (0.5 mM, 0.11 ± 0.07 µmol min^{-1} mg $protein^{-1}$, $p < 0.001$, *n* = 3, Figure 4.5) was reversed by exposure to cold light (8 Mlx) for 1.5 h (0.27 ± 0.07 µmol min^{-1} mg $protein^{-1}$, $p < 0.01$, *n* = 3, Figure 4.5).

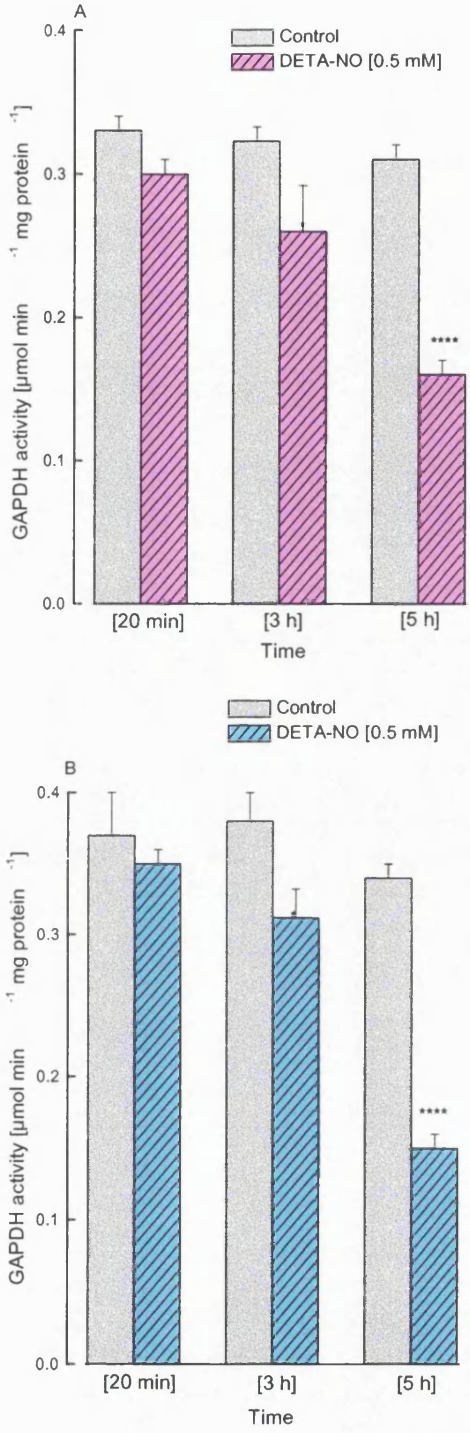

Figure 4.3 DETA-NO (0.5 mM) inhibited in GAPDH activity in (A) Jurkat and (B) L929 cells. Statistical analysis: * cells treated with DETA-NO versus the control. * = $p < 0.05$; **** = $p < 0.001$ ($n = 4$).

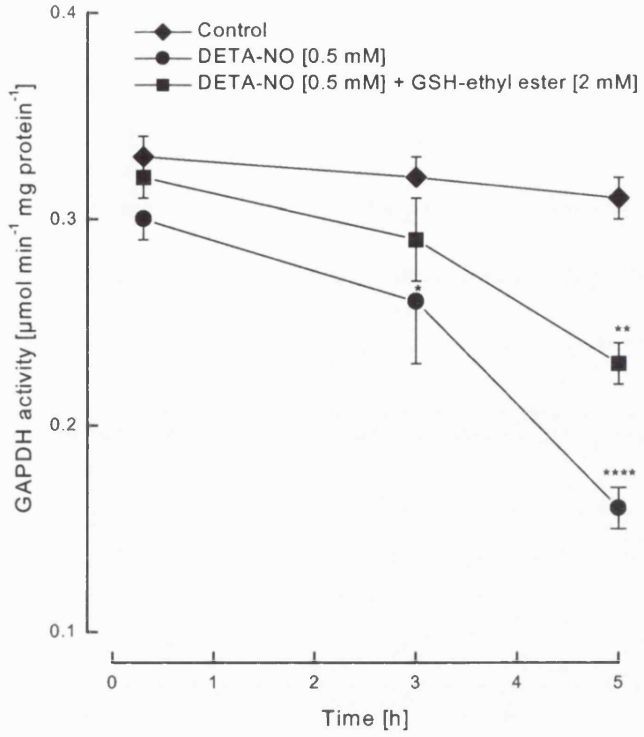

Figure 4.4 Activity of GAPDH in Jurkat cells with and without DETA-NO (0.5 mM) and GSH-ethyl ester (2 mM). Statistical analysis: * cells treated with DETA-NO versus the control. * = $p < 0.05$, **** = $p < 0.001$ ($n = 4$).

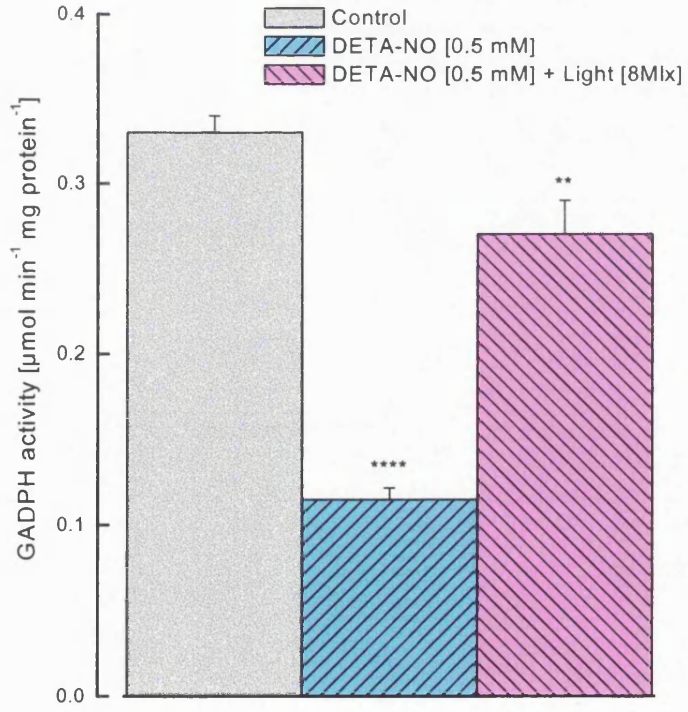

Figure 4.5 DETA-NO (0.5 mM) inhibits GAPDH activity at 5 h. A source of cold light (8 Mlx) for 1.5 h reversed the activity. Statistical analysis: * cells treated with DETA-NO versus the control. ** = $p < 0.01$; **** = $p < 0.001$ ($n = 3$).

4.4 DISCUSSION

These results show that prolonged exposure of different cells (i.e. Jurkat and L929) to exogenous NO leads to a decrease in the intracellular GSH, that precedes the inhibition of the glycolytic enzyme GAPDH and complex I activity. This is accordance with previous studies suggesting an important role of GSH in modulating NO-mediated cellular damage (Radi *et al.*, 1991; Bolanos *et al.*, 1995; Bolanos *et al.*, 1996; Almeida *et al.*, 1998; Clementi *et al.*, 1998). The addition of GSH-ethyl ester or exposure to cold light restored the ability of oxyHb to reverse the NO-dependent blockade of the oxygen consumption. In addition, increasing the number of cells prevented this irreversible inhibition, presumably by increasing the concentration of GSH and reducing the concentration of NO per cell. The photolability of the irreversible inhibition, together with its sensitivity to thiols (i.e. addition of GSH-ethyl ester), was consistent with the formation of a nitrosothiol (Bauer *et al.*, 1995). Glutathione can physiologically react with NO, producing in aerobic conditions, S-nitrosoglutathione (GSNO; Singh *et al.*, 1996), which is involved in many physiological and pathological processes. S-nitrosoglutathione can, after having achieved its role of carrier of NO (Hogg *et al.*, 1996), generate glutathione conjugates in the presence of GSH, resulting in a depletion of cellular GSH (Singh *et al.*, 1996). This decrease could result in the nitrosylation of other thiols in the cells by NO. The inhibition of complex I activity presumably by S-nitrosylation may represent a turning point in the cell transforming the physiological regulation of complex IV (Brown & Cooper, 1994) by NO to pathophysiological inhibition by NO. S-nitrosylation has been proposed to be an important signalling event in the

cell (Stamler, 1994) playing an important role in many processes ranging from signal transduction (Lander *et al.*, 1997, delaTorre *et al.*, 1998), host defence (Gobert *et al.*, 1998; Persichini *et al.*, 1998), ion channel regulation (Broillet, 1999; Broillet & Firestein, 1997) and neurotransmission (Lei *et al.*, 1992; Broillet, 1999). However, it is not clear whether S-nitrosylation is a physiological mechanism or whether it only occurs in cells exposed to oxidative stress. Following long term exposure of DETA-NO (0.5 mM), the concentration of GSH was reduced by 20-40 % by 1 h and decreased further over the time course of the experiment. This decrease in GSH concentration preceded the inhibition of glutathione reductase activity (Beltran *et a*l. 2000), complex I and GAPDH. This is in accordance with a previous study in which, following 5 h incubation of J774 cells with DETA-NO, irreversible (by oxyHb) inhibition of respiration at the level of complex I activity was observed after a decrease in GSH concentration. Furthermore, the irreversible inhibition occurred earlier when the cells were previously incubated with L-BSO for 18 h (Clementi *et al.*, 1998).

In conclusion, following long term exposure of cells to NO, GSH is a key factor in determining the pathological and physiological status of the cell.

CHAPTER 5

PARADOXICAL EFFECT OF HIGH CONCENTRATIONS OF NITRIC OXIDE: INHIBITION OF CELLULAR OXYGEN CONSUMPTION AND INCREASE OF EXTRACELLULAR OXYGEN CONSUMPTION

5 PARADOXICAL EFFECT OF HIGH CONCENTRATIONS OF NITRIC OXIDE: INHIBITION OF CELLULAR OXYGEN CONSUMPTION AND INCREASE OF EXTRACELLULAR OXYGEN CONSUMPTION

5.1 SUMMARY

This study investigates the effect of a higher concentration of NO on oxygen consumption in J774 (murine macrophage) and Jurkat (human T lymphocyte) cells.

Exogenous NO (1-8 μM) generated by DETA-NO (0.5 mM-10 mM) inhibited cellular respiration over 5 h, which was reversed by oxyhaemoglobin (oxyHb). However, the ability of oxyhaemoglobin to reverse the effect of NO lessened with time and was completely ineffective after 3-5 h in both cells.

The use of different substrates and inhibitors of the respiratory chain demonstrated that the site of inhibition by NO was downstream of the citric acid cycle and upstream of ATP synthesis. The study further demonstrated that NO caused an oxyHb-reversible inhibition of complex IV and an oxyHb-irreversible inhibition of complex I activity. Direct measurement of complex I activity in J774 cells, showed a decrease in activity after 3 h incubation with DETA-NO (5 mM).

Exposure of DETA-NO 5 mM to J774 cells decreased the concentration of the intracellular reduced glutathione. This decrease preceded the inhibition of complex I activity.

An increase in oxygen consumption by the extracellular medium was also observed at high concentrations of exogenous NO and following activation of

J774 cells with cytokines and bacterial products, indicating that endogenously released NO leads to similar cellular changes. The extracellular oxygen consumption was dependent on the presence of viable cells during the DETA-NO incubation or during the activation period, and dependent on the presence of proteins (< 30 KDa) in the extracellular medium. The oxygen consumption by the extracellular medium was photosensitive and temperature-dependent and increased by addition of GSH.

This study shows that a higher concentration of NO resulted in a progressive inhibition of respiration, similar to that observed at lower concentrations of NO (Chapter 3 and 4). This inhibition of respiration was accompanied by a paradoxical increase in oxygen consumption in the extracellular medium.

This indicates that the amounts of NO generated during pathological conditions may contribute to tissue hypoxia via two distinct mechanism, by inhibiting cell respiration and by promoting removal of oxygen from the extracellular medium.

5.2 INTRODUCTION

Despite the clear importance of iNOS expression in the cytotoxicity of activated cells, the role of NO itself on the respiratory enzymes is less clear. Short-term exposure to exogenous NO, of the rat skeletal muscle mitochondria, inhibits mitochondrial oxygen consumption in a reversible manner, by competing with oxygen at the level of complex IV (Cleeter *et al.*, 1994). Whereas long-term exposure of exogenous NO to neurones inhibits complex I, II-III and IV (Bolanos *et al.*, 1996). In this latter study there is a decrease in glutathione concentration associated with inhibition of cellular

respiration (Bolanos *et al.*, 1996). Furthermore, a similar profile of inhibition is observed in Jurkat and L929 cells (Chapter 3 and 4), suggesting that the inhibition of respiration following exposure of cells to pathological concentrations of NO (i.e. 1.5 μM) is a general mechanism that is associated with the concentration of glutathione.

In certain pathological conditions such as brain ischaemia (Tominaga *et al.*, 1994); (Brorson *et al.*, 1999) and septic shock (Singer & Brealey, 1999) the tissue concentration of NO to which cells may be exposed approaches even higher concentrations of NO (up to 10 μM). In view of this, the effect of high concentrations of NO, on cellular oxygen consumption and glutathione concentrations, was examined using J774 (murine macrophage) and Jurkat (human lymphocyte) cells.

5.3 RESULTS

The NO donor, (z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2 diolate, (DETA-N0, 0.5, 3, 5 and 10 mM), dissolved in the incubation medium, generated increasing concentrations of NO, which reached a plateau after about 20 min and remained constant throughout the time course of the experiments. In the incubation medium the concentration of NO generated at the plateau (20 min) by DETA-NO (0.5, 3, 5 and 10 mM) were 1.2 ± 0.3; 2.8 ± 0.5; 5.6 ± 0.6; and 7.8 ± 0.9 μM respectively, as detected by the NO electrode (Figure 5.1). This release was constant for the 5 hours of the experiment.

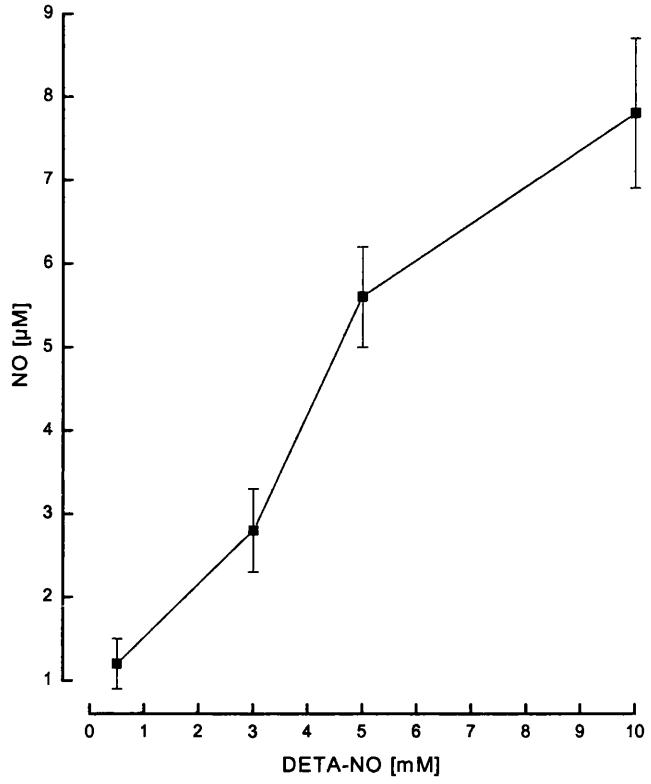

Figure 5.1 NO (µM) released from DETA-NO (mM) dissolved in the incubation medium at the plateau (20 min, *n* = 4).

5.3.1 EFFECTS OF EXOGENOUS NITRIC OXIDE ON OXYGEN CONSUMPTION

The oxygen consumption in J774 and Jurkat cells (at 0 h: 27.6 ± 2.1 and 34.1 ± 2.2 nmol min^{-1} 10^7 $cells^{-1}$ and at 5 h: 23.7 ± 4 and 33.6 ± 1.2 nmol min^{-1} 10^7 $cells^{-1}$, respectively) did not alter significantly over 5 h in either of the cells.

The addition of DETA-NO (0.5-10 mM) to the cells inhibited oxygen consumption by 70-80 % within 20 min ($p < 0.001$, *n* = 6 for each, Figure 5.2 A, B).

Paradoxically the initial inhibitory effect of NO on oxygen consumption decreased over time, becoming a stimulatory effect at higher concentrations. This increase in oxygen consumption was dependent on the concentration of DETA-NO (n = 6 for each, Figure 5.2 A, B).

The effect of DETA-NO was not the consequence of a non-specific, toxic effect since the cells were viable at the end of the experiment (5 h) as assessed by trypan blue exclusion (n = 6 for each, Table 5.1). Decomposed DETA-NO (0.5 -10 mM) did not produce any significant changes in the oxygen consumption of both cells (0.4 ± 0.02 % and 0.3 ± 0.02 % in J774 and Jurkat cells respectively, NS, n = 4 for each).

Table 5.1 Viability assessed by trypan blue exclusion of (A) J774 and (B) Jurkat cells during 5 h of incubation with and without DETA-NO (0.5-10 mM, *n* = 6 for each).

A

Time	Control	DETA-NO [0.5 mM]	DETA-NO [3 mM]	DETA-NO [5 mM]	DETA-NO 10 mM]
0 h	95 ± 4 %	95 ± 4 %	94 ± 3 %	96 ± 5 %	94 ± 2 %
1 h	93 ± 4 %	94 ± 5 %	91 ± 5 %	92 ± 2 %	92 ± 4 %
2 h	88 ± 4%	89 ± 4 %	88 ± 2 %	87 ± 4 %	88 ± 3 %
3 h	86 ± 3 %	87 ± 2 %	86 ± 4 %	85 ± 2%	83 ± 5 %
4 h	85 ± 2 %	83 ± 3 %	83 ± 5 %	83 ± 3 %	81 ± 5 %
5 h	82 ± 4 %	81 ± 5 %	82 ± 3 %	80 ± 5 %	79 ± 5 %

(100 % = all the cells were viable)

B

Time	Control	DETA-NO [0.5 mM]	DETA-NO [3 mM]	DETA-NO [5 mM]	DETA-NO 10 mM]
0 h	95 ± 3 %	96 ± 2 %	93 ± 4 %	94 ± 2 %	93 ± 4 %
1 h	93 ± 5 %	92 ± 4 %	92 ± 3 %	91 ± 3 %	91 ± 3 %
2 h	91 ± 2%	89 ± 3 %	89 ± 2 %	88 ± 5 %	87 ± 4%
3 h	87 ± 4 %	87 ± 5 %	86 ± 3 %	86 ± 2%	82± 5 %
4 h	84 ± 3 %	86 ± 4 %	84 ± 5 %	84 ± 2 %	80 ± 3 %
5 h	82 ± 5 %	85 ± 3 %	81 ± 3 %	81 ± 4 %	78 ± 2 %

(100 % = all the cells were viable)

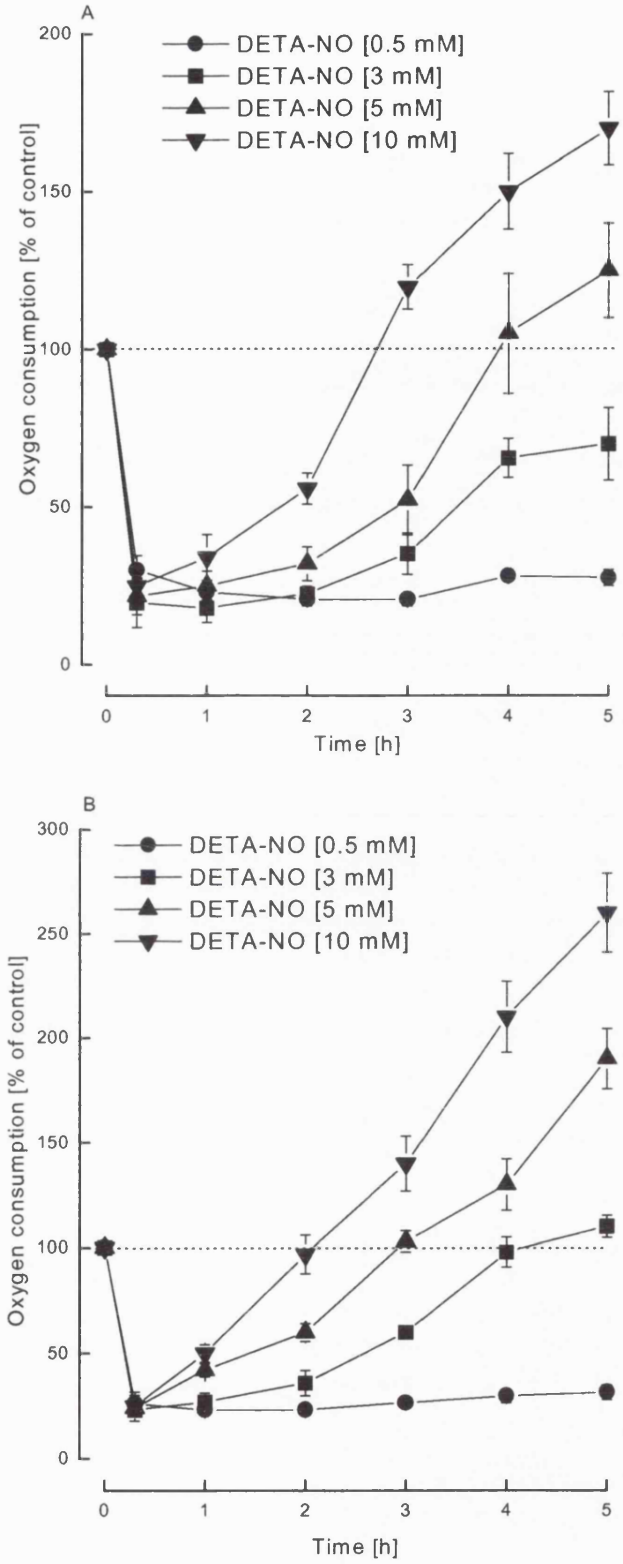

Figure 5.2 Inhibition of oxygen consumption by DETA-NO (0.5-10 mM) and its reversal over time in (A) J774 and (B) Jurkat cells (n = 6 for each).

5.3.1.1 Oxygen consumption of cells and their extracellular medium

To investigate whether the paradoxical increase in oxygen consumption was due to an action of NO on the cells or due to some other biochemical/chemical reaction, the cells were separated from the incubation medium and oxygen consumption of both cells and extracellular medium analysed separately (Figure 5.3).

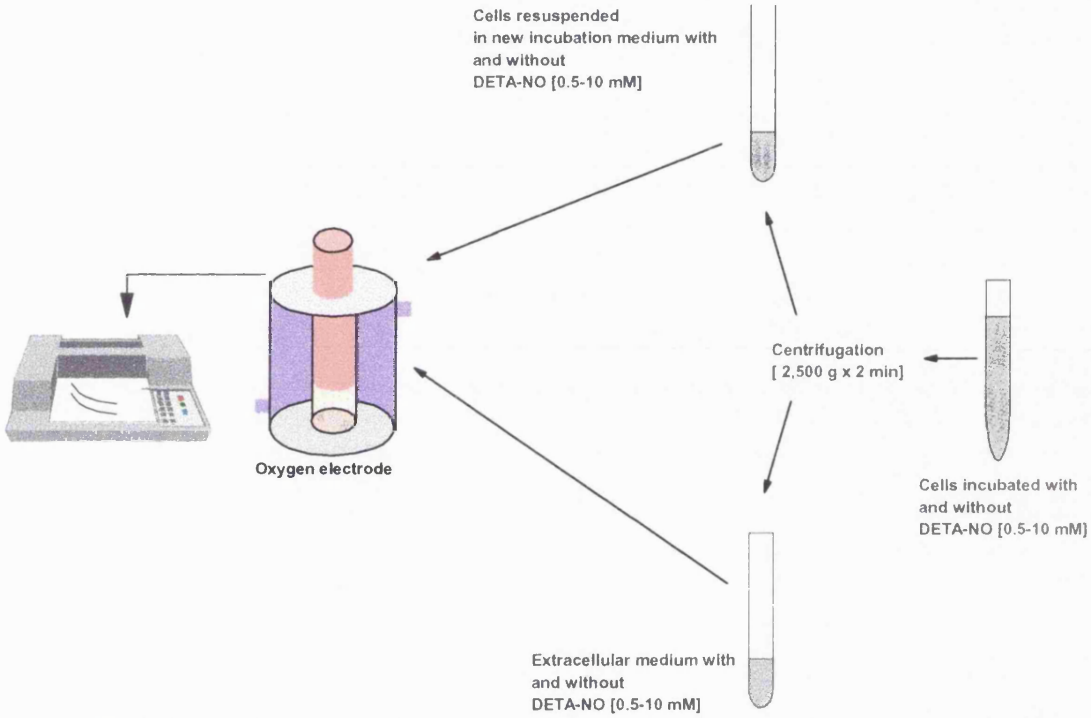

Figure 5.3 Schematic representation of the oxygen consumption measurement of the extracellular medium and the cells incubated with and without DETA-NO (0.5 -10 mM). At specific time points the cells were centrifuged. The oxygen consumption of the extracellular medium and the cells were analysed immediately with or without DETA-NO (at the same concentration present during the incubation).

5.3.1.1.1 Oxygen consumption of cells

As mentioned above, the cells were separated from the incubation medium by centrifugation at the time-points indicated, resuspended in incubation

medium with DETA-NO (at the same concentration present during the incubation) and oxygen consumption was analysed immediately (Figure 5.4).

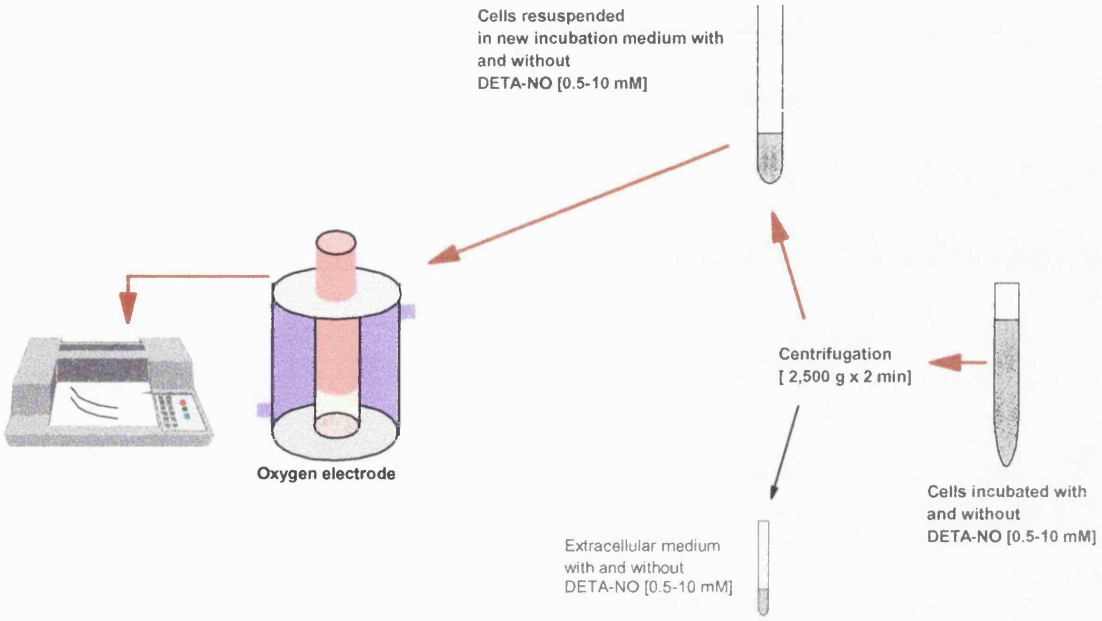

Figure 5.4 Schematic representation of the oxygen consumption measurement of the cells incubated with and without DETA-NO (0.5-10 mM). At specific time points the cells were centrifuged. The oxygen consumption of the cells resuspended in new medium with and without DETA-NO (0.5-10 mM) was immediately analysed.

The oxygen consumption of J774 and Jurkat cells was inhibited by all concentrations of DETA-NO (0.5, 5 and 10 mM) and this inhibition persisted throughout the duration of the experiments (Figure 5.6 A and Figure 5.7 A; J774 and Jurkat cells respectively n = 6 for each). The inhibition was initially fully reversible by addition of oxyHb (8-24 μM, at 20 min, 98 ± 1.3, 96 ± 2.1 and 96 ± 2.3 with 0.5, 5 and 10 mM DETA-NO, respectively in J774 cells and 97.6 ± 2.9, 96.7± 3.1 and 99 ± 2.3 with 0.5, 5 and 10 mM DETA-NO, respectively, in Jurkat cells, n = 3 for each). However after 1 hour of incubation the ability of oxyHb to reverse the inhibition decreased

progressively until it became ineffective. The inability of oxyHb to reverse this inhibition appeared earlier with higher concentrations of DETA-NO (5 ± 0.5; 2.5 ± 0.2 and 1.5 ± 0.2 h with 0.5, 5 and 10 mM DETA-NO, respectively, in J774 cells; 5 ± 0.2; 2 ± 0.5; 1 ± 0.4 h with 0.5, 5 and 10 mM DETA-NO, respectively, in Jurkat cells, *n* = 3 for each).

5.3.1.1.2 Oxygen consumption of extracellular medium

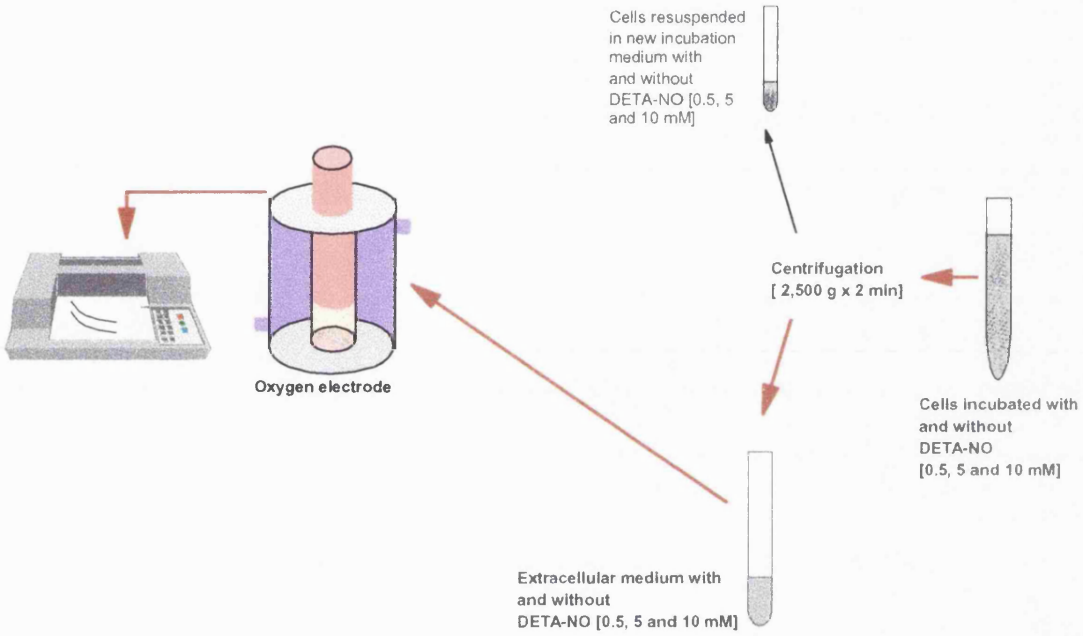

Figure 5.5 Schematic representation of the oxygen consumption of the extracellular medium of cells exposed to DETA-NO (0.5, 5 and 10 mM). At specific time points the cells were centrifuged and the oxygen consumption of the extracellular medium was immediately analysed.

Oxygen consumption of the extracellular medium, measured immediately after separation from the cells (Figure 5.5), progressively increased. This increase was dependent on the concentration of DETA-NO in which cells were incubated and also on the time of exposure (Figure 5.6 B and Figure 5.7 B, *n* = 6 for each). Oxygen consumption of the extracellular medium of the cells exposed to DETA-NO (5 mM) was increased at 3 and 2 h in J774

and Jurkat cells, respectively, (*n* = 6 for each, Figure 5.6 B and Figure 5,7 B). Oxygen consumption of the extracellular medium of the cells exposed to DETA-NO (10 mM) was increased at 2 and 1 h in J774 and Jurkat cells, respectively, (*n* = 6 for each, Figure 5.6 B and Figure 5,7 B).

Oxyhaemoglobin (24 μM), at 3-4h, reduced the oxygen consumption of the extracellular medium of the cells incubated with DETA-NO (5 mM, by 94 ± 23 and 91 ± 29 %, *n* = 4 for each; 10 mM, by 92 ± 37 %, 88 ± 31%, in J774 and Jurkat cells, respectively, *n* = 4 for each). Whereas at 3-4 h, oxyHb (24 μM) had no effect on the oxygen consumption of the extracellular medium of J774 and Jurkat cells that were not exposed to DETA-NO.

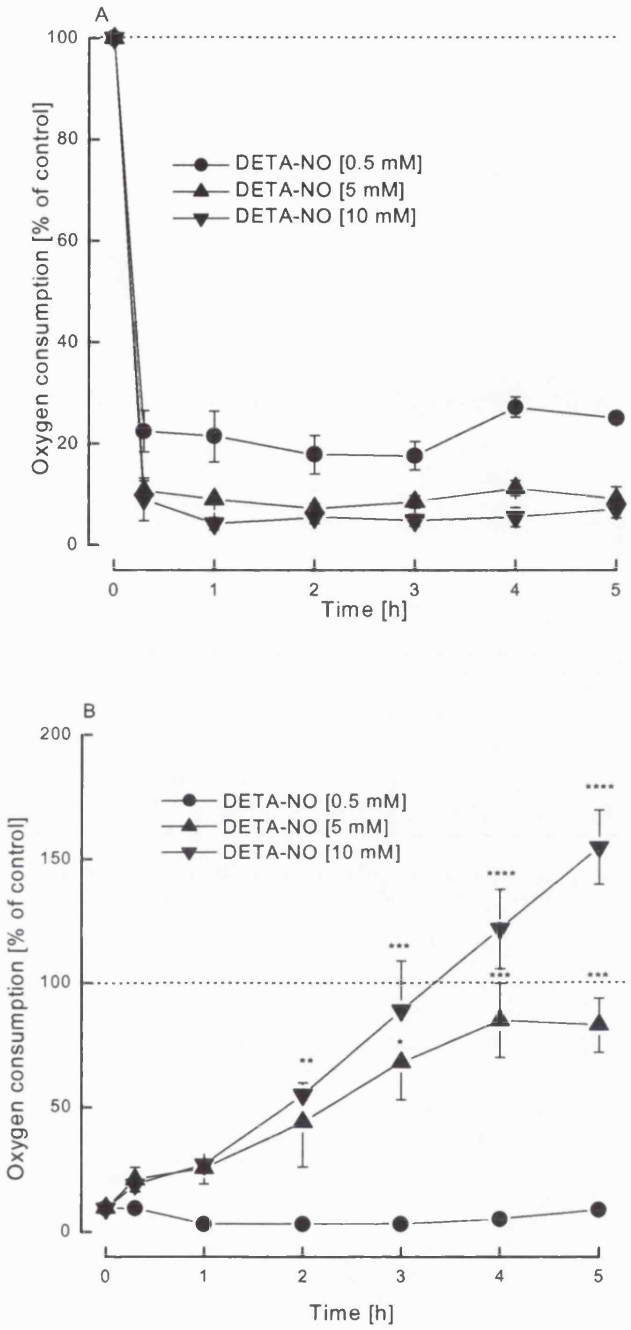

Figure 5.6 DETA-NO (0.5, 5 and 10 mM) inhibited cellular oxygen consumption in (A) J774 cells whereas DETA-NO (5, 10 mM) increased oxygen consumption of (B) the extracellular medium. Statistical analysis: within the same group versus time 20 min. * = $p < 0.05$, ** = $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$ ($n = 6$).

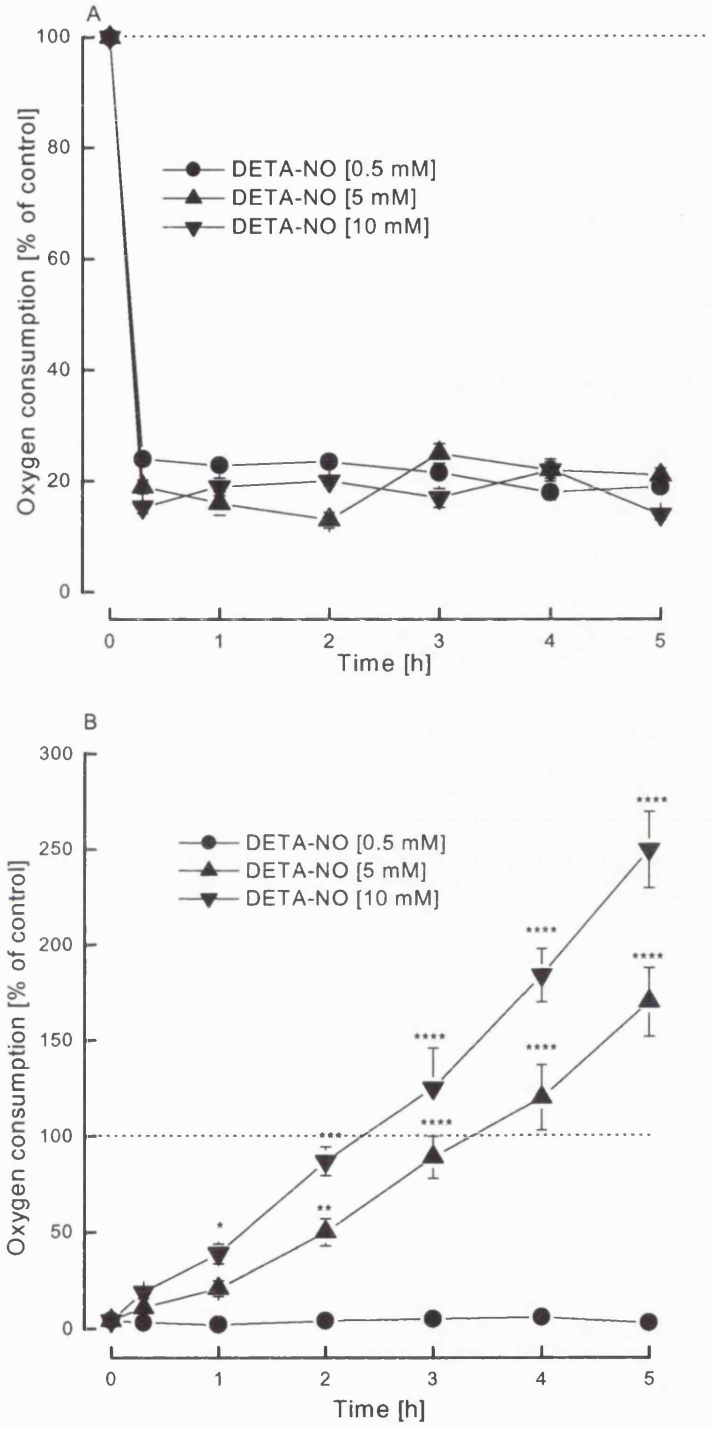

Figure 5.7 DETA-NO (0.5, 5 and 10 mM) inhibited cellular oxygen consumption in (A) Jurkat cells, whereas DETA-NO (5, 10 mM) increased oxygen consumption of (B) the extracellular medium. Statistical analysis: within the same group versus time 20 min. * = $p < 0.05$, **= $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$, ($n = 6$).

5.3.2 STUDY OF THE CELLS

5.3.2.1 Cellular oxygen consumption

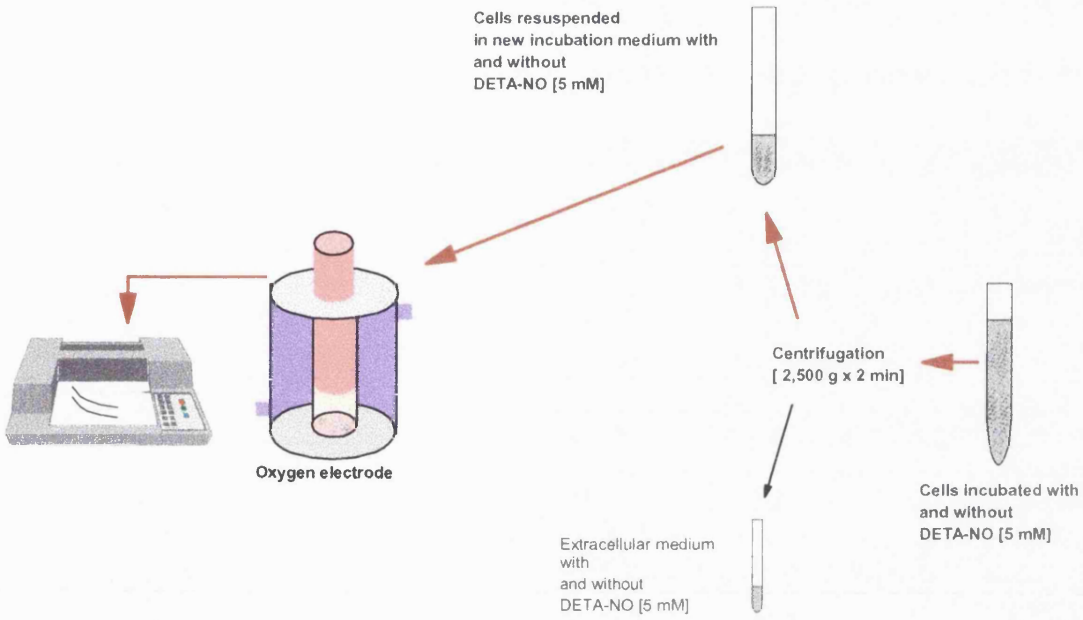

Figure 5.8 Schematic representation of the oxygen consumption of the J774 cells incubated with and without DETA-NO (5 mM). At specific time points the cells were centrifuged and the oxygen consumption of the cells resuspended in new medium without and with DETA-NO (5 mM) and immediately analysed.

As mentioned above (Section 5.3.1.1) J774 cells were incubated in the presence of DETA-NO (5 mM) at the time-points indicated and separated from the incubation medium by centrifugation and resuspended in fresh medium with DETA-NO (5 mM).

5.3.2.1.1 Targets of the inhibition of cellular respiration

Oxygen consumption was measured in the presence of inhibitors and substrates of the respiratory chain to investigate whether a high concentration of NO (up to 6 μM) generated by DETA-NO (5 mM) inhibits

cellular respiration in the same manner as that observed with a lower concentration of NO (1.2 ± 0.3 μM generated by DETA-NO 0.5 mM; Chapter 3).

Addition of 3-nitropropionic acid (0.6 mM) and β-hydroxybutyrate (6 mM) to J774 cells in the absence of DETA-NO did not significantly inhibit the cellular oxygen consumption (at 5 h from 23.7 ± 4.0 to 25.6 ± 3.8 nmol min $^{-1}$ 10^7 cells^{-1}, *n* = 3-6).

Addition of DETA-NO (5 mM) to J774 cells inhibited oxygen consumption (at 5 h from 23.7 ± 4.0 to 2.3 ± 0.9 nmol min $^{-1}$ 10^7 cells^{-1}, *n* = 3-6, $p < 0.001$). Addition of 3-nitropropionic acid (0.6 mM) and β-hydroxybutyrate (6 mM) did not significantly alter the cellular oxygen consumption of J774 cells exposed to DETA-NO (5 mM, at 5 h from 2.3 ± 0.9 to 4.8 ± 2.1 nmol min $^{-1}$ 10^7 cells^{-1}, *n* = 3-6). Furthermore addition of oxyHb (24 μM), to scavenge NO, did not reverse the inhibition of cellular oxygen consumption at 5 h (at 5 h from 4.8 ± 2.1 to 4.6 ± 1.7 nmol min $^{-1}$ 10^7 cells^{-1}, Table 5.2, *n* = 3-6).

Addition of the protonophore, carbonyl cyanide p-(trifluoro-methoxy) phenylhydrazone (FCCP, 1 μM, an uncoupler of the flow of electrons from the synthesis of ATP) increased oxygen consumption in J774 cells (from 23.7 ± 4.0 to 42.8 ± 4.2 nmol min^{-1} 10^7 cells^{-1}, *n* = 3-6 $p < 0.005$).

Addition of DETA-NO (5 mM) to J774 cells inhibited oxygen consumption. Addition of FCCP (1 μM) had no effect on the oxygen consumption (from 2.3 ± 0.9 to 2.6 ± 0.09 nmol min $^{-1}$ 10^7 cells^{-1}, *n* = 3-6) in J774 cells exposed to DETA-NO (5 mM) for 5 h.

Addition of myxothiazol (0.5 μM), *N,N,N'N'*-tetramethyl-*p*-phenylene diamine (TMPD, 80 μM) and ascorbic acid (4 mM) to J774 cells, did not significantly alter cellular oxygen consumption (at 5 h from 23.7 ± 4.0 to 21.4 ± 2.1 nmol min^{-1} 10^7 $cells^{-1}$, *n* = 3-6).

Addition of DETA-NO (5 mM) to J774 cells inhibited oxygen consumption (at 5 h from 23.7 ± 4.0 to 2.3 ± 0.9 nmol min^{-1} 10^7 $cells^{-1}$, *n* = 3, $p < 0.001$). Addition of myxothiazol (0.5 μM) and TMPD (80 μM) / ascorbic acid (4 mM, *n* = 3-6) did not significantly alter the oxygen consumption of J774 cells incubated in the presence of DETA-NO (5 mM) for 5 h (at 5 h from 2.3 ± 0.9 to 5.8 ± 3.2 nmol min^{-1} 10^7 $cells^{-1}$, *n* = 3-6). Whereas addition of oxyHb (24 μM), to scavenge NO, reversed the inhibition of respiration (from 5.8 ± 3.2 to 22.3 ± 4.9 nmol min^{-1} 10^7 $cells^{-1}$, *n* = 3-6, $p < 0.005$).

Addition of rotenone (2 μM) and succinate (6 mM) to J774 cells did not significantly alter the oxygen consumption after 5 h incubation (at 5 h, from 23.7 ± 4.0 to 22.4 ± 3.2 nmol min^{-1} 10^7 $cells^{-1}$, *n* = 3-6).

Addition of DETA-NO (5 mM) to J774 cells inhibited oxygen consumption (at 5 h, from 23.7 ± 4.0 to 2.3 ± 0.9 nmol min^{-1} 10^7 $cells^{-1}$, *n* = 3-6, $p < 0.001$). Addition of rotenone (2 μM) and succinate (6 mM) did not significantly alter the oxygen consumption of J774 cells exposed to DETA-NO (5 mM) over 5 h (at 5 h, from 2.3 ± 0.9 to 1.3 ± 1.1 nmol min^{-1} 10^7 $cells^{-1}$, *n* = 3-6). Whereas further addition of oxyHb (24 μM) reversed the inhibition of oxygen consumption (at 5 h from 1.3 ± 1.1 to 21.8 ± 4.1 nmol min^{-1} 10^7 $cells^{-1}$ Table 5.2, *n* = 3-6, $p < 0.005$).

Table 5.2 Effects of DETA-NO (5 mM) on cellular oxygen consumption using different inhibitors and substrates. Cellular oxygen consumption was measured before and after the addition of inhibitor, substrates and oxyHb. The values are expressed as a percentage of the oxygen consumption observed in control cells that were treated with the same inhibitors and substrates but without DETA-NO (*n* = 3).

Treatment	Pathways	20min	20min + oxyHb [24 µM]	5 h	5 h + oxyHb [24 µM]
DETA-NO [5 mM]		8.75 ± 3	86.6 ± 4.4	9 ± 1.8	6 ± 5.7
3-Nitropropionic acid [0.6 mM] + β Hydroxybutyrate [6 mM]	Complexes I-III-IV	28.7 ± 3.2	99.3 ± 2.3	19.3 ± 1.2	18 ± 1.7
Myxothiazol [0.5 µM]+ Ascorbate [4 mM] / TMPD [80 µM]	Complex IV	21.5 ± 3.5	106.5 ± 9.5	30 ± 9.1	105.3 ± 15
Rotenone [2 µM] + Succinate [6 mM]	Complexes II-III-IV	23.7 ± 5	110 ± 4.4	4.2 ± 3.0	95.7± 8.9

5.3.2.2 Effect of nitric oxide on complex I activity

Complex I activity, measured as the rate of NADH oxidation, was analysed in J774 cell homogenates incubated with and without DETA-NO (5 mM) for 5 h. The activity of complex I of J774 cells was constant over the 5 h time

course of the experiment (9.9 ± 0.7, 9.9 ± 0.5 and 9.8 ± 1.2 nmol min^{-1} mg $protein^{-1}$, at 20 min, 3 h and 5 h, respectively, *n* = 4, Figure 5.9). The activity of complex I was inhibited by rotenone (2 μM, 4.6 ± 0.5, 4.3 ± 0.3 and 4.9 ± 0.6 nmol min^{-1} mg $protein^{-1}$, at 20 min, 3 h and 5 h, respectively, *n* = 4, Figure 5.9).

Incubation with DETA-NO (5 mM) for 20 min did not inhibit complex I activity (9.6 ± 0.5 nmol min^{-1} mg $protein^{-1}$, *n* = 4, Figure 5.9). Whereas incubation with DETA-NO (5 mM) for 3 and 5 h inhibited the activity of complex I (4.4 ± 0.5 and 4.7 ± 0.6 nmol min^{-1} mg $protein^{-1}$ at 3 h and 5 h respectively, Figure 5.9, *n* = 4).

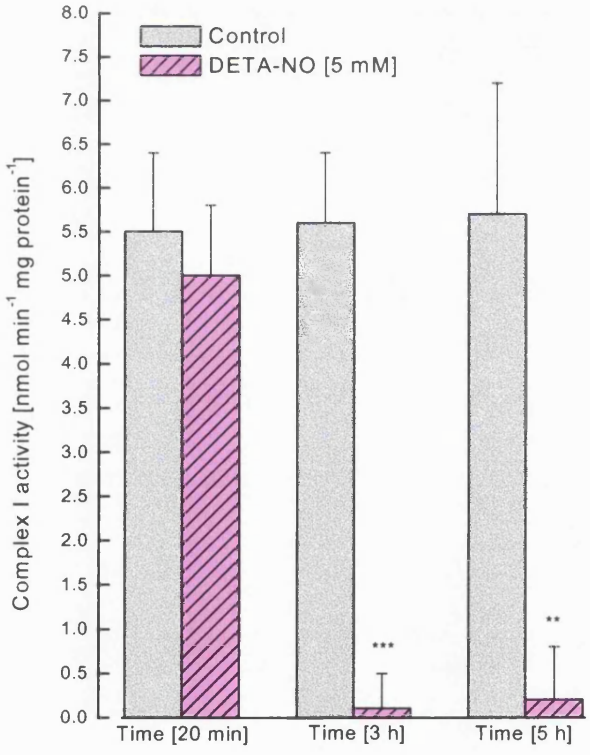

Figure 5.9 Effect of DETA-NO (5 mM) on complex I activity in J774 cells. Statistical analysis: * cells treated versus the control cells at the same time point. ** $p < 0.01$; *** $p < 0.005$, (*n* = 4).

5.3.2.3 Measurement of glutathione

Glutathione (GSH) concentration in J774 cells over a 5 h time course was not significantly decreased (32.2 ± 1.1 and 27 ± 1.8 nmol mg protein $^{-1}$ at 0 h and 5 h, respectively, Figure 5.10, n = 4). Incubation of J774 cells with DETA-NO (5 mM) resulted in a significant decrease of intracellular GSH concentration within 10 minutes ($p < 0.001$, n = 4, Figure 5.10) without any further significant decrease thereafter ($p < 0.001$, n = 4, Figure 5.10). Incubation for 5 h of J774 cells with decomposed DETA-NO (5 mM) did not have any significant effect on the intracellular GSH concentration (31.4 ± 3.1 and 28 ± 1.2 nmol mg protein $^{-1}$ at 0 h and 5 h, respectively, NS, n = 3).

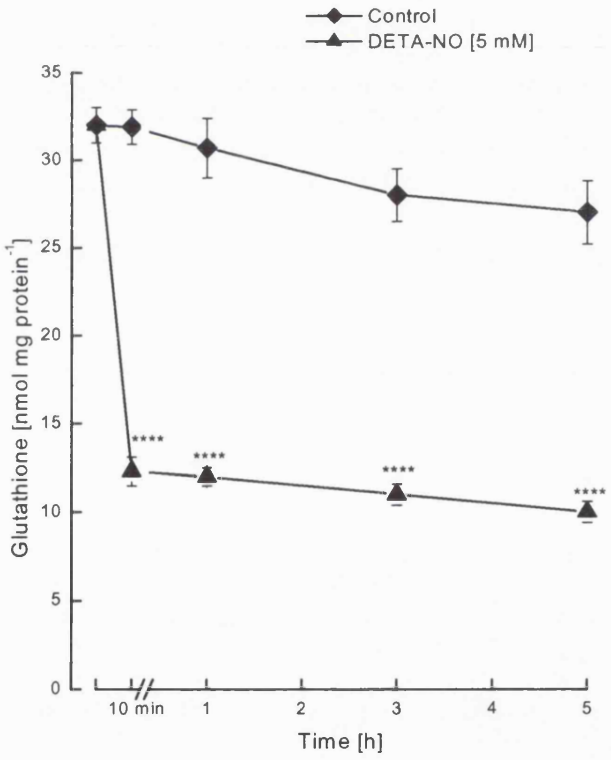

Figure 5.10 Intracellular GSH concentration in J774 cells incubated with and without DETA-NO (0.5 and 5 mM). Statistical analysis: * cells treated versus the control cells at the same time point. *= $p < 0.05$; ** = $p < 0.01$; *** $p < 0.005$; **** = $p < 0.001$ (n = 4).

5.3.2.3.1 Role of GSH on cellular oxygen consumption

To investigate the role of GSH on the inhibition of complex I by NO, cells were either treated with the membrane permeable GSH analogue, GSH-ethyl ester (2 mM) or exposed to cold light (8 Mlx). Following incubation of the cells with DETA-NO (5 mM) for 4 h, cells were centrifuged (2.500 × g 2 min) and resuspended in new medium with DETA-NO (5 mM) and incubated in the presence of either GSH-ethyl ester (2 mM) or exposed to light (8 Mlx) for 1.5 hours. GSH-ethyl ester (2 mM) restored oxygen consumption (from 8.8 ± 3.4 to 78 ± 4 % of that observed in control cells). Exposure to cold light (8 Mlx) for a further 1.5 h also reversed the inhibition of oxygen consumption (from 8.8 ± 3.4 to 74 ± 8 % of that observed in control cells, n = 3).

5.3.3 STUDY OF THE EXTRACELLULAR MEDIUM

5.3.3.1 Effect of exogenous nitric oxide on extracellular oxygen consumption

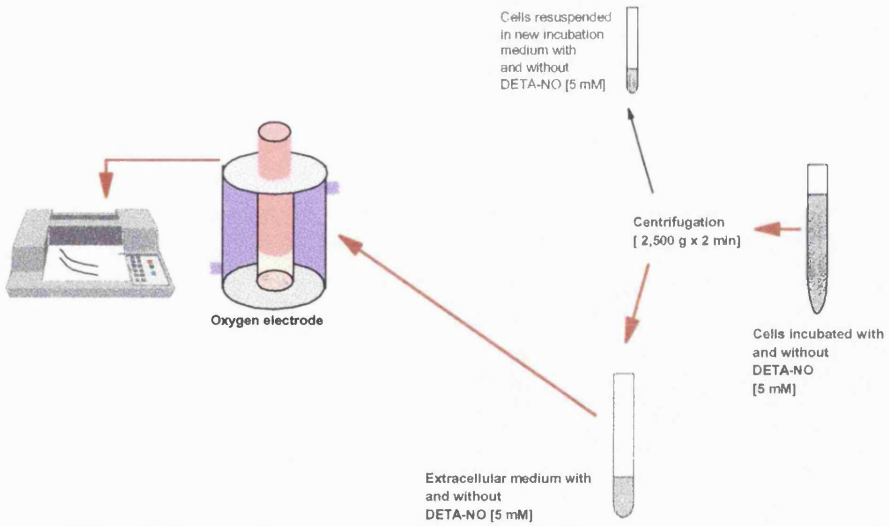

Figure 5.11 Schematic representation of the oxygen consumption of the cells incubated with and without DETA-NO (0.5-10 mM) and of the extracellular medium. At specific time points the cells were centrifuged. The oxygen consumption of the extracellular medium was immediately analysed.

Experiments have been performed in order to investigate the biochemical characteristics, of the extracellular oxygen consumption of J774 cells incubated with DETA-NO (5 mM) over 4 h. J774 cells incubated for 4 h with and without DETA-NO were centrifuged and the oxygen consumption of the extracellular medium was examined (Figure 5.11).

The oxygen consumption of extracellular medium from cells exposed to DETA-NO (5 mM) increased over the 4 h incubation (1.2 ± 0.9 and 24 ± 6.4 nmol min^{-1} at 20 min and 4 h, respectively, Figure 5.12).

The oxygen consumption of extracellular medium from cells exposed to DETA-NO for 4 h (24 ± 6.4 nmol min^{-1}) was significantly higher than that from cells not exposed to DETA-NO (1.4 ± 0.9 nmol min^{-1}, Figure 5.12).

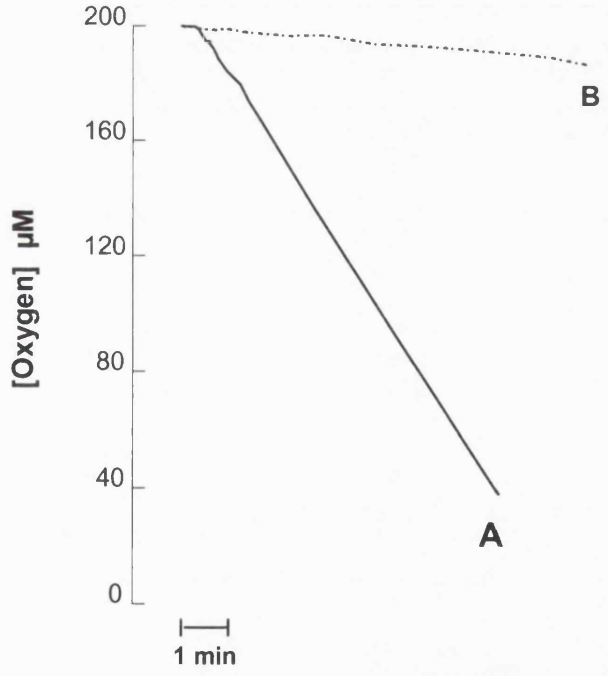

Figure 5.12 Example of the extracellular oxygen consumption after 4h incubation with (A) and without (B) DETA-NO (5 mM).

5.3.3.1.1 Requirement of viable cells during DETA-NO incubation for extracellular oxygen consumption

Oxygen consumption of the extracellular medium after incubation of cells without DETA-NO was constant over 4 h of the experiment (1.4 ± 0.6 and 1.4 ± 0.9 nmol min^{-1} ml^{-1} at 20 min and 4 h, respectively).

Oxygen consumption of the incubation medium only (without cells) with and without DETA-NO (5 mM) was constant over the 4 h of experiment (1.1 ± 1.3 1.1 ± 0.8 nmol min^{-1} ml^{-1} with DETA-NO at 20 min and 3 h, respectively; 1.2 ± 0.8 and 1.5 ± 0.8 nmol min^{-1} ml^{-1} without DETA-NO at 20 min and 4 h, respectively).

Oxygen consumption of the extracellular medium after incubation of cells with decomposed DETA-NO (5 mM) was constant over the 4 h of experiment (1.1 ± 0.3 and 0.9 ± 1.3 nmol min^{-1} ml^{-1} at 20 min and 4 h, respectively).

Oxygen consumption of the extracellular medium after incubation of dead cells (freeze-thawing) with DETA-NO (5 mM) was constant over the 4 h of experiment (1 ± 0.5 and 1.1 ± 0.6 nmol min^{-1} ml^{-1} at 20 min and 4 h, respectively).

These results suggested that the increase in oxygen consumption of the extracellular medium required the presence of viable cells and exposure of the cells to NO.

5.3.3.1.2 Presence of proteins in the extracellular medium

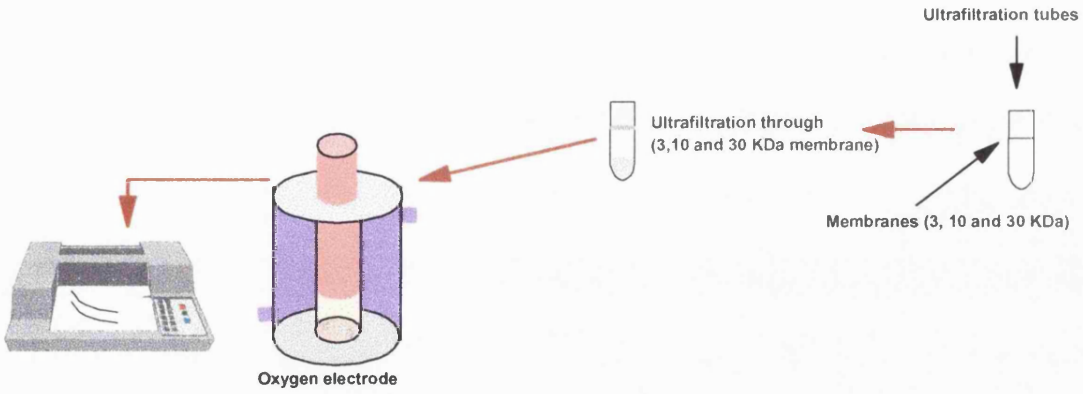

Figure 5.13 Schematic representation of ultrafiltration of the extracellular medium through 3-10 and 30 KDa membranes and immediate measurement of oxygen consumption.

The extracellular medium from J774 cells incubated with and without DETA-NO (5 mM) was ultrafiltrated through membrane device filters (3-30 KDa) in order to verify the presence of molecules of a certain size in the extracellular medium (Figure 5.13).

The extracellular medium of cells incubated for 4 h with DETA-NO (5 mM) ultrafiltrated through membranes, trapping at different molecular weight (3, 10 and 30 kDa) decreased oxygen consumption (18 ± 0.4, 16 ± 1.1 and 13 ± 2.7 nmol min^{-1} ml^{-1}, respectively, n = 3). Whereas the extracellular medium of cells incubated for 4 h with DETA-NO (5 mM) ultrafiltrated through filter device membranes (trapping molecules with a molecular weight higher than 30 kDa) abolished the oxygen consumption of the extracellular medium (1.3 ± 0.4 nmol min^{-1} ml^{-1} n = 3).

These results suggested the presence of molecules (up to 30 KDa) in the extracellular medium after incubation of the cells with NO.

To study the nature of these molecules the protein concentrations were measured in the extracellular medium at 20 min and 4 h, after incubation of J774 cells, with and without DETA-NO (5 mM).

The concentration of protein in the extracellular medium after incubation of cells with DETA-NO (5 mM) increased over the 4 h of incubation (0.40 ± 0.03 and 1.20 ± 0.31 μg protein μl^{-1} at 20 min and 4 h, respectively, *n* = 3). There was no increase in the protein concentration, in the extracellular medium of cells without DETA-NO treatment (0.31 ± 0.03 and 0.48 ± 0.09 μg μl^{-1} at 0h and 4 h, respectively, *n* = 3).

5.3.3.1.3 Biochemical properties

The following experiments were performed to determine whether the oxygen consumption of the extracellular medium of J774 cells incubated with DETA-NO (5 mM) for 4 hours was temperature (experiments performed in the dark) and photosensitive (Figure 5.14)

Oxygen consumption of the extracellular medium (24 ± 0.2 nmol min^{-1} ml^{-1}) of cells incubated with DETA-NO (5 mM) for 4 hours was reduced after 1 h incubation at room temperature (21°C; 21.2 ± 0.1 nmol min^{-1} *n* = 3, Figure 5.14 and 5.15) and at 37°C (19.2 ± 0.3 nmol min^{-1} ml^{-1}, *n* = 3, Figure 5.14 and 5.15) and was abolished after incubation at 98°C for 30 min (1.1 ± 0.4 nmol min^{-1}, *n* = 3, Figure 5.14 and 5.15) or following illumination (8 Mlx) for 1 h (1.4 ± 0.9 nmol min^{-1}, *n* = 3, Figure 5.14 and 5.15).

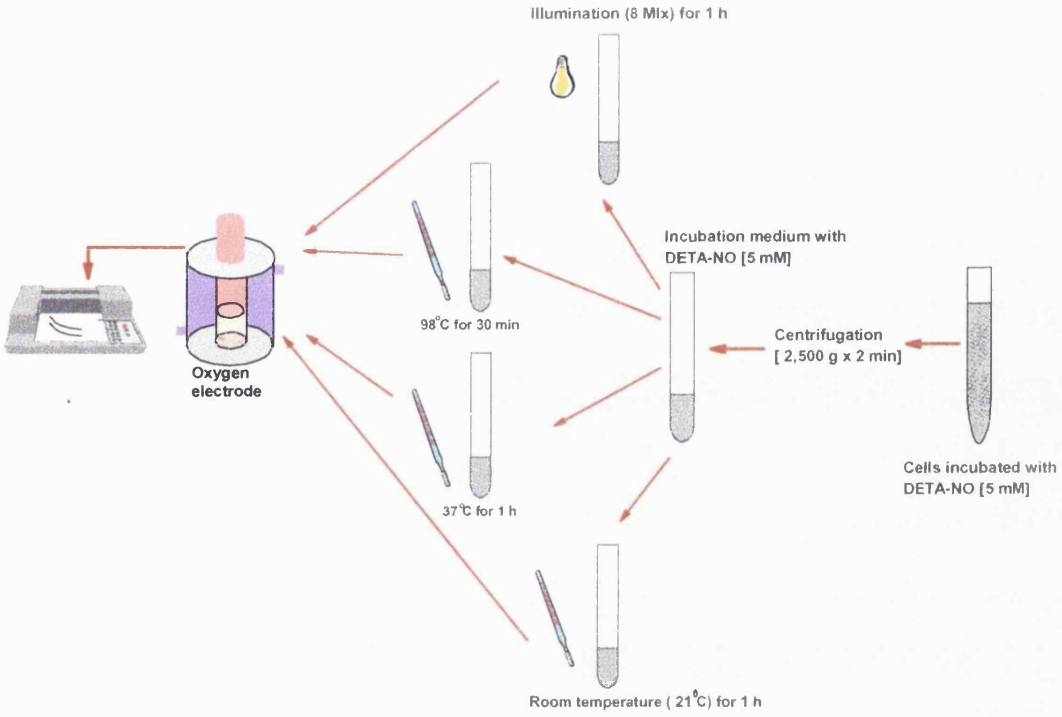

Figure 5.14 Schematic representation of the oxygen consumption measurement of the extracellular medium following temperature and light incubation.

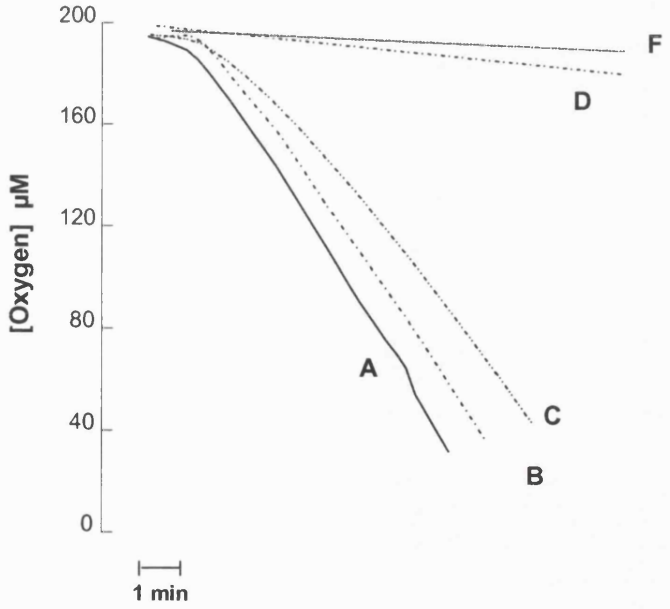

Figure 5.15 Example of extracellular oxygen consumption after incubation with DETA-NO (5 mM; A) and after storage at room temperature (B), at 37°C (C), at 98°C (D) and following illumination at room temperature (F).

5.3.3.1.4 Effect of GSH on extracellular oxygen consumption

In the extracellular medium of J774 cells incubated DETA-NO (5 mM), an increase in GSH (21 ± 7 µM and 108 ± 26 µM at 0 h and 4 h respectively, *n* = 4) was observed after 4 h of incubation.

Furthermore the effect of the addition of GSH on the extracellular medium was analysed. Oxygen consumption (24 ± 6.4 nmol min^{-1} ml^{-1}) of the extracellular medium of cells treated with DETA-NO (5 mM) for 4 h was increased by addition of GSH in a concentration dependent manner (to 45 ± 4.5 nmol min^{-1} ml^{-1}, at 4 h after addition of GSH 0.5 mM, *n* = 4, Figure 5.16 and Table 5.3).

Addition of GSH (0.5 mM) to the extracellular medium of J774 cells incubated with DETA-NO (5 mM) for 4 h increased the concentration of NO (by 1.4 ± 0.2 µM, *n* = 2).

Addition of GSH (0.5 mM) to the extracellular medium of cells incubated for 4 h without DETA-NO did not significantly alter the extracellular oxygen consumption (from 1.4 ± 0.9 nmol min^{-1} ml^{-1} to 1.6 ± 0.9 nmol min^{-1} ml^{-1}, *n* = 4, Figure 5.16).

Addition of GSH (0.5 mM) to the incubation medium only (without cells) with DETA-NO (5 mM) did not significantly alter the oxygen consumption (from 1.1 ± 0.8 nmol min^{-1} ml^{-1} to 1.3 ± 1.1 nmol min^{-1} ml^{-1}, *n* = 4).

Table 5.3 GSH increased the extracellular oxygen consumption of cells exposed to DETA-NO (5 mM) for 4 h in a concentration dependent manner (n = 4).

GSH [mM]	Oxygen consumption [nmol min^{-1} ml^{-1}]
0	24 ± 0.2
0.5	45 ± 5
1	54 ± 5
3	74 ± 6
10	110 ± 16

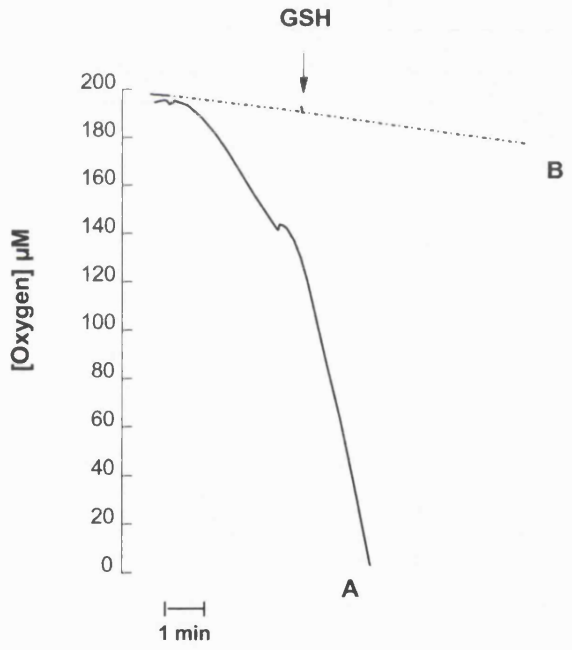

Figure 5.16 Example of extracellular oxygen consumption after 4 h incubation with (A) and without (B) DETA-NO (5 mM) followig addition of GSH.

5.3.3.2 Effects of endogenous nitric oxide on extracellular oxygen consumption

5.3.3.2.1 Activation of iNOS

In order to investigate whether the effects, observed with high concentrations of NO (up to 8 μM), could occur in pathophysiological conditions, when NO is generated by NOS, preliminary experiments were performed with activated J774 cells. The cells were activated for 18 h with IFN γ (50 units ml^{-1}) and endotoxin (LPS, 10 μg ml^{-1}) in the presence or absence of the NO synthase substrate, L-arginine (0.5 mM).

The oxygen consumption of the extracellular medium of non-activated J774 cells and of activated J774 cells was 0.7 nmol min^{-1} ml^{-1} and 9.1 nmol min^{-1} ml^{-1}, respectively (n = 2).

The oxygen consumption of extracellular medium obtained from activated J774 cells in the absence of the NO synthase substrate, L-arginine was 0.8 nmol min^{-1} (n = 2).

These results suggested that endogenous NO was required to generate the increased extracellular oxygen consumption.

In order to investigate whether the oxygen consumption of the extracellular medium of activated cells was similar to that of cells incubated with DETA-NO (5 mM) preliminary experiments were performed.

5.3.3.2.2 Presence of proteins in the extracellular medium

Ultrafiltration of the extracellular medium of J774 cells activated for 18 h in the presence of L-arginine (0.5 mM) with a filter membrane device (trapping molecules < 30 kDa) reduced the oxygen consumption (from 9.1 nmol min^{-1} ml^{-1} to 7.1 nmol min^{-1}, *n* = 2). Whereas ultrafiltration of extracellular medium from activated J774 cells with the filter membrane device (trapping molecules > 30 kDa) abolished the oxygen consumption (from 9.1 to 0.7 nmol min^{-1} ml^{-1}, *n* = 2).

5.3.3.2.3 Biochemical properties

Oxygen consumption of the extracellular medium of J774 cells activated for 18 h in the presence of L-arginine (0.5 mM) was photosensitive and temperature dependent.

Extracellular oxygen consumption of J774 cells activated for 18 h in the presence of L-arginine (0.5 mM), was reduced after incubation for 1 h at 37°C (from 9.1 to 7.4 nmol min^{-1}, *n* = 2) and at room temperature (21 °C, from 9.1 to 8.2 nmol min^{-1}, *n* = 2) and was abolished after 30 min at 98°C (0.8 nmol $min^{-1}ml^{-1}$, *n* = 2) and after illumination (8 Mlx) for 1 h (0.7 nmol $min^{-1}ml^{-1}$, *n* = 2).

Extracellular oxygen consumption of J774 cells activated for 18 h in the absence of L-arginine (0.8 nmol $min^{-1}ml^{-1}$, *n* = 2), was not altered by incubation for 1 h at 37°C (0.9 nmol min^{-1}, *n* = 2) and at room temperature (21 °C, 0.7 nmol min^{-1}, *n* = 2) and after 30 min at 98°C (0.8 nmol $min^{-1}ml^{-1}$, *n* = 2) and after illumination (8 Mlx) for 1 h (0.7 nmol $min^{-1}ml^{-1}$, *n* = 2).

5.3.3.2.4 Effect of GSH on extracellular oxygen consumption

Addition of GSH (0.5 -10 mM) to the extracellular medium of J774 cells activated, for 18 h in the presence of L-arginine, caused an increase in the oxygen consumption of the extracellular medium in a concentration dependent manner (Table 5.4, $n = 2$).

Addition of GSH (10 mM) did not cause an increase in the oxygen consumption of the extracellular medium obtained from J774 cells activated for 18 h in the absence of L-arginine (from 0.8 to 0.9 nmol min^{-1} ml^{-1}, $n = 2$).

Table 5.4 GSH increased the extracellular oxygen consumption of J774 cells activated for 18 h in the presence of L-arginine in a concentration dependent manner ($n = 4$).

GSH [mM]	Oxygen consumption [nmol min^{-1} ml^{-1}]
0	9.1 ± 1.5
0.5	16.4 ± 1.3
1	20 ± 1.2
3	28 ± 1.4
10	42 ± 2.8

5.4 DISCUSSION

These results show that high concentrations of exogenous NO (~ 5.6 μM) inhibited the cellular oxygen consumption with a similar profile to that observed with lower concentrations of NO (~1.3 μM, Chapters 3 and 4). There are pathological conditions, such as septic shock (Singer & Brealey, 1999) and brain ischaemia (Tominaga *et al.*, 1994), which may lead to the generation of such high concentrations of endogenous NO.

In this study, incubation of J774 and Jurkat cells with high concentrations of DETA-NO (5 mM ≅ 5.6 μM NO) caused a decrease in GSH concentration that preceded the inhibition of the respiratory chain.

Studies, using different inhibitors and substrates of the respiratory chain, showed that the inhibition of complex IV was always reversible by oxyHb whereas the inhibition of complex I became irreversible by oxyHb after several hours. This inhibition was confirmed by direct measurement of the activity of the complex I. The inhibition of complex I was reversed by addition of GSH and treatment with light. These results are in agreement with previous studies (Chapters 3 and 4) using Jurkat and L929 cells exposed to lower concentrations of NO. This suggests that the mechanism of inhibition of respiration by NO is similar in several cell types over a range of pathological concentrations of NO. These results in non-activated J774 cells exposed to high concentrations of NO for long periods exhibit a sequential inhibition of only two enzymes of the respiratory chain whereas, in activated macrophages, a variety of enzymes have been reported to be inhibited (Granger & Lehninger, 1982). Although in both situations the inhibition is NO-dependent, it is likely that the activated and non-activated cells provide a different biochemical environment in which NO exerts its actions, leading to differential inhibitory effects.

Following incubation of J774 cells in the presence of high concentrations of NO a paradoxical extraction of oxygen by the extracellular medium was observed. This oxygen consumption was dependent on the time of exposure of the cells to NO and on the NO concentration. This phenomenon requires

the presence of metabolically active cells, since either incubation with DETA-NO (5 mM) alone in the medium or with cells killed by freeze-thawing did not result in oxygen consumption in the extracellular medium. This event was caused by the presence in the extracellular medium of one or more proteins with an apparent molecular weight up to 30 kDa. The consumption of oxygen was reduced by oxyHb, abolished by exposure to light and high temperature (98°C) and increased by addition of GSH. The increase in oxygen by the extracellular medium is not restricted to cells exposed to exogenous NO since it was also observed after activation of the cells with IFN-γ and endotoxin. In this case extracellular oxygen consumption was entirely dependent on the endogenous generation of NO since it required the presence of L-arginine (Hibbs, Jr. *et al.*, 1987; Tucker *et al.*, 1991); (Assreuy *et al.*, 1993; Zhang *et al.*, 1994b). The increase in oxygen consumption by the extracellular medium of activated J774 cells showed the same characteristics as observed with DETA-NO treated cells. This suggests that extracellular oxygen consumption is an event specifically dependent on NO and not due to other actions of DETA-NO.

The oxygen consumption in the extracellular medium may be explained in part by the breakdown of nitrosothiol(s) either spontaneously or metal-catalysed or following the interaction with thiols. These reactions are accompanied by the release of either NO or nitroxyl ion, both of which can further react with dissolved oxygen to form nitrite and peroxynitrite (Xin & Bloomfield, 1999).

Furthermore the presence of GSH in the supernatant may explain, in part, the increase in oxygen consumption, by its reaction with nitrosothiol(s)

(Singh *et al.*, 1996). However the ultrafiltration of the low molecular weight fraction did not significantly reduce the oxygen consumption in the extracellular medium. The dramatic increase in oxygen consumption on addition of exogenous GSH is likely to be caused by an enhanced decomposition of nitrosothiol(s), leading to a burst of NO and possibly NO^- release and consequent oxygen consumption (Feelisch personal communication, 1999).

An additional explanation for the extracellular oxygen consumption might be related to the presence of thioredoxin, a multifunctional intracellular and extracellular protein (~13 kDa), in the extracellular medium. Thioredoxin may react with nitrosothiol(s) such as GSNO, found in the supernatant generating GSH (Nikitovic & Holmgren, 1996). Further studies are required to identify the presence of thioredoxin in the extracellular medium.

Thus, following exposure to exogenous or endogenous NO for several hours there is a decrease in oxygen utilisation due to two distinct actions, one being the inhibition of cellular oxygen consumption and the other, the generation of a hypoxic microenvironment due to the oxygen consumption by the extracellular medium. Whether such a situation occurs *in vivo* needs to be investigated since such a synergistic deleterious effect may be responsible for the progression of pathology in some clinical conditions, including septic shock.

CHAPTER 6

PHYSIOLOGICAL REGULATION OF CELLULAR RESPIRATION BY NITRIC OXIDE *EX VIVO*

6 PHYSIOLOGICAL REGULATION OF CELLULAR RESPIRATION BY NITRIC OXIDE *EX VIVO*

6.1 SUMMARY

This study investigates the role of NO on myocardial and hepatic mitochondrial respiration under basal conditions using eNOS and iNOS mutant and wild type mice.

Baseline hepatic oxygen consumption was greater than the myocardial oxygen consumption in the eNOS and iNOS mutant and wild type mice.

Baseline myocardial and hepatic oxygen consumption were not significantly different between eNOS mutant and wild type mice and iNOS mutant and wild type mice.

Addition of L-N^G-methylarginine (L-NMMA, 300 μM) *in vitro* significantly increased oxygen consumption in the heart and liver of the eNOS and iNOS wild type, and iNOS mutant mice but not in the eNOS mutant mice.

This study demonstrates that the generation of NO under basal conditions plays a role in the physiological regulation of mitochondrial respiration *ex vivo*.

6.2 INTRODUCTION

Several studies *in vitro* have provided evidence that nitric oxide, binding to the same site of complex IV of the respiratory chain as oxygen, acts as a physiological regulator of cellular respiration (Brown & Cooper, 1994); (Cleeter *et al.*, 1994). Indeed, inhibition of NO synthase increases cellular oxygen consumption in isolated alveolar type II cells (Miles *et al.*, 1996) and

in endothelial cells (Clementi *et al.*, 1999). Several studies *in vivo* have also indicated that NO may have a physiological role in regulating cellular respiration. Administration of an inhibitor of NO synthase (L-N-nitroarginine) in conscious dogs produces a rapid and sustained increase in whole body oxygen consumption (Shen *et al.*, 1994) and administration of L-N-nitroarginine to exercising dogs causes an increase in myocardial oxygen consumption (Ishibashi *et al.*, 1998). All these studies suggest that NO may play a role in regulating mitochondrial respiration *in vitro* and *in vivo.*

The present study investigates whether NO synthase regulates myocardial and hepatic mitochondrial respiration *ex vivo* using eNOS and iNOS mutant and wild type mice.

6.3 RESULTS

6.3.1 DETERMINATION OF LINEARITY OF TISSUE WEIGHT AND OXYGEN CONSUMPTION

The myocardial and hepatic oxygen consumption determined by a Clark type oxygen electrode was linearly proportional with tissue weight (30-45 mg) in eNOS and iNOS mutant and wild type mice (Figure 6.1 A, B, Figure 6.2 A, B, Figure 6.3 A, B, Figure 6.4 A, B).

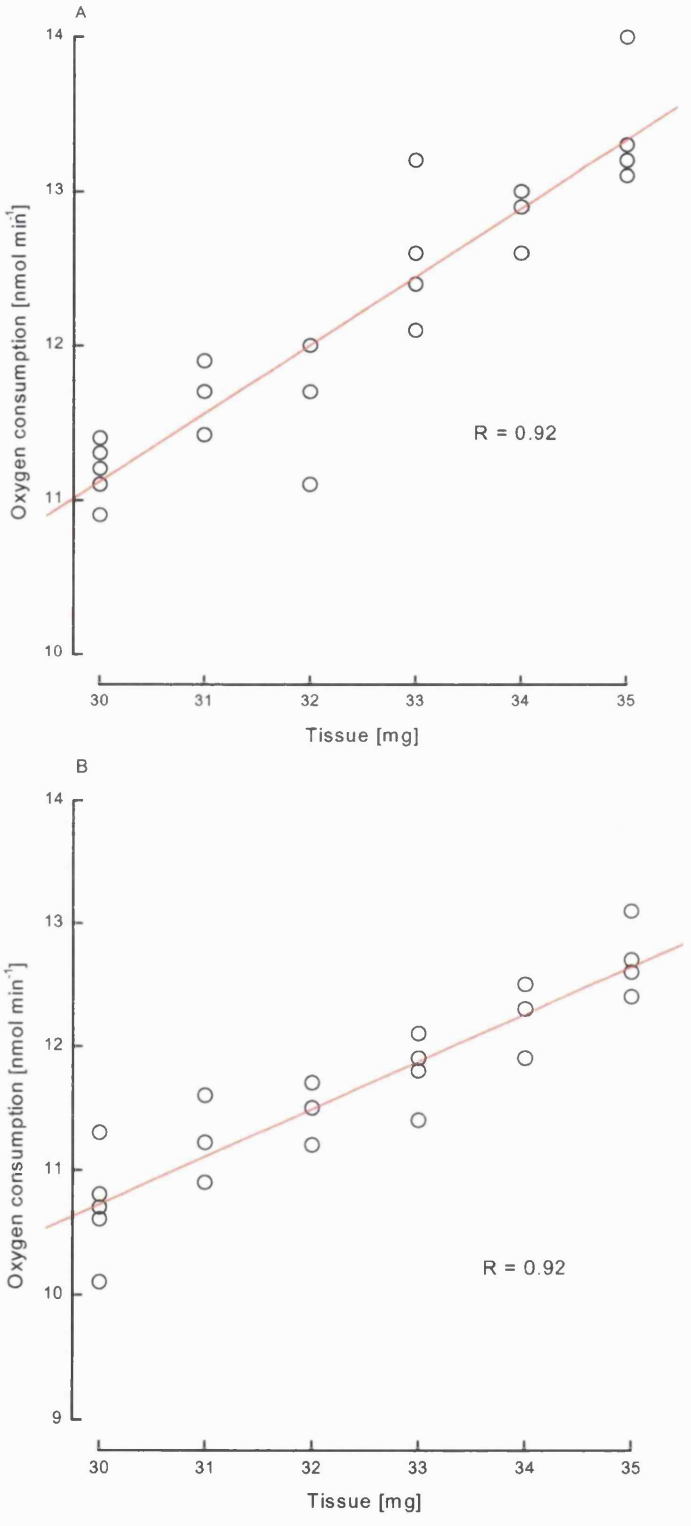

Figure 6.1 Myocardial oxygen consumption is linearly proportional to tissue weight. Different quantities of heart tissue (mg) of (A) eNOS mutant and (B) eNOS wild type mice were weighed and their oxygen consumption measured using a Clark type oxygen electrode.

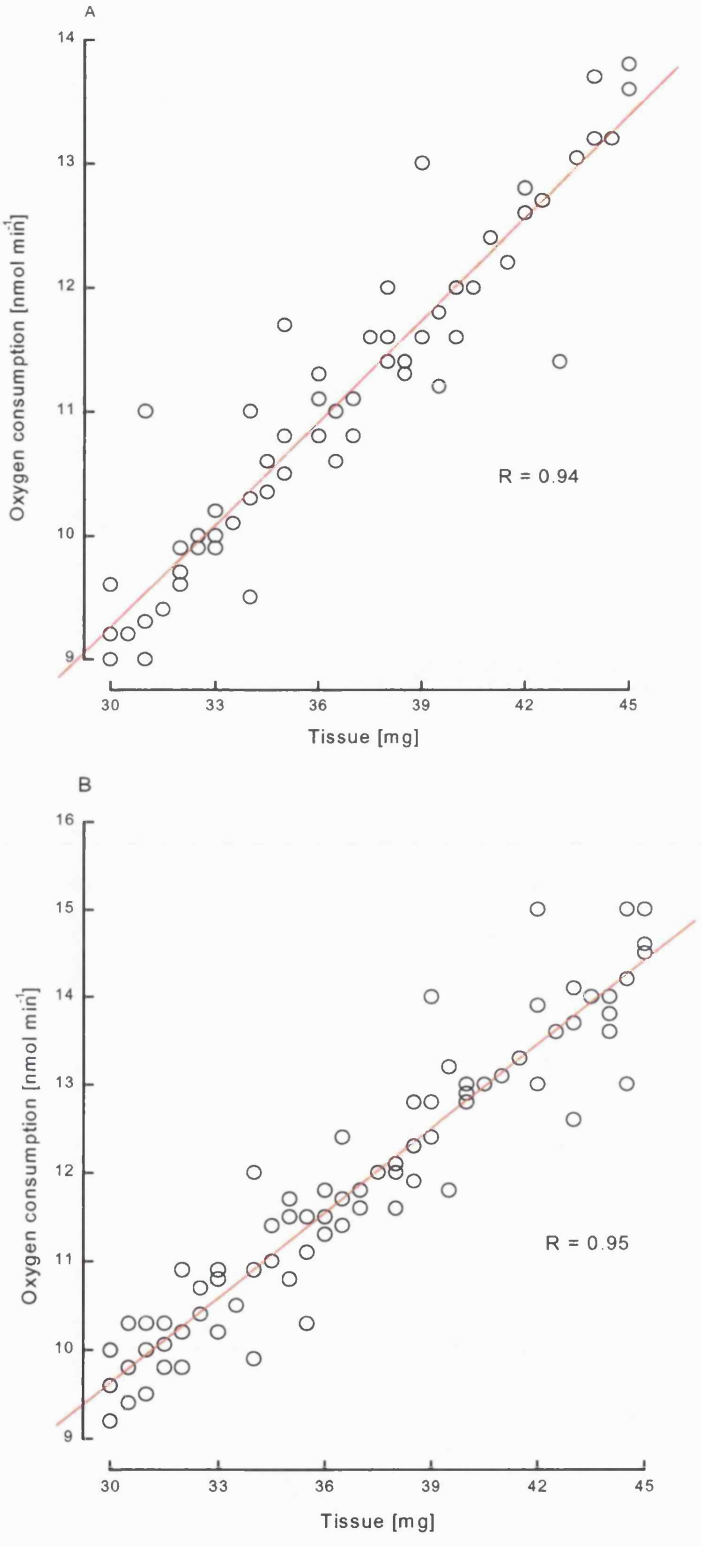

Figure 6.2 Myocardial oxygen consumption is linearly proportional to tissue weight. Different quantities of heart tissue (mg) of (A) iNOS mutant and (B) iNOS wild type mice were weighed and their oxygen consumption measured using a Clark type oxygen electrode.

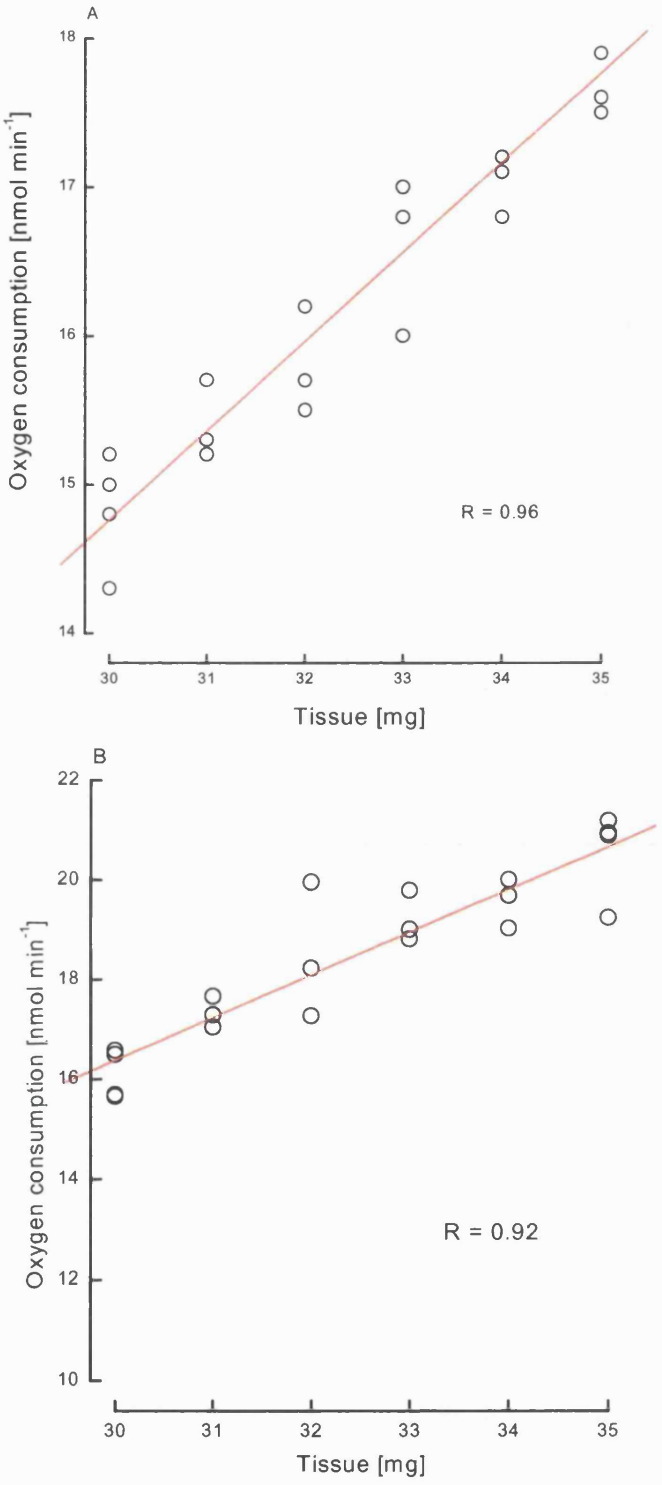

Figure 6.3 Hepatic oxygen consumption is linearly proportional to tissue weight. Different quantities of liver tissue (mg) of (A) eNOS mutant and (B) wild type mice were weighed and their oxygen consumption measured using a Clark type oxygen electrode.

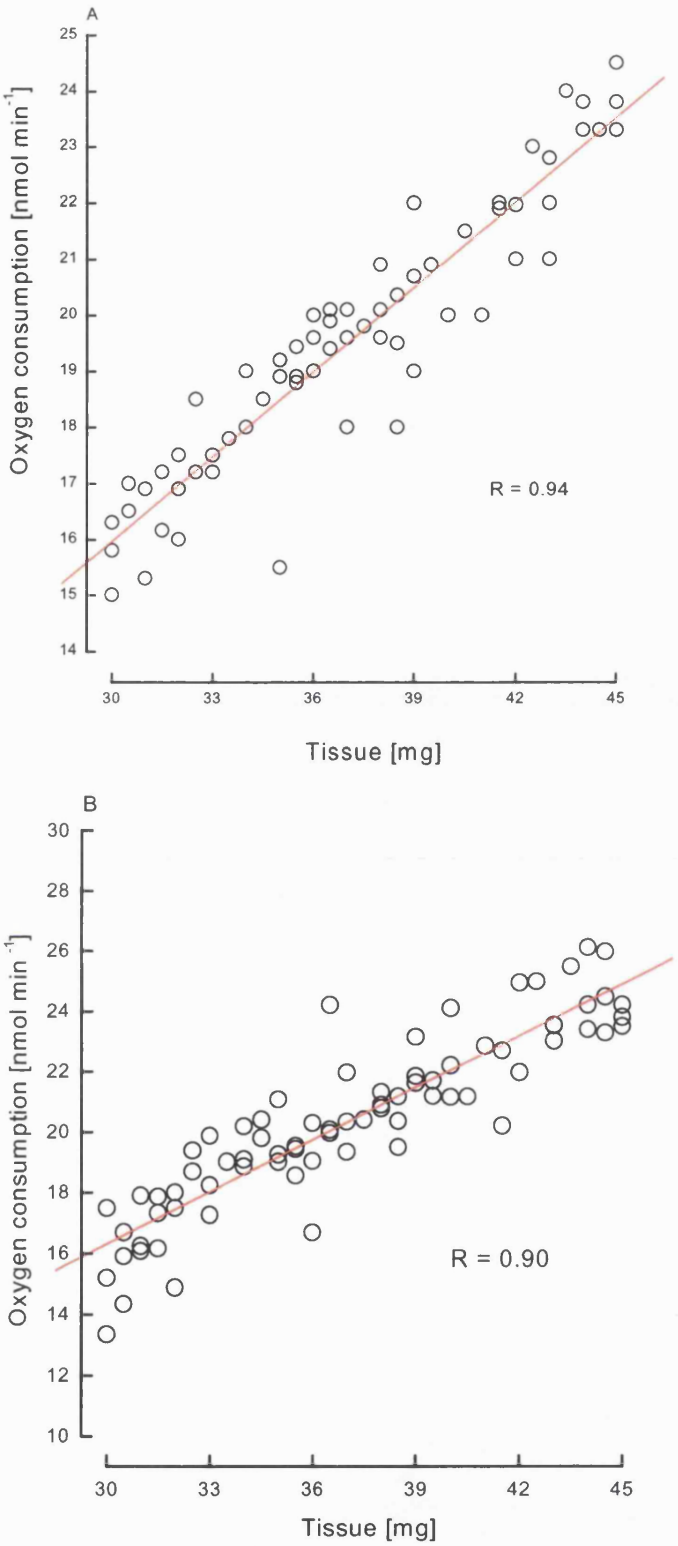

Figure 6.4 Hepatic oxygen consumption is linearly proportional to tissue weight. Different quantities of liver tissue (mg) of (A) iNOS mutant and (B) iNOS wild type mice were weighed and their oxygen consumption measured using a Clark type oxygen electrode.

6.3.2 DETERMINATION OF OXYGEN CONSUMPTION AS A MARKER OF THE RESPIRATORY CHAIN ACTIVITY

Addition of myxothiazol (1-100 µM) *in vitro*, an inhibitor of complex III of the respiratory chain, reduced hepatic oxygen consumption in a concentration dependent manner in iNOS mutant and wild type mice (n = 4-6, Figure 6.5 A, B). Myxothiazol (100 µM) reduced hepatic oxygen consumption by 90 ± 3 % and 84 ± 3 % in the iNOS mutant and wild type mice, respectively (n = 4-6, Figure 6.5 A, B). Myxothiazol (100 µM) reduced hepatic oxygen consumption by 87 ± 3 % and 82 ± 1 % in the eNOS mutant and wild type mice, respectively (n = 3, Figure 6.6).

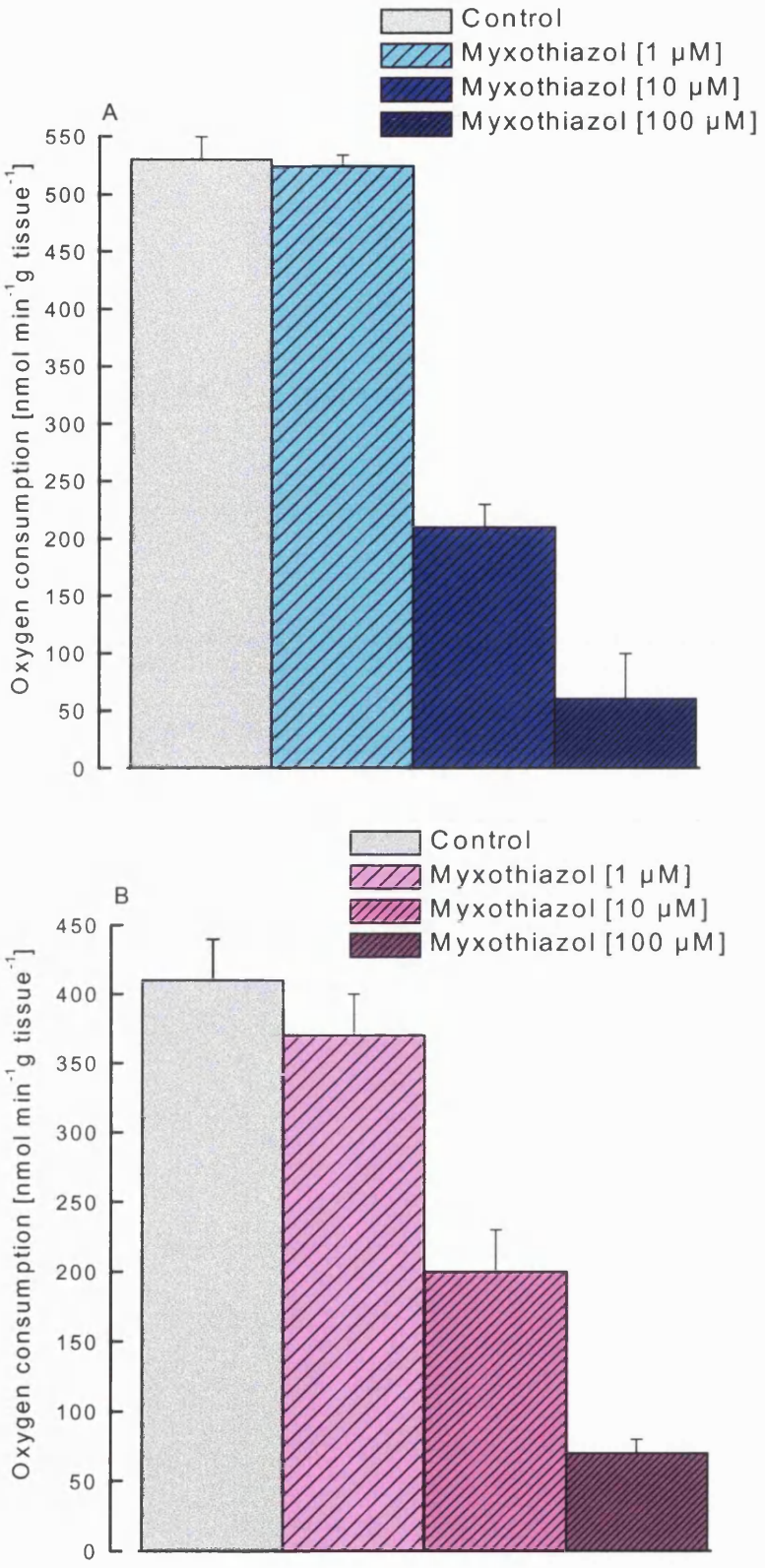

Figure 6.5 Myxothiazol reduces hepatic oxygen consumption of (A) iNOS mutant and (B) wild type mice (n = 4-6).

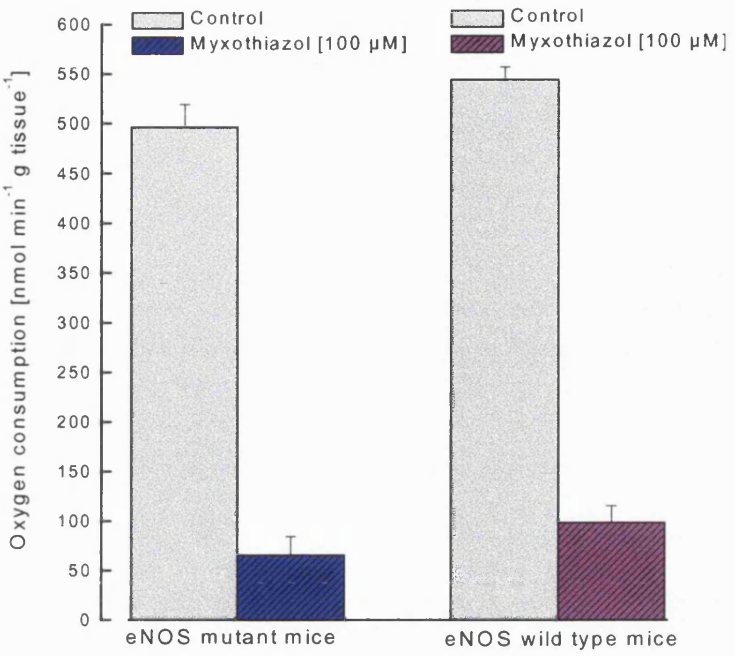

Figure 6.6 Myxothiazol reduces hepatic oxygen consumption of eNOS mutant and wild type mice (n = 3).

6.3.3 BASAL GENERATION OF NITRIC OXIDE REGULATES TISSUE RESPIRATION

6.3.3.1 Endothelial NOS mutant and wild type mice

Baseline oxygen consumption was lower in the heart than the liver, in both the eNOS mutant mice (376 ± 15 and 496 ± 23 nmol min^{-1} g $tissue^{-1}$, heart and liver respectively, $p < 0.005$, n = 6, Figure 6.7 A) and wild type mice (362 ± 30 and 544 ± 13 nmol min^{-1} g $tissue^{-1}$, heart and liver respectively, $p < 0.001$, n = 6, Figure 6.7 B). Baseline oxygen consumption was not significantly different between mutant and wild type mice ($p > 0.05$, Figure 6.7 A, B). Addition of L-N^G-methylarginine HCl (L-NMMA; 300 µM) *in vitro* did not increase the oxygen consumption in the heart (by 1.9 ± 0.2 nmol min^{-1} g $tissue^{-1}$, NS, n = 4, Figure 6.7 A) or in the liver of eNOS mutant mice (by 1.1

± 0.4 nmol min^{-1} g $tissue^{-1}$,NS, *n* = 4, Figure 6.7 A). Addition of L-NMMA (300 μM) *in vitro* significantly increased oxygen consumption in the heart (by 76 ± 3 nmol min^{-1} g $tissue^{-1}$, $p < 0.001$, *n* = 4, Figure 6.7 B) and in the liver of wild type mice (by 82 ± 2 nmol min^{-1} g $tissue^{-1}$, $p < 0.001$, *n* = 4, Figure 6.7 B).

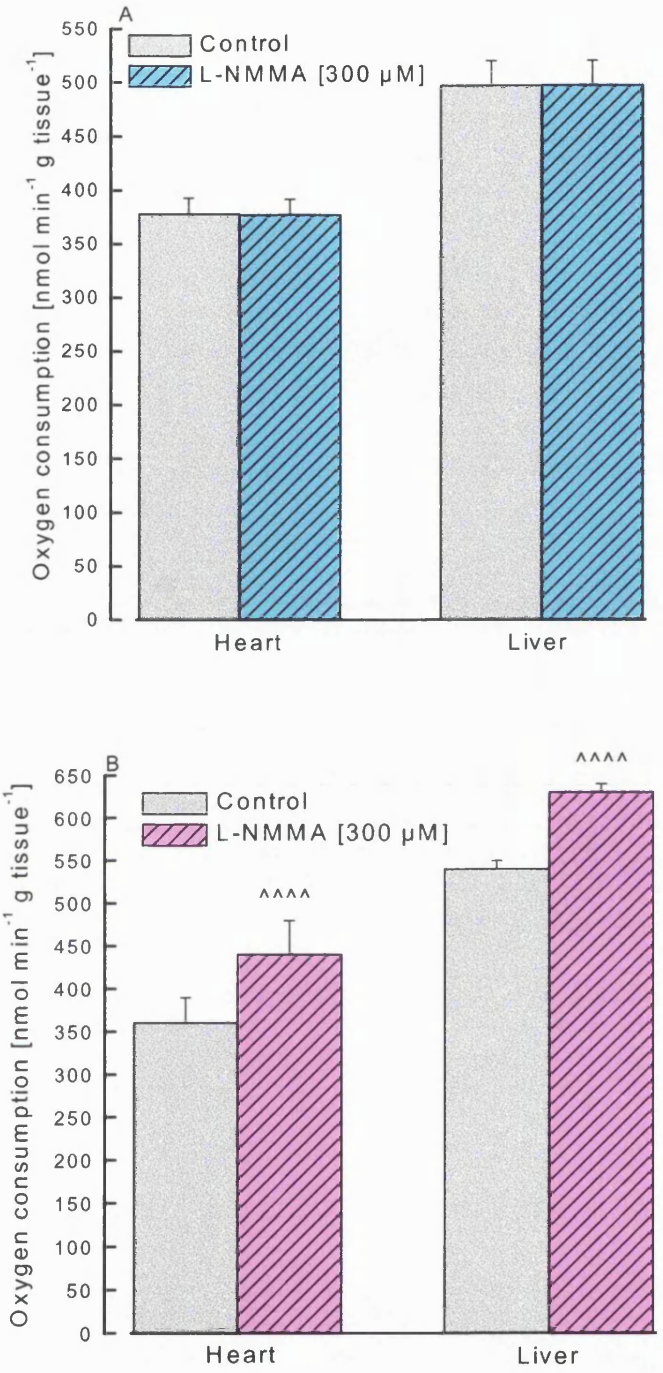

Figure 6.7 Addition of L-NMMA (300 µM) *in vitro* did not increase myocardial and hepatic oxygen consumption of (A) eNOS mutant, but did increase myocardial and hepatic oxygen consumption of (B) eNOS wild type mice. Statistical analysis: within the same group. ^^^^ = $p < 0.001$ ($n = 4$).

6.3.3.2 Inducible NOS mutant and wild type mice

Baseline oxygen consumption was lower in the heart than in the liver in both the iNOS mutant mice (300 ± 20 and 530 ± 30 nmol min^{-1} g $tissue^{-1}$, heart and liver, respectively, $p < 0.001$, $n = 6$, Figure 6.8 A) and wild type mice (320 ± 20 and 550 ± 30 nmol min^{-1} g $tissue^{-1}$, heart and liver respectively, $p < 0.001$, $n = 6$, Figure 6.8 B). Baseline oxygen consumption was not significantly different between the iNOS mutant and wild type mice (NS, $n = 6$, Figure 6.8 A, B). Addition of L-NMMA (300 μM) *in vitro* significantly increased oxygen consumption in the heart (by 50 ± 1 and 30 ± 2 and nmol min^{-1} g $tissue^{-1}$, $p < 0.001$, $n = 6$, Figure 6.8 A, B), and liver (by 50 ± 2 and 30 ± 3 nmol min^{-1} g $tissue^{-1}$, $p < 0.001$, $n = 6$, Figure 6.8 A, B) of iNOS mutant and wild type mice, respectively.

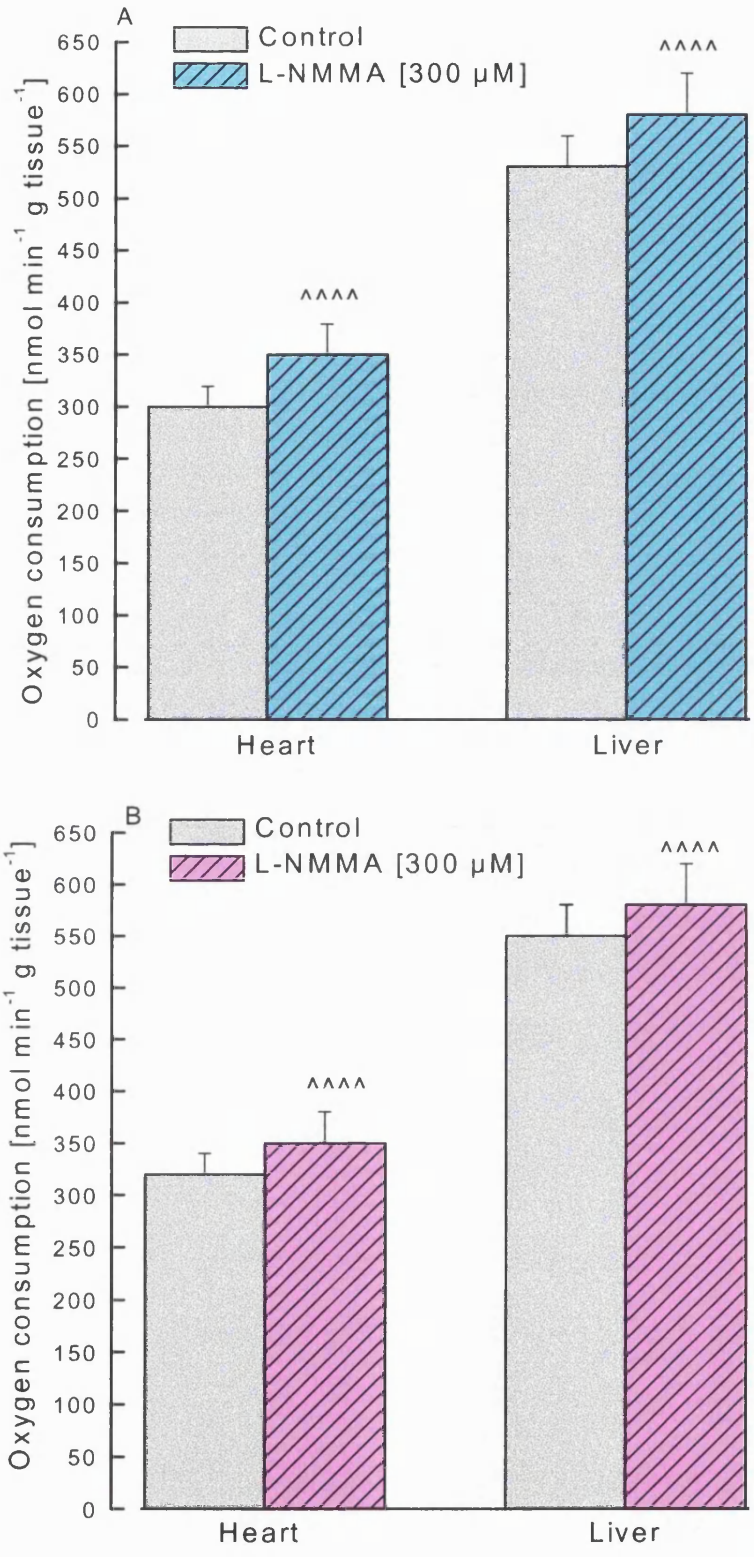

Figure 6.8 Addition of L-NMMA (300 µM) *in vitro* increases myocardial and hepatic oxygen consumption of (A) iNOS mutant and (B) iNOS wild type mice. Statistical analysis: within the same group. ^^^^ = $p < 0.001$ ($n = 6$).

6.4 DISCUSSION

In these studies myocardial and hepatic oxygen consumption in eNOS and iNOS mutant and wild type mice were measured in the physiological oxygen tension range of the microvasculature (5-40 mmHg). Oxygen consumption was linear with tissue weights used in all groups of mice. In addition, confirmation was gained that the oxygen consumption measured was principally due to mitochondrial respiration since it could be inhibited by addition of myxothiazol *in vitro*, a known inhibitor of complex III of the respiratory chain.

Greater baseline oxygen consumption in the liver compared to the heart was observed in all groups of mice, suggesting a greater energy demand in the liver than the heart. However the use of a Clark-type oxygen electrode to measure oxygen consumption in a non-beating heart in the absence of blood may not truly reflect the physiological myocardial oxygen consumption *in vivo*. Thus a beating heart *in vivo* may indeed have greater oxygen consumption than that observed in these experiments.

Addition of L-NMMA *in vitro* significantly increased myocardial and hepatic oxygen consumption of eNOS and iNOS wild type and iNOS mutant mice but not of eNOS mutant mice. In this regard there was a trend that myocardial oxygen consumption of the eNOS mutant was higher than those observed in the eNOS wild type mice although this difference did not reach significance. This disparity between removal of NO by chemical (i.e. L-NMMA) and genetic means may be due to compensatory mechanisms independent of NO during development of the eNOS mutant mice. Indeed the basal oxygen consumption of both tissues was similar for the iNOS

mutant and their wild type counterparts. These results suggest that the basal generation of NO produced by eNOS regulates mitochondrial respiration physiologically *ex vivo*. This is in accordance with previous studies showing that inhibition of NO synthase by L-NAME increased oxygen consumption in alveolar type II (Miles *et al.*, 1996) and in endothelial cells (Clementi *et al.*, 1999). Furthermore, administration of an inhibitor of NO synthase (L-N-nitroarginine) increases myocardial oxygen consumption in dogs during exercise (Ishibashi *et al.*, 1998) and in whole body oxygen consumption in conscious dogs (Shen *et al.*, 1994) independently of blood flow. In this latter study the vasopressor, methoxamine, had no significant effect on oxygen consumption. This suggests that basal NO generation has an inhibitory effect on mitochondrial oxygen consumption *in vivo* (Shen *et al.*, 1994). In addition heart muscle slices incubated with bradykinin or carbachol *in vitro* caused a NO-dependent inhibition of tissue respiration that was not mimicked by 8-bromo-cGMP, a cell-permeable cGMP analogue. This suggests that endothelial derived NO directly inhibits the mitochondrial respiratory chain (Xie *et al.*, 1996) independent of guanylate cyclase activation. More recent experiments have shown that, bradykinin induced a significant decrease in myocardial oxygen consumption from iNOS mutant mice and eNOS wild type mice, but not from eNOS mutant mice (Loke *et al.*, 1999). However, Loke and co-workers, failed to determine an increase in myocardial oxygen consumption after addition of L-NAME, which differs from this study and may be due to several factors including the wide age-range of mice used in their experiments (8-39 weeks), the time of measurement and the different NOS inhibitor. Indeed preliminary experiments in this study

showed that the age of the mice was important to clearly determine the changes in oxygen consumption (range 6-12 weeks). In addition, the myocardial oxygen consumption in Loke's study was measured after 2 hours of incubation compared to 10 min in the present study. Furthermore, in the present study the oxygen consumption was followed for several minutes, while in Loke' s study the effect of L-NAME was measured immediately after addition. Since L-NAME is reported to have a slower onset of effect than L-NMMA (Rees *et al.*, 1990a) this may be another contributing factor.

The present study demonstrates that basal NO plays a physiological role in regulating mitochondrial respiration. However further studies are required to demonstrate the source of NO, which may be generated from eNOS or nNOS located in close proximity to the cells (Andries *et al.*, 1998) or directly in the mitochondria (Bates *et al.*, 1995; Kobzik *et al.*, 1995, Giulivi, 1998). The latter possibility may be more plausible since the half life of NO is relatively short *in vivo* and NO produced by a calcium-dependent mitochondrial NOS, would be more likely to regulate respiration. In this regard the role of NO may be important by acting as a limiting step in the consumption of oxygen in physiological conditions. This "NO-break" of respiration may become less efficient in conditions in which more ATP would be required such as during exercise, since under these conditions the muscle contracts and calcium is sequestered, with less available for other activities such as constitutive NOS activation. This may suggest that cell types that are required to increase their ATP levels rapidly may be under a greater tonic control of basal NO. Thus controlling mechanisms of NO synthase such as calcium sequestration would rapidly decrease NO

concentration resulting in a rapid increase in ATP concentrations. However further studies are required to investigate this potentially novel regulatory mechanism.

CHAPTER 7

REVERSIBLE INHIBITION OF TISSUE RESPIRATION BY NITRIC OXIDE IN SUB-LETHAL ENDOTOXIN SHOCK

7 REVERSIBLE INHIBITION OF TISSUE RESPIRATION BY NITRIC OXIDE IN SUB-LETHAL ENDOTOXIN SHOCK

7.1 SUMMARY

This study investigates the role of NO on myocardial and hepatic mitochondrial oxygen consumption in sub-lethal endotoxin shock using iNOS mutant and wild type mice.

Following administration of a low dose of endotoxin (6 mg kg^{-1} *iv.*) in iNOS wild type mice there was a fall in blood pressure leading to established shock at 12 h (> 40 mmHg decrease). All the mice survived over the 24 h of observation.

The low dose of endotoxin (6 mg kg^{-1} *iv.*) elevated the concentration of nitrite/nitrate in iNOS wild type mice reaching a maximum at 12 h and declining thereafter. This was associated with a decrease in myocardial and hepatic oxygen consumption reaching a minimum at 12 h and reversing thereafter. L-N^G-methylarginine (L-NMMA) reversed the decrease in oxygen consumption.

The low dose of endotoxin (6 mg kg^{-1} *iv.*), administrated to iNOS mutant mice, produced a fall in blood pressure, which did not progress into established shock and increased plasma nitrite/nitrate at 1 h only. Endotoxin (6 mg kg^{-1} *iv.*) had no significant effect on oxygen consumption in iNOS mutant mice. All the mice survived over the 24 h of observation.

These results demonstrate that following a sub-lethal dose of endotoxin (6 mg kg^{-1} *iv.*) the production of NO leads to established shock with a reversible inhibition of mitochondrial respiration.

7.2 INTRODUCTION

Septic shock is defined as " severe sepsis with hypotension (a reduction of > 40 mmHg from baseline) in the absence of other causes for hypotension despite adequate fluid resuscitation" (Hinds & Watson, 1996). Septic shock is characterised by microbial invasion, hypotension, vascular damage and disseminated intravascular coagulation leading to multiple organ failure and eventually death. In the early 1960's, septic shock was characterised simply as hypotension; in the 1970's and 1980's the definition expanded to include an imbalance between oxygen delivery (Powers & Powell, Jr., 1973; Perret *et al.*, 1975) and oxygen demand (Clemens *et al.*, 1981; Cain, 1984; Fink *et al.*, 1989). In the 1990's the complexity of septic shock increased by the recognition of a primary defect in the ability of the tissues to consume oxygen (Manthous *et al.*, 1993; Hayes *et al.*, 1993; Pilas *et al.*, 1995), although mitochondrial dysfunction had been implicated for several years (Schumer *et al.*, 1970; Mela *et al.*, 1971; Nicholas *et al.*, 1972; Poderoso *et al.*, 1978). During this period several studies reported an association between an elevation in plasma nitrite/nitrate concentration and a low systemic vascular resistance in septic shock patients (Ochoa *et al.*, 1991; Evans *et al.*, 1993). A relationship between the loss of vascular tone and induction by endotoxin of an inducible NO synthase was also recognised (Rees *et al.*, 1990b). More recently NO has been suggested to cause an alteration in the mitochondrial respiration in human septic shock (Singer & Brealey, 1999). Thus overproduction of NO has been suggested to provide a common mechanism by which microbial products and various cytokines bring about the vascular and metabolic defects that lead to multiple organ

failure and death (Ochoa *et al.*, 1991; Evans *et al.*, 1993; Vallance & Moncada, 1993; Petros *et al.*, 1994; Thiemermann, 1994; Rees, 1995a).

The present study investigates the role of NO on mitochondrial respiration in a model of sub-lethal septic shock *ex vivo*. Myocardial and hepatic oxygen consumption was determined using iNOS wild type and mutant mice following administration of a low dose of endotoxin.

7.3 RESULTS

7.3.1 INDUCTION OF SUB-LETHAL ENDOTOXIN SHOCK AND SURVIVAL PROFILE

A preliminary series of experiments were carried out to study the survival profile following the administration of endotoxin (*E. coli i.v.*). Following endotoxin (6 mg kg^{-1} *i.v)* the mice showed characteristic " shock-like" symptoms (i.e. piloerection and lethargy), however, there were no deaths during the 24 h of observation in iNOS mutant and wild type mice (n = 6, Chapter 8).

7.3.2 BLOOD PRESSURE PROFILE

After recovery from surgery (18-24 h) basal mean arterial blood pressure (MABP) of untreated mice remained constant throughout the following 24 h of observation in both groups of mice (112 ± 3 and 105 ± 2 mmHg at 0 h and 118 ± 2 and 105 ± 3 mmHg at time 24 h, in iNOS mutant and wild type mice, respectively, *n* = 6, Figure 7.1).

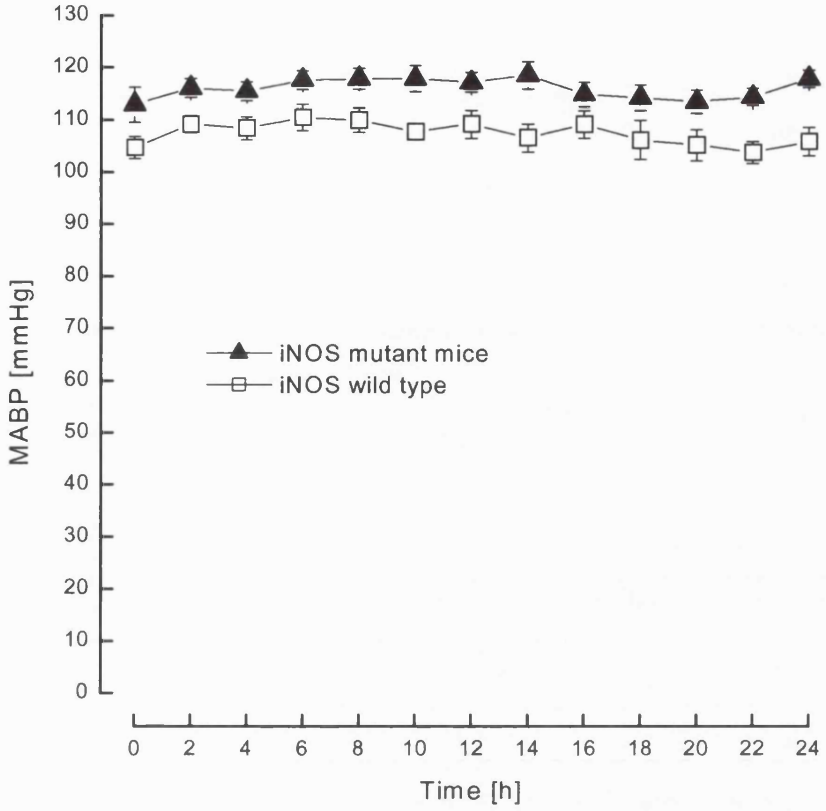

Figure 7.1 Basal mean arterial blood pressure (MABP) over 24 h period in iNOS mutant and wild type mice (n = 6).

Administration of endotoxin (*E. coli*, 6 mg kg $^{-1}$) in wild type mice caused a small fall in blood pressure that reversed after 2 h. Thereafter there was a progressive fall in blood pressure after approximately 4 h, (from a basal MABP of 105 ± 1 mmHg to 64 ± 3 mmHg at 24 h, n = 6, Figure 7.2), leading to established shock (fall in MABP > 40 mmHg). The fall in blood pressure was significantly reduced in iNOS mutant mice (from a basal MABP of 117 ± 3 mmHg to 93 ± 4 mmHg at 24 h, n = 6, Figure 7.2) which did not lead to established shock.

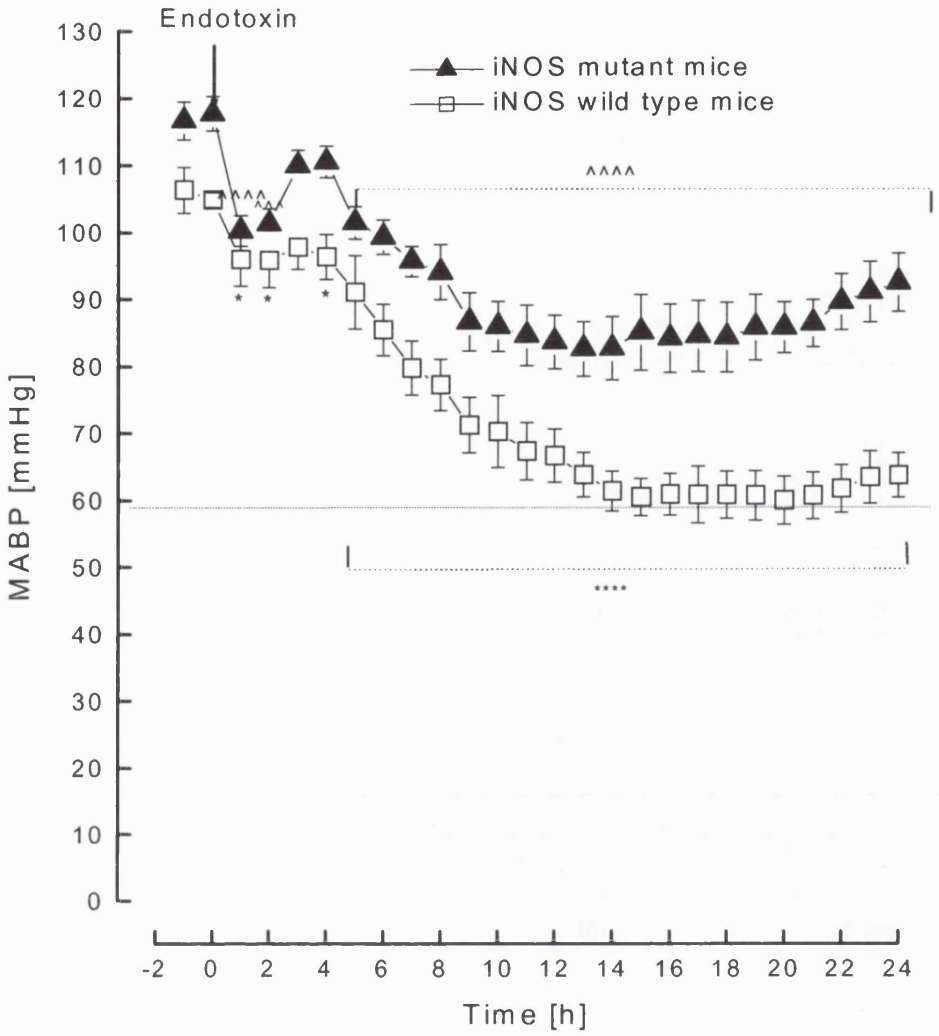

Figure 7.2 Effect of low dose endotoxin (*E.coli,* 6 mg kg^{-1}) on mean arterial blood pressure (MABP) in conscious iNOS mutant and wild type mice. Statistical analysis; within group analysis using one way ANOVA for repeated measures followed by Bonferroni test; * $p < 0.05$; ^^^ $p < 0.01$; ****,^^^^ $p < 0.001$ ($n = 6$) Sustained MABP below 60 mmHg is associated with death.

7.3.3 ELEVATION OF PLASMA NITRITE/NITRATE

The basal concentration of nitrite/nitrate in the plasma was 28 ± 1 µM ($n = 6$) in wild type mice and 30 ± 2 µM ($n = 6$) in iNOS mutant mice. The low dose of endotoxin (6 mg kg^{-1} *i.v.*) elevated the concentration of nitrite/nitrate (170 ± 7 µM at 4 h, $p < 0.001$, $n = 6$, Figure 7.3) reaching a maximum at 12 h (505

± 36 µM, $p < 0.001$, $n = 6$, Figure 7.3) and declining thereafter (285 ± 23 µM at 24 h; $p < 0.001$, $n = 6$) in wild type mice. The low dose of endotoxin (6 mg kg^{-1} *i.v.*) slightly elevated the concentration of nitrite/nitrate only at 1 h (46 ± 7 µM at 4 h, $p < 0.01$, $n = 6$ Figure 7.3) in the iNOS mutant mice.

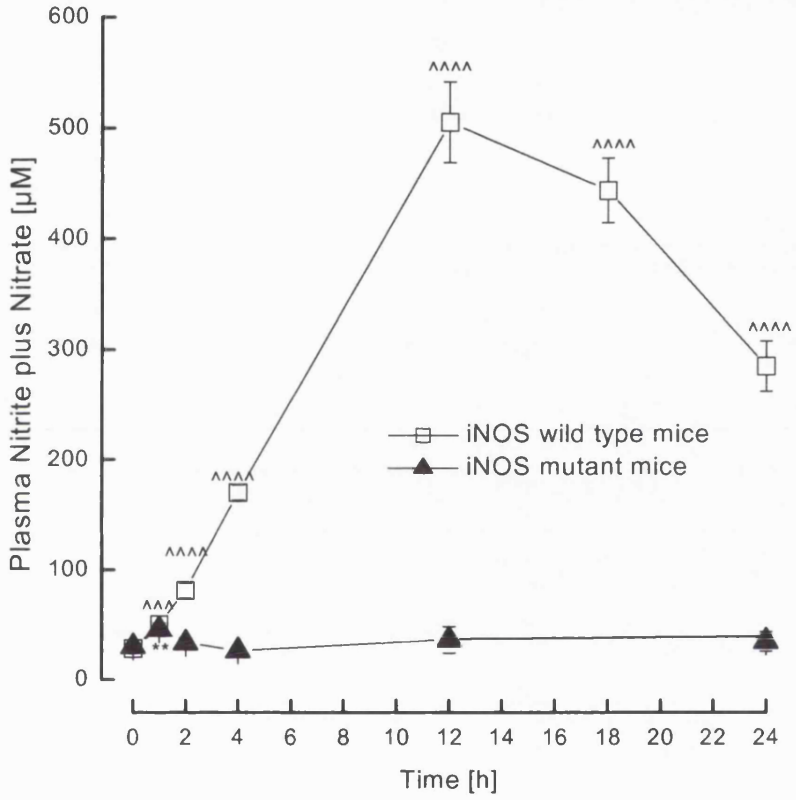

Figure 7.3 Effect of endotoxin (*E.coli* 6 mg kg^{-1}) on the concentration of plasma nitrite and nitrate in iNOS mutant and wild type mice. Statistical analysis within group analysis: Student t test: ^^ ** = $p < 0.01$, ^^^ $p < 0.005$, ^^^^ = $p < 0.001$ ($n = 6$).

7.3.4 REVERSIBLE INHIBITION OF TISSUE RESPIRATION

Following administration of the low dose of endotoxin (6 mg kg^{-1} *i.v.),* myocardial (320 ± 20 nmol min^{-1} g $tissue^{-1}$, $n = 6$, Figure 7.4 A) and hepatic oxygen consumption (550 ± 30 nmol min^{-1} g $tissue^{-1}$, $n = 6$, Figure 7.4 B)

decreased after 4 h (190 ± 20 and 260 ± 10 nmol min^{-1} g $tissue^{-1}$, heart and liver, respectively, $p < 0.001$, $n = 6$, Figure 7.4 A, B) reaching a minimum at 12 h (150 ± 10 and 200 ± 10 nmol min^{-1} g $tissue^{-1}$, heart and liver respectively; $p < 0.001$, $n = 6$, Figure 7.4 A, B and Figure 7.6) and reversing thereafter (290 ± 20 and 540 ± 60 nmol min^{-1} g $tissue^{-1}$, heart and liver, respectively at 24 h, NS, $n = 6$, Figure 7.4 A, B) in the wild type mice. N^G-monomethyl-L-arginine (300 µM) reversed the decrease in oxygen consumption *in vitro* in both tissues at all time points analysed ($n = 6$, Figure 7.4 A, B). The addition of D-NMMA did not alter the oxygen consumption in either the heart (by 1.7 ± 0.3 and 1.5 ± 0.3 nmol min^{-1} g $tissue^{-1}$ at 0 h and by 1.4 ± 0.5 and 1.6 ± 0.4 nmol min^{-1} g $tissue^{-1}$ at 24 h, $n = 4$) or the liver (by 1.9 ± 0.5 and 1.2 ± 0.3 nmol min^{-1} g $tissue^{-1}$ at 0 h and by 1.5 ± 0.6 and 1.1 ± 0.5 nmol min^{-1} g $tissue^{-1}$ at 24 h, wild type and mutant mice, respectively, $n = 4$). Endotoxin had no significant effect on oxygen consumption in the liver and the heart of the iNOS mutant mice ($n = 6$, Figure 7.5 A and B). When compared to baseline, L-NMMA (300 µM) had no significant effect over the time course of the experiment in the heart and liver of the iNOS mutant mice ($n = 6$, Figure 7.5 A and B).

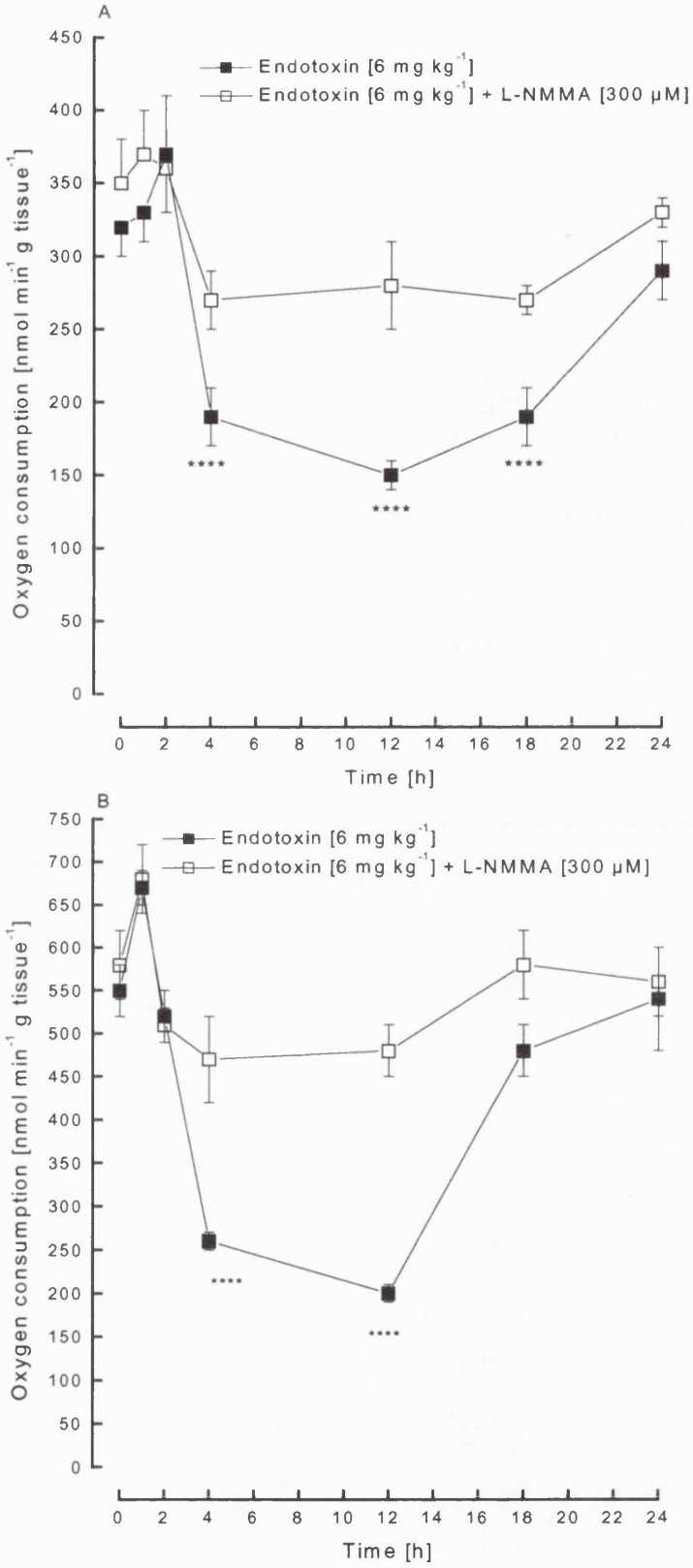

Figure 7.4 L-NMMA reversed the endotoxin (*E.coli,* 6 mg kg^{-1}) induced decrease in oxygen consumption in the (A) heart and (B) liver of iNOS wild type mice. Statistical analysis; within group analysis using one way ANOVA followed by Bonferroni test; **** $p < 0.001$, ($n = 6$).

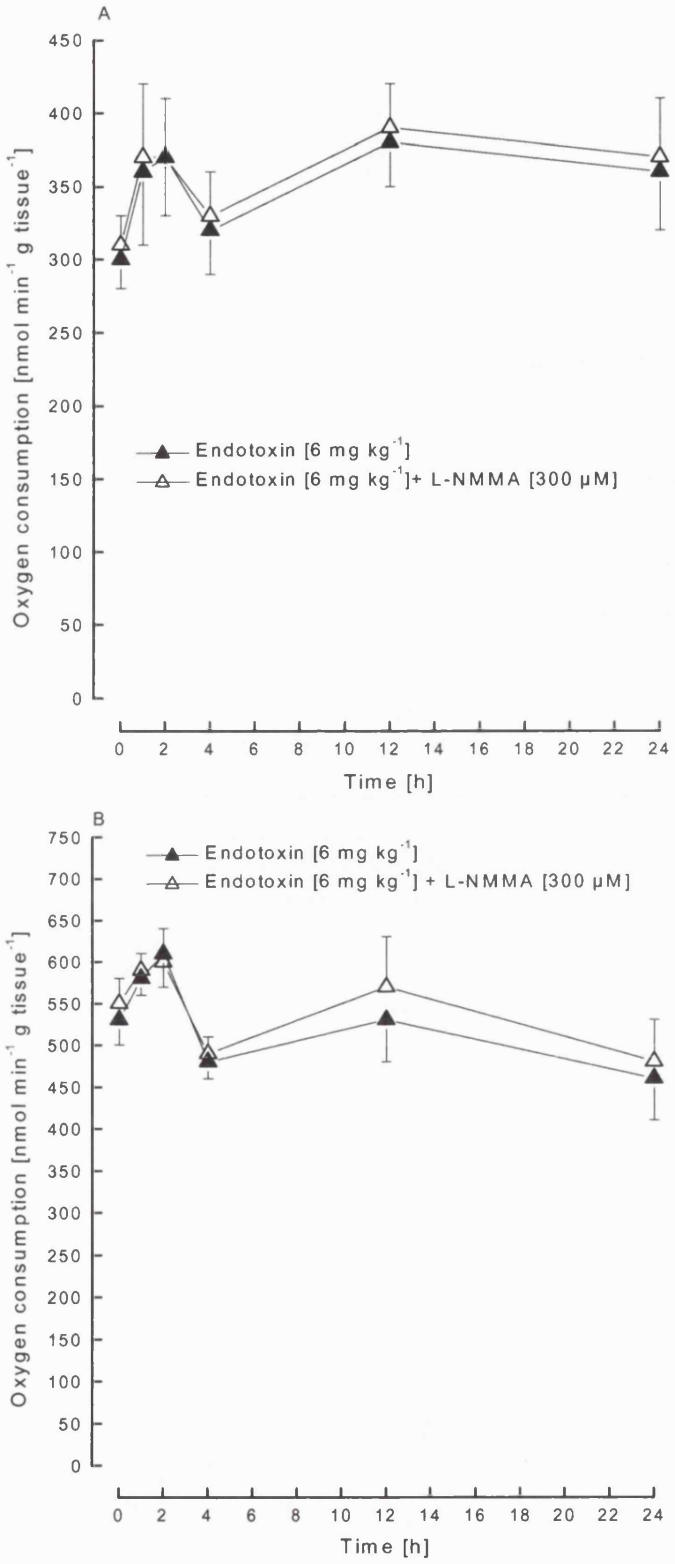

Figure 7.5 Effect of endotoxin (*E.coli,* 6 mg kg^{-1}) alone and with L-NMMA (300µM) on oxygen consumption (A) of the heart and (B) liver of iNOS mutant mice (n = 6).

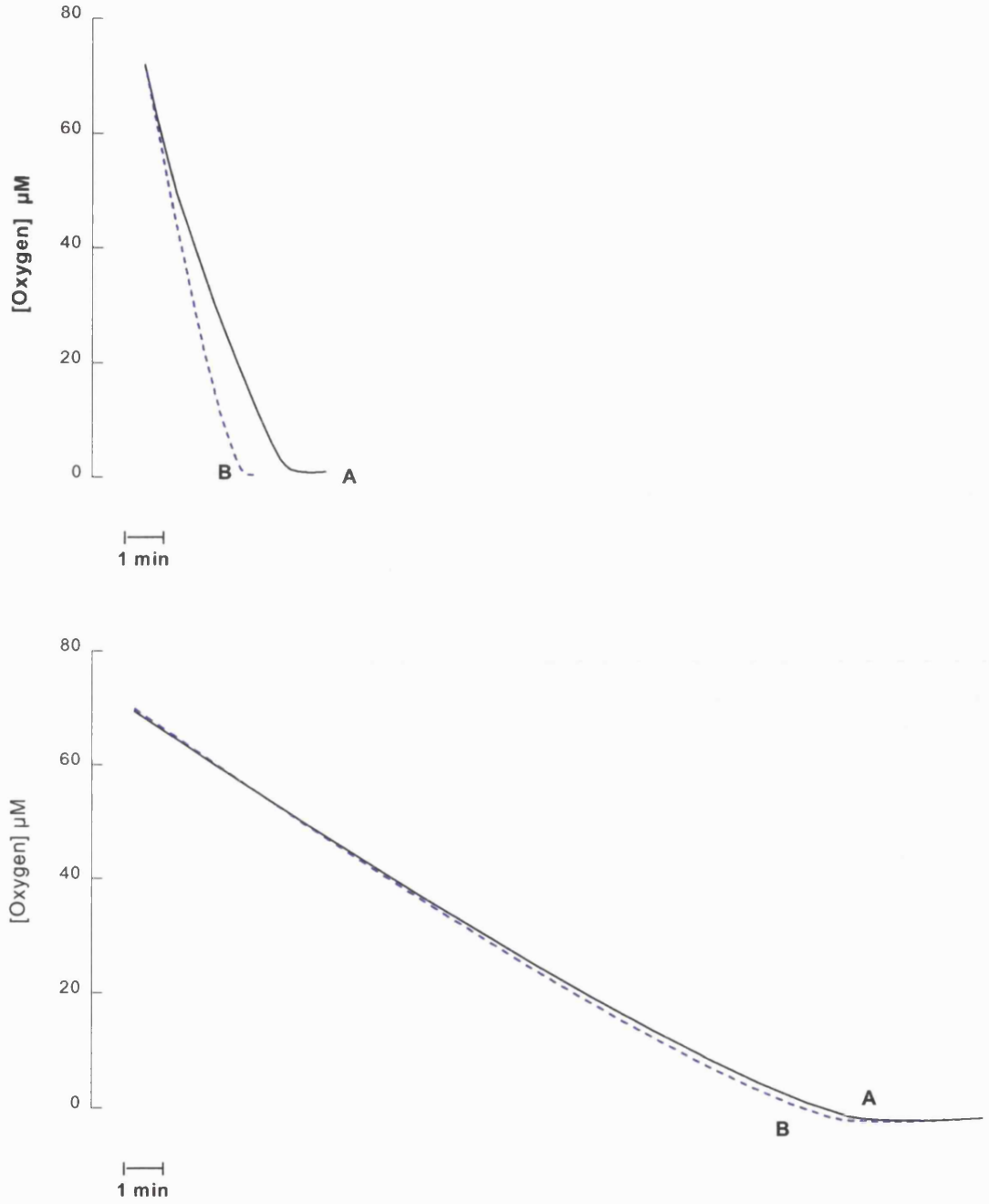

Figure 7.6 Example of oxygen consumption of the heart of iNOS wild type mice (A) in the absence and (B) in the presence of L-NMMA at time 0 h (upper panel) and 12 h (lower panel) following endotoxin (E.coli 6 mg kg^{-1}) administration.

7.3.5 INVOLVEMENT OF COMPLEX I FOLLOWING ENDOTOXIN ADMINISTRATION

Addition of L-NMMA (300 μM) alone increased baseline hepatic oxygen consumption in iNOS wild type mice (from 513 ± 15 to 543 ± 30 nmol min^{-1} g $tissue^{-1}$, $p < 0.001$, $n = 4$, Figure 7.7). Succinic acid (100 μM) alone also significantly increased baseline hepatic oxygen consumption in the iNOS wild type mice (from 513 ± 15 to 558 ± 14 nmol min^{-1} g $tissue^{-1}$, $p < 0.001$, $n = 4$, Figure 7.7). Addition of L-NMMA (300 μM) and succinic acid (100 μM) in combination further increased baseline hepatic oxygen consumption (from 513 ± 15 to 580 ± 22 nmol min^{-1} g $tissue^{-1}$; at 0 h, $p < 0.001$, $n = 4$, Figure 7.7) in iNOS wild type mice.

Following the low dose of endotoxin (6 mg kg^{-1}) at 18 h, addition of L-NMMA (300 μM) reversed the decrease in hepatic oxygen consumption (from 480 ± 20 nmol min^{-1} g $tissue^{-1}$ to 580 ± 4 nmol min^{-1} g $tissue^{-1}$, $n = 4$, Figure 7.7) in iNOS wild type mice. By contrast, succinic acid (100 μM) *in vitro* at 18 h had no significant effect on hepatic oxygen consumption (from 480 ± 20 to 497 ± 20 nmol min^{-1} g $tissue^{-1}$, $n = 4$, Figure 7.7) in iNOS wild type mice. Addition of L-NMMA (300 μM) and succinic acid (100 μM) in combination had no greater effect (589 ± 12 nmol min^{-1} g $tissue^{-1}$) than L-NMMA (300 μM) alone ($n = 4$, Figure 7.7) in iNOS wild type mice.

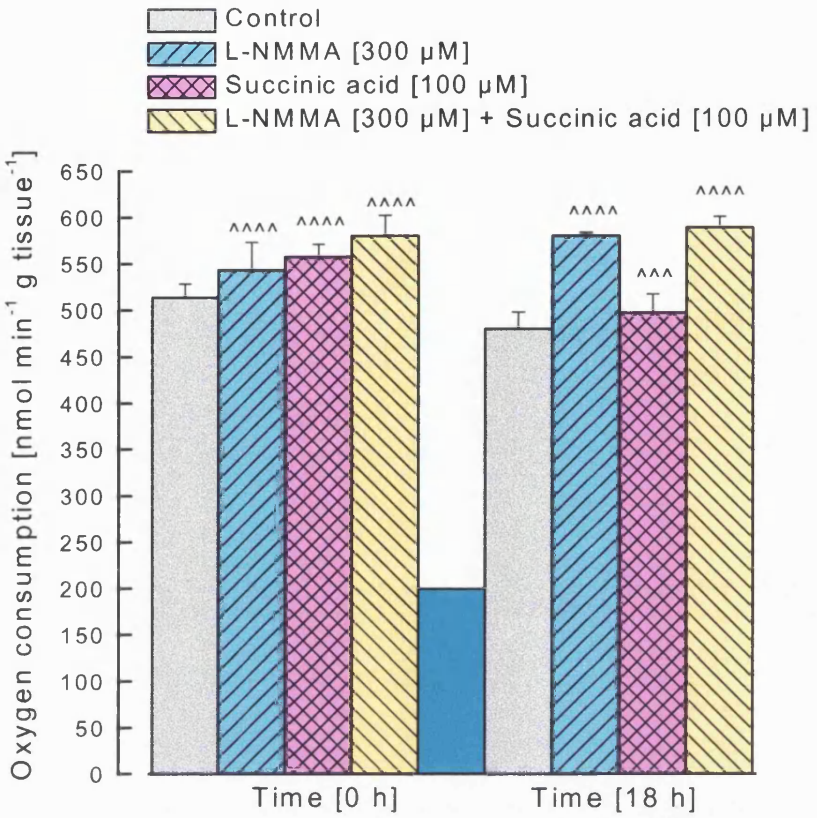

Figure 7.7 Effect of endotoxin (*E.coli,* 6 mg kg^{-1}) alone and in combination with L-NMMA (300 µM) and/or succinic acid (100 µM) on the oxygen consumption in the iNOS wild type mice. Statistical analysis; within the same group using Student t test. ^^^= $p < 0.005$, ^^^^ = $p < 0.001$ ($n = 4$).

7.4 DISCUSSION

A low dose of endotoxin caused a transient fall in blood pressure over the first two hours both in wild type and iNOS mutant mice, associated with an increase in plasma nitrate and nitrite and an increase in oxygen consumption. The observed early-stage increase in oxygen consumption is in agreement with previous studies showing a transient increase in respiratory activity in septic rats (Ohtoshi *et al.*, 1984) and in pigs with peritonitis (Hirai *et al.*, 1984). All these results are in accordance with previous studies in man showing that in the early phase of shock humoral,

haematological, metabolic and endocrine alterations occur (Suffredini *et al.*, 1989) resulting in a transient decrease in blood pressure and increase in oxygen consumption. These alterations generate an increase in eNOS activity (Salvemini *et al.*, 1989) and an increase in the metabolic rate (Fong *et al.*, 1990) and in the hypothalamic pituitary adrenal axis resulting in an increased production of adrenocorticotrophic hormone, cortisol, growth hormone and catecholamines (Wolff, 1973; Elin *et al.*, 1981). In addition, humoral inflammatory factors are generated including tissue plasminogen activator, coagulation proteins and activation of the kallikrein-kinin system leading to activation of inflammatory and hemodynamic responses (Suffredini *et al.*, 1989). Furthermore, cellular events occur involving the activation of mast cells, polymorphonuclear cells (neutrophils, eosinophils and basophils) and mononuclear cells (monocytes and lymphocytes). The chemotatic stimulation and phagocytosis of neutrophils activate the respiratory burst oxidase causing an increase in oxygen consumption (Baggiolini & Wymann, 1990). In the early stages of endotoxin shock the increase in nitrite/nitrate concentration in the plasma suggests that NO may have protective role such as maintaining the physiological vasodilator tone, during the catecholamine (Rees, 1995a) and oxygen bursts.

After the initial transient fall in blood pressure, there was a progressive fall in blood pressure leading to established shock (fall in MABP > 40 mmHg) in iNOS wild type mice, which appeared to reverse to some extent after 20 hours. There was a less dramatic fall in blood pressure in iNOS mutant mice (fall in MABP < 40 mmHg), which did not develop into established shock and

reversed after 20 hours to a larger extent. All iNOS wild type and mutant mice survived over the 24 hours, time course of the experiment.

The low dose of endotoxin (6 mg kg^{-1} *iv.*) elevated the concentration of plasma nitrite/nitrate reaching a maximum at 12 hour and declining thereafter in iNOS wild type mice. By contrast, in iNOS mutant mice a low dose of endotoxin (6 mg kg^{-1} *iv.*) elevated the concentration of plasma nitrite/nitrate only at 1 h. This increase in plasma nitrite/nitrate in the early stage was probably due to an increase in eNOS activity (Salvemini *et al.*, 1989).

These results confirm a previous study demonstrating that endotoxin induced the expression of iNOS leading to an increase in plasma nitrite/nitrate and a progressive fall in blood pressure (Rees *et al.*, 1998). Interestingly the iNOS mutant mice showed a fall in blood pressure albeit significantly reduced, indicating that other mediators were also involved in the cardiovascular changes following endotoxemia. Importantly the progression into established septic shock was dependent on the induction of iNOS following the low dose of endotoxin.

The elevation of plasma nitrite/nitrate was associated with a reversible decrease in myocardial and hepatic oxygen consumption. Addition of L-NMMA *in vitro* reversed the decrease in oxygen consumption, indicating that this inhibition was reversible over the time course of the experiment in iNOS wild type mice.

This low dose of endotoxin administered to iNOS mutant mice did not alter the concentration of nitrite and nitrate in the plasma (apart from the small increase at 1h) or the profile of the oxygen consumption. All these results

suggest that administration of low dose of endotoxin leads to an increase in NO production resulting in a fall in blood pressure and inhibition of myocardial and hepatic oxygen consumption.

Addition of L-NMMA alone or in combination with succinic acid, a substrate of complex II, reversed the inhibition of respiration over the 24 h of the experiments, suggesting a reversible inhibition of respiration by NO. This is in agreement with previous studies *in vitro* showing that short-term exposure of NO to isolated synaptosomes inhibited complex IV in a reversible manner (Brown & Cooper, 1994). Furthermore, NO added to isolated complex IV caused an immediate inhibition of oxygen consumption that was completely reversed when NO was removed (Brown & Cooper, 1994). In addition, short-term exposure of the skeletal muscle mitochondria to exogenous NO inhibited oxygen in a reversible manner (Cleeter *et al.*, 1994).

In summary the gradual and short-term generation of NO from iNOS following administration of a low dose of endotoxin is associated with a reversible increase in plasma nitrite/nitrate, accompanied by a reversible inhibition of mitochondrial respiration resulting in non-lethal shock.

CHAPTER 8

PATHOLOGICAL INHIBITION OF TISSUE RESPIRATION BY NITRIC OXIDE IN LETHAL ENDOTOXIN SHOCK

8 PATHOLOGICAL INHIBITION OF TISSUE RESPIRATION BY NITRIC OXDE IN LETHAL ENDOTOXIN SHOCK

8.1 SUMMARY

This study investigates the role of NO on myocardial and hepatic oxygen consumption in lethal endotoxin shock using iNOS mutant and wild type mice.

Following a high dose of endotoxin (12.5 mg kg^{-1} *iv.*) deaths occurred in both iNOS mutant and wild type mice. Most of the deaths in the iNOS mutant mice occurred at an earlier time point (12-16 h) than the wild type mice (12-24 h). However the overall mortality was greater in the wild type mice.

Administration of the high dose of endotoxin produced a progressive fall in blood pressure leading to established shock (fall in MABP > 40 mmHg) in the wild type mice and borderline shock in the iNOS mutant mice.

Administration of the high dose of endotoxin to wild type mice produced a rapid and sustained increase in the plasma concentration of nitrite/nitrate. This was associated with a progressive decrease in myocardial and hepatic oxygen consumption. The ability of L-NMMA to reverse the decrease in oxygen consumption lessened with time and was abolished by 18 h. By contrast at 18 h, addition of L-NMMA with succinic acid, a substrate of complex II, reversed the decrease in oxygen consumption, while the addition of succinic acid alone had no significant effect.

The high dose of endotoxin had no significant effect on plasma nitrite/nitrate concentration or on the decrease in oxygen consumption in iNOS mutant mice.

These results demonstrate that following a high dose of endotoxin, the overproduction of NO causes pathological inhibition of tissue oxygen consumption due, at least in part, to inhibition of complex I of the respiratory chain. This was associated with a significant number of deaths.

8.2 INTRODUCTION

Despite improvement in intensive care and antibiotic therapy in sepsis patients, mortality has remained relatively constant over the last few decades. It has been estimated that approximately one million patients develop sepsis each year in the USA alone. More than 50 % of these patients develop septic shock with an associated mortality of 40-60 % (Brun-Buisson, 2000). The pathological mechanism for the development of multiple organ failure and subsequent death is still poorly understood due to the complexity of the inflammatory mediators released. Different theories have been postulated to explain why organs fail during septic shock including localised defects in organ blood flow as a result of hypotension and the obstruction of nutrient capillaries by micro-thrombi and aggregated neutrophils (Lam *et al.*, 1994), whereas others suggest that there is a direct cellular inability to consume oxygen despite an adequate supply (Fink & Payen, 1996). It is likely that defects of both systems contribute to the development of multiple organ dysfunction and death. Indeed as detailed previously (Chapter 7) a low dose of endotoxin leads to the development of shock associated with a short-term elevation of plasma nitrite and nitrate, a short-term inhibition of tissue respiration and low blood pressure. Yet no deaths were observed over the time course of the studies (Chapter 7). This

suggests that more dramatic changes are required to the cardiovascular and cellular respiratory systems to progress into multi organ failure and death.

The present study investigates the role of NO on mitochondrial respiration in a lethal septic shock model *ex vivo*. Myocardial and hepatic oxygen consumption was determined using iNOS wild type and mutant mice, following administration of a lethal dose of endotoxin.

8.3 RESULTS

8.3.1 INDUCTION OF LETHAL ENDOTOXIN SHOCK AND SURVIVAL PROFILE

A preliminary series of experiments were carried out to study the survival profile following the administration of a low and high dose of endotoxin (*E. coli i.v.* 6 and 12.5 mg kg^{-1}). Following administration of the low dose of endotoxin (6 mg kg^{-1} *i.v)* there were no deaths during the 24 h of observation in iNOS mutant and wild type mice (Chapter 7, Figure 8.1). Following administration of a higher dose of endotoxin (12.5 mg kg^{-1} *i.v.*) deaths occurred in both iNOS mutant and wild type mice. However, deaths occurred earlier in the iNOS mutant mice (12-16 h) than the wild type mice (12-24 h, Figure 8.1). By contrast, only 7 % of wild type mice survived after 24 h whereas 52 % of the iNOS mutant mice survived after 24 h (Figure 8.1).

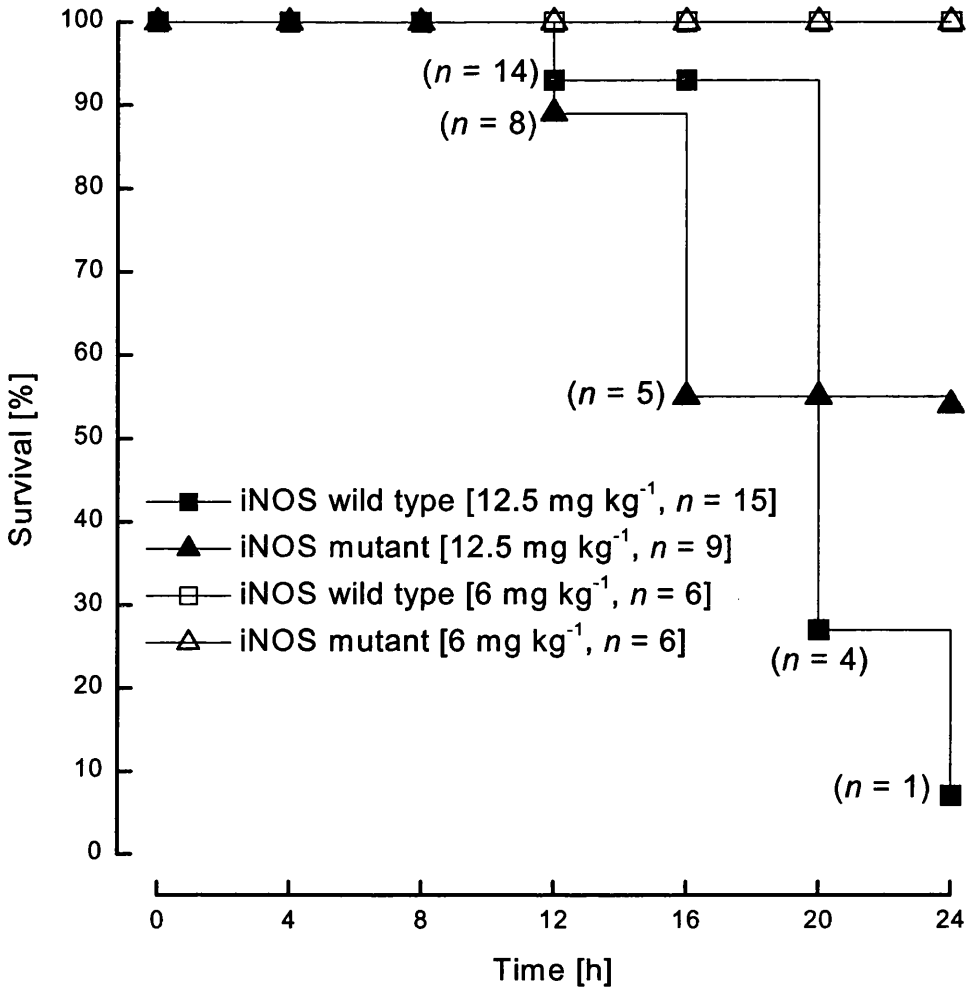

Figure 8.1 Kaplan-Meier plot of the effect of endotoxin (*E.coli* 6 mg kg^{-1} and 12.5 mg kg^{-1}) on the survival profile of iNOS mutant and wild type mice.

8.3.2 BLOOD PRESSURE PROFILE

Administration of endotoxin (12.5 mg kg^{-1}) in wild type mice caused a small but rapid fall in blood pressure with 2 h. Thereafter there was a progressive fall in blood pressure after ~ 4 h, from the basal MABP of 109 ± 4 mmHg to 54 ± 3 mmHg at 24h (n = 6, $p < 0.001$, Figure 8.2) leading to established shock (fall in MABP > 40 mmHg). The fall in blood pressure was significantly reduced in iNOS mutant mice from the basal MABP of 114 ± 3 mmHg to 74

± 4 mmHg at 24 h (n = 6, $p < 0.001$, Figure 8.2) such that these animals progressed into only "borderline" shock.

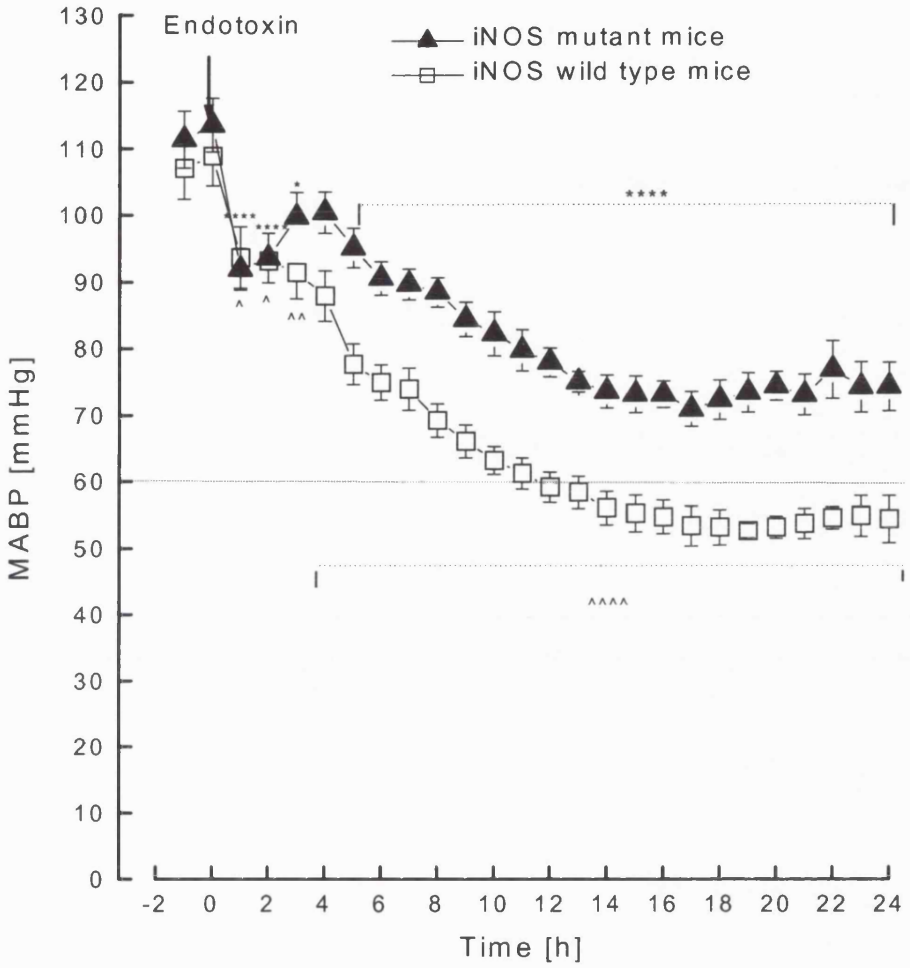

Figure 8.2 Effect of endotoxin (*E.coli* 12.5 mg kg^{-1}) on mean arterial blood pressure (MABP) in conscious iNOS mutant and wild type mice. Statistical analysis; within group analysis using one way ANOVA followed by Bonferroni test; *,^ $p < 0.05$; ^^ $p < 0.01$ and ; ****,^^^^ $p < 0.001$ (n = 6). MABP below 60 mmHg is associated with death.

8.3.3 SUSTAINED ELEVATION OF PLASMA NITRITE/NITRATE

Following administration of the high dose of endotoxin (12.5 mg kg^{-1} *i.v*) the elevation of the concentration of nitrite/nitrate was more rapid (239 ± 4 µM at

4 h, $p < 0.001$, n = 4-6, Figure 8.3) and sustained (596 ± 28 µM at 18 h, $p < 0.001$, n = 4-6, Figure 8.3) compared to the low dose (6 mg kg^{-1} *i.v,* Chapter 7). There was no elevation in plasma nitrite/nitrate concentration in the iNOS mutant mice (n =4-6, Figure 8.3).

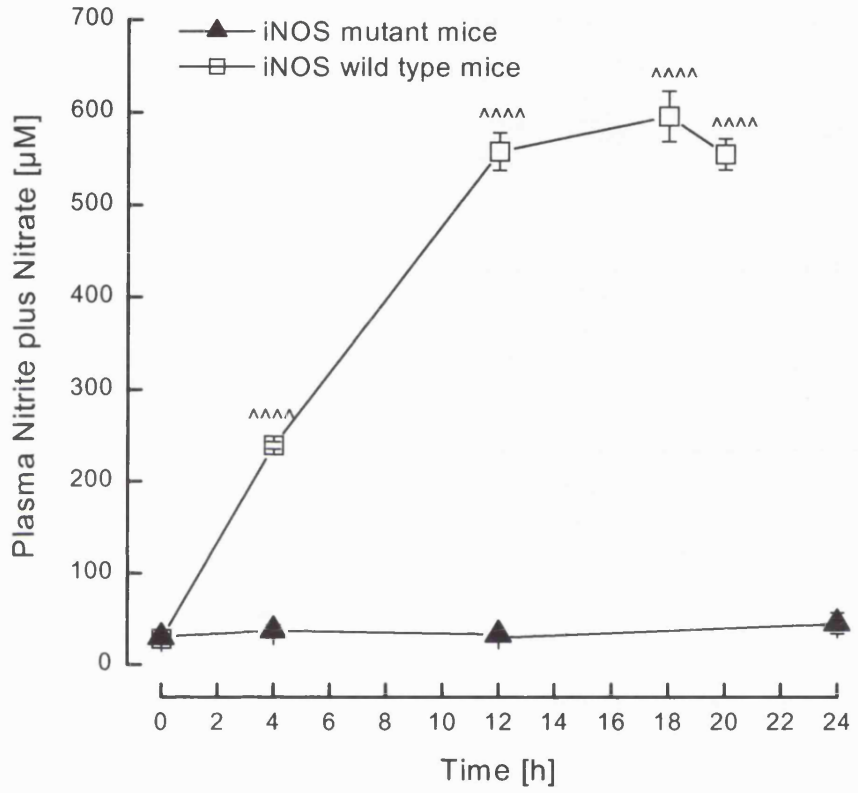

Figure 8.3 Effect of endotoxin (*E.coli*, 12.5 mg kg^{-1}) on the concentration of plasma nitrite and nitrate in iNOS mutant and wild type mice. Statistical analysis: ^ within group analysis, ^^^^ = $p < 0.001$ (n =4-6).

8.3.4 IRREVERSIBLE INHIBITION OF TISSUE RESPIRATION

Following the lethal dose of endotoxin (12.5 mg kg^{-1} *i.v.*) myocardial (320 ± 20 nmol min^{-1} g $tissue^{-1}$, n = 4-6, Figure 8.4 A) and hepatic oxygen consumption (550 ± 30 nmol min^{-1} g $tissue^{-1}$, n = 4-6, Figure 8.4 B)

decreased progressively (220 ± 10, and 360 ± 30 nmol min^{-1} g $tissue^{-1}$ at 4 h, $p < 0.001$, 90 ± 10 and 180 ± 6 nmol min^{-1} g $tissue^{-1}$ at 20 h, $p < 0.001$ for heart and liver, respectively, *n* = 4-6, Figure 8.4 A, B and Figure 8.6) in the wild type mice.

The ability of L-NMMA (300 μM) to reverse the decrease in oxygen consumption lessened with time (260 ± 20 and 410 ± 20 nmol min^{-1} g $tissue^{-1}$ at 4 h, heart and liver respectively; 100 ± 10 and 170 ± 10 nmol min^{-1} g $tissue^{-1}$ at 20 h, heart and liver, respectively, *n* = 4-6, Figure 8.4 A, B).

Addition of D-NMMA did not significantly alter the oxygen consumption in either the heart (by 1.2 ± 0.4 and 0.9 ± 0.5 nmol min^{-1} g $tissue^{-1}$ at 4 h and by 1.3 ± 0.6 and 1.2 ± 0.4 nmol min^{-1} g $tissue^{-1}$ at 12 h, NS, *n* = 4) or the liver (by 1.4 ± 0.5 and 1.1 ± 0.3 nmol min^{-1} g $tissue^{-1}$ at 4 h and by 1.4 ± 0.6 and 1.6 ± 0.7 nmol min^{-1} g $tissue^{-1}$ at 12 h, NS, *n* = 4) of the wild type and mutant mice, respectively.

There was no significant decrease in oxygen consumption in the iNOS mutant mice (260 ± 20 and 480 ± 20 nmol min^{-1} g $tissue^{-1}$ at 4 h, NS; 290 ± 30 and 490 ± 20 nmol min^{-1} g $tissue^{-1}$ at 24 h, heart and liver, respectively, *n* = 6, Figure 8.5 A, B). Addition of L-NMMA (300 μM) did not increase oxygen consumption in the iNOS mutant mice at any time point when compared to baseline (*n* = 6, Figure 8.5 A, B).

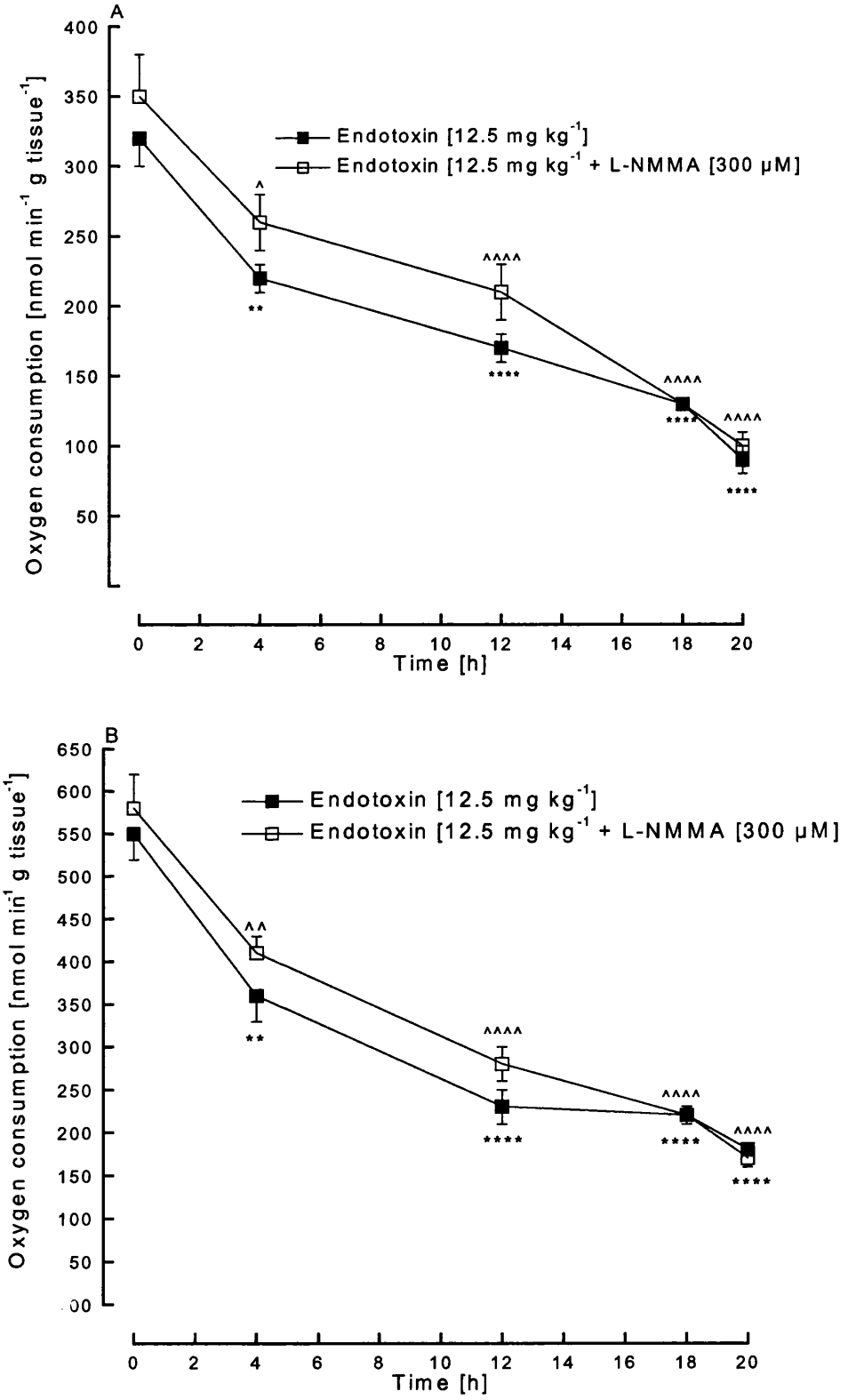

Figure 8.4 The inability of L-NMMA to reverse the endotoxin-induced decrease (*E.coli,* 12.5 mg kg^{-1}) in oxygen consumption in the heart (A) and the liver (B) of iNOS wild type mice. Statistical analysis; within group analysis using one way ANOVA followed by Bonferroni test; ^ $p < 0.05$; ^^ $p < 0.01$ and; ****,^^^^ $p < 0.001$ (n = 4-6).

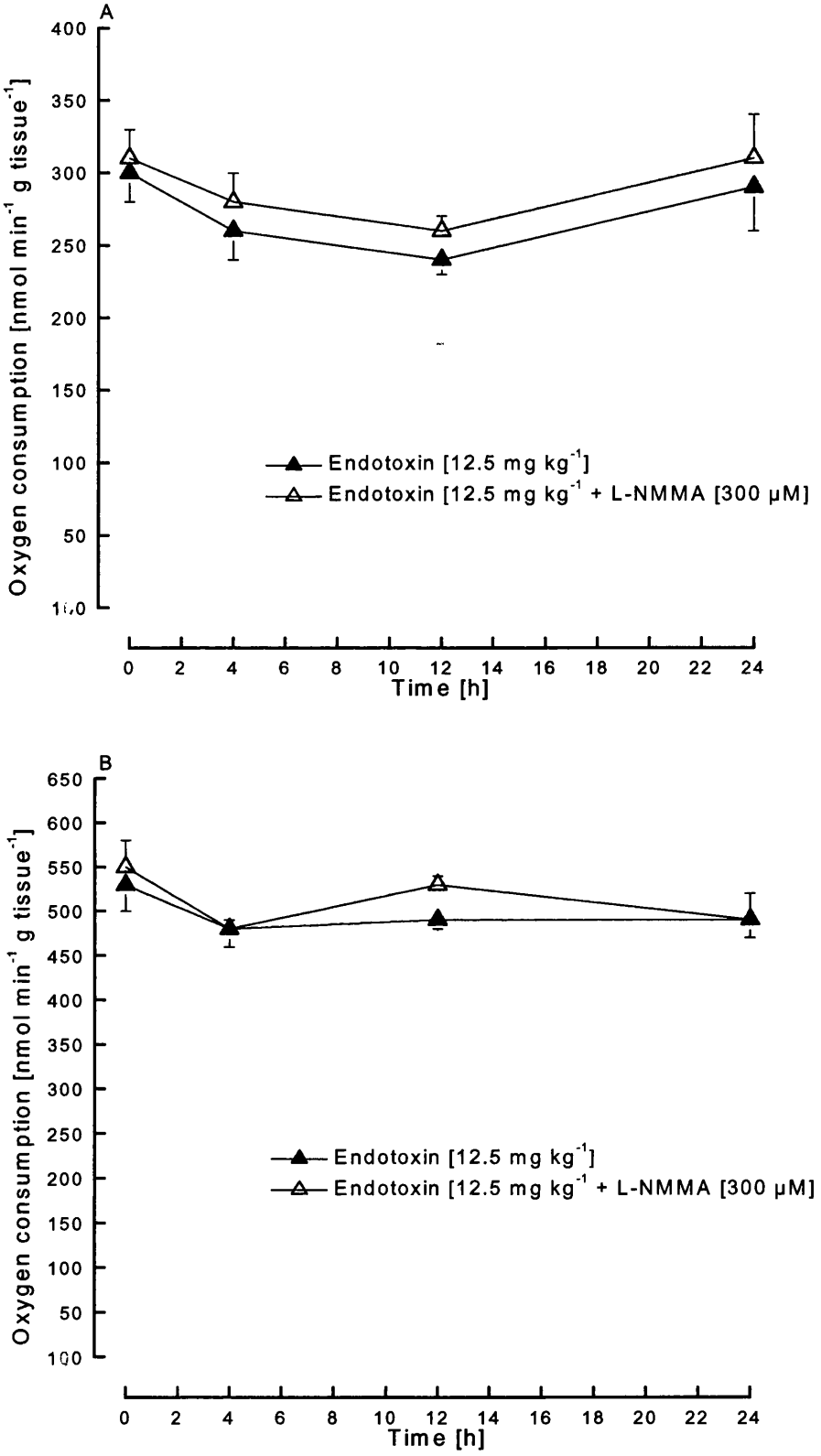

Figure 8.5 Effect of endotoxin (*E.coli* 12.5 mg kg^{-1}) on the oxygen consumption of the heart (A) and the liver (B) of iNOS mutant mice (n = 6).

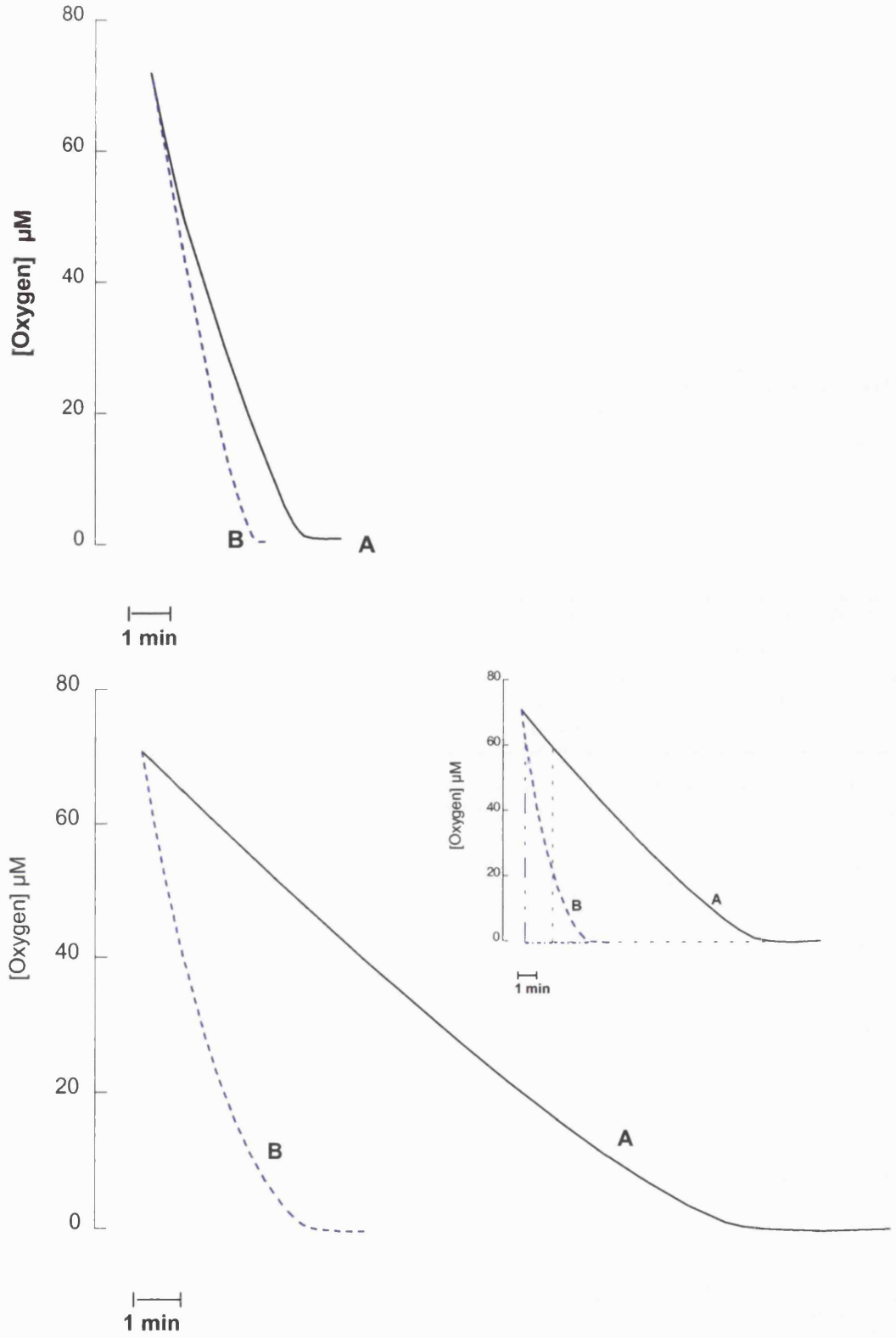

Figure 8.6 Example of oxygen consumption of the chopped heart of iNOS wild type mice (A) in the absence and (B) in the presence of L-NMMA at time 0 h (upper panel) and 18 h (lower panel) following endotoxin (E.coli 12.5 mg kg^{-1}) administration.

8.3.5 INHIBITION OF COMPLEX I FOLLOWING ENDOTOXIN ADMINISTRATION

Addition of L-NMMA (300 μM) alone or in combination with succinic acid (100 μM) increased baseline hepatic oxygen consumption in iNOS wild type mice (Chapter 7, Figure 8.7).

Following the lethal dose of endotoxin (12.5 mg kg^{-1}), neither L-NMMA (300 μM) nor succinic acid (100 μM) alone had any significant effect on the hepatic oxygen consumption at 18 h (220 ± 20, 220 ± 4 and 225 ± 40 nmol min^{-1} g $tissue^{-1}$, in control, L-NMMA, and succinic acid treated respectively, *n* = 4, Figure 8.7) in iNOS wild type mice. Whereas, addition of L-NMMA (300 μM) and succinic acid (100 μM) in combination at 18 h reversed the decrease in hepatic oxygen consumption (from 220 ± 20 to 428 ± 6 nmol min^{-1} g $tissue^{-1}$, *n* = 4, Figure 8.7) in iNOS wild type mice.

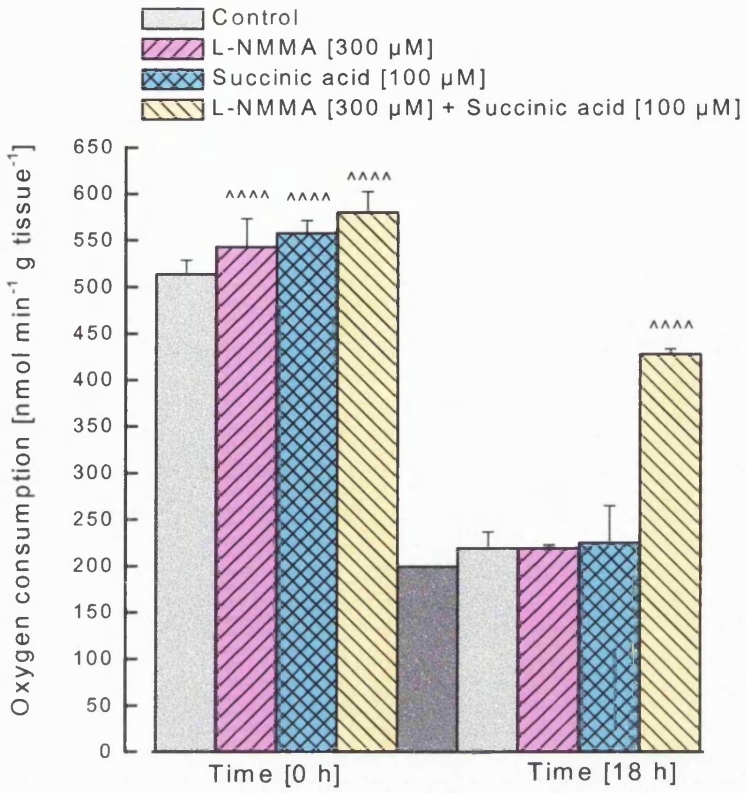

Figure 8.7 Effect of endotoxin (*E.coli,* 12.5 mg kg^{-1}) alone and in combination with L-NMMA (300 µM) and/or succinic acid (100 µM) on the oxygen consumption in iNOS wild type. Statistical analysis; within the same group using Student t test: ^^^= $p < 0.005$, ^^^^ = $p < 0.001$ ($n = 4$).

8.3.6 INHIBITION OF TISSUE RESPIRATION AFTER DEATH

In an attempt to assess the alterations in oxygen consumption after death of the mice, measurements were performed at various time points after cervical dislocation. The hepatic oxygen consumption after cervical dislocation decreased with time (550 ± 30, 250 ± 9, 185 ± 9, 147 ± 11 and 60 ± 6 nmol min^{-1} g $tissue^{-1}$ at 0 h, 45 min, 1 h, 1.5 h and 3 h respectively, $n = 4$, Figure 8.8) in the wild type mice.

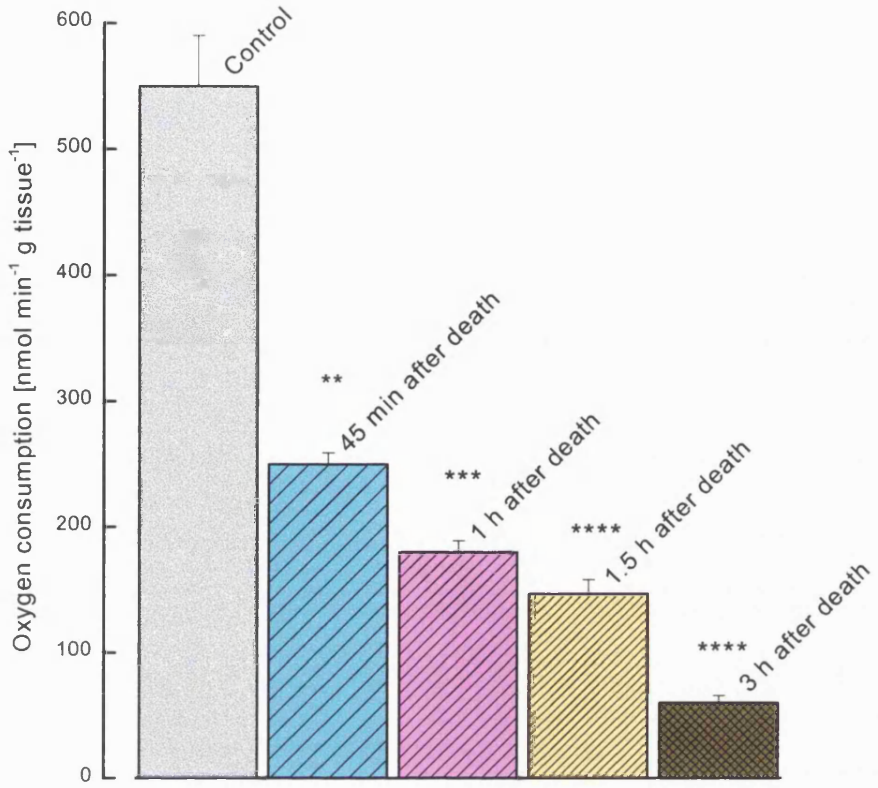

Figure 8.8 Hepatic oxygen consumption of iNOS wild type mice, at 0 h, 45 min, 1 h and 1.5 h after death, Statistical analysis versus the control using Student t test, ** = $p < 0.01$, *** = $p < 0.005$ and **** = $p < 0.001$ ($n = 4$).

The myocardial oxygen consumption after cervical dislocation also decreased with time (320 ± 20 and 88 ± 20 nmol min^{-1} g $tissue^{-1}$ at 0 h and 1 h, respectively , $n = 4$, Figure 8.9) in the wild type mice. The myocardial and hepatic oxygen consumption 1h after cervical dislocation ($n = 4$, Figure 8.8) is similar to that observed 20 h after the administration of the high dose of endotoxin (90 ± 10 and 180 ± 6 nmol min^{-1} g $tissue^{-1}$ in the heart and liver, respectively, $n = 4$, Figure 8.9). The addition of L-NMMA (300 μM) 1 h after cervical dislocation did not increase the myocardial and hepatic oxygen consumption (from 88 ± 20 to 89 ± 30 nmol min^{-1} g $tissue^{-1}$ in the heart and from 185 ± 9 to 184 ± 20 nmol min^{-1} g $tissue^{-1}$ in the liver, $n = 4$).

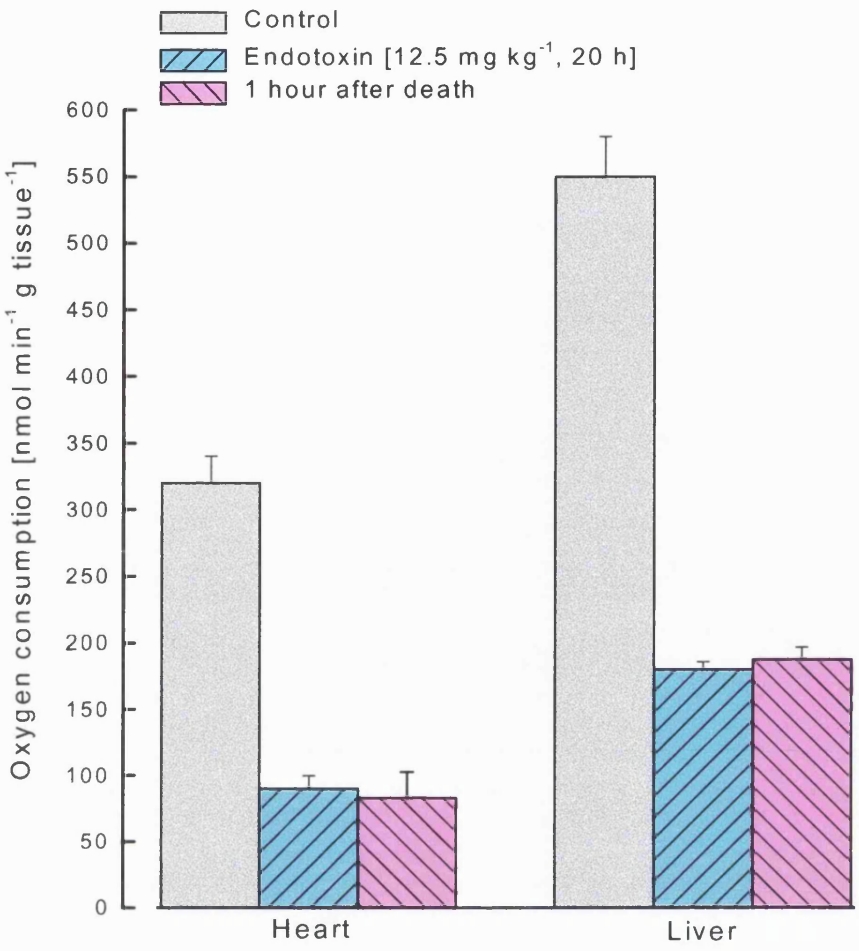

Figure 8.9 Comparison of oxygen consumption in the heart and the liver of iNOS wild type mice, immediately after death (control 0 h), 1 h after death and 20 h after endotoxin (12.5 mg kg^{-1}, n = 4).

8.4 DISCUSSION

Administration of a high dose of endotoxin in the iNOS mutant and wild type mice generated an initial fall in blood pressure, which is possibly associated with the activation of eNOS and other mediators (Chapter 7). Thereafter there was a progressive fall in blood pressure leading to established shock (fall > 40 mmHg) in wild type mice. The fall in blood pressure in iNOS mutant mice was reduced and progressed only into "borderline" shock,

suggesting that after a high dose of endotoxin a component of the fall in blood pressure was due to the release of several mediators such as histamine, serotonin and platelet activating factor, whereas a large component of the fall in blood pressure was due to overproduction of NO from iNOS. These results are in agreement with a previous study showing a decrease in blood pressure following endotoxin (8.75 mg kg^{-1}; Rees, 1999a) whereby deaths were associated with a sustained fall in blood pressure below 60 mmHg. Interestingly, following the high dose of endotoxin deaths occurred in both iNOS mutant and wild type mice. The deaths occurred earlier in the iNOS mutant mice than in the wild type. A possible explanation for this may be that iNOS mutant mice suffer excessive vasoconstriction of some vascular beds in the early stages of shock due to the lack of the counterbalancing vasodilator action of NO. However further studies are required to investigate this. Interestingly more deaths occurred in the wild type mice, which appeared to be due, in part, to a sustained low blood pressure.

Administration of a high dose of endotoxin caused a rapid and sustained increase in the concentrations of nitrite and nitrate in the plasma over the study period. This was associated with an irreversible inhibition of the respiration both in the heart and the liver of the wild type mice. The ability of L-NMMA to reverse the inhibition of myocardial and hepatic oxygen consumption lessened with time until it became ineffective at 18-20 hours, suggesting an irreversible inhibition of the respiratory chain by NO. These results and those presented in chapter 7 indicate that the concentration and the duration of NO production is important in determining whether reversible

inhibition of cellular respiration and survival occurs or whether irreversible inhibition of cellular respiration and increased mortality occurs. Interestingly both doses of endotoxin lead to a progression into established shock suggesting that sustained inhibition of cellular respiration is the key factor that determines mortality.

All these results are in agreement with previous studies in humans, reporting differences in oxygen consumption depending on the stage and the status of septic shock. Patients with non-lethal septic shock (survivors) have a greater oxygen extraction and consumption, despite a lower cardiac index and lower oxygen delivery than more severely ill patients (non-survivors) (Kreymann *et al.*, 1993). In addition, survivors are characterised by an ability to increase both the oxygen delivery and consumption. By contrast, non-survivors mostly failed to increase oxygen consumption not only following fluid resuscitation, but also when delivery was enhanced with aggressive inotropic support with dobutamine (Bouchier-Hayes *et al.*, 1997). This provides further evidence that the inability to increase oxygen consumption in lethal septic shock is probably related to an impairment in oxygen extraction and an inhibition of the respiratory chain. This is confirmed by an earlier study using muscle mitochondria of septic shock patients, which showed an inhibition of cellular respiration at the level of complex I (Poderoso *et al.*, 1978). In addition endotoxin-induced shock has been associated with inhibition of complex I and II in the heart of baboons (Gellerich *et al.*, 1999).

In the present study, 18 hours after administration of a lethal dose of endotoxin, the addition of succinic acid, a substrate of complex II, did not

increase the oxygen consumption suggesting that the respiration was inhibited downstream of complex II. Addition of L-NMMA alone did not reverse these effects, whereas the addition of L-NMMA in combination with succinic acid significantly reversed the inhibition. This suggests that overproduction of NO caused an irreversible inhibition in the proximity of complex I and a reversible inhibition possibly at the level of complex IV. The inability of L-NMMA and succinic acid to completely reverse the inhibition of respiration may suggest the involvement of other inhibitory mediators such as reactive oxygen species released during endotoxin shock.

These results are in accordance with a previous study showing that in muscle biopsies and blood samples taken from septic shock patients there was an increase in NO production and a depression of mitochondrial enzyme activity, predominantly in complex I and complex IV (Singer & Brealey, 1999).

Thus it is likely that following a low dose of endotoxin the gradual and short-term production of NO led to a reversible inhibition of cellular respiration and survival (Chapter 7). Whereas following a high dose of endotoxin the rapid and more sustained production of NO led to irreversible inhibition of cellular respiration and death. These results are in agreement with previous studies showing that short-term exposure of exogenous NO to isolated synaptosomes (Brown & Cooper, 1994) and to skeletal muscle mitochondria inhibits oxygen consumption in a reversible manner (Cleeter *et al.*, 1994). Whereas long-term exposure to NO of Jurkat, L929 and J774 cells results in a reversible inhibition of complex IV and an irreversible inhibition of complex I activity (Chapters 3, 4 and 5). The irreversible inhibition is proceeded by a

decrease in GSH (Chapters 4 and 5) and interestingly a decrease in GSH has been reported in septic shock patients (Brealey *et al.*, 1999). This suggests that GSH plays an important role in modulating NO-mediated inhibition of cellular respiration *in vitro* (Chapters 4 and 5) and *ex vivo* (Chapter 8). Furthermore the addition of GSH to cells exposed to NO can prevent or reverse the inhibition of respiration (Chapter 4 and 5). This suggests that GSH administration in combination with NO synthase inhibitors may be the optimum therapy to reverse the fall in blood pressure and the cytopathic dysoxia associated with inhibition of the respiratory chain and may lead to an improvement in the survival profile.

Thus the fall in blood pressure and the cytopathic dysoxia characteristic of septic shock is due at least in part to overproduction of NO, that leads to a defect in both oxygen delivery, from inadequate blood flow, and oxygen extraction, by suppression of cellular respiration. If the maldistribution of blood flow and the cytopathic dysoxia is prolonged this may lead to multiple organ failure and ultimately death.

CHAPTER 9

GENERAL DISCUSSION

9 GENERAL DISCUSSION

Over the last decade nitric oxide has been shown to play a wide range of physiological and pathological roles including regulation and inhibition of the mitochondrial respiratory chain. NO has been shown to compete with oxygen at complex IV and to reversibly inhibit this enzyme activity, even at nanomolar concentrations (Brown & Cooper, 1994). Studies *in vitro* show that NO causes rapid and oxygen-dependent inhibition of respiration at complex IV in neurones (Brown & Cooper, 1994), astrocytes (Brown *et al.*, 1995) and pancreatic β-cells (Laffranchi & Spinas, 1996). In addition, inhibition of NO synthase in lung alveolar type II cells increases cellular oxygen consumption, while an NO donor produces an opposing effect, suggesting that constitutive NO production inhibits respiration (Miles *et al.*, 1996). Furthermore, the basal release of NO, in primary cultures of endothelial cells, is sufficient to partially inhibit endothelial cell respiration, to the extent that inhibition of NO synthase led to an increase of respiration (Clementi *et al.*, 1999). These results suggest that physiological NO regulates the cellular respiration *in vitro*.

In pathological conditions macrophages (Hibbs, Jr. *et al.*, 1988) and several other cells including astrocytes (Bolanos *et al.*, 1994), hepatocytes (Stadler *et al.*, 1991), smooth muscle cells (Geng *et al.*, 1992) and myocytes (Oddis & Finkel, 1995) activated with cytokines and endotoxin produce cytotoxic substances that inhibit cellular respiration. Although these activated cells produce a variety of potentially toxic substances their toxic proprieties have been associated with the expression of iNOS leading to a sustained NO

production. Indeed inhibitors of NOS or scavengers of NO prevent this cytotoxic activity (Hibbs, *et al.*, 1988; Drapier *et al.*, 1988; Stuehr & Nathan, 1989). However, it is difficult to define the specific mechanism underlying the inhibition of the respiration because activated cells release other toxic mediators.

In an attempt to clarify the specific pathological role of NO in inhibiting respiration, cells were exposed to exogenous NO over long periods. Studies in this thesis show that long-term exposure of exogenous NO to different cells (Jurkat, L929 and J774) inhibits cellular respiration reversibly by oxyHb at the level of complex IV (Chapter 3 and Chapter 5) and irreversibly by oxyHb at the level of complex I. These results are in accordance with previous studies in which long-term exposure of neurones to exogenous NO inhibited complexes IV II-III and I (Bolanos *et al.*, 1996). Long-term exposure of NO to the cells leads to a decrease in reduced glutathione concentration that precedes the inhibition at the level of complex I (Chapter 4 and Chapter 5). This is accordance with previous studies suggesting an important role of GSH in modulating NO-mediated cellular damage (Radi *et al.*, 1991; Bolanos *et al.*, 1995; Bolanos *et al.*, 1996, Almeida *et al.*, 1998, Clementi *et al.*, 1998). The inhibition is reversed by addition of reduced glutathione or cold light, suggesting that S-nitrosylation of thiols is necessary for the activity of the enzyme. This is in accordance with a previous study in which incubation of neurones for 24 h with S-nitroso-N-acetylpenicillamine caused a decrease in GSH concentrations accompanied by inhibition of respiration (Bolanos *et al.*, 1996).

The inhibition of cellular respiration observed following exposure of cells to high concentrations of NO is accompanied by an increased oxygen consumption of the extracellular medium (Chapter 5). This increase is not restricted to cells exposed to exogenous NO since it is also observed after activation of the cells with IFN-γ and endotoxin in the presence of L-arginine. This suggests that extracellular oxygen consumption is an event specifically dependent on NO (Chapter 5). Thus, following exposure of cells to exogenous or endogenous NO for several hours there is a decrease in oxygen utilisation due to two distinct actions, one being the inhibition of cellular oxygen consumption and the other, the generation of a hypoxic microenvironment due to the oxygen consumption by the extracellular medium. Whether such a situation occurs *in vivo* requires further investigation since such a synergistic deleterious effect may play a part in some clinical conditions including septic shock. However if this situation occurred *in vivo* it would be relatively localised since systemic PO2 may be normal or even elevated in septic shock patients (Boekstegers *et al.*, 1991). Previous studies have provided evidence that physiological NO regulates the respiratory chain *in vitro*. Studies in this thesis have investigated the physiological role of NO as a regulator of cellular respiration using an inhibitor of NO synthase in eNOS and iNOS mutant and wild type mice (Chapter 6). Myocardial and hepatic oxygen consumption significantly increase following addition of L-NMMA *in vitro* in eNOS and iNOS wild type and iNOS mutant mice but not in eNOS mutant mice. These results suggest that the basal generation of NO produced by eNOS regulates the mitochondrial respiratory chain physiologically *ex vivo* (Chapter 6). This is in

accordance with previous studies *in vitro* as described above and with studies *in vivo.* In this regard administration of an inhibitor of NO synthase (L-N-nitroarginine) increases myocardial oxygen consumption in dogs during exercise (Ishibashi *et al.*, 1998) and in whole body oxygen consumption in conscious dogs (Shen *et al.*, 1994) that is independent of blood flow changes. More recent experiments have shown that, bradykinin (presumably by stimulating the release of NO from eNOS) induces a significant decrease in myocardial oxygen consumption from iNOS mutant mice and eNOS wild type mice, but not from eNOS mutant mice (Loke *et al.*, 1999).

Thus basal NO plays a physiological role in regulating mitochondrial respiration (Chapter 6). However further studies are required to demonstrate the source of NO, which may be generated from eNOS or nNOS (Andries *et al.*, 1998) or directly from the mitochondria (Bates *et al.*, 1995; Kobzik *et al.*, 1995, Giulivi *et al.*, 1998). The latter is an interesting possibility since the half life of NO is relatively short *in vivo* and NO produced by a calcium dependent type mitochondrial NOS, would be more likely to regulate respiration. In this regard the role of NO may be important by acting as a limiting step in the consumption of oxygen in physiological conditions. This "NO-break" of respiration may become less efficient in conditions in which more ATP would be required such as during exercise, since under these conditions the muscle contracts and calcium is sequestered, with less available for other activities such as constitutive NOS activation. It will be interesting to see whether tissues, such as skeletal muscle and the heart, that have the ability to increase their ATP

concentrations rapidly have a greater basal generation of NO and thus a higher degree of "active respiratory chain suppression". If this is the case it would provide a sensitive control system by which the cells can rapidly alter the generation of ATP.

Although relatively low concentrations of NO have been shown to physiologically regulate cellular respiration *in vitro* (Brown & Cooper, 1994) and *ex vivo* (Chapter 6), higher concentrations of NO have been shown to pathologically inhibit cellular respiration *in vitro* (Chapter 3, Chapter 4 and Chapter 5). Studies in this thesis have demonstrated that high concentrations of NO generated in endotoxin shock pathologically inhibit cellular respiration (Chapter 7 and Chapter 8). A low dose of endotoxin causes a transient fall in blood pressure over the first two hours both in wild type and iNOS mutant mice, associated with a small increase in NO concentration and a small increase in oxygen consumption (Chapter 7). This early increase in oxygen consumption has been reported previously and possibly reflects the generation of a variety of mediators or cytokines that leads to a respiratory burst. After the initial transient fall in blood pressure, the administration of the low dose of endotoxin to iNOS wild type mice leads to a short-term generation (up to 12 h) of high concentrations of NO associated with a short-term inhibition (up to 12 h) of mitochondrial respiration resulting in non-lethal shock. These results confirm a previous study demonstrating that endotoxin induces the expression of iNOS leading to an increase in NO concentrations and a progressive fall in blood pressure (Rees *et al.*, 1998). Interestingly the iNOS mutant mice do not show any changes in NO production or changes in cellular respiration whereas they

exhibit a fall in blood pressure (albeit significantly reduced), indicating that other mediators are involved in the hypotension following endotoxemia. Importantly the progression into established septic shock was dependent on the induction of iNOS (Chapter 7).

Administration of a high dose of endotoxin in the iNOS mutant and wild type mice also generates an initial fall in blood pressure, which is possibly associated with the activation of eNOS and other mediators (Chapter 8). Thereafter there is a progressive fall in blood pressure leading to lethal established shock (fall > 40 mmHg) in wild type mice. The fall in blood pressure in iNOS mutant mice progresses only into "borderline" shock. Interestingly, following the high dose of endotoxin, deaths occurred in both iNOS mutant and wild type mice. The deaths occurred earlier in the iNOS mutant mice than in the wild type mice. Surprisingly the deaths occurred without a substantial fall in the blood pressure in iNOS mutant mice. A possible explanation for this may be that iNOS mutant mice suffer excessive vasoconstriction of some vascular beds, however further studies are required to investigate this. Administration of a high dose of endotoxin caused a sustained increase in the concentrations of NO associated with a prolonged inhibition of the respiration, at the level, at least in part, of complex I in the wild type mice. Thus administration of the low dose of endotoxin generates a short-term increase in NO production associated with a short-term inhibition of cellular respiration, a fall in blood pressure and survival (Chapter 7). Whereas the high dose of endotoxin (Chapter 8) leads to sustained elevation in NO concentrations and a sustained inhibition of cellular respiration associated with increased mortality. Interestingly,

although both doses of endotoxin lead to progression into established shock, it appears that the sustained inhibition of cellular respiration is the key component in determining mortality (Chapter 7 and Chapter 8). These results are in agreement with a previous study of muscle biopsies and blood of septic shock patients, showing an increase in NO production associated with a decrease in cellular respiration and a decrease in GSH (Brealey *et al.*, 1999).

Thus the fall in blood pressure and the cytopathic dysoxia (Chapter 7 and Chapter 8) characteristic of septic shock is associated at least in part with the overproduction of NO, that leads to a defect in oxygen delivery due to maldistribution of blood flow and a defect in oxygen extraction by suppression at cellular respiration (Chapter 7 and Chapter 8). A further complication may involve a localised extracellular oxygen consumption similar to that observed *in vitro* (Chapter 5). It is likely that if the maldistribution of blood flow and the cytopathic dysoxia is prolonged this will lead to multiple organ failure and death (Figure 9.1).

The inhibition of cellular respiration in these studies is likely to be preceded by a decrease in GSH concentration as observed *in vitro* following long-term exposure to different cells to exogenous NO (Chapter 4 and Chapter 5). It will be interesting to determine whether this depletion occurs under these *in vivo* experimental conditions.

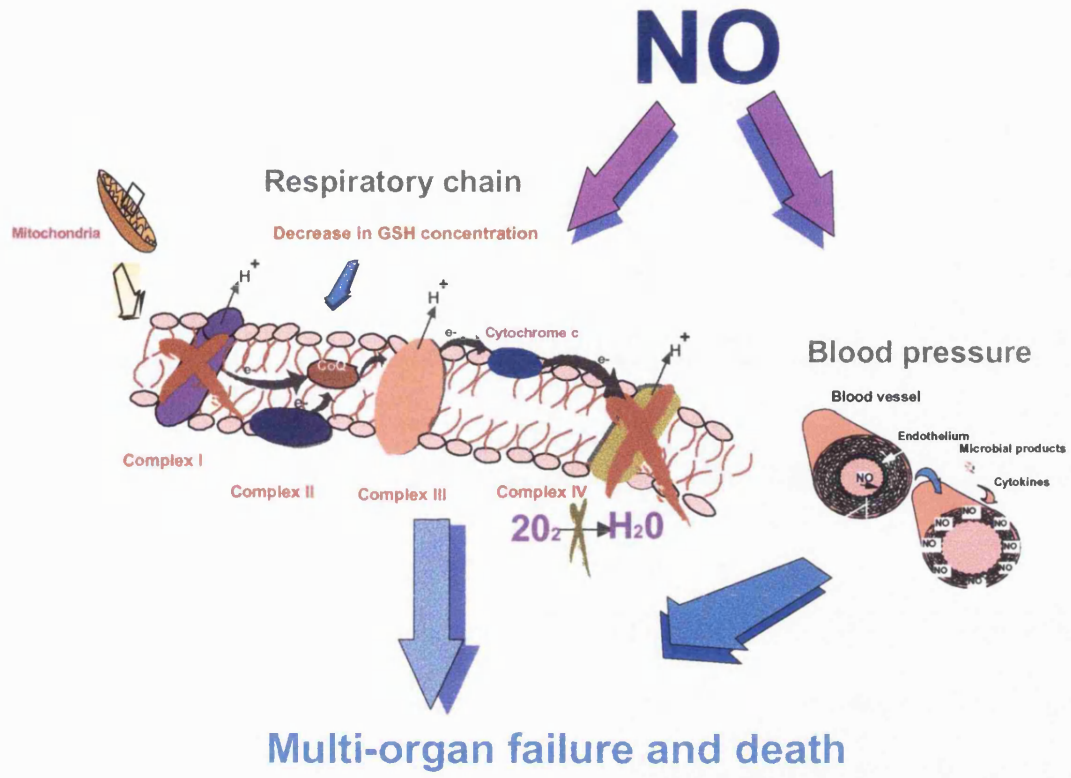

Figure 9.1 Overproduction of NO as a common mechanism leading to cytopatic dysoxia and fall in blood pressure, characteristic of septic shock, and the progression into multiple organ failure and death.

All this data suggests that GSH or N-acetyl-L-cysteine in combination with a NO synthase inhibitor would be of therapeutic benefit to reverse the fall in blood pressure and the cytopathic dysoxia associated with inhibition of the respiratory chain and may lead to an improvement in the survival profile. Indeed GSH or N-acetyl-L-cysteine administration could play an important role in the regulation of energy metabolism and in protecting against respiratory chain damage (Singer & Brealey, 1999); (Ortolani *et al.*, 2000). A number of studies have already been performed to determine whether the inhibition of iNOS could have any additional benefit over conventional vasopressor therapy in septic shock patients and have suggested possible advantages including limiting the increase in microvascular permeability

(Laszlo *et al.*, 1995; Whittle *et al.*, 1995). Selective inhibitors of iNOS are in development and may have a more useful therapeutic role by blocking excessive pathological NO generation without affecting the physiological generation of NO from eNOS and nNOS. One such compound is 7W93, which has no effect on normal blood pressure in a conscious mouse but reverses an endotoxin-induced decline in blood pressure (Rees, 1999a). Several other approaches have been clinically evaluated including modified haemoglobin compounds. As the understanding of the mechanism underlying the cytopathic dysoxia and hypotension of septic shock emerges the potential for the development of novel therapeutic agents for this condition will increase. It will be interesting to see if a combination of glutathione supplementation and NO inhibition will be successful.

9.1 CONCLUSIONS

This thesis demonstrates that NO inhibits tissue respiration both in physiological and pathological conditions. In addition the sequence of events that lead from physiological regulation to pathological inhibition and the biochemical conditions necessary for this process to take place are examined.

This thesis demonstrates that

- Following long term exposure to exogenous NO, the reversible inhibition of complex IV and the irreversible inhibition of complex I (by oxyHb) is a general mechanism observed in different cells and is preceded by a decrease in GSH concentration.

- Long-term exposure of the cells to NO decreases oxygen utilisation due to two distinct actions, one being the inhibition of cellular oxygen consumption and the other, the generation of a hypoxic microenvironment due to the oxygen consumption by the extracellular medium.
- Inhibition of basal NO generation, with a NO synthase inhibitor increases cellular respiration in iNOS mutant and wild type mice, but not in eNOS mutant mice.
- Administration of a low dose of endotoxin to the mice leads to a short-term generation of NO associated with a short-term inhibition of mitochondrial respiration resulting in non-lethal shock.
- Administration of a high dose of endotoxin leads to a sustained generation of NO accompanied by a persistent inhibition of mitochondrial respiration, at the level (at least in part) of complex I, resulting in lethal shock.

These results suggest that NO can be considered as a physiological regulator and a pathological inhibitor of tissue respiration. The basal release of small amounts of NO acts as a regulating mechanism, whereby cells respond to changes in their environment and modify ATP production. By contrast the generation of high concentrations of NO over a sustained period decreases reduced glutathione concentration and pathologically inhibits cellular respiration leading to multiple organ failure and ultimately death.

CHAPTER 10

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10 REFERENCES

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CHAPTER 11

PUBLICATIONS

11 PUBLICATIONS

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