NITRIC OXIDE REACTIVITY AND TOXICITY IN BRAIN TISSUE *IN VITRO*

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ABSTRACT

The role of nitric oxide (NO) in brain physiology and pathology is governed by its concentration, a function of the balance between synthesis and breakdown. Following cerebral ischaemia NO may play a protective or destructive role, and the literature is plagued by contradictory findings. Contributing to the confusion is a lack of knowledge as to what constitutes a toxic concentration of NO, how NO is inactivated *in vivo*, and a large number of potential pitfalls.

Measured using an NO-sensitive electrode, most of the NO delivered using a NONOate donor was removed by reaction with tissue culture medium. The main constituent responsible was Hepes buffer, which consumed NO in a superoxide dismutase-sensitive manner, indicating formation of peroxynitrite from superoxide and NO. Given the widespread use of Hepes, the reaction may contribute artifactually to multiple effects of NO observed *in vitro.*

The hypothesis that NO mediates neurodegeneration arising from NMDA receptor activity was then re-examined using organotypic slice cultures of rat hippocampus. The NO-cGMP signaling pathway was well preserved in such cultures but no component of NMDA-induced cell death was attributable to NO. At the same time, the tissue was remarkably resistant to exogenous NO at up to 1000-fold higher concentrations. Together, these results seriously question the proposed role of NO in NMDA receptor-mediated excitotoxicity.

An avid NO consumption mechanism in rat cerebellar cells and brain homogenate had been previously described. A combination of transition metals and ascorbate was shown to be responsible for a component of this consumption. When this mechanism of consumption was inhibited dispersed rat brain preparations continued to consume NO by another powerful, as yet undetermined, mechanism.

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This thesis is dedicated to all my parents.

In loving memory of Mum, Pere-Pere and Grandma.

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ABBREVIATIONS

aCSF	Artificial cerebrospinal fluid
CaM	Calmodulin
CBF	Cerebral blood flow
cGK	cGMP-dependent protein kinase
cGMP	Guanosine 3',5'-cyclic monophosphate
CNG	Cyclic nucleotide gated
CNS	Central nervous system
CREB	cAMP response element binding protein
DAF-2	4,5-diaminofluorescein
EDRF	Endothelial derived relaxing factor
eNOS	Endothelial NO synthase
GMP	Guanosine monophosphate
GTP	Guanosine 5'-triphosphate
H_2O_2	Hydrogen peroxide
HIF-1	Hypoxia inducible factor
Hz	Hertz
IFN-γ	Interferon-γ
iNOS	Inducible NO synthase
L-NAME	N^G-nitro-*L*-arginine methyl ester
L-NNA	Nitro-*L*-arginine
$LOO^{\bullet}$	Lipid peroxyl radical
LPS	Lipopolysaccharide
MAO	Monoamine oxidase
MMP	Matrix metalloproteinase
MPT	Mitochondrial permeability transition
NO	Nitric oxide
$NO_{GC}R$	Guanylyl cyclase coupled nitric oxide receptor
nNOS	Neuronal NO synthase
NOS	Nitric oxide synthase
NO_2^-	Nitrite
NO_3^-	Nitrate

OGD	Oxygen glucose deprivation
$OH^{\bullet}$	Hydroxyl radical
$ONOO^{-}$	Peroxynitrite
$O_2^{\bullet-}$	Superoxide
PARP-1	Poly(ADP-ribose) polymerase-1
PDE	Phosphodiesterase
PSD-95	Post synaptic density protein-95
RBCs	Red blood cells
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
$RO_2^{\bullet}$	Peroxyl radical
SIN-1	3-morpholinosydnonimine
SNAP	*S*-nitroso-*N*-acetylpenicillamine
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
VEGF	Vascular endothelial growth factor
7-NI	7-nitroindazole
1400 W	*N*-(3-(aminomethyl)benzyl)acetamidine
3-NT	3-nitrotyrosine

CHAPTER 1: INTRODUCTION

1.1 THE DISCOVERY OF NO

Nitric oxide (nitrogen monoxide radical; NO) had a colourful beginning when, as a contaminant of the laughing gas nitrous oxide, it proved almost fatal when inhaled by Sir Humphrey Davy, whose pioneering work concerned the administration of medicinal airs (Smith, 1967). Since these early days, and from its reputation as an environmental pollutant in smog and cigarette smoke, this unique signalling molecule has come far, being named "molecule of the year" by the journal *Science* in 1992 (Culotta & Koshland, Jr., 1992), and earning the Nobel Prize for Physiology or Medicine for a trio of American researchers in 1998. NO is freely diffusible, a property unique for a signalling molecule. The neurotransmitter glutamate, for example, is typically stored in, and released from vesicles into the synapse, from where specific uptake mechanisms are in place to control its concentration. In contrast NO is synthesised when needed, and to date there are no clear-cut routes established for its degradation. Furthermore, NO is very unstable in biological systems, which, in addition to its reputation as a toxic molecule, contributed to the difficulties inherent in its identification and isolation. It took evidence from several lines of biological enquiry for researchers to begin to understand the incredibly diverse role NO plays in both physiological signalling and cytotoxicity.

The vasodilating properties of nitroglycerin had been used for many decades in the treatment of hypertension before it was realised that its actions were mediated through the liberation of NO. Release of NO from this, and other nitrovasodilators (including sodium nitroprusside and sodium azide), stimulates the enzyme guanylate cyclase (GC) to produce cyclic guanosine 3'-5' monophosphate (cGMP) from 5' guanosine triphosphate (GTP) (Arnold *et al.*, 1977; Katsuki *et al.*, 1977). Several years later, an investigation into the cGMP-dependent vasodilating actions of acetylcholine (ACh) *in vitro,* observed that unintentional rubbing of the intimal surface of rabbit thoracic aorta removed the endothelial cells, thereby rendering the vessels unresponsive (Furchgott & Zawadzki, 1980). In response to ACh

then, a diffusible endothelium-derived relaxing factor (EDRF) was produced that could permeate the smooth muscle leading to relaxation. Notably the nitrovasodilators could elicit muscle relaxation in the absence of endothelial cells. The identity of EDRF proved difficult to pin down, until, following suggestions during a meeting in 1986 by Robert Furchgott, and then Louis Ignarro, that EDRF was NO, it was demonstrated that vascular endothelial cells could synthesize NO in quantities sufficient to relax smooth muscle (Palmer *et al.*, 1987).

Meanwhile, experiments in the CNS examined the response of cerebellar tissue to stimulation with the excitatory neurotransmitter glutamate, or the GC activator sodium nitroprusside. Using selective lesioning of the tissue, this work concluded that the subsequent cGMP increases did not occur in the same cells stimulated by glutamate, but in other cell types. It was suggested that an unstable factor might be diffusing intercellularly (Garthwaite & Garthwaite, 1987). Subsequently this factor was shown to behave identically to EDRF. It caused aortic smooth muscle relaxation and like EDRF its release was Ca^{2+}-dependent. Moreover responses could be increased in the presence of superoxide dismutase (SOD), an enzyme known to prolong the life of EDRF (Garthwaite *et al.*, 1988). Subsequently, as had already been shown in macrophages (see below), and endothelial cells (Palmer *et al.*, 1988), the precursor required for this novel activity was reported to be *L*-arginine. The diffusible factor was, of course, NO (Garthwaite *et al.*, 1989).

Whilst the novel field of NO signalling was born, a third area of research had connected NO to cellular toxicity. Measurement of urinary nitrate (NO_3^-) had revealed that more NO_3^- was excreted than was consumed in the diet by both rats and humans (Green *et al.*, 1981a; Green *et al.*, 1981b). Synthesis of NO_3^- could be increased remarkably following injection of rats with the inflammatory stimulator lipopolysaccharide (LPS) (Wagner *et al.*, 1983). Further investigation found that the major source of NO_3^- (and NO_2^-) in LPS treated mice was from macrophages (Stuehr & Marletta, 1985). The cytotoxic properties of the activated macrophages were found to be dependent upon *L*-arginine (Hibbs, Jr. *et al.*, 1987), and finally the effector molecule reported to be NO (Hibbs, Jr. *et al.*, 1988).

1.2 NITRIC OXIDE PRODUCTION

Nitric oxide synthase, catalytic mechanism

In the space of a year, three diverse cell types (endothelial cells, cerebellar granule cells and macrophages) had been recognised to synthesise NO from *L*-arginine, so the subsequent identification and molecular characterisation of three distinct genes for differing NO synthase (NOS) isoforms was perhaps not surprising. Termed endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS), the active enzymes exist as a homodimer composed of subunits of between 130–160 kDa. eNOS and nNOS are constitutively expressed and calcium-dependent, while iNOS expression is induced by a number of cytokines and is calcium-independent. The enzymes contain relatively tightly bound cofactors (6*R*)-5,6,7,8-tetrahydrobiopterin (BH_4), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), haem and calmodulin (CaM). The overall reaction is depicted below (figure 1.1) and, though not yet fully understood, it involves donation of electrons by NADPH to the reductase domain, which proceed *via* FAD and FMN to the oxygenase domain.

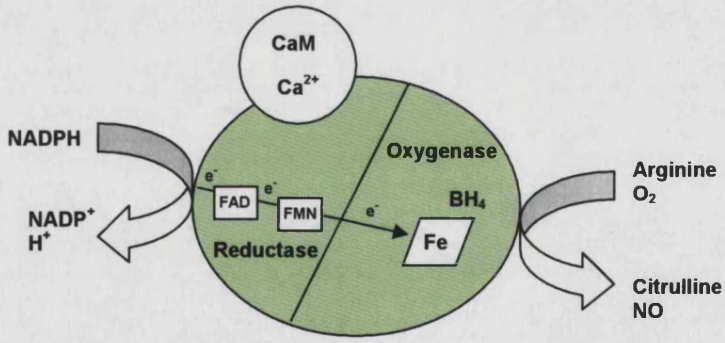


Figure 1.1 Overall reaction catalysed by NOS (from Alderton *et al.*, 2001)

There they interact with the haem iron and BH_4 at the active site to catalyse the reaction of oxygen with *L*-arginine, forming N^G-Hydroxy-L-arginine as an intermediate, and finally generating citrulline and NO as products (Alderton *et al.*, 2001). In elucidating the mechanism by which NOS generates NO, comparisons have been made with cytochrome P450 reductase, which

donates electrons to the cytochrome P450 enzyme family involved in fatty acid oxidation in the endoplasmic reticulum. This reductase shares close homology to the reductase domain of NOS (Bredt *et al.*, 1991).

The possible existence of a further NOS isoform has been discussed in the literature since immunocytochemical techniques localized eNOS staining to (non-synaptosomal) rat brain and liver mitochondria (Bates *et al.*, 1995). A functional enzyme (mitochondrial NOS; mtNOS) was then reported in intact liver mitochondria, where activity was associated with the inner membrane (Ghafourifar & Richter, 1997). Recent studies indicate that mtNOS is a splice variant of nNOS (Elfering *et al.*, 2002; Giulivi, 2003), though its identity, and relevance, continues to be debated (Lacza *et al.*, 2003).

The regulation of NOS by CaM was first noted when nNOS was purified from the cerebellum (Bredt & Snyder, 1990). The Ca^{2+} sensitivity of both nNOS and eNOS (EC_{50} ~250-350 nM) is now understood to require the presence of a 45 amino acid long 'autoinhibitory loop' inserted into the middle of the FMN binding domain. At resting Ca^{2+} levels in the cell (< 100 nM) CaM does not bind to NOS. However when Ca^{2+} levels rise Ca^{2+}/CaM binds and the loop is displaced from its docking site elsewhere on the protein, causing a conformational change, and relieving the inhibitory effect (probably impeded electron transfer) (Lane & Gross, 2000). The lack of such a sequence in iNOS renders it Ca^{2+}-independent. However iNOS binds CaM tightly, so is active irrespective of changes in Ca^{2+} concentration. The consequence of such regulation is that nNOS can respond to Ca^{2+} transients with a 'puff' of NO, while iNOS derived NO may reach higher concentrations since the enzyme is persistently activated for longer durations. The role of BH_4 in NOS activity is complicated in contrast to that of CaM. Many different mechanisms of action have been suggested including a role in dimerisation, or redox activity during catalysis (see Alderton *et al*, 2001).

nNOS Localization

On a regional scale the brain contains vastly differing NOS activities, with the highest being found in the cerebellum (Salter *et al.*, 1995). NADPH diaphorase staining has proved a useful tool for NOS localization, though it

does not discriminate between isoforms. After paraformaldehyde fixation, and in the presence of NADPH, NOS reduces the dye nitroblue tetrazolium into a dark blue formazan product. The staining co-localizes remarkably well with nNOS immunohistochemistry and, in combination, these techniques have localized nNOS to discrete, but widespread, neuronal populations, though they do not match any neurotransmitter exactly (Dawson *et al.*, 1991a; Southam & Garthwaite, 1993). Staining is densest in the cerebellar molecular layer and granule cell layers. High immunoreactivity is also found in the olfactory bulb, inferior and superior colliculi and dentate gyrus of the hippocampus. eNOS is also found in the CNS, where it is located exclusively in endothelial cells (Topel *et al.*, 1998).

nNOS is localized to synaptic membranes via a PDZ domain on its N-terminal, which interacts with the postsynaptic density proteins PSD-95 and PSD-93 (Brenman *et al.*, 1996). This discovery is significant because NR2 subunits of the NMDA-type glutamate receptor also interact with PSD-95 (Kornau *et al.*, 1995). A ternary complex is thus formed with NMDA receptors, PSD-95 and nNOS (Christopherson *et al.*, 1999). In this way nNOS is conveniently placed close to Ca^{2+} influx during glutamate stimulation, thus enhancing the potential of NO to act in a spatially discrete manner. Additional regulation may occur through interaction of the PDZ domain of nNOS with the recently identified protein CAPON. Overexpression of CAPON results in the loss of PSD-95/nNOS complexes in transfected cells (Jaffrey *et al.*, 1998). CAPON has recently also been reported to form a complex with nNOS and synapsin, which is enriched in presynaptic terminals (Jaffrey *et al.*, 2002). Several studies have confirmed the synaptic co-localization of nNOS and PSD-95 using detailed immunohistochemical methods (Aoki *et al.*, 1998; Burette *et al.*, 2002; Martinelli *et al.*, 2002).

Splice variants of the full-length nNOS gene (nNOSα) have been detected. nNOSβ and nNOSγ are found in the brains of nNOSα knockout mice where they are responsible for residual activity, probably explaining why the knockouts do not have serious pathology. nNOSβ has a similar activity to nNOSα *in vitro*, though nNOSγ is considerably less active (Brenman *et al.*, 1996). In agreement with a more prominent role for nNOSβ, this variant

undergoes 2-3 fold upregulation in the cortex and striatum of knockouts while nNOSγ mRNA is not detected (Eliasson *et al.*, 1997a). Notably nNOSβ lacks the PDZ domain of nNOSα and does not bind PSD-95. Though despite being separated from NMDA receptors it remains catalytically active.

1.3 NO INACTIVATION

The principle means by which NO is broken down in aqueous solution is through reaction with O_2 (autoxidation, see chapter 3.1). Autoxidation of NO is slow, however, and cannot account for NO breakdown in biological systems. Surprisingly little is known about NO inactivation in the brain, though several potential NO inactivation mechanisms have been proposed, these are discussed in chapter 6.1.

1.4 NO SIGNAL TRANSDUCTION

Guanylyl cyclase coupled receptors

It is widely recognised that NO exerts most of its physiological effects by activation of guanylyl cyclase (GC)-coupled receptors ($NO_{GC}R$), which catalyse the generation of cGMP from GTP (Denninger & Marletta, 1999; Hobbs, 1997). The active enzyme usually exists as an obligate heterodimer composed of one of two α and one of two β subunits, though a fifth subunit, variant $\beta 2$ ($v\beta 2$), has recently been identified which may be active as a homodimer (Gibb *et al.*, 2003; Koglin *et al.*, 2001). Of the four subunit combinations possible; $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 1\beta 2$ and $\alpha 2\beta 2$, only two have been shown to exist at the protein level. $\alpha 1\beta 1$ is widely expressed and was first isolated from bovine lung, and $\alpha 2\beta 1$ was originally identified in the human placenta. Notably these isoforms have by far the greater catalytic activity upon transfection into COS-7 cells (Gibb *et al.*, 2003). $NO_{GC}Rs$ are typically around 150 kDa in mass, require Mg^{2+} or Mn^{2+} for activation, and bind NO by virtue of a prosthetic haem in the N-terminal region. The haem moiety is associated with the β subunit of $NO_{GC}R$ via an axial ligand provided by a histidine residue, His105 (Wedel *et al.*, 1994). The catalytic domain is at the

C-terminal region, with a dimerization domain, thought to mediate the subunit association, sandwiched in-between (Figure 1.2).

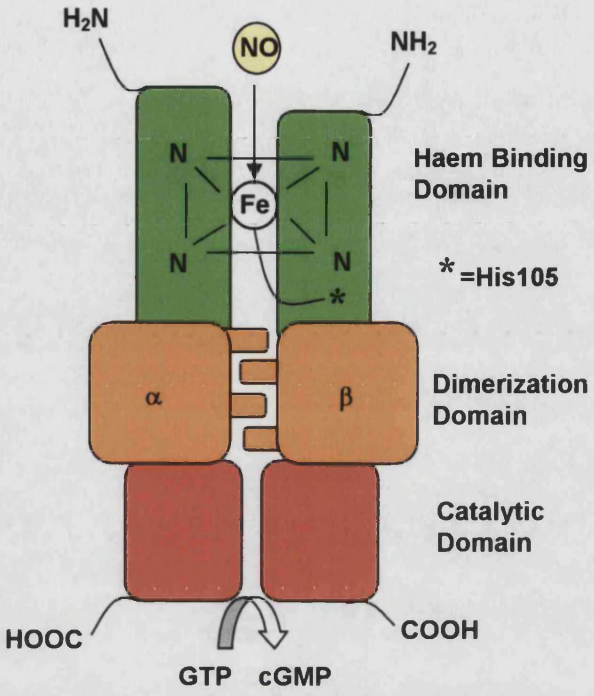


Figure 1.2 Schematic representation of a $NO_{GC}R$ $\alpha\beta$ heterodimer (after Hobbs, 1997)

The mechanism of $NO_{GC}R$ activation is unclear, and indeed the number of NO binding sites has been a matter of some debate (Bellamy *et al.*, 2002b; Zhao *et al.*, 1999). It is thought that upon NO binding the His105 bond breaks, triggering a conformational change and exposing the catalytic site (Sharma & Magde, 1999). Recent methodological advances have allowed the generation of steady-state NO concentrations *in vitro*, a requisite for ascertaining the potency of NO at its receptor targets. Using such methods it was found that NO activates $NO_{GC}Rs$ with EC_{50} values of <2 nM in intact cells (Gibb *et al.*, 2003; Griffiths *et al.*, 2003), a value substantially lower than earlier estimates of 250-300 nM (Russwurm *et al.*, 1998; Stone & Marletta, 1996).

An important characteristic underlying the actions of NO as a neurotransmitter is the ability of native $NO_{GC}Rs$ (compared to purified enzyme) to desensitize within seconds upon sustained activation (Bellamy *et al.*, 2000; Bellamy & Garthwaite, 2001b; Wykes *et al.*, 2002). This

mechanism (in combination with phosphodiesterase activity, see below) may underlie the diversity of cellular cGMP responses. Although the mechanism of desensitization is as yet unknown, it appears to be regulated by cGMP (Wykes *et al.*, 2002).

Previously localization studies have concentrated upon the expression of either the $\alpha 1$ or the $\beta 1$ subunit, and it was not until recently that expression of all four subunits was compared in the rat brain by in situ hybridisation (Gibb & Garthwaite, 2001). Broadly speaking the results of this study found that in some areas, e.g. caudate-putamen and nucleus accumbens, $NO_{GC}Rs$ exist mainly as the $\alpha 1\beta 1$ heterodimer. In other areas such as the hippocampus and olfactory bulb $\alpha 2\beta 1$ is dominant. Interesting recent developments have now altered the long held perception of GC as a soluble enzyme. The $\alpha 2\beta 1$ isoform was demonstrated, by co-precipitation from brain homogenates, to interact with PSD-95. Similarly to NOS this interaction occurs at a PDZ domain, thus forming a signalling microdomain composed of Ca^{2+} permeable NMDA receptors, Ca^{2+}-dependent nNOS and $\alpha 2\beta 1$ $NO_{GC}R$ (Russwurm *et al.*, 2001). Other work has confirmed this membrane localization in various cell types and further found that it may be Ca^{2+}-sensitive (Zabel *et al.*, 2002).

Regulating cGMP

Cyclic nucleotide phosphodiesterase (PDE) enzymes catalyse the hydrolysis of cyclic nucleotides (cAMP or cGMP) to 5' monophosphates (5'AMP or 5'GMP). Their major function is often thought to be simple termination of the cyclic nucleotide second messenger signal, though since $NO_{GC}R$ activity does not vary between the two major heterodimers (see above) it may be left to PDEs to modulate both the amplitude and duration of the signal. A large variety of PDE gene families (totalling 11) have now been classified according to their primary sequence and regulation (see table 1.1 Beavo, 1995; Fawcett *et al.*, 2000; Soderling & Beavo, 2000). Nearly all the PDEs are homodimers with monomer molecular weights of 50-135 kDa. Most families have been shown to have more than one gene product and splice variants have been found in many cases. All PDEs contain a core of ~270

amino acids that are highly conserved and form the C-terminal catalytic domain. The N-terminal region contains the regulatory segment such as a CaM binding (PDE1) or cGMP binding domain (PDE2, 5, 6, 10 and 11).

Family	Preference	Tissue distribution	Comments
1	cGMP + cAMP	Widespread	Ca^{2+}/CaM-activated
2	cAMP + cGMP	Adrenal cortex, brain, platelets, heart	cGMP-activated
3	CAMP	Smooth muscle, platelets, heart, liver, lymphocytes	cGMP-inhibited
4	CAMP	Widespread	'cAMP-specific'
5	CGMP	Smooth muscle, platelets, brain, kidney	cGMP-binding, 'cGMP-specific'
6	CGMP	Retina, pineal gland	Retinal rod photoreceptor PDE
7	CAMP	Lymphocytes (PDE7A), widespread (7B)	'cAMP-specific'
8	CAMP	Testis, eye, liver, kidney, skeletal muscle	PAS domain (protein and ligand interactions)
9	CGMP	Widespread	High affinity cGMP hydrolysis
10	cAMP + cGMP	Heart, brain, placenta, kidney	cGMP-binding
11	cAMP + cGMP	Widespread	cAMP-inhibited? cGMP-binding?

Table 1.1 Properties of the phosphodiesterase families. From (Beavo, 1995; Fawcett *et al.*, 2000; Soderling & Beavo, 2000).

The localization of PDEs families in the brain has been well characterised (Cherry & Davis, 1999; Kotera *et al.*, 2000; Repaske *et al.*, 1993; van Staveren *et al.*, 2001), although much of this work relies on *in situ* hybridisation, which does not provide evidence of protein function. An example is PDE1B1, whose mRNA is high in the striatum (Polli & Kincaid, 1994) whereas, in contradiction, activity in this area was found to be mainly related to PDE2 (Wykes *et al.*, 2002). The distinct expression profiles (and activities) of different families, highlighted recently by a study on PDEs 2, 5 and 9 (van Staveren *et al.*, 2003), is likely to have a strong bearing on the resultant cGMP profiles in the respective cells. In the cerebellum, for example, cGMP accumulates to a steady plateau over two minutes under the influence of PDE5 and, surprisingly, some PDE4 activity (Bellamy & Garthwaite, 2001a). In contrast striatal cells (containing PDE2) exhibit a more transient cGMP profile within the same time scale (Wykes *et al.*, 2002).

Downstream of cGMP

There are three main classes of effector proteins mediated by cGMP: PDEs, cGMP-dependent protein kinases (cGKs) and cyclic nucleotide gated (CNG) ion channels.

As seen in table 1.1, PDEs 2 and 3 are both regulated by cGMP, which either activates or inhibits the enzyme. Taking PDE3 first, cGMP binding inhibits the degradation of cAMP (thus raising cAMP levels) simply due to the less efficient hydrolysis of cGMP at the catalytic site (Degerman *et al.*, 1997). In contrast PDE2 (and indeed PDEs 5 and 6, 10 and 11) contains two modulatory binding sites for cGMP known as GAF domains A and B (Martinez *et al.*, 2002). When cGMP binds the GAFB domain of PDE2 the enzymes rate and affinity of the catalytic site for both cAMP and cGMP is increased by allosteric interactions. Similarly, cGMP binding to the GAFA domain on PDE5 increases cGMP hydrolysis at the catalytic site (Rybalkin *et al.*, 2003). Subsequent phosphorylation of the enzyme by cGMP-dependent protein kinase I (cGKI) further enhances its activity (Mullershausen *et al.*, 2003). In summary then, cGMP can influence not only its own hydrolysis but also that of cAMP, providing potential for PDE-dependent 'crosstalk' between the two second messenger molecules.

Two genes for cGKs have been identified in mammals, cGKI (which has α and β splice variants) and cGKII. The translated proteins are homodimers with the 76 kDa cGKI located primarily in the cytosol and the 86 kDa cGKII membrane bound, by virtue of a myristoylation site on its N-terminal. cGKs also contain N-terminal dimerization domains, autophosphorylation sites, an autoinhibitory region (cGKI), two cGMP binding sites (high and low affinity) and a C-terminal catalytic domain with ATP and peptide binding regions (Lohmann *et al.*, 1997; Lucas *et al.*, 2000). In the CNS cGKI appears to be located almost exclusively in the Purkinje cells of the cerebellum while cGKII is more widespread (de Vente *et al.*, 2001; El Husseini *et al.*, 1999). Despite the fact that over 40 distinct CNS proteins may be phosphorylated by cGKs (Wang & Robinson, 1995) relatively few have been thoroughly characterised. Best understood is G-substrate, which (like cGKI) is mainly localized in Purkinje cells (Detre *et al.*, 1984). Upon

phosphorylation, G-substrate (and a similar protein DARPP-32) inhibits protein phosphotase-1 activity (Hall *et al.*, 1999). cGMP signalling *via* this pathway has been proposed to mediate cerebellar long-term depression (LTD) (Feil *et al.*, 2003), and hippocampal long-term potentiation (Kleppisch *et al.*, 2003).

Cyclic nucleotide gated (CNG) channels were first discovered in the visual and olfactory systems. Upon binding of intracellular cAMP or cGMP a conformational change in the C-terminal region is coupled to the opening of the channel pore which is preferentially permeable to Ca^{2+} ions (Flynn *et al.*, 2001). The nomenclature for these channels has been somewhat confusing, but recently a consensus has been reached. There are 6 members of the gene family grouped into two subtypes, A and B. The channels form as tetramers of A and B subunits (each containing six transmembrane domains) surrounding the central pore. The prototypical rod channel consists of CNGA1 and CNGB1, while the cone channel consists of CNGA3 and CNGB3, finally the olfactory channel is made up from CNGA2, CNGA4 and a splice variant, CNGB1b (Kaupp & Seifert, 2002; Matulef & Zagotta, 2003). CNG channels are known to be expressed in a range of non-sensory tissues and have been identified in the brain mainly by mRNA detection. Both CNGA1 and CNGA2 have been described in the CNS where they are localised to discrete neuronal types (El Husseini *et al.*, 1995; Kingston *et al.*, 1996; Kingston *et al.*, 1999; Strijbos *et al.*, 1999). Functional channels have been described in the hippocampus where activation and Ca^{2+} influx may be involved in synaptic plasticity (Bradley *et al.*, 1997).

1.5 THE PHYSIOLOGICAL ROLE OF NO

In vitro NO is well known to reversibly inhibit the terminal enzyme of the mitochondrial respiratory chain, cytochrome *c* oxidase, in competition with oxygen (Brown & Cooper, 1994; Koivisto *et al.*, 1997). O_2 binding occurs at the haem a_3 of the binuclear haem a_3/Cu_B centre when the enzyme is reduced (a_3^{2+} and Cu^{+}). NO can bind to either metal, but when the complex is under high electron flux (more reduced) NO binds the ferrous haem (a_3^{2+}), and at low electron flux (when the binuclear centre is oxidised), NO binds to

the Cu^{2+} and is rapidly converted to NO_2^-, see (Cooper, 2002). Respiratory inhibition by NO in this manner has been suggested to regulate mitochondrial respiration by increasing the K_m of cytochrome *c* oxidase for oxygen (Brown, 1995), a consequence of which might be to increase O_2 supply to surrounding tissues (Thomas *et al.*, 2001). Several lines of evidence have found that endogenously produced NO can decrease O_2 consumption *in vitro* (Brown *et al.*, 1998; Clementi *et al.*, 1999; Loke *et al.*, 1999; Poderoso *et al.*, 1998; Shen *et al.*, 1995) and *in vivo* (Shen *et al.*, 1994; Shen *et al.*, 1995), though it remains possible that cGMP may mediate some of these effects (Balligand *et al.*, 1993; Gong *et al.*, 1998; Shen *et al.*, 1995). In addition the sensitivity of $NO_{GC}R$ to NO is at least two orders of magnitude higher than that of cytochrome *c* oxidase (Bellamy *et al.*, 2002a). The important question of whether NO regulates cellular respiration *in vivo* thus remains unresolved (Brown, 2001; Moncada & Erusalimsky, 2002).

Perhaps the most widely researched correlate of NO physiology in the brain is its role in synaptic plasticity, a process involved in memory formation. Following brief, high frequency synaptic stimulation, LTP (usually studied in slices of the hippocampus) is a resultant long-lasting (hours or days) increase in the efficacy of synaptic transmission. Long-term depression (LTD) in contrast is a decrease (hours) in synaptic strength. As befits the NO field, the evidence for its role in LTP is conflicting. Some studies find LTP is completely blocked by NOS inhibitors (Bohme *et al.*, 1991; O'Dell *et al.*, 1991), while others show only partial (Haley *et al.*, 1993; Holscher, 1999), or no effect (Cummings *et al.*, 1994). Much of this controversy may stem from the variations in the stimulation intensities applied by different labs, with stronger stimulations being NO-independent (Haley *et al.*, 1993), while temperature may also be an issue. When evidence is positive for a role of NO, experiments point to the involvement of cGMP in this pathway (Boulton *et al.*, 1995; Kleppisch *et al.*, 2003).

1.6 NO AND NEUROPATHOLOGY

NO continues to be of great interest in the pathogenesis of chronic disease states including Parkinson's (Hirsch & Hunot, 2000; Liberatore *et al.*, 1999),

Alzheimer's (Dorheim *et al.*, 1994; Gahtan & Overmier, 1999) and multiple sclerosis (Smith & Lassmann, 2002), though its role in the acute death associated with cerebral ischaemia has received the most attention to date. The remainder of this chapter concerns the role of NO in ischaemic brain injury. Much of the literature is contradictory, but remains fascinating since NO may play either a destructive or protective role. There are many good reviews of NO in ischaemia (Iadecola, 1997; Samdani *et al.*, 1997a; Strijbos, 1998) which together with the exhaustive 'Ischaemic Cell Death In Brain Neurones' (Lipton, 1999) and other selected reviews concerning NO induced cell death (Brown & Borutaite, 2002; Murphy, 1999; Virag *et al.*, 2003) provide an excellent background to the field.

Global ischaemia (for example caused by cardiac arrest) and focal ischaemia (following thrombosis of a specific artery) are characterised by diminished blood flow, the concomitant O_2 and nutrient deprivation initiating the cascade of events leading to neuronal death. Germane to the hypothesis that NO contributes to cell death following an ischaemic episode is the post-ischaemic release of 'excitotoxic' glutamate which causes sustained activation of the NMDA type of glutamate receptor. Since NMDA receptors are physically linked to nNOS via PSD-95 (Brenman *et al.*, 1996) it has been suggested that NO mediates cell death following NMDA receptor activity (Dawson *et al.*, 1991b; Sattler *et al.*, 1999). Much of the impetus for this hypothesis has stemmed from experiments using a brief NMDA stimulation to activate this pathway in dispersed neuronal cultures. Remarkable protection was found following inhibition of NOS (Dawson *et al.*, 1991b; Izumi *et al.*, 1992; Strijbos *et al.*, 1996) or disruption of the nNOS gene (Dawson *et al.*, 1996). Characteristically for this field, however, other reports showed no protection by NOS inhibition (Demerle-Pallardy *et al.*, 1991; Hewett *et al.*, 1993; Pauwels & Leysen, 1992).

Further evidence that NO is damaging in ischaemia has been accumulated from various models *in vitro* and *in vivo* utilising NOS inhibitors and knockout mice, microsensor detection of NO, or measurement of end products such as nitrite and nitrate (NOx^-) or nitrotyrosine. However, results have again been conflicting, and are subject to many pitfalls as detailed below. The current dogma is that nNOS-generated NO may reach toxic

levels (low μM) in focal ischaemia, and particularly during the reperfusion following global ischaemia, thereby contributing to a neuronal death that starts to manifest 6-12 hrs post-insult. iNOS is also upregulated following global or focal ischaemia (probably in glia or invading leukocytes respectively) peaking at 24 – 48 hrs after the initial insult. Therefore NO also impacts upon the later stages of a progressive degeneration that may continue for up to a week, see (Iadecola, 1997; Lipton, 1999; Samdani *et al.*, 1997a; Strijbos, 1998).

Of the mechanisms put forward to explain NO-dependent cell death, there are two leading candidates. NO alone is relatively unreactive, though it can bind in competition with O_2 to cytochrome *c* oxidase, thereby directly inhibiting respiration, at least *in vitro* (Bal-Price & Brown, 2001; Brorson *et al.*, 1999; Brown & Borutaite, 2002). Perhaps more attractive is the diffusion-limited reaction of NO with superoxide ($O_2^{\bullet-}$), which is increased during and after ischaemia, to form the highly toxic species peroxynitrite ($ONOO^-$), a strong oxidant (Beckman & Koppenol, 1996).

Paradoxically, NO generation may also be neuroprotective, either *via* cGMP-mediated mechanisms such as increased vasodilation and platlet disaggregation (Iadecola, 1997; Samdani *et al.*, 1997a; Strijbos, 1998), inhibition of apoptosis (Fiscus, 2002), stimulation of angiogenesis (Ando *et al.*, 2002; Maulik & Das, 2002; Zhang *et al.*, 2003), or more directly by inhibition of lipid peroxidation (Hogg & Kalyanaraman, 1999; O'Donnell & Freeman, 2001). Evidence, particularly from studies with NOS inhibitors, suggests that post-ischaemic NO generated by eNOS may indeed be protective. Furthermore recent evidence has implicated NO as a protective factor in ischaemic pre-conditioning, and also in the neurogenic response that follows several days after the insult. These proposed destructive and protective roles of NO in cerebral ischaemia are summarised in figure 1.3.

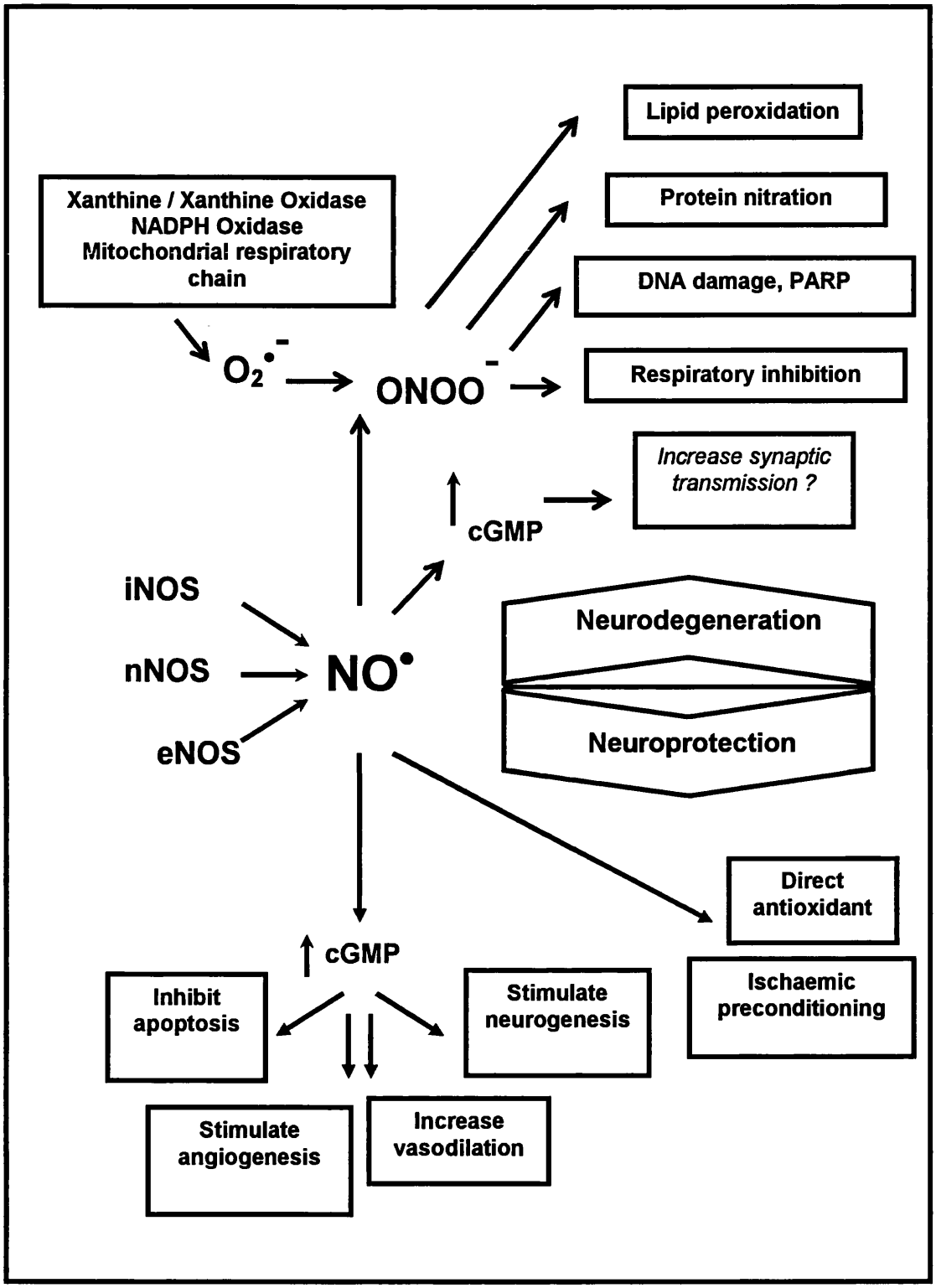


Figure 1.3 Summary diagram of the possible degenerative and protective roles of NO in cerebral ischaemia (see text for details).

1.7 IDENTIFYING POTENTIAL ARTIFACTS

Tissue culture experiments

Many of the discrepancies in the literature concerning NO pathology may have arisen from problems associated with culturing cells. It has long been observed that the numbers of nNOS-positive neurones may change when culture conditions are altered. For example plating neurones on glial feeder layers compared with a polyornithine matrix has been associated with an impoverishment of nNOS containing neurones (Samdani *et al.*, 1997b). Equally important may be the developmental age of the culture, and the timing and intensity of the insult (Aizenman *et al.*, 1998).

It is often overlooked that cultured cells may be abnormally vulnerable to oxidative and nitrosative stress. Media are frequently deficient in antioxidants (vitamin E is insoluble and vitamin C unstable) and are actually pro-oxidant unless trace metals are deliberately scavenged. Furthermore in air-equilibrated media (about 200 μM O_2, compared to 25 μM *in vivo*) cultured cells may generate more reactive oxygen species (ROS; such as $O_2^{\bullet-}$) because the O_2 concentration is not limiting, for discussion see (Halliwell, 2003).

A good alternative to primary dissociated cultures may be organotypic slice cultures. Such slices survive for longer in culture (up to a month) and they retain their complex cellular organization and normal electrophysiological properties (Gahwiler, 1981; Stoppini *et al.*, 1991). Hippocampal slice cultures are regularly used for the study of ischaemic and excitotoxic insults and show a similar regional vulnerability to that found *in vivo* (Pringle *et al.*, 1997; Strasser & Fischer, 1995; Vornov *et al.*, 1994; Vornov *et al.*, 1991). Surprisingly there have been no reports of the effects of NO following such stimulations.

NOS inhibition

Initial experiments *in vivo* using competitive inhibitors such as nitro-*L*-arginine (L-NNA) and its pro-drug N^G-nitro-*L*-arginine methyl ester (L-NAME) gave conflicting results. These compounds are non-selective and the resulting

eNOS inhibition is likely to have led to unwanted effects such as reduction of cerebral blood flow and constriction of pial arterioles, leading to increases in infarct volume, reviewed in (Iadecola, 1997). L-NAME may also have non-specific effects, such as muscarinic antagonism (Buxton *et al.*, 1993).

More recently the compound 7-nitroindazole (7-NI) has been used as a selective nNOS inhibitor (Adachi *et al.*, 2000; Lei *et al.*, 1999) though this may be misguided as, at the level of isolated enzyme, no selectivity is seen (Alderton *et al.*, 2001). 7-NI can indeed reduce focal infarct volume without increasing blood pressure (Yoshida *et al.*, 1994), but this may have other explanations, such as a selectivity for neuronal *vs.* endothelial cells (Alderton *et al.*, 2001). Recently, 7-NI was reported to protect gerbils from 5-min global ischaemia by an unknown mechanism distinct from nNOS inhibition (Lei *et al.*, 1999). Besides its NOS inhibitory actions, 7-NI may act as a monoamine oxidase (MAO) inhibitor *in vivo* (Desvignes *et al.*, 1999). Following global ischaemia in rats, accumulated catecholamines may produce H_2O_2 *via* MAO, although the use of MAO inhibitors or knockout animals offered no protection (Holschneider *et al.*, 1999; Simonson *et al.*, 1993). Nevertheless, a protective combination of NOS/MAO inhibition by 7-NI cannot be ruled out as the mechanism of action for this compound. Data obtained with another partially selective nNOS inhibitor ARL 17477 (23-fold selective *vs.* eNOS) have shown protection against transient ischaemia *in vivo* (O'Neill *et al.*, 2000; Zhang *et al.*, 1996b), but this compound is only five-fold selective *vs.* iNOS, the inhibition of which was not monitored.

Similar problems with selectivity have occurred with studies using the iNOS selective inhibitor aminoguanidine. This compound has approximately 10-fold selectivity *vs.* eNOS, but very little *vs.* nNOS (Alderton *et al.*, 2001). Although protective in some studies (Iadecola *et al.*, 1995; Iadecola *et al.*, 1996; Zhang *et al.*, 1996a) such effects could be caused by nNOS inhibition or be related to the inhibition of polyamine oxidase activity (Ivanova *et al.*, 1998). Furthermore, aminoguanidine may have other effects, such as catalase inhibition (Ou & Wolff, 1993) and antioxidant properties (Giardino *et al.*, 1998; Yildiz *et al.*, 1998).

The only inhibitors to exhibit high selectivity are against iNOS: *N*-(3-(aminomethyl)benzyl)acetamidine (1400W), GW273629 and GW274150 are

greater than 50-fold selective over both the constitutive NOS isoforms (Alderton *et al.*, 2001). Importantly, 1400W has recently been successful in reducing infarct volume in focal ischaemia (Parmentier *et al.*, 1999). Unfortunately, the development of highly selective inhibitors of nNOS and eNOS remains elusive; they would be very useful in addressing ambiguities plaguing the literature.

Exogenous NO

Cell death due to the application of exogenous NO to neurones is one of the key criteria used to support the hypothesis that endogenous NO is toxic. Although there is little knowledge of what constitutes a pathological NO concentration, various NO-releasing chemicals have been applied in many models (Bonfoco *et al.*, 1995; Brorson *et al.*, 1999; Dawson *et al.*, 1996; Izumi *et al.*, 1993). Historically the most commonly used donors, sodium nitroprusside (SNP), S-nitroso-N-acetylpenicillamine (SNAP) and 3-morpholinosydnonimine (SIN-1) are now recognised not to produce NO as their primary species (Feelisch *et al.*, 1989; Lipton & Stamler, 1994). The advent of the NONOates, which release authentic NO with predictable kinetics, has simplified matters (Keefer *et al.*, 1996) and the resulting NO concentrations in biological buffers can be predicted (Schmidt *et al.*, 1997), see section 3.1. Unfortunately errors are still made, such as comparing NO levels released from NONOates at room temperature with neuronal toxicity of these compounds at 37°C (Brorson *et al.*, 1999). Importantly the rates of NO release from NONOates may also be influenced by metal ions and have a striking dependence upon pH (Davies *et al.*, 2001), and so they should be used with a degree of caution.

Measuring NO

Changes in NO following ischaemic insults have been monitored by several methods, including electron paramagnetic resonance (Tominaga *et al.*, 1993; Wei *et al.*, 1999), or microdialysis (Adachi *et al.*, 2000; Fassbender *et al.*, 2000; Lei *et al.*, 1999); unfortunately these methods give readings that are difficult to translate into brain NO concentrations. Monitoring NO directly with time using a microsensor should be a good solution, but results using this

technique have been variable. In one often quoted study (Malinski *et al.*, 1993) NO rose from undetectable levels to a 1-4 μM peak in the time immediately following middle cerebral artery occlusion. Alternatively, a much lower peak concentration (< 20 nM) has been recorded (Lin *et al.*, 1996). Microsensor data may well be compromised since the electrodes are susceptible to interference from various compounds including tyrosine and ascorbate (Lin *et al.*, 1996; Stingele *et al.*, 1998). Perhaps the most sensitive method of measuring NO in the brain is to use $NO_{GC}Rs$ as a biosensor and follow the production of cGMP. Recent data obtained in this manner from striatal slices agrees with the lowest microsensor estimates of post-ischaemic NO, in that NO concentrations were less than 1 nM and rose only modestly (2-fold) following simulated ischaemia (Griffiths *et al.*, 2002a).

Formation of $ONOO^-$ is thought to be one of the major routes by which NO may elicit neuronal death. *In vitro*, $ONOO^-$ may nitrate tyrosine residues at physiological pH (Beckman & Koppenol, 1996; Reiter *et al.*, 2000) and the presence of 3-nitrotyrosine (3-NT) staining post-ischaemia is often taken as strong evidence of the formation of $ONOO^-$ in toxic quantities (Forman *et al.*, 1998; Tanaka *et al.*, 1997). However such data should be treated with caution, firstly because tyrosine nitration may be indicative of a number of reactive nitrogen species (RNS) *in vivo* and secondly, because the assays used to detect 3-NT appear unreliable (Halliwell *et al.*, 1999).

The recent development of a series of fluorescent NO indicators (Kojima *et al.*, 1998a) has culminated in an increasing use of the compound 4,5-diaminofluorescein (DAF-2). In the presence of O_2, NO and NO-related RNS nitrosate DAF-2 to yield the highly fluorescent DAF-2 triazole. DAF-2 and its cell permeable analogue DAF-2 DA have been used to image NO following NMDA stimulation in the CA1 region of acute hippocampal slices (Kojima *et al.*, 1998b), and in organotypic hippocampal slices subjected to ischaemia (Morrison *et al.*, 2002). However, the specificity of these compounds has been questioned following a report that their fluorescence may be enhanced by Ca^{2+}, Mg^{2+} or incident light (Broillet *et al.*, 2001). Although further investigation blamed these effects upon increased NO release from the donor *S*-nitrosocysteine, other donors used in the original

study (DETA/NO and SNP) have no such reactivity (Suzuki *et al.*, 2002). Other groups have since highlighted DAF-2 reactivity with ascorbic acid (Zhang *et al.*, 2002b) or $ONOO^-$ (Roychowdhury *et al.*, 2002). Use of these fluorescent compounds should therefore only be undertaken with stringent controls, and in the knowledge that results are unlikely to be quantitative for NO.

1.8 DOES NO CONTRIBUTE TO ISCHAEMIC DAMAGE?

The initial evidence of a role for NO in ischaemic damage has been reviewed many times, the focus here is on recently published data investigating the role of endogenously produced NO.

In vitro

NO from nNOS

Data from dissociated cultures are conflicting. Further evidence for NO-dependent cell death includes an interesting set of experiments in which NMDA-stimulated NO formation (measured by EPR spin trapping) was correlated with cerebellar granule cell death. When levels of methylarginines (thought to be endogenous competitive inhibitors of NOS) were raised, NO-mediated excitotoxicity was suppressed (Cardounel & Zweier, 2002). Confirming previous findings, several groups have also been able to evoke NO release and subsequent death (blocked by the addition of L-NAME) by stimulating cortical cultures with glutamate (Almeida & Bolanos, 2001; Ruiz *et al.*, 2000). Conversely, NOS inhibition failed to protect neurones from hypoxia-induced cell death (Demerle-Pallardy *et al.*, 2000) and, in agreement with earlier findings that NO is not toxic (Hewett *et al.*, 1993), nitrotyrosine staining was not detected in NMDA stimulated cortical neurones (Trackey *et al.*, 2001). No attempt was made to quantify NO levels in these studies.

Interestingly there are no new reports of NO contributing to damage in intact brain slice models. However, recent studies have monitored the production of NO following hypoxia (Meini *et al.*, 2003) and stimulated ischaemia (Oxygen glucose deprivation: OGD) in the striatum (Griffiths *et al.*,

2002a) and have confirmed previous findings *in vivo* (Toung *et al.*, 1999) indicating that NO rises only very modestly (2-fold) following such insults, perhaps by as little as 35 %, from 0.6 to 0.8 nM (Griffiths *et al.*, 2002a).

NO from iNOS

Several groups have investigated the toxicity of NO generated from iNOS *in vitro*. Expression of functional iNOS may be induced by stimulating glia with a combination of a bacterial endotoxin (lipopolysaccharide, LPS) and a cytokine (interferon-γ, IFN-γ). Astrocytic (Stewart *et al.*, 2000; Stewart *et al.*, 2002) microglial (Golde *et al.*, 2002) or mixed glial (Bal-Price & Brown, 2001) cultures stimulated in this way have all been shown able to cause death of co-cultured neurones through NO release. Unfortunately the relevance of these findings is difficult to gauge since glia proliferate in culture while neuronal numbers remain relatively constant. The numbers of iNOS positive cells (and NO level generated) may therefore be markedly enhanced compared to *in vivo*.

In addition to glia, iNOS expression in the ischaemic brain has also been detected in cerebral endothelial cells (Iadecola *et al.*, 1996) which *in vitro* can produce NO after treatment with proinflammatory cytokines (Bonmann *et al.*, 1997). Following ischaemia, cerebral endothelial cell death may contribute to secondary injuries, such as breakdown of the blood brain barrier. Recent work has shown that OGD induces iNOS in cultured endothelial cells, leading to an NO-dependent cell death (Xu *et al.*, 2000). However, the relevance of NO toxicity in such an unphysiological scenario is unclear.

Compared with dissociated cultures, organotypic slices of hippocampus appear a better model in which to examine iNOS generated NO toxicity. Neuronal/glia ratios are akin to those seen *in vivo*, and the progression of cell death may be followed for many days. In agreement with a recent study in glial cultures (Sola *et al.*, 2002) it has been confirmed in our lab that iNOS activation is confined to microglia in LPS/IFN-γ stimulated hippocampal slice cultures. However, in contrast to another study (Lee *et al.*, 2003), when NO levels (measured by monitoring accumulated NO_x^-) were

significantly increased compared to control, no neuronal cell death was seen up to six days post-stimulation [Dr S. Duport, personal communication].

In vivo

Further experiments using 7-NI treated rats or nNOS deficient mice have confirmed that NO generated from nNOS increases post-ischaemic infarct volumes (Goyagi *et al.*, 2001). However the picture remains complicated as 7-NI did not protect neurones when administered during reperfusion following focal ischaemia, leading to the suggestion that eNOS generated NO may contribute to cellular demise at that time (Gursoy-Ozdemir *et al.*, 2000). A recent study also indicates that changes in intracellular pH during ischaemia may have a strong bearing on the variable results previously seen with 7-NI, and other inhibitors (Coert *et al.*, 2003).

Concerning the role of iNOS *in vivo,* microdialysis studies in rat striatum did not detect a major generation of NO during acute cerebral ischaemia whereas, 2 days following the insult, nitrite levels doubled. This delayed NO generation was inhibited by aminoguanidine, indicating iNOS dependence (Fassbender *et al.*, 2000). In agreement, the iNOS selective inhibitor 1400W reduced infarct size by 36 %, ameliorated the neurological score, and attenuated the weight loss of rats normally seen 3 days following transient focal ischaemia (Parmentier *et al.*, 1999). Antisense oligodeoxynucleotides against iNOS have also decreased lesion volume and nitrotyrosine staining following transient focal ischaemia in rats (Parmentier-Batteur *et al.*, 2001). Furthermore, aminoguanidine-treated mice displayed reduced infarct volumes similar to those seen in iNOS knockout mice (Sugimoto & Iadecola, 2002), whilst L-arginine exacerbated the infarct size in wild-type but not iNOS knockout mice, supporting a deleterious effect of NO (Zhao *et al.*, 2003). Expression of iNOS *per se*, however, may not be damaging because local or systemic LPS injection caused the expression of iNOS in microglia *in vivo*, but no neuronal degeneration (Han *et al.*, 2002; Morimoto *et al.*, 2002). Perhaps, in ischaemia, iNOS-derived NO is damaging because it adds on to other ongoing pathological mechanisms (see below).

1.9 HOW DOES NO CONTRIBUTE TO ISCHAEMIC DAMAGE?

Respiratory inhibition

The inhibition of mitochondrial respiration by NO has been the subject of some excellent recent reviews (Brown & Borutaite, 2002; Moncada & Erusalimsky, 2002; Stewart & Heales, 2003). Briefly, NO may inhibit respiration reversibly at cytochrome *c* oxidase (see above), or irreversibly, after prolonged exposure, at multiple sites including complex I (Clementi *et al.*, 1998), possibly after the conversion of NO to other RNS such as $ONOO^-$ (Riobo *et al.*, 2001). Alternatively NO may shift the mitochondrial electron transport chain into a more reduced state, enhancing $O_2^{\bullet-}$ formation (Poderoso *et al.*, 1996). At lower NO levels this $O_2^{\bullet-}$ increase will result in H_2O_2 production while higher NO levels are likely to scavenge the $O_2^{\bullet-}$ resulting in $ONOO^-$ production. Several different pathways to cell death can occur. Apoptosis or necrosis may ensue dependent upon the glycolytic capacity of the cell (Bal-Price & Brown, 2000). ATP depletion may also lead to further excitotoxicity in neurones (Bal-Price & Brown, 2001; Stewart *et al.*, 2002). Alternatively NO, or more likely RNS, can cause induction of the MPT and apoptosis (Borutaite *et al.*, 2000).

It is important to note that inhibition of respiration by NO is in competition with O_2. At the hyperoxic O_2 concentrations (approximately 200 μM) in which air equilibrated *in vitro* experiments are undertaken, the IC_{50} of NO for cytochrome *c* oxidase may be up to 450 nM (Koivisto *et al.*, 1997) compared with an IC_{50} of 120 nM at physiological tissue O_2 (Bellamy *et al.*, 2002a). This difference in potency highlights the need to conduct *in vitro* experiments at more physiological O_2 concentrations.

Elegant studies in dissociated neuronal cultures have recently implicated both reversible (Bal-Price & Brown, 2001) and irreversible (Stewart *et al.*, 2000) respiratory inhibition, and subsequent excitotoxicity (Bal-Price & Brown, 2001; Stewart *et al.*, 2002), dependent upon neuronal maturation state (Golde *et al.*, 2002), as the cause of death following expression of iNOS in co-cultured glia. Similarly, glutamate receptor-mediated activation of nNOS in cultured cortical neurones may trigger either

apoptosis (by transient inhibition of ATP synthesis and MPT pore opening) or necrosis (by oxidative stress and persistent inhibition of ATP synthesis) (Almeida & Bolanos, 2001).

Although direct inhibition of respiration by NO is an appealing hypothesis, the only study to address this possibility *in vivo* gave evidence to the contrary (De Visscher *et al.*, 2002). In addition, although NO production following expression of iNOS in organotypic hippocampal slices may be toxic (Lee *et al.*, 2003), this is not always the case (Dr S.Duport, personal communication). In agreement, LPS injection was only seen to be toxic *in vivo* when paired with an excitotoxic stimulus, in this case injection of ibotenate (Morimoto *et al.*, 2002).

$ONOO^-$ formation

$ONOO^-$ is a powerful oxidant, more toxic than NO and $O_2^{\bullet-}$ individually, that is formed when the rate of NO generation is sufficient to out-compete superoxide dismutase (SOD) for $O_2^{\bullet-}$ (Beckman & Koppenol, 1996). Under ischaemic conditions the $O_2^{\bullet-}$ required for this reaction may be generated by leakage from the mitochondrial electron transport chain, particularly during reperfusion (Piantadosi & Zhang, 1996). Amongst other mechanisms, enzymatic $O_2^{\bullet-}$ generation may occur by the xanthine/xanthine oxidase pathway or from plasma membrane NADPH oxidase in microglia and other cells (see chapter 6.1). The reactions of $ONOO^-$ are affected by intracellular pH and antioxidant status, and death may be initiated by many mechanisms, including lipid peroxidation, protein nitration, DNA damage, or the irreversible inhibition of respiration (recently reviewed in Szabo, 2003; Virag *et al.*, 2003).

Unfortunately the production of $ONOO^-$ is difficult to detect unequivocally. As before, recent studies have monitored 3-nitrotyrosine increases in both focal (Han *et al.*, 2002; Parmentier-Batteur *et al.*, 2001; Gursoy-Ozdemir *et al.*, 2000) and global (Alonso *et al.*, 2002) ischaemia, though the specific mechanism of death was not investigated. Recent work has demonstrated that $ONOO^-$ may be generated *in vitro* by microglia activated with LPS/IFN-γ (to stimulate iNOS) and phorbol 12-myristate 13-acetate (to generate $O_2^{\bullet-}$) though the quantities involved were not enough to

cause neuronal death over the short timecourse of these experiments (Bal-Price *et al.*, 2002).

$ONOO^-$-dependent DNA damage and subsequent activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP-1) has been well studied in ischaemia. PARP-1 uses NAD (an important co-enzyme in energy metabolism) as its substrate. Excessive PARP-1 activation leads to decreased NAD levels and thus subsequent ATP depletion (Pieper *et al.*, 1999). Both inhibition of PARP-1 (Plaschke *et al.*, 2000) or knockout of the PARP-1 gene (Eliasson *et al.*, 1997b) have shown significant protection in experimental ischaemia. Consistent with the theory that NO and $ONOO^-$ are involved in PARP-1 activation, PARP-1 was not activated after NMDA administration in mice lacking the nNOS gene (Mandir *et al.*, 2000).

Other mechanisms

One means by which low NO levels may cause damage is through enhanced synaptic transmission. *In vivo*, most hippocampal CA1 neurones destined to die following ischaemic reperfusion were found to exhibit potentiated synaptic transmission (Gao *et al.*, 1999) and, following inhibition of mitochondrial respiration *in vitro*, endogenous NO (acting *via* cGMP) potentiates hippocampal synaptic transmission during low frequency stimulation (Bon & Garthwaite, 2001). It may be proposed, therefore, that NO aids synaptic potentiation and, hence, delayed excitotoxicity by operating through physiological cGMP-dependent pathways. Consistent with this hypothesis, transient anoxia can elicit a long term potentiation-like phenomenon that can be blocked by NO synthase inhibition in the CA1 hippocampus *in vitro* (Huang & Hsu, 1997). Other ways in which low (nanomolar) concentrations of NO could contribute to damage include a cGMP-dependent enhancement of neurotransmitter release (Trabace & Kendrick, 2000) or activation of ion channels (Kaupp & Seifert, 2002).

Alternative mechanisms include matrix metalloproteinases (MMPs), a class of endopeptidase whose levels are increased in experimental ischaemia and which may be deleterious. Recent findings suggest that activation of extracellular soluble or membrane bound MMPs may occur by *S*-nitrosation reactions following ischaemia (Gu *et al.*, 2002). Another

interesting possibility is that NO may induce intracellular Ca^{2+} mobilisation from the endoplasmic reticulum following hypoxia (Meini *et al.*, 2003).

Summary I (NO-dependent death)

The conflicting data generated in dissociated cultures continues to muddy the issue of whether endogenous NO generated from nNOS causes cell death post-ischaemia. There has been no confirmation that nNOS generates the high (μM) levels of NO measured previously (Malinski *et al.*, 1993). Indeed, NO levels appear to rise so modestly that it is difficult to understand how they could become directly toxic (Griffiths *et al.*, 2002a). While blocking nNOS *in vivo* with 7-NI appears protective there remain several unresolved issues concerning this compound (see artifacts section above). The possibility remains however, that low NO levels generated by nNOS can contribute to damage by enhancing synaptic transmission, or through formation of $ONOO^-$.

Although dissociated cultures exposed to LPS/IFN-γ can die in response to iNOS-generated NO, a similar activation of endogenous microglia is not always sufficient to cause neuronal death in more complex tissue models. Meanwhile, data generated *in vivo* with selective inhibitors and knockout mice does strengthen the case for iNOS-dependent death that is delayed several days post-insult. The most likely cause of iNOS-dependent cell death in ischaemia is through formation of $ONOO^-$. It shall be interesting to see if direct targeting of this oxidant using the novel $ONOO^-$ decomposition catalyst recently described (Szabo *et al.*, 2002) offers therapeutic benefit.

1.10 IS NO PROTECTIVE IN ISCHAEMIA?

In vitro

Historically, *in vitro* experiments have been used to examine the toxic role of NO in cerebral ischaemia. However the subsequent use of NOS inhibitors *in vivo* quickly uncovered evidence that NO could be protective through mechanisms such as vasodilation and the inhibition of platelet aggregation or leukocyte adhesion (Iadecola, 1997; Samdani *et al.*, 1997a; Strijbos, 1998).

Although other mechanisms of NO protection have been suggested (see below), only a few groups have addressed them in excitotoxic or ischaemic models *in vitro.* Recently NO donors were reported to reduce NMDA-induced neuronal injury in mixed cortical cultures (Vidwans *et al.*, 1999), and in oligodendrocytes undergoing oxidative stress (Rosenberg *et al.*, 1999). NO is also involved in ischaemic preconditioning in dissociated cultures subjected to OGD (Gonzalez-Zulueta *et al.*, 2000), hippocampal slices undergoing anoxic preconditioning (Centeno *et al.*, 1999), and a macrophage-like cell line (RAW264) preconditioned against an NO induced death (Yoshioka *et al.*, 2003).

In vivo

A selection of studies has continued to address eNOS expression in ischaemia. eNOS protein expression starts to increase in all striatal microvessels between 6 and 24 hrs following transient focal ischaemia, and continues for at least a week (Veltkamp *et al.*, 2002). Similarly eNOS staining increased during the week following permanent focal ischaemia and, interestingly, appeared in both neuronal and non-neuronal cell types, particularly in the penumbra (Leker *et al.*, 2001). Furthermore, during the early phase of global ischaemia NO release appears to derive prominently from eNOS, although this isoform contributes to only a fraction of total brain NOS activity (Wei *et al.*, 1999).

NO is protective following transient ischaemia in gerbil striatum, since damage is aggravated by L-NNA; the similar effect of 7-NI implies that nNOS may be responsible for protection rather than eNOS (Adachi *et al.*, 2000). Non-transcriptional activation of eNOS in response to corticosteroid treatment augments cerebral blood flow and reduces infarct size in a mouse model of transient cerebral ischaemia (Limbourg *et al.*, 2002). In contrast, other experiments using 7-NI and L-NNA have concluded that eNOS-generated NO may be damaging during reperfusion (Gursoy-Ozdemir *et al.*, 2000). In agreement with the hypothesis that NO is protective, NO donors reduced infarct volume in a rat models of global (Mason *et al.*, 2000) and transient focal (Pluta *et al.*, 2001) ischaemia. Finally, other recent evidence

indicates that NO is involved in ischaemic preconditioning (Atochin *et al.*, 2003), angiogenesis (Zhang *et al.*, 2003) and neurogenesis (Zhang *et al.*, 2001; Zhu *et al.*, 2003) (see below).

1.11 HOW IS NO PROTECTIVE IN ISCHAEMIA?

NO as an antioxidant

Although much of the literature has focused on the reaction of NO with $O_2^{\bullet-}$ (and formation of deleterious $ONOO^-$) NO is extremely reactive with other species such as peroxyl ($RO_2^\bullet$) and hydroxyl ($OH^\bullet$) radicals. By these reactions, or chelation of redox active metal ions such as iron to form iron nitrosyl complexes, NO may achieve antioxidant effects (Halliwell *et al.*, 1999; Kagan *et al.*, 2001; Wink *et al.*, 2001). There is extensive evidence that ROS formation and consequently lipid peroxyl radicals ($LOO^\bullet$) are deleterious in stroke, and a good degree of neuroprotection is seen upon application of lipid peroxidation inhibitors (Hall *et al.*, 1997; Huh *et al.*, 2000). NO reacts potently with $LOO^\bullet$ and, by acting as a chain-breaking antioxidant, prevents the propagation of lipid peroxides with a greater efficacy than the endogenous antioxidant α-tocopherol (vitamin E) (O'Donnell *et al.*, 1997). The inhibition of lipid peroxidation by NO has been well reviewed recently (Hogg & Kalyanaraman, 1999; O'Donnell & Freeman, 2001) and, though there is no direct evidence that NO is protective by this manner in ischaemia, the protective effects of NO release from donors post-ischaemia correlate with a decrease in levels of reactive O_2 species (Mason *et al.*, 2000; Pluta *et al.*, 2001). Finally, it is important to remember that NO is not solely an antioxidant: its actions will greatly depend upon the redox environment of the cell and the levels of other endogenous antioxidants, such as glutathione, SOD, catalase and vitamin E.

Increase in cerebral blood flow

Perhaps the clearest mechanism by which NO exerts protective effects in ischaemic tissue is through the maintenance of cerebral blood flow (Iadecola, 1997; Samdani *et al.*, 1997a; Strijbos, 1998). Recently, NOS inhibitors have

been used to assess the relative contributions of the different NOS isoforms to blood flow during forebrain ischaemia and the results indicate that the contribution of nNOS to vasodilation in hippocampus, striatum and cortex is greater than that of eNOS (Santizo *et al.*, 2000). However, this phenomenon may be model-dependent since earlier studies with knockout mice implicated eNOS in improved perfusion following focal ischaemia (Lo *et al.*, 1996). More recent work in eNOS knockout mice supports the idea that eNOS activity is critical for augmenting blood flow during acute L-arginine infusion (Yamada *et al.*, 2000).

Stimulation of angiogenesis

Angiogenesis, the process by which new blood vessels are formed from pre-existing ones, may be regulated by several growth factors, including vascular endothelial growth factor (VEGF), which aids the recruitment and proliferation of endothelial cells. VEGF promotes NO production and induces eNOS and iNOS expression in vascular endothelial cells *in vitro* (Hood *et al.*, 1998; Kroll & Waltenberger, 1998). NO may also act upstream of VEGF and enhance its synthesis by stabilising the activity of hypoxia-inducible factor (HIF-1) (Dulak & Jozkowicz, 2003). NO is known to stimulate angiogenesis in both ischaemic cardiac tissue (Maulik & Das, 2002) and ischaemic retina *in vivo* (Ando *et al.*, 2002), and principally exerts its actions through cGMP-dependent pathways (Donnini & Ziche, 2002). Cerebral eNOS may play a predominant role in VEGF-induced angiogenesis and vascular permeability *in vivo* (Fukumura *et al.*, 2001) and, in the ischaemic penumbra, eNOS expression appears to temporally and anatomically co-localise with VEGF expression (Leker *et al.*, 2001). Though few groups have directly examined NO-induced angiogenesis in ischaemic brain, recent experiments using DETA/NO support the conclusion that the NO/cGMP pathway is involved (Zhang *et al.*, 2003).

Inhibition of apoptosis

Apoptosis, or programmed cell death, is directly associated with the activation of caspase proteases and mitochondrial cytochrome *c* release, and is one of the end stage mechanisms by which delayed neuronal death

manifests following ischaemia (Lipton, 1999; Nicotera *et al.*, 1999). Using antagonists of the $NO_{GS}Rs$, NO donors, or synthetic cGMP analogues, it has been found that NO, at low non-toxic concentrations, potently inhibits apoptosis in a cGMP/cGK-dependent manner in many cell types (reviewed in Fiscus, 2002). Indeed, basal cGMP appears to be requisite for survival of cerebellar granule cells in culture (Ciani *et al.*, 2002c). This, and other studies using a PC12 cell line (Ha *et al.*, 2003) have indicated a role for the PI3K/Akt pathway in NO-mediated protection from apoptosis, possibly by phosphorylation and inhibition of the pro-apoptotic protein BAD. Alternatively cGK-dependent phosphorylation of another transcription factor, cAMP response element binding protein (CREB) may be involved (Ciani *et al.*, 2002a; Ciani *et al.*, 2002b).

Ischaemic preconditioning

Ischaemic preconditioning is the ability of brief (sublethal) insults to protect the tissue from a subsequent severe ischaemia. Preconditioning may be rapid (min to hrs between insults) or delayed, with hrs to days between insults and requiring new protein synthesis. Though the molecular mechanisms are not fully understood a growing body of literature supports the role of NO in cerebral ischaemic preconditioning (Centeno *et al.*, 1999; Gonzalez-Zulueta *et al.*, 2000). This includes a reported lack of rapid cerebral preconditioning in nNOS and eNOS knockout mice compared to wildtype (Atochin *et al.*, 2003). The NO-dependent signalling cascades responsible for neuronal ischaemic preconditioning were recently reviewed (Nandagopal *et al.*, 2001), and are thought to involve NMDA receptor-dependent NOS activation, the Ras/Erk signalling cascade and ultimately the activation of CREB, leading to increased production of neuroprotective molecules.

Stimulation of neurogenesis

Neurogenesis is now known to occur in the adult rat dentate gyrus after simulated stroke (Jin *et al.*, 2001; Liu *et al.*, 1998) suggesting it may be a critical element of brain repair. Recently it was reported that NO released from DETA/NO may induce neurogenesis and reduce functional deficits in post-ischaemic rat dentate gyrus (Zhang *et al.*, 2001). In agreement, another

group attributed a 3-fold increase in new post-ischaemic granule neurones in rat dentate gyrus to NO release since neither iNOS knockout mice nor aminoguanidine-treated rats showed such increases (Zhu *et al.*, 2003). Similar neurogenic effects, and reduced neurological deficits, are seen when cGMP levels were increased following application of sildenafil (an inhibitor of cGMP breakdown by the phosphodiesterase PDE5) to post-ischaemic rats (Zhang *et al.*, 2002a).

NMDA receptor modulation

An often-quoted mechanism by which NO may have protective actions is through direct action at a redox modulatory site on the NMDA receptor (Lipton *et al.*, 1993; Lipton & Stamler, 1994). However this theory is controversial (Aizenman *et al.*, 1998) and to date there is no convincing evidence that it occurs *in vivo.*

Summary II (NO protection)

A good deal of evidence supports a protective role for NO in cerebral ischaemia, and our knowledge of the mechanisms by which NO can be protective has advanced substantially. There appears to be little debate that eNOS-generated NO is protective through blood flow augmentation. Both nNOS and eNOS may be involved in the process of preconditioning, while iNOS looks to be important in subsequent repair. A substantial literature has continued to highlight the potential for NO to act as a direct antioxidant or, through cGMP, to inhibit apoptosis or stimulate angiogenesis. These mechanisms remain to be examined thoroughly in cerebral ischaemia models *in vivo*.

1.12 CONCLUSIONS

The flood of conflicting evidence concerning the involvement of NO in post-ischaemic neurodegeneration or protection may now be evaluated in light of the many pitfalls to have beset the field. With this in mind, the last few years have seen little evidence that, following cerebral ischaemia *in vivo*,

endogenous nNOS produces concentrations of NO sufficient to have direct cytotoxic effects. That nNOS knockout mice show less ischaemia-evoked damage may reflect alternative (possibly physiological) mechanisms engaged by NO. The use of a new generation of selective inhibitors has confirmed that iNOS-generated NO can contribute to ischaemic damage; although the mechanism remains to be established, direct respiratory inhibition appears unlikely. In general the tide is turning towards a better appreciation of the protective and restorative actions of NO and, with regard to cerebral ischaemia, we are only beginning to understand the many ways by which such protection may be mediated. Elucidating the molecular mechanisms by which NO exerts protection may help identify new potential therapeutic targets.

1.13 GENERAL AIMS

Many doubts remain as to the validity of studies designed to examine NO toxicity. Particularly lacking is a consensus as to the toxic NO concentration generated following pathological insult, meaning that experiments using exogenous donor compounds are difficult to understand in context. This NO concentration is ultimately a balance between production and breakdown, though surprisingly little is known about the latter. The following chapters explore the reactivity and toxicity of NO in a complex tissue culture model (organotypic slices). In later chapters a recently described mechanism by which NO is consumed in brain preparations (Griffiths & Garthwaite, 2001) has been further characterised.

CHAPTER 2: MATERIALS AND METHODS

2.1 MATERIALS

Compound	Abbreviation	Source
D(-)-2-Amino-5-phosphonopentanoic acid	D-AP5	Tocris
Ascorbate	-	Sigma
Ascorbate Oxidase	AO	Sigma
B27 supplement (without antioxidants)	B27	Life-Tech
Bovine serum albumin (fraction V)	BSA	Sigma
Cuprizone	-	Sigma
Guanosine 3',5'-cyclic monophosphate	cGMP	Sigma
Guanosine triphosphate	GTP	Sigma
Deferoxamine-mesylate	-	Sigma
Deoxyribonuclease	DNase	Sigma
Diaminobenzadine	DAB	Vector
Diethylamine NONOate	DEA/NO	Alexis
Diethylenetriamine NONOate	DETA/NO	Alexis
Diethylenetriaminepentaacetic acid	DTPA	Sigma
Dimethyl sulfoxide	DMSO	Sigma
Dithiothreitol	DTT	Sigma
Dizocilpine maleate	MK-801	Tocris
Deoxyribonuclease	DNase	Sigma
Dulbecco's modified eagle medium	DMEM; 41965	Life-Tech
Ethylenediaminetetraacetic acid	EDTA	Sigma
Ethylene glyco-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid	EGTA	Sigma
Hank's balanced salt solution	HBSS	Life-Tech
Haemoglobin	Hb	Sigma
Haemoglobin beads	Hb-beads	Sigma
Horse serum (heat inactivated)	HS	Life-Tech
N-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid	Hepes	Sigma

1-Hydroxy-2-oxo-3-(*N*-ethyl-2-aminoethyl)-3-ethyl-1-triazene	NOC-12	Alexis
3-Isobutyl-1-methylxanthine	IBMX	Sigma
Minimal essential medium (with Hepes)	MEM 10012	Life-Tech
Minimal essential medium (with $NaHCO_3$)	MEM 31095	Life-Tech
Minimal essential medium vitamin mix	Vits	Life-Tech
Myxothiazol	-	Sigma
Neurobasal medium	NBM 21103	Life-Tech
L-nitroarginine	L-NNA	Tocris
7-nitroindazole	7-NI	Tocris
Neocuproine	-	Sigma
N-Methyl-D-aspartic acid	NMDA	Sigma
Penicillin/streptomycin	P/S	Life-Tech
Propidium Iodide	PI	Sigma
Poly-*D*-Lysine	-	Sigma
Riboflavin	Rib	Sigma
RPMI-1640	No. 31870	Life-Tech
Sodium hydroxide	NaOH	BDH
Sodium nitrite	$NaNO_2$	BDH
Sodium cyanide	NaCN	Sigma
Soluble guanylate cyclase (from bovine lung)	sGC	Alexis
Soybean trypsin inhibitor	-	Sigma
Superoxide dismutase	SOD	Sigma
Triton X-100	-	Sigma
Tris(hyroxymethyl)aminomethane	Tris	Sigma
6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid	Trolox	Sigma
Trypan blue	-	Sigma
Trypsin	-	Sigma
Trypsin/EDTA	-	Life-Tech
Uric acid	-	Sigma

Table 2.1 General materials

KEY

BDH:	BDH/Merck, Poole, Dorset, UK.
Calbiochem	Calbiochem, CN Biosciences UK, Nottingham, UK.
Life-Tech:	Life-Technologies Ltd (GIBCO-BRL), Paisley, UK.
Sigma:	Sigma-Aldrich Company LTD, Poole, Dorset, UK.
Tocris:	Tocris: Tocris Cookson Ltd, Avonmouth, Bristol, UK
Vector:	Vector Labs, Inc, Burlingame, CA 94010, US.

1st Antibody	1st Host	Conc.	2nd Host	Conc.	Linked
cGMP (gift from Dr. J. de Vente, Maastricht, Netherlands)	Sheep	1:8000	Donkey	1:200	Biotin
GFAP (Dako, Cambridgeshire, UK)	Rabbit	1:1000	Donkey	1:200	Rhodamine
nNOS (gift from Dr. P.C. Emson, Cambridge, UK.)	Sheep	1:10000	Donkey	1:200	Biotin
eNOS (BD Transduction Labs, Kentucky, USA)	Mouse	1:1000	Horse	1:300	Biotin

Table 2.2 Antibodies for immunohistochemistry

2.2 GENERAL SOLUTIONS

NO donors

Donor stocks were prepared freshly each day in 10 mM NaOH, and kept on ice to prevent decomposition.

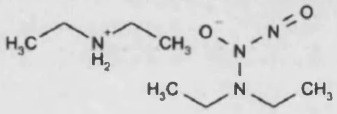
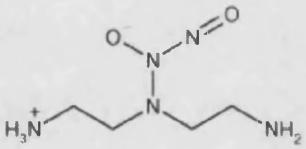
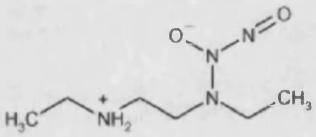
Donor	Structure	Half-life (pH 7.4, 37°C)
DEA/NO		2.1 min
DETA/NO		20 h
NOC-12		100 min

Table 2.3 NO donor compounds

Tissue inactivation buffer

50 mM Tris, 4 mM EDTA at pH 7.4, heated to 90°C 5 min prior to use

Haemoglobin preparation

1 mM Hb (type 1) was incubated with 10 mM Na^+ dithionate for 4 hours at room temperature. 10 ml of Hb/dithionate solution was dialysed in 1 L of water at 4°C overnight (water was changed after 1 hour), and stored in aliquots at –20°C until use.

2.3 GENERAL METHODS

Tissue Inactivation

GC reaction mix or hippocampal slices were inactivated by immersion in an Eppendorf tube containing 150-200 µl tissue inactivation buffer, pre-heated to 90°C for 5 min. Inactivated enzyme or tissue was homogenised and frozen at –20°C until use.

cGMP radioimmunoassay

Inactivated tissue was homogenised by sonication, a sample was removed for protein determination and the remainder centrifuged at 10,000g for 5 min at 4°C. The supernatant was analysed for cGMP content using radioimmunoassay (details available from Amersham plc, Buckinghamshire, UK) and expressed as pmol cGMP/mg protein.

Protein Determination

Sample protein content was determined using the bicinchoninic acid method. Briefly, 10 µl samples or BSA standards (0-1 mg/ml; prepared in inactivation buffer) was added to 200 µl of bicinchoninic acid reagent (Pierce biotechnology, Rockford, US.). Samples were incubated at 37 °C for 30 min and absorbance read at 642 nm. Sample protein content was determined against the BSA standard curve.

Measurement of NO and O_2.

Continuously stirred samples (1 ml) were measured in tissue culture medium, cell incubation buffer, Tris (25 mM), or Hepes (25 mM), at pH 7.4 in a sealed chamber maintained at 37°C. In some experiments the chamber remained open to the air. The chamber was equipped with a Clark type O_2 electrode (Rank Brothers, Bottisham, Cambs, U.K.) and an NO sensitive electrochemical probe (ISO-NOP, World Precision Instruments, Stevenage, U.K., see figure 2.1). The NO probe was calibrated using the chemical reduction of $NaNO_2$ (0.1-10 µM) in the presence of 0.1 M H_2SO_4 and 0.1 M KI. The O_2 electrode was zeroed by addition of excess Na^+ dithionate, and O_2 content of air-equilibrated solution assumed to be 185 µM. Compounds

were injected into the chamber through a rubber seal using a Hamilton syringe (maximum volume, 10 µl). NO and O_2 probe signals were sampled at 1 Hz using Duo 18 version 1.1^{TM} (World Precision Instruments). At the termination of each experiment the probes were baselined by the addition of excess Hb or Na^+ dithionate as appropriate.

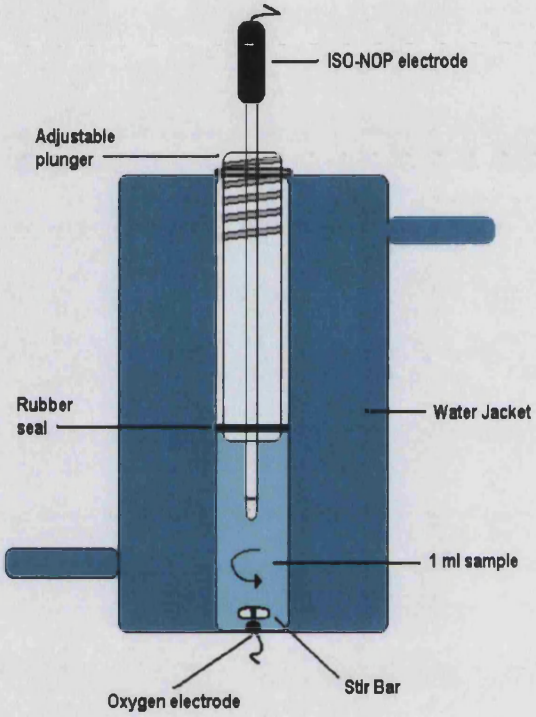


Figure 2.1 NO and O_2 electrode setup

CHAPTER 3: IDENTIFYING A CONFOUNDING ARTIFACT

3.1 INTRODUCTION

One of the key criteria to be met in order to sustain the hypothesis that NO is an endogenous toxin is that exogenous NO, at concentrations relevant to those produced endogenously, should be able to elicit cell death. Unfortunately, there is little knowledge of what constitutes a pathological concentration of NO when it is generated endogenously *in vivo*. As previously mentioned (chapter 2), various NO-releasing chemicals have been used in various concentrations to investigate the toxic potential of NO (Bonfoco *et al.*, 1995; Brorson *et al.*, 1999; Dawson *et al.*, 1996). Historically the most commonly used ones include sodium nitroprusside (SNP), S-nitroso-N-acetylpenicillamine (SNAP) and 3-morpholinosydnonimine (SIN-1). However, the primary species produced by these donors may not be NO. For example, SIN-1 decomposition is associated with $O_2^{\bullet-}$ release and $ONOO^-$ generation (Feelisch *et al.*, 1989). Furthermore reactive nitrogen species that act as nitrosonium ion (NO^+) donors have been implicated in the actions of SNAP and SNP (Butler *et al.*, 1995; Lipton & Stamler, 1994).

The advent of the NONOates, which release authentic NO with predictable kinetics, has simplified matters. The rate of NO release from the NONOates is a function of pH, temperature and the identity of the nucleophile carrier (Keefer *et al.*, 1996). In aqueous, aerobic solutions, NO is inactivated by reaction with O_2 in a process termed autoxidation (Ford *et al.*, 1993). Knowing the kinetics of NO release and inactivation, the resulting NO concentrations in biological buffers can be predicted (Schmidt *et al.*, 1997).

In aqueous, aerobic solutions, NO reacts with molecular O_2 to ultimately form NO_2^- according to the reaction sequence described below.

(1) $2NO + O_2 \rightarrow 2NO_2$

(2) $2NO_2 + 2NO \rightarrow 2N_2O_3$

(3) $2N_2O_3 + 2H_2O \rightarrow 4NO_2^- + 4H^+$

This autoxidation reaction is summarised in equation (4) below, where *k* is the rate constant of inactivation.

$$(4)\quad \frac{d[NO]}{dt} = k[O_2][NO]^2$$

By monitoring the appearance of NO_2^-, disappearance of NO, or formation of H^+, several groups have studied the kinetics of this reaction and made various estimates of *k* at 22-25°C, pH 7.4 (Goldstein & Czapski, 1995; Kharitonov *et al.*, 1994; Lewis & Deen, 1994; Wink *et al.*, 1993). It is clear from these studies, and equation (4) above, that the autoxidation reaction is second order with respect to NO and first order with respect to O_2. Thus, though relatively stable at low nM concentrations, higher NO concentrations are more rapidly inactivated. This is illustrated clearly when following NO profiles generated by the NONOates, autoxidation is negligible in the initial phase but becomes progressively effective as NO concentrations rise (Figure 3.1). Using their reported value for *k* at 37°C (13.6×10^6 M^{-2} s^{-1}) the mathematical model of Schmidt *et al.,* has been used to illustrate the differing NO profiles derived from donor compounds used in this thesis.

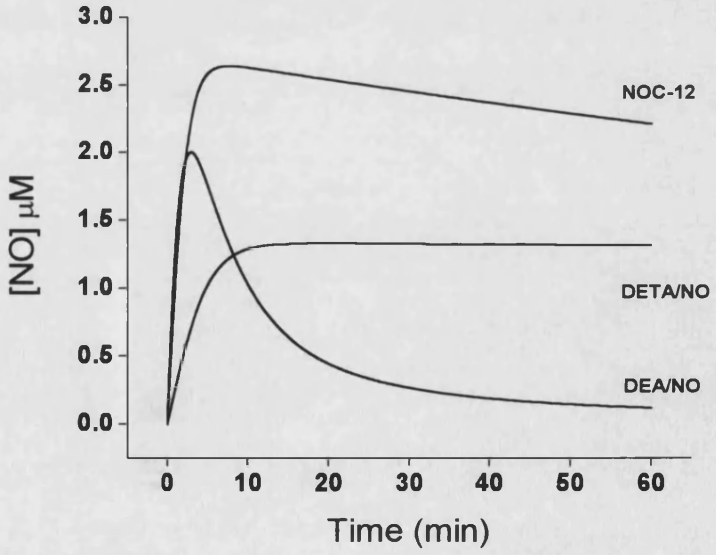


Figure 3.1 Predicted NO profiles for NOC-12 (100 µM), DETA/NO (300 µM) and DEA/NO (3 µM) at pH 7.4, 37°C, see Schmidt *et al.*, 1997.

Aims

In the course of studies originally aimed at determining the toxicity of exogenous NO towards brain neurones maintained in primary culture, it was decided to use the donor compound diethylenetriamine NONOate (DETA/NO), since it has a long half-life (20 h). In simple buffer solutions, the NO generated by DETA/NO accumulates to a steady-state concentration when the rate of autoxidation is equal to the rate of NO release, making it potentially useful for studies of the cytotoxicity of NO at defined concentrations and over long time periods in tissue culture. On measuring the concentrations attained in the tissue culture media itself, however, a large and unexpected difference was found compared with predictions. This chapter reports on the origin of the discrepancy and the potentially serious implications this has for working with NO in tissue culture media, or even simple buffer solutions.

3.2 METHODS

Measuring NO concentration

NO concentrations were measured in tissue culture medium, Tris (25 mM) or Hepes (25 mM), all 1 ml at pH 7.4, 37°C, using the electrochemical probe (ISO-NO) placed in a sealed vessel (see chapter 2.3). The tissue culture media tested were: minimal essential medium buffered with Hepes or $NaHCO_3$, Neurobasal medium, Dulbecco's modified eagle medium, and RPMI-1640. DETA/NO was prepared fresh each day in 10 mM NaOH and kept on ice until use. Where indicated, SOD was added to give an activity of 1000 U/ml (Beckman & Koppenol, 1996). The metal chelators and radical scavengers DTPA, neocuproine, cuprizone, uric acid, and deferoxamine were added from at least 100-fold concentrated stock solutions to Tris or Hepes buffer and, where appropriate, the pH was re-adjusted to 7.4. Vitamins (100x minimal essential medium vitamin mix), or riboflavin (0.1 – 0.2 mg/l) were added to Tris or Hepes buffer. Experiments designed to examine the effect of laboratory lighting used a lightproof cardboard box covering the entire apparatus, whose lid could be opened and closed as desired. Upon opening the lid the apparatus was exposed, at a distance of approximately 2 meters, to standard laboratory lighting conditions (4 fluorescent strip bulbs). All experiments were undertaken at least 3 times and statistical differences analysed using one-way or multiple comparison ANOVA with Dunnett's post-hoc test; *P* values of <0.05 were regarded as significant.

Determination of $NO_{GC}R$ activity

Enzyme activity was measured at 37°C in 1.5 ml Eppendorf tubes. Purified $NO_{GC}R$ from bovine lung was diluted in buffer (pH 7.4) containing Tris (10 mM), DTT (1 mM) and BSA (0.05%) to give a stock concentration of 5 μg/ml and stored on ice. DETA/NO (3 μM) was pre-equilibrated at 37°C for 3 hours in Tris or Hepes (25 mM), $MgCl_2$ (3 mM), and BSA (0.05%) with or without the addition of SOD (1000 U/ml). Substrate (GTP, 1 mM) was added to the reaction 10 s before the addition of $NO_{GC}R$ to give a final concentration of 0.05 μg/ml. Aliquots of the reaction mix were removed after 10 min, inactivated by addition to boiling buffer (50 mM Tris, 3 mM EDTA) and cGMP

levels measured by radioimmunoassay. Results are given as means ± S.E.M. and were analysed by one-way ANOVA with Dunnett's post-hoc test.

3.3 RESULTS

Inactivation of NO in tissue culture medium

Addition of the slow releasing NO donor DETA/NO (300 μM) to simple Tris buffer (25 mM, pH 7.4) resulted in a build up of NO, which reached a plateau of around 1 μM after about 10 min (Figure 3.2A,B) as predicted by the kinetics of autoxidation (Schmidt *et al.*, 1997). Subsequent addition of SOD (1000 U/ml) had no significant effect. When a similar test was performed in MEM (Life Technologies catalogue number 10012), which forms the basis of many different cell culture media, the NO plateau was much lower, about 0.2 μM. Addition of SOD then resulted in an increase in the NO concentration to a value (about 0.8 μM) close to, but still significantly less than, that found in Tris buffer. These findings suggest that, in Tris, NO is being consumed largely by autoxidation whereas, in MEM, reaction with $O_2^{\bullet-}$ is mainly responsible. Broadly, MEM contains 4 main ingredients: inorganic salts, amino acids, vitamins and other compounds (D-glucose, Hepes, phenol red, sodium succinate).

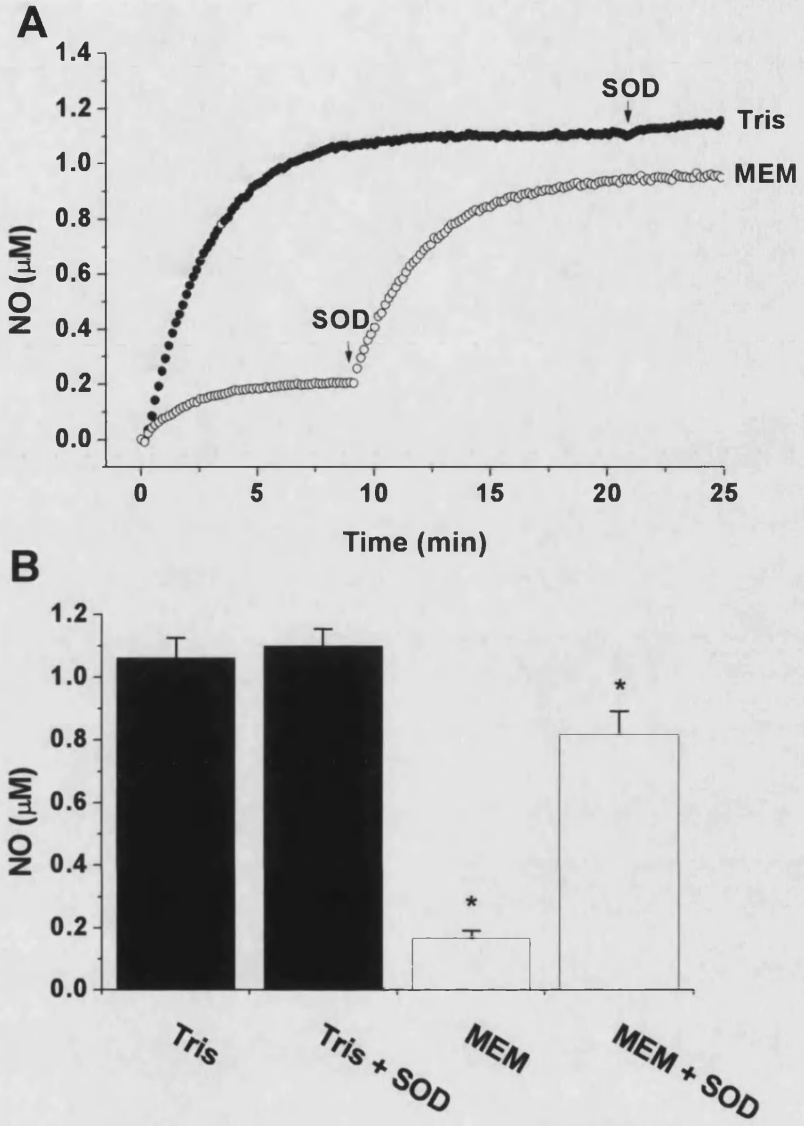


Figure 3.2 Inactivation of NO by MEM

(A), Representative traces of NO accumulation upon addition of DETA/NO (300 μM) to Tris buffer (filled circles) or MEM (open circles); subsequent additions of SOD (1000 U/ml) are marked by arrows. **(B)** Summary data from 3 independent experiments; * $P<0.05$ versus the control NO concentration in Tris buffer.

Importance of the buffer

Initially, Hepes and Tris buffers (25 mM) were compared (Figure 3.3A,B). In response to 300 μM DETA/NO, the mean steady-state NO concentration attained in Hepes was consistently less than in Tris (1.0 ± 0.12 μM vs 1.19 ± 0.09 μM; n =3). Addition of SOD to Hepes restored NO to the concentration found in Tris (1.17 ± 0.09 μM). As the DETA/NO concentration was lowered to 100 μM and then to 30 μM, a greater proportion of the NO was consumed in the Hepes buffer (reversible by SOD) such that, at 30 μM DETA/NO, the NO plateau was reduced by about 75% (Figure 3.3B). The difference in absolute NO concentrations at 30 μM DETA/NO, however, was less (0.13 μM) than at 100 μM (0.2 μM) or 300 μM (0.2 μM). With a fixed DETA/NO concentration (300 μM), the consumption of NO increased with increasing Hepes concentrations over the range 12.5-200 mM (Figure 3.3C)

The influence of Hepes at DETA/NO concentrations lower than 30 μM could not be measured easily because the resulting NO concentration approached the detection limit of the electrochemical probe (10 nM). To determine if Hepes-dependent NO consumption occurred at NO concentrations relevant to physiological signalling, the activity of the NO receptor enzyme, $NO_{GC}R$, was measured. Activation of $NO_{GC}R$ by NO catalyses the production of cGMP from GTP, and occurs at low nanomolar NO concentrations (Bellamy *et al.*, 2000; Griffiths & Garthwaite, 2001). In Tris-buffered reaction mix, DETA/NO (3 μM) stimulated cGMP accumulation from $NO_{GC}R$ at a rate of 10.1 ± 1.6 μmol/min/mg and there was no significant change in the rate in the presence of SOD (Figure 3.3D). In contrast, in Hepes-buffered reaction mix, $NO_{GC}R$ activity was reduced by approximately 65% compared to that seen in Tris, and the control activity could be restored by SOD.

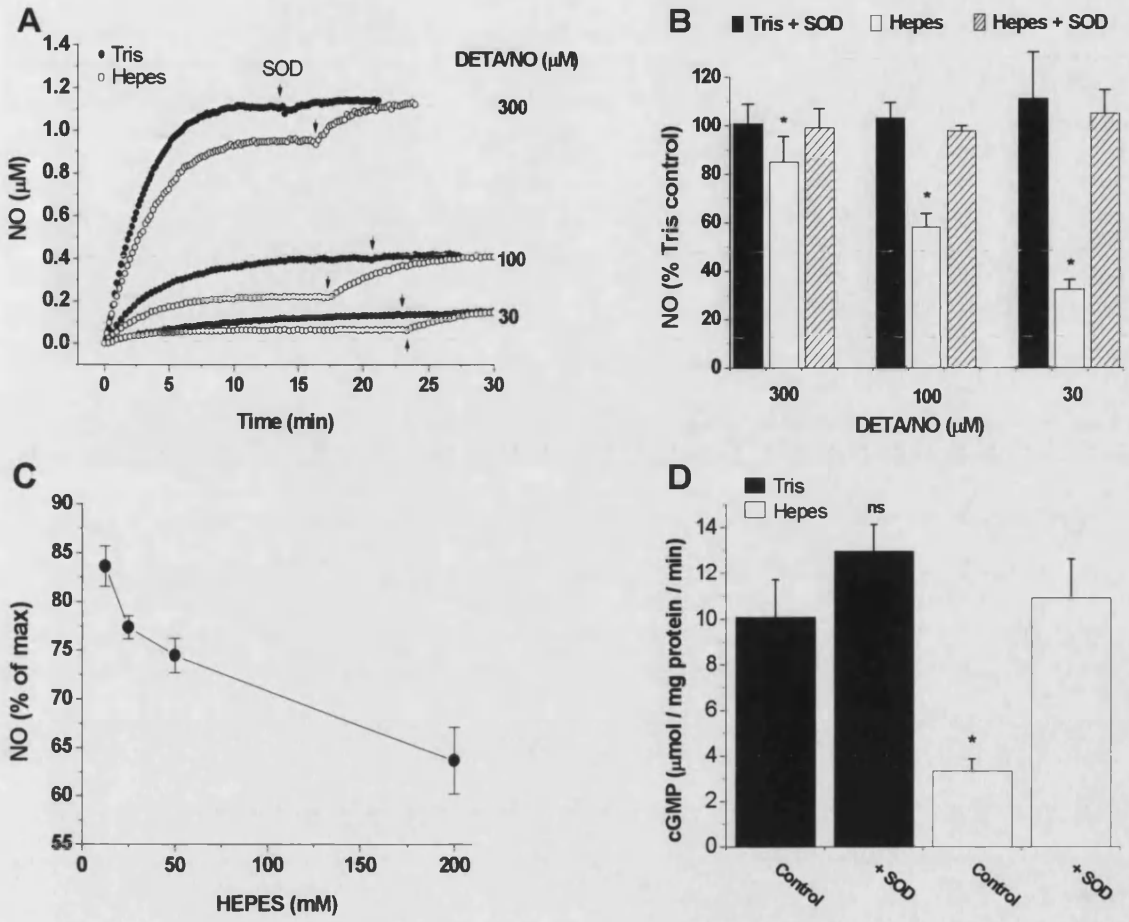


Figure 3.3 Inactivation of NO by Hepes buffer

(A) Representative traces of NO accumulation upon addition of DETA/NO (30-300 μM) to Tris (filled circles) or Hepes (open circles); subsequent additions of SOD (1000 U/ml) are marked by arrows. **(B)**, summary data from 3 independent experiments; *P<0.05 versus the control NO concentration in Tris buffer. Key: solid bars, Tris + SOD; open bars, Hepes; hatched bars, Hepes + SOD. **(C)**, summary data of steady-state NO concentrations generated from DETA/NO (300 μM) in different Hepes concentrations (12.5 – 200 mM). Data are expressed as a percentage of the maximum NO signal (approximately 1 μM in all cases) generated following further addition of SOD (1000 U/ml). **(D)**, sCG was stimulated by addition to a reaction mix pre-equilibrated with DETA/NO (3 μM) and buffered with either Tris or Hepes in the presence or absence of SOD as indicated. Data are means ± S.E.M. from 6 independent experiments; *P<0.05 versus Tris control, ns = not significant versus Tris control.

The source of $O_2^{\bullet-}$

The inhibition of NO consumption in Hepes buffer by SOD suggests an involvement of $O_2^{\bullet-}$, which reacts extremely rapidly with NO to form $ONOO^-$ (Beckman & Koppenol, 1996). A possible source of $O_2^{\bullet-}$ would be the autoxidation of contaminating metal ions, particularly iron and copper ions (Halliwell, 1992). To examine this possibility, DETA/NO (100 μM) was added to Tris or Hepes buffer (25 mM) containing the Cu^+ chelator neocuprione (100 μM), the Cu^{2+} chelator cuprizone (100 μM), or the iron chelators DTPA (100 μM) or deferoxamine (300 μM). Addition of neocuproine, cuprizone or DTPA had no effect on NO levels in Hepes (all remained significantly lower than in Tris controls), and subsequent additions of SOD restored NO to the concentrations found in Tris (Figure 3.4). In the presence of deferoxamine, however, NO levels attained in Hepes buffer were the same as those formed in Tris buffer, and subsequent additions of SOD had no further effect. A similar result was obtained in the presence of the $ONOO^-$ scavenger uric acid (300 μM; Figure 3.4).

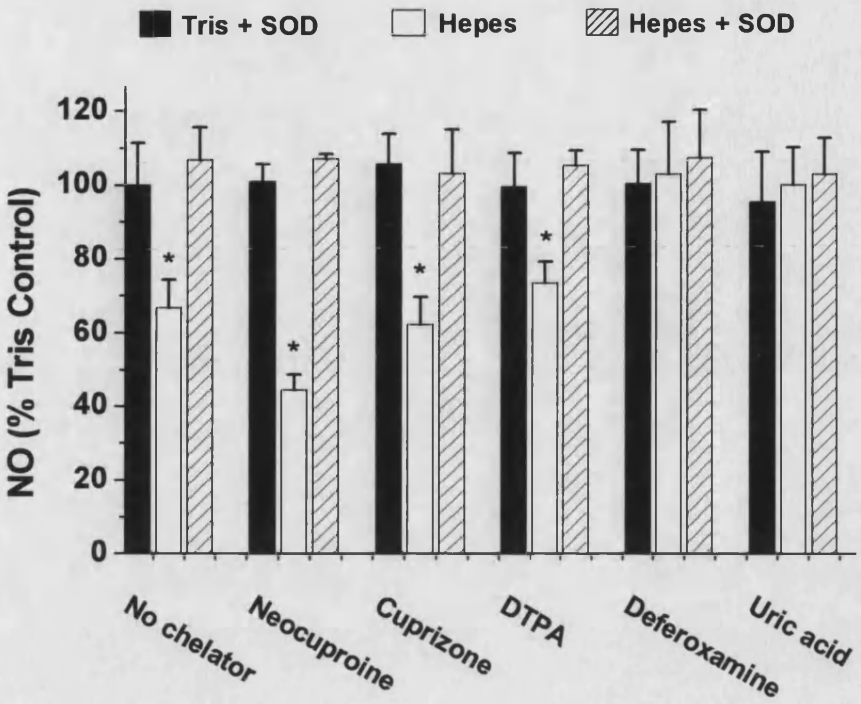


Figure 3.4 Effect of metal chelators and uric acid on NO concentrations

DETA/NO (100 μM) was added to Tris or Hepes (25 mM) in the presence of DTPA, neocuproine or cuprizone (all at 100 μM), deferoxamine (300 μM) or uric acid (100 μM). Once a steady-state NO concentration was achieved, SOD (1000 units/ml) was added and the recording continued until the NO concentration was again steady. Data are from 3-6 independent experiments and show the test NO concentrations expressed as a percentage of the control NO concentration found in Tris buffer (approximately 300 nM). *P<0.05 versus control.

Effect of vitamins

The consumption of NO in Hepes buffer alone cannot explain the extent of NO inactivation observed when 300 μM DETA/NO was added to MEM. In pursuit of the other ingredients responsible, the mix of vitamins present in MEM was examined (Figure 3.5A). In Tris buffer, the vitamins decreased the maximum level of NO achieved by 300 μM DETA/NO by about 0.2 μM and NO levels were restored by the addition of SOD (Figure 3.5A,B). With the vitamins in Hepes buffer, however, the NO signal was almost abolished. SOD restored the NO concentration under these conditions to about 80% of control.

Of the components of the vitamin mix present (D-Ca pantothenate, choline chloride, folic acid, i-inositol, nicotinamide, pyroxidal HCl, riboflavin, thiamine HCl), riboflavin was considered a likely candidate because it is capable of generating $O_2^{\bullet-}$ (Joshi, 1985). When DETA/NO (300 μM) was added to Tris in the presence of riboflavin at the concentration found in MEM (0.1 mg/l; Figure 3.5C) the maximum concentration of NO was reduced by 0.18 ± 0.03 μM (n = 3). SOD restored NO to the control level (1.1 ± 0.1 μM). Increasing the riboflavin concentration led to an approximately proportional increase in NO consumption that was, in all cases, inhibited by SOD (Figure 3.5D). When examined in Hepes buffer, the NO concentration reached in the presence of riboflavin was below detection levels, consistent with the very low concentration found in the vitamin mix. Again, SOD restored the NO concentration to approximately 80% of the control.

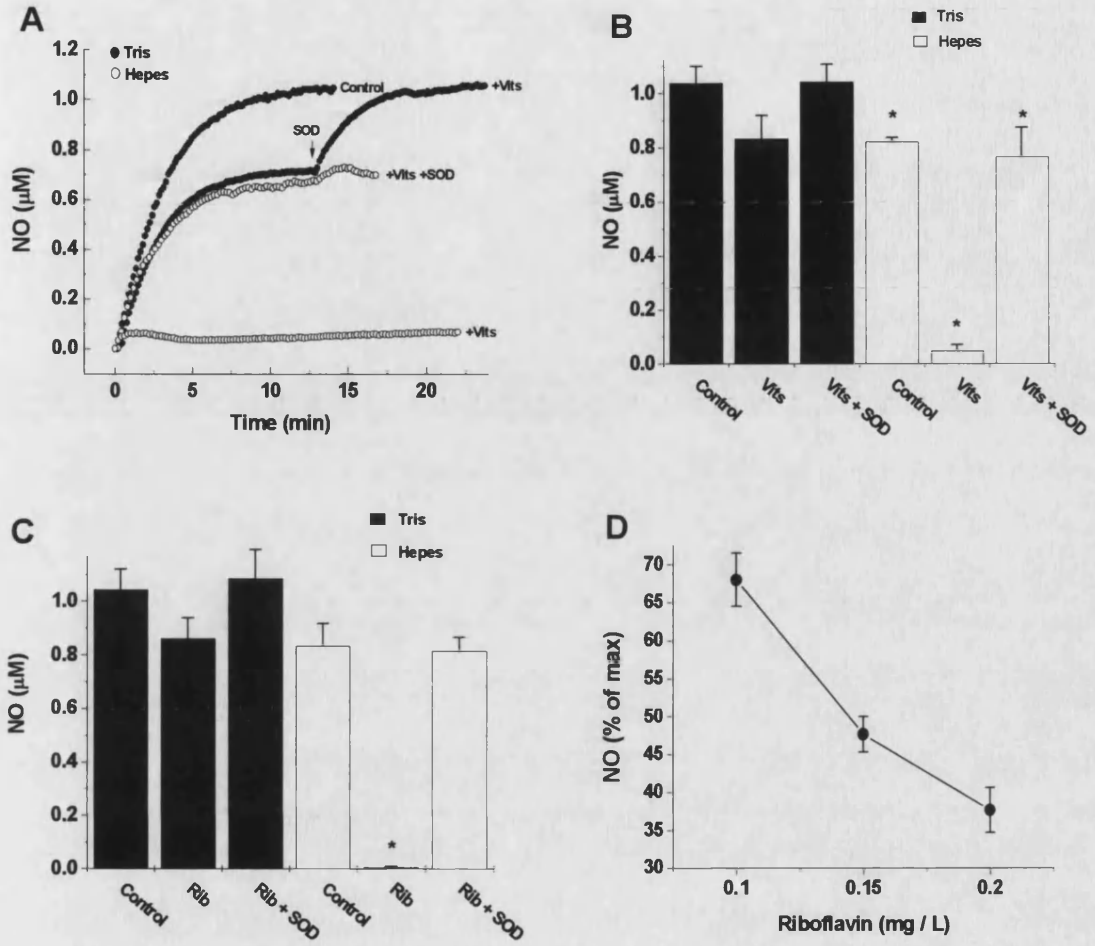


Figure 3.5 Inactivation of NO by vitamins

(A) Representative traces of NO accumulation upon addition of DETA/NO (300 μM) to Tris (filled circles) or Hepes (hollow circles) in the presence of vitamins (+Vits). SOD (1000 U/ml) was added as indicated by the arrow or, with Hepes, was present throughout. The control trace (labelled "control") contained no additions. **(B)**, summary data from 3 independent experiments; *P<0.05 versus Tris control. **(C)**, summary data from similar experiments (n = 3-4) using riboflavin (Rib, 0.1 mg/l) instead of the mixture of vitamins; *P<0.05 vs Tris control. **(D)**, Steady-state NO concentrations generated from DETA/NO (300 μM) in different riboflavin concentrations (0.1 - 0.2 mg/L) in Tris buffer. Data (n = 3 - 4) are expressed as a percentage of the maximum NO signal (approximately 1 μM in all cases) generated following a further addition of SOD (1000 U/ml).

Influence of light

Riboflavin is photosensitive and its presence in culture media has been reported to contribute to phototoxic effects (Zigler, Jr. *et al.*, 1985). To investigate whether the consumption of NO was due to photosensitisation of riboflavin the apparatus was enclosed in a light-proof box, the lid of which could be opened or closed as desired. As before, in the light (Figure 3.6A,D), the presence of riboflavin reduced the maximum NO concentration achieved with DETA/NO (300 μM) in Tris buffer by about 20%. In the dark, no effect of riboflavin in Tris buffer was observed (Figure 3.6B,D). In Hepes buffer in the dark, riboflavin did not decrease the NO concentration further (Figure 3.6C,D). On opening the box, however, the NO concentration fell by about 0.5 μM within 10 min and then continued to fall more gradually (Figure 3.6C,D). On closing the box, NO abruptly increased and, within 20 min, regained the concentration previously achieved. In Hepes buffer without riboflavin light did not alter the NO concentration (Figure 3.6C).

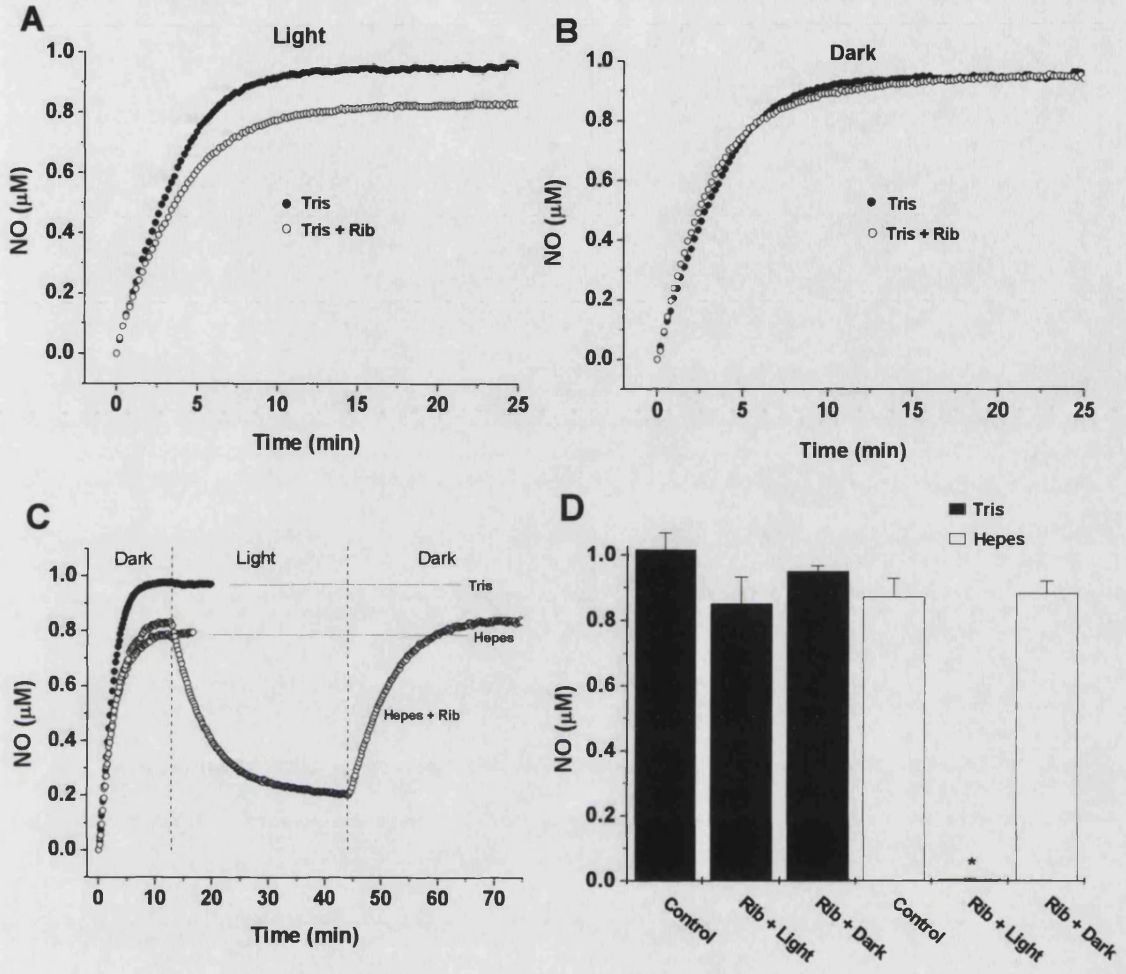


Figure 3.6 Effects of light on the consumption of NO in Tris or Hepes in the presence or absence of riboflavin

(A) and **(B)** show representative traces of NO accumulation upon addition of DETA/NO (300 μM) to Tris buffer (filled circles) or Tris + riboflavin (Rib, 0.1 mg/l, open circles) in the light **(A)** or dark **(B)**. **(C)**, trace showing the accumulation of NO in Tris buffer alone (filled circles) or Hepes buffer (open circles) in the absence or presence of riboflavin (Rib), as indicated. The apparatus was initially kept in the dark but was exposed to light during the interval indicated. **(D)**, summary data from 3-6 experiments; *P<0.05 versus Tris control.

NO consumption by different tissue culture media

Several commonly used tissue culture media containing various Hepes and riboflavin concentrations (MEM 11012 and 31095, Neurobasal medium, DMEM and RPMI-1640) were tested for their ability to consume NO released by DETA/NO (100 μM) in the light or dark. In the dark, only MEM 11012 (which contains 25 mM Hepes) was effective whereas in the light, NO concentrations attained in all the media tested were below the detection limit (Table 3.1).

Media	Life-Tech Catalogue Number	Hepes (mM)	Riboflavin (mg/L)	NO in dark (μM)	NO in light (μM)
MEM	11012	25	0.1	0.14 ± 0.01 *	<0.01 *
MEM	31095	--	0.1	0.29 ± 0.03	0.01 ± 0.005 *
RPMI-1640	31870	--	0.2	0.25 ± 0.03	<0.01 *
DMEM	41965	--	0.4	0.24 ± 0.02	<0.01 *
Neurobasal	21103	10	0.4	0.24 ± 0.02	<0.01 *

Table 3.1 Steady-state NO concentrations in different tissue culture media

The data (n = 3 - 10) represent the mean steady-state NO concentration formed following addition of 100 μM DETA/NO to various tissue culture media in the light or dark.

*P<0.05 vs Tris control (0.28 ± 0.01 μM).

3.4 DISCUSSION

The study in this chapter shows that, over a range of concentrations covering those encountered *in vitro* following constitutive or inducible NO synthase activity (Bal-Price & Brown, 2001; Clementi *et al.*, 1999; Lewis *et al.*, 1995), NO can be consumed as a result of the presence of two common constituents of cell culture media: riboflavin and Hepes buffer. The combination of the two ingredients under normal laboratory lighting conditions leads to a greatly amplified quench of the NO signal. Moreover, the sensitivity of the quench to SOD in all cases implies the participation of $O_2^{\bullet-}$ and subsequent generation of $ONOO^-$, which can be a powerful oxidant and cytotoxin (Beckman & Koppenol, 1996).

Hepes is a very commonly-used biological buffer and the finding that NO was consumed by this buffer in a SOD-sensitive manner, implies a continuous formation of $O_2^{\bullet-}$ that interacts with NO. Moreover, the effect remained marked down at the NO concentrations relevant to $NO_{GC}R$ activation, which lie in the low nanomolar range (Bellamy *et al.*, 2000; Griffiths & Garthwaite, 2001). Consequently, Hepes buffer could introduce artifacts associated with $O_2^{\bullet-}$ formation over a wide range of biologically-relevant NO concentrations.

As no such effect was observed in Tris buffer, the $O_2^{\bullet-}$ generation is not simply due to contaminants, such as metal ions. A likely mechanism comes from a study (Kirsch *et al.*, 1998) showing that $O_2^{\bullet-}$ was generated upon addition of $ONOO^-$ to Hepes. It was suggested that $ONOO^-$ (or any strong oxidant derived from it) oxidises the piperazine ring of Hepes. Following deprotonation, reaction with O_2 leads to the formation of $O_2^{\bullet-}$ which then goes on to form hydrogen peroxide *via* dismutation. In our experiments, the inhibition of the Hepes-dependent NO consumption by SOD and by the $ONOO^-$ scavenger uric acid would be consistent with this reaction being responsible.

For such a scheme to explain the results, there would need to be a source of $O_2^{\bullet-}$ to initiate $ONOO^-$ formation. One possible source considered was the autoxidation of trace metals (for more detail see chapter 6), the most likely candidates being iron and copper. Neither of the copper chelators had

a significant effect on the Hepes-dependent NO quench, which could indicate a lack of involvement of copper ions. However, Cu^{2+} can form an active complex with Hepes (Hegetschweiler & Saltman, 1986; Simpson *et al.*, 1988) which could render the ion unavailable for chelation. The iron chelators DTPA and deferoxamine gave differing results, with only deferoxamine inhibiting NO consumption. Again these results are difficult to interpret clearly. A lack of effect of DTPA could be explained by the iron-DTPA complex continuing to catalyse oxygen radical generation (Egan *et al.*, 1992). Conversely, the effect of deferoxamine may be explained by the ability of this compound to scavenge free radicals, including $O_2^{\bullet-}$ (Davies *et al.*, 1987), rather than sequester iron. Thus, whilst metal contaminants are still good candidates, unambiguous evidence for their involvement is difficult to obtain. Nevertheless, it is likely that there is a background formation of $O_2^{\bullet-}$ that would react with NO at a diffusion-controlled rate (Koppenol, 1998) to form $ONOO^-$ which, by oxidising Hepes, would lead to more $O_2^{\bullet-}$, more $ONOO^-$, and so on (Figure 3.7). Adding further complications, the reaction of $ONOO^-$ with Hepes buffer may also result in the formation of NO donors with unknown additional reactivity (Schmidt *et al.*, 1998). Obviously, it would have been desirable to have measured the rates of formation of $O_2^{\bullet-}$ and/or $ONOO^-$ directly but the usual methods for doing so result in the consumption of these species, which would have the effect of inhibiting the reaction (c.f. effect of SOD or uric acid).

The other ingredient found to be responsible for NO consumption was riboflavin, when exposed to laboratory lighting. The generation of $O_2^{\bullet-}$ by illuminated riboflavin is well documented (Joshi, 1985; Naseem *et al.*, 1988). Indeed, riboflavin is used to produce $O_2^{\bullet-}$ for determining the efficiency of $O_2^{\bullet-}$ scavengers (Roubaud *et al.*, 1997). However $O_2^{\bullet-}$ may not be the only species involved in riboflavin photosensitisation. Reactions involving riboflavin are complex, and light-sensitised riboflavin may also produce singlet oxygen ($^1O_2$), which may further react with organic compounds (Spikes, 1989). Deleterious effects of riboflavin on mammalian cells in culture have been known for many years and have been ascribed to various mechanisms. Work by Griffin *et al* suggested that just 3 h exposure of

riboflavin-containing medium to bench top levels of cold white fluorescent light could produce significant toxicity to a leukemic cell line (Griffin *et al.*, 1981). The principal mechanism of phototoxicity is thought to involve the generation of $^1O_2$ and probable subsequent oxidation of the amino acids tryptophan and tyrosine (Griffin *et al.*, 1981; Stoien & Wang, 1974). It has since been demonstrated that fluorescent light may cause build up of toxic H_2O_2 levels in Dulbecco's modified Eagle's medium or RPMI 1640 medium, both of which contain riboflavin (Wang & Nixon, 1978; Zigler, Jr. *et al.*, 1985).

In the present experiments, the SOD-sensitive quench of the NO signal produced by riboflavin alone (in the light) in Tris buffer increased in proportion with riboflavin concentration, as expected from a simple generation of $O_2^{\bullet-}$. At the concentrations found in MEM (11012) the NO quench exerted by riboflavin (0.1 mg/L) was similar to that produced by Hepes (25 mM) implying similar rates of NO consumption (about 20% of the rate of release from the donor) under these two conditions. The quench of NO down to undetectable levels observed with the combination of Hepes and riboflavin (in the light) implies a much greater than additive effect. This may be explained by there being disproportionately more $O_2^{\bullet-}$ ultimately available as a result of its additional formation following Hepes oxidation (Figure 3.7).

In comparison with the simple combination of Hepes and riboflavin in the light, the complete MEM containing the same concentrations of these ingredients quenched NO less (80% versus 100% with 300 μM DETA/NO), suggesting that other components of MEM may act as radical scavengers or suppress radical generation. A likely contributor here is the pH indicator phenol red, which quenches photoexcited riboflavin (Grzelak *et al.*, 2001). At a lower DETA/NO concentration normally generating about 300 nM NO, however, light exposure effectively eliminated the NO signal in Hepes-containing MEM (Table 3.1). Under the same conditions, other common media (lacking Hepes) behaved similarly, suggesting that, in the light, the riboflavin content alone is sufficient to consume all the available NO, when released at this rate (approximately 100 nM/min; Griffiths & Garthwaite, 2001).

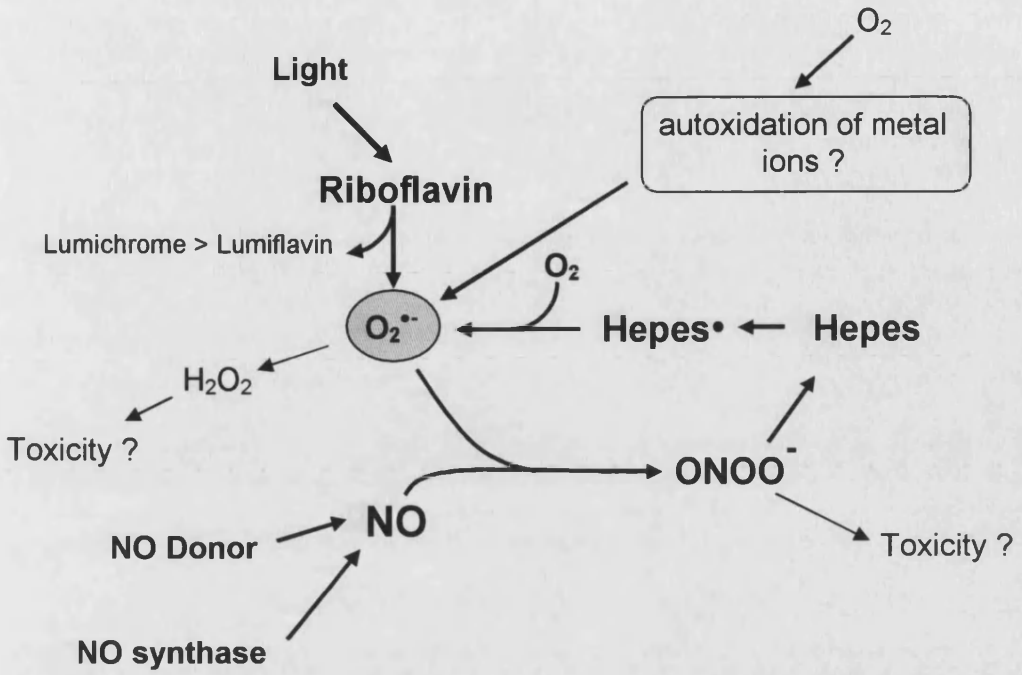


Figure 3.7 Possible interactions between NO, Hepes, riboflavin and light

$O_2^{\bullet-}$, initially generated by riboflavin and/or from the presence of metal contaminants, reacts with NO to form $ONOO^-$, which then oxidises Hepes buffer forming the Hepes radical. This radical fuels further $O_2^{\bullet-}$ generation from molecular O_2. By this means Hepes sustains $O_2^{\bullet-}$ generation and the subsequent consumption of NO. The products H_2O_2 and/or $ONOO^-$ formed artifactually in this way could exert biological actions, including cytotoxicity.

Overall, the results point to potential sources of artifact that may have unwittingly complicated many *in vitro* studies. Since the inclusion of Hepes buffer is an option in most standard media formulations (MEM, RPMI-1640, DMEM) and is inherent in others (Neurobasal Medium) its presence is not always published. Likewise, as the frequency and intensity of light exposure is rarely mentioned, it must be assumed that the potential problems associated with NO being present with either Hepes buffer or light-exposed riboflavin, despite being deducible from information in the literature, are not generally appreciated. The exaggerated effect of the two together multiplies the potential problems. The findings with Hepes are likely to apply to other "Good" buffers equipped with a piperazine ring, such as MOPS, PIPES and EPPS (Grady *et al.*, 1988; Kirsch *et al.*, 1998), and they should therefore be only used with caution, particularly in experiments with NO.

3.5 CONCLUSION

In the absence of the requisite information, the extent to which media factors might have generated spurious results in published studies cannot be evaluated. Blockade of cell death by NO synthase inhibitors and SOD is typically taken to indicate mediation by endogenously-generated $ONOO^-$. It now becomes plausible that inadvertent reactions between NO and constituents of the bathing medium are responsible. Interestingly, the cytotoxicity of dopamine has recently been attributed to artifactual oxidative reactions occurring in tissue culture media (Clement *et al.*, 2002) although the ingredients responsible were not identified. Alternatively, the consumption of NO by the medium may render NO non-toxic, depending on its fate therein. Whether NO appears to participate in a toxic (or other) process in cultured cells or not may depend on a simple experimental variable, such as the choice of buffer or the degree of exposure to light.

CHAPTER 4: HIPPOCAMPAL SLICE CULTURES AND DAMAGE BY ENDOGENOUS NO

4.1 INTRODUCTION

Following ischaemic episodes central neurones degenerate, often with a delayed time-course of up to several days. A contributor to the damage is glutamate release, leading to a sustained activation of NMDA receptors (Choi, 1992; Meldrum & Garthwaite, 1990; Strijbos *et al.*, 1996). NMDA receptors are physically linked to the Ca^{2+}-dependent neuronal NO synthase nNOS (Brenman *et al.*, 1996), and NO produced following prolonged NMDA receptor activation may act as a toxin that contributes to neuronal death (Dawson *et al.*, 1991; Sattler *et al.*, 1999). The mechanisms usually invoked include inhibition of mitochondrial respiration, and combination with $O_2^{\bullet-}$ to form the oxidant species, $ONOO^-$. Evidence that endogenous NO contributes directly to damage through these or other mechanisms, however, remains controversial (for discussion see chapter 1).

Glutamate is an excitotoxin

Glutamate is the major fast excitatory neurotransmitter in the mammalian brain. Over three decades ago it was discovered that application of glutamic acid was toxic to neurones of the mouse retina (Lucas & Newhouse, 1957) and may result in cell death of different brain regions (Olney, 1969). The term excitotoxicity was later christened to describe the phenomenon that "glutamate toxicity is a direct effect of its excitatory actions on neurones".

For neurones to transmit information at a high rate, and to prevent possible toxicity, it is necessary for glutamate's postsynaptic actions to be terminated rapidly. To this end a family of high-affinity excitatory amino acid transporters have evolved with both glial (EAAT1-2) and neuronal (EAAT3-5) subtypes. The most widespread are EAAT2 and 3, with other subtypes being more tissue specific (Gegelashvili & Schousboe, 1998). The uptake of glutamate into astrocytes is quantitatively superior to that into neurones, hence it is a combination of astrocytic uptake, and diffusion away from the

synaptic cleft that enables glutamate concentrations to return to ambient levels within milliseconds (Bergles *et al.*, 1999). Glutamate is transported against a steep concentration gradient by the co-transport of three Na^+ and one H^+ ions into the cell, while one K^+ is transported out (Levy *et al.*, 1998). The maintenance of glutamate concentrations is therefore much at the mercy of ion pumps such as the Na^+/K^+ ATPase.

Glutamate release in ischaemia

The initial consequence of a block in cerebral blood flow (CBF) is a decrease in tissue O_2 and therefore cessation of aerobic respiration. As ATP falls in the affected tissue ATP-dependent processes (including the Na^+/K^+ ATPase) cease functioning. The following rise in extracellular K^+ and intracellular Na^+ triggers an 'anoxic depolarization' (Szatkowski & Attwell, 1994), that is enhanced by glutamate transporters reversing, pumping glutamate out of cells (Rossi *et al.*, 2000; Szatkowski *et al.*, 1990). Glutamate release has been detected following *in vitro* ischaemia (Mitani *et al.*, 1991; Roettger & Lipton, 1996) and excitotoxicity (Strijbos *et al.*, 1996), or *in vivo* (Benveniste *et al.*, 1984; Obrenovitch *et al.*, 1993), where it can accumulate to high levels (~100 μM) sufficient to trigger neurodegeneration. Although other mechanisms for glutamate release have been proposed (Ca^{2+}-dependent vesicular release, and release through swelling activated anion channels), 'reversed uptake', occurring mainly via the neuronal transporters, appears to be the major mechanism by which glutamate is released during ischaemia (Jabaudon *et al.*, 2000; Rossi *et al.*, 2000).

A vicious cycle leads to delayed neuronal death

Surprisingly, despite intensive research, the mechanisms underlying glutamate induced neuronal death are not fully understood. Such death may be either acute, or delayed, depending upon the duration and intensity of glutamate exposure. Moreover, the different classes of glutamate receptor (NMDA, AMPA and metabotropic) do not participate equally in the ensuing death, hence the receptor profile of the affected tissue is critical (Meldrum & Garthwaite, 1990). Following ischaemic glutamate release, NMDA receptor activation is of central importance, and its pharmacological blockade is

neuroprotective in various models (Fujisawa *et al.*, 1993; Kaku *et al.*, 1993). Blockade of AMPA receptors can be equally protective (Fujisawa *et al.*, 1993) though it does not protect all regions (Gill *et al.*, 1992), and the protective actions of the AMPA antagonist NBQX may simply have been mediated through lowering of core temperature *in vivo* (Nurse & Corbett, 1996). Less comprehensive research into metabotropic receptor antagonism has been undertaken. The available evidence therefore points towards ionotropic glutamate receptors, particularly the NMDA receptor, as primarily responsible for excitotoxicity. Following sustained NMDA receptor activation the consequent rises in intracellular Ca^{2+} may trigger events such as calpain and lipase activation as well as free radical generation and activation of Ca^{2+}-ATPase, which will further deplete energy (Meldrum & Garthwaite, 1990).

In models of excitotoxicity, prolonged exposure to NMDA elicits neuronal death rapidly, whereas brief exposure produces a delayed death more akin to that seen following ischaemia (Dawson *et al.*, 1991; Strijbos *et al.*, 1996). Mechanistically it has been proposed that a cycle may be set in motion linking initial (non-toxic) NMDA receptor stimulation, with Na^{+}-channel activation, further glutamate release, and eventually cell death as a consequence of persistent NO production see Fig 4.1.

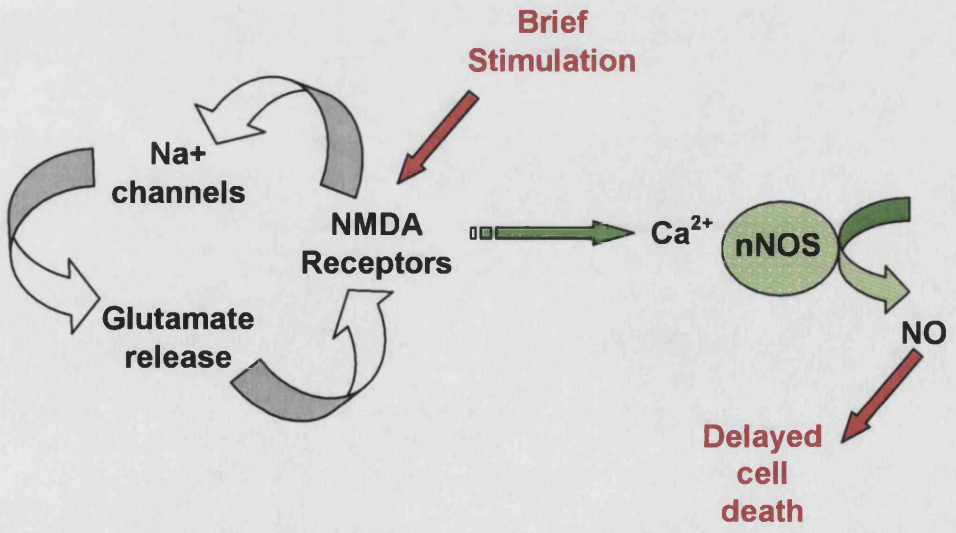


Figure 4.1 A vicious cycle to neuronal death, from Strijbos *et al*, 1996

This cycle was first proposed to occur in cultured striatal neurones which were found to release glutamate for about 8 h following a transient (5 min) NMDA stimulation (Strijbos *et al.*, 1996). The glutamate increase was prevented either by blocking NMDA receptors, or voltage-gated Na^+ channels. The voltage-gated Na^+ channel generates action potentials in excitable cells, but its blockade by compounds such as lamotrigine or tetrodotoxin confers neuroprotection in many models of ischaemia (Probert *et al.*, 1997; Smith & Meldrum, 1995; Weber & Taylor, 1994; Wiard *et al.*, 1995). Limiting Na^+ influx in this way may have several beneficial effects including; a reduction in cellular energy expenditure, an increase in the voltage-sensitive block of the NMDA-operated channels by Mg^{2+}, or preventing reversal of the glutamate transporter as outlined above. Cell death in the striatal model was delayed, becoming maximal only after 16 h, and could be inhibited by post-insult application of the voltage-gated Na^+ channel inhibitor tetradotoxin (TTX), NMDA receptor blockade with MK801, or NOS inhibition with L-NNA (Strijbos *et al.*, 1996).

The role of NO in excitotoxicity

As described above, excitotoxicity is most commonly investigated by challenging dispersed neuronal cultures briefly with glutamate or NMDA and then monitoring cell survival over the next day (Taylor & Meldrum, 1995). Some studies showed clear neuroprotection by NOS inhibition (Dawson *et al.*, 1991; Izumi *et al.*, 1992b; Strijbos *et al.*, 1996) or knockout of the nNOS gene (Dawson *et al.*, 1996), but others found no causal relationship between NO and cell death (Demerle-Pallardy *et al.*, 1991; Hewett *et al.*, 1993; Pauwels & Leysen, 1992). The potential causes of such discrepancies have been discussed previously (see chapter 1).

More limited experiments have used freshly-prepared brain slices, which should be more representative of the intact brain, but the results have been similarly discordant. One study on hippocampal slices found NOS inhibition to be protective (Izumi *et al.*, 1992a), while another, using seemingly identical methods, found no protection (Garthwaite & Garthwaite, 1994). An obvious limitation here is the short recovery period allowed (typically 90 min), which may not allow time for a more delayed NO-

dependent death to become manifest. Unfortunately, experiments undertaken *in vivo* do not clarify matters, (see Moncada *et al.*, 1992; Fujisawa *et al.*, 1993; Lerner-Natoli *et al.*, 1992; Globus *et al.*, 1995). A complication here is the non-selectivity of the NOS inhibitors influencing blood flow through inhibition of the endothelial NOS (eNOS).

Aims

In view of the unresolved contradictions and the potential problems with experimental models used beforehand, this study has re-examined the role of NO in NMDA-induced neurodegeneration using organotypic hippocampal slice cultures as the model. Such cultures are increasingly used because they retain their complex cellular organization and electrophysiological properties, and can survive for weeks to months (Gahwiler, 1981; Stoppini *et al.*, 1991). Moreover, hippocampal slice cultures show a similar regional vulnerability to that found *in vivo* in response to ischaemic and excitotoxic insults (Pringle *et al.*, 1997; Strasser & Fischer, 1995; Vornov *et al.*, 1991). Consequently, provided that the endogenous NO signalling pathway remains intact in such preparations, they should represent good experimental material with which to test the hypothesis that NO contributes to excitotoxic damage.

4.2 METHODS

Hippocampal slice culture preparation

Slice cultures were prepared according to the method of (Stoppini *et al.*, 1991). Sprague-Dawley rats (7-8 days old, from Charles River, UK Limited) were killed by decapitation and associated exsanguination as approved by the British Home Office and local ethics committee. The brains were immersed in ice-cold minimal essential medium supplemented with 10 mM Tris, and penicillin/streptomycin (100 U/ml and 100 μg/ml respectively). Hippocampi were rapidly dissected out and 400 μm transverse sections prepared on a McIlwain tissue chopper (Mickle Laboratory Engineering Ltd, Surrey, UK). Slices were separated mechanically and randomised before being placed onto culture inserts (Millicell-CM: Millipore, Watford, UK, 4 slices per insert). Culture inserts were incubated in 6-well plates with 1 ml media consisting of minimal essential medium (50%), heat-inactivated horse serum (25%), Hank's balanced salt solution (25%), and penicillin/streptomycin (as above), buffered to pH 7.3 with Tris (5 mM) and $NaHCO_3$ (0.35 g/100 ml). Cultures were incubated at 37°C in 5% CO_2 for 4 days and subsequently at 33°C in 5% CO_2 until use at 12-14 days *in vitro.* Inserts were transferred to fresh media after 1, 4, 7, and 10 days.

General protocols

Serum may protect against glutamate excitotoxicity in cortical neurones and cerebellar cultures (Dux *et al.*, 1992; Wood *et al.*, 1997) while HEPES buffer contributes to $O_2^{\bullet-}$-dependent NO consumption, presumably forming $ONOO^-$ (see chapter 3), experiments were therefore performed on cultures pre-incubated for 1 h in serum-free/HEPES-free medium (SFM) consisting of: minimal essential medium without HEPES (74 %), Hank's balanced salt solution (24 %), B27 supplement without antioxidants (2 %) penicillin/streptomycin (as above) and glucose (0.5 g/l). $O_2^{\bullet-}$-dependent NO consumption is also brought about by the action of laboratory lighting on riboflavin (see chapter 3) a universal constituent of culture media. Care was therefore taken at all times to keep exposure of the slices to light to a

minimum by performing all media changes with the laboratory lighting switched-off and limiting microscopic observation to the beginning and end of the experiments; separate cultures were therefore used for each time point.

NMDA stimulations

Cultures were transferred to SFM containing NMDA (0.1-1 mM) for 15 min. After a brief wash in fresh SFM, cultures were replaced in the same SFM used for preincubation and recovered for 0-72 h. The NOS inhibitors L-nitroarginine (L-NNA) and 7-nitroindazole (7-NI) were present 15 min prior to addition of NMDA and throughout the stimulation and recovery. MK801 (10 μM) was added during the recovery period only.

cGMP accumulation

Cultures were exposed to the NO donor DEA/NO and/or NMDA for 2-15 min in the presence of the general phosphodiesterase inhibitor IBMX, 1 mM, (10 min preincubation). When used, the NOS inhibitors L-NNA or 7-NI were pre-incubated with the slices for 15 min, and remained present during NMDA stimulation. Individual slices were inactivated by immersion in boiling buffer (Tris 50 mM, EDTA 4 mM, pH 7.5, 150 μl/slice). Protein was determined by the bicinchoninic acid method and cGMP quantified by radioimmunoassay. Alternatively slices were fixed for immunohistochemistry (see below). Statistical differences were analysed using one-way ANOVA with Dunnett's *post hoc* test; P values of < 0.05 were regarded as significant.

Immunohistochemistry

Slices were fixed (4% paraformaldehyde in 0.1 M phosphate buffer) for at least 30 min (overnight for cGMP staining) before being permeabilized with 0.1% Triton X-100 in Tris-buffered saline for 20 min. Non-specific binding was reduced by incubation (1 h) in 5-10% serum of the secondary antibody host (see Table 2.2). Primary antibodies were applied for 48 h at appropriate concentrations (Table 2.2). Slices were further washed in Tris-buffered saline before being incubated overnight in the appropriate biotinylated or fluorescent secondary antibody (Chemicon International Ltd, Harrow, UK or

Vector Labs, Peterborough, UK). Biotin staining was developed using a standard avidin-peroxidase complex (Vectastain Elite ABC kit; Vector Labs), with diaminobenzidine as the substrate. For cGMP co-localisation studies, biotin staining was visualised with avidin/fluoroscein (Vector Labs). Slices were mounted on gelatin-coated slides and images captured on a PC running KS300 Imaging System (Imaging Associates, Thame, UK) using a JVC KY-F55B colour video camera (JVC Professional Products; London, UK) attached to a Leica DMRD microscope (Leica, Cambridge, UK). Slices double-labelled with cGMP/GFAP were imaged using a Leica TCS SP confocal microscope.

Assessment of neuronal damage

Propidium iodide (PI) is a highly polar fluorescent dye that can enter dying or injured cells and intercalate with their DNA and RNA to produce a red/yellow fluorescence at excitation 493 nm and emission 630 nm (Newell *et al.*, 1995). Cultures were placed into SFM containing PI (5 μg/ml) 1 h prior to stimulation and viability was assessed after 30 minutes. Any cultures in which neuronal damage was observed were discarded (< 5 % of slices). PI remained in the medium throughout the experimental and recovery period. At the termination of experiments fluorescence images (focussed through the brightest plane of the slice) were captured using a Leica DM IRB inverted microscope. Analysis was undertaken using Scion Image Version 4.0.2. (written by Wayne Rasband at the U.S. National Institutes of Health and available as free download from www.scioncorp.com). To aid identification of the areas of CA1, CA3 and the dentate gyrus all slices underwent a further overnight incubation at 4°C and images were re-captured. Maximal fluorescent signal (100 % death) was determined from those slices that had undergone treatment giving close to maximal death (1 mM NMDA or 3 μM myxothiazol). The area of PI fluorescence above background in each region was determined using the 'density slice' function contained in Scion Image. To ensure areas of PI correlated with areas of neuronal death, cultures were fixed overnight in 4 % paraformaldehyde and stained with the Nissl stain thionin (data not shown; Pringle *et al.*, 1997; Strasser & Fischer, 1995; Vornov *et al.*, 1991). Statistical differences were analysed using one-way

ANOVA with Bonferroni correction for multiple comparisons; *P* values of < 0.05 were regarded as significant.

RESULTS

Despite the increasing use of organotypic hippocampal slice cultures, the extent to which the NO signalling pathway is preserved in these preparations is unknown; accordingly, the first objective was to address this question.

Immunohistochemical characterisation

In good agreement with findings *in vivo* (Valtschanoff *et al.*, 1993) immunohistochemistry revealed nNOS-positive staining in all regions of the hippocampal slice cultures after 12-14 days *in vitro* (Fig. 4.2A-C). The majority of this staining was concentrated in the dentate gyrus (DG) and subiculum where a proportion of cells may form back projections to CA1 *in vivo* (Seress *et al.*, 2002). The pyramidal layers (CA1-3) contained only a small proportion of the total nNOS positive cell bodies. nNOS positive cells appeared mainly rounded in shape, consistent with their primary colocalization with GABA in interneurones (Valtschanoff *et al.*, 1993), although occasional bipolar neurones were stained in the CA3 area (Fig. 4.2A, arrow). Notably there was no appreciable staining in the CA1 pyramidal cells themselves, consistent with a majority of studies (Lin & Totterdell, 1998). A network of nNOS positive fibres was evident throughout the slice, however, particularly in CA2 (not shown).

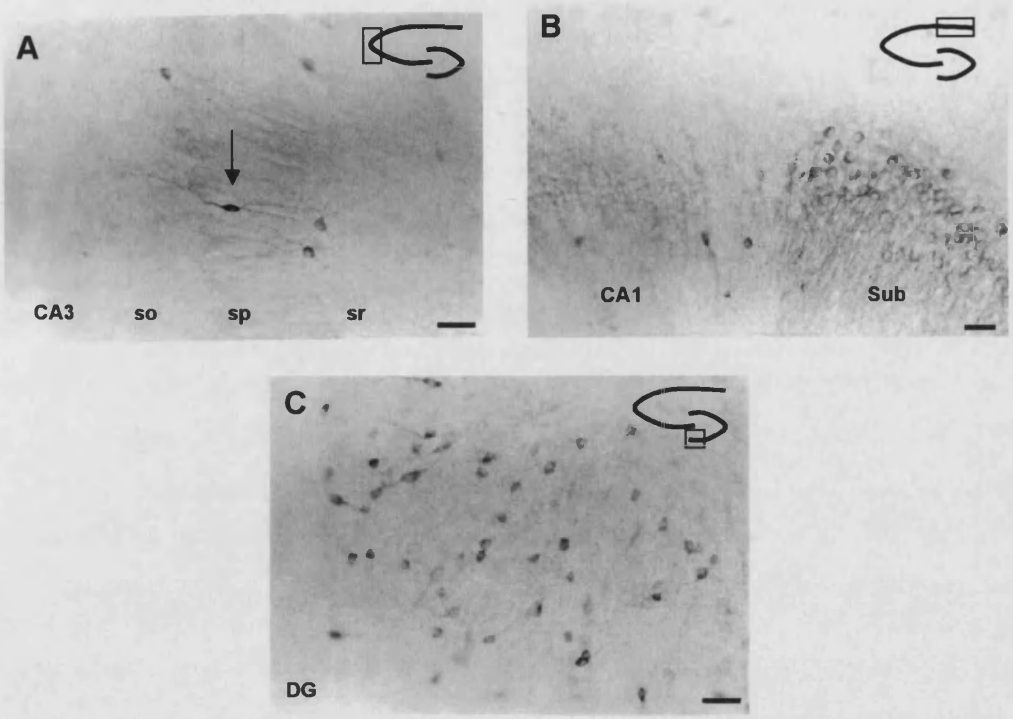


Figure 4.2 Distribution of nNOS

Photomicrographs of nNOS staining in hippocampal slice cultures. **(A)** positive neurones can be found in all layers of CA3; stratum oriens (so), stratum pyramidale (sp) and stratum radiatum (sr). Positive cells in the sp are clearly bipolar in shape (arrow) and not pyramidal. **(B)** A clear demarcation may be seen between CA1 (little staining) and the subiculum (many nNOS positive cells). **(C)** Small nNOS positive neurones are evenly distributed throughout the dentate gyrus. Scale bars = 50 μm.

In vivo, eNOS staining is found in endothelial cells at all stages of rat brain development (Topel *et al.*, 1998). Accordingly, eNOS labelling in the slices was evident in capillaries, which remained as a network at 12 days *in vitro* (Figs 4.3A, B) but degenerated after a month in culture to leave only a few isolated eNOS positive cells (not shown). Although eNOS staining has been reported in CA1 pyramidal neurones, this has now been considered an artifact (Demas *et al.*, 1999); in agreement, we detected no eNOS staining in CA1 pyramidal neurones.

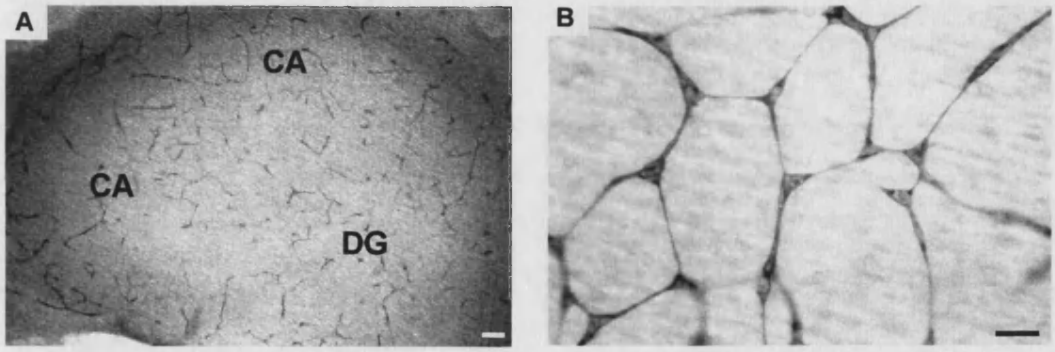


Figure 4.3 Distribution of eNOS (A) Low power (scale bar = 100 μm) and **(B)** high power (scale bar = 50 μm) photomicrographs of eNOS-positive endothelial cell networks in hippocampal slice cultures.

We next sought to determine the existence of the downstream components of the endogenous NO signalling pathway. Upon binding NO, the guanylyl cyclase (GC)-coupled NO receptors synthesise cGMP from GTP (Ignarro, 1991; Waldman & Murad, 1987). In control slices (incubated with IBMX to inhibit phosphodiesterase activity) the unstimulated level of cGMP amounted to about 20 pmol/mg protein. Following a 5 min stimulation of slices with increasing concentrations of the NO donor DEA/NO (1-100 μM), cGMP accumulation increased to reach a maximum of about 90 pmol/mg protein (Fig. 4.4A). Immunohistochemistry following this maximum DEA/NO stimulation revealed a large, but non-uniform, increase of cGMP staining throughout the slice in comparison to IBMX controls (Figs 4.4B, C). This staining was evident in neuronal-like cell bodies throughout CA3 and the DG (arrows, Fig. 4.4C), but was more diffuse in the region of CA1 (Fig. 4.4C). In other regions (CA3, Fig. 4.4D) a network of cGMP-positive fibres was evident, the origin of which appeared neuronal because in some cases they could be traced back to neurone-like cell bodies. The findings are in good agreement with the pattern of cGMP staining observed in acute hippocampal preparations (Teunissen *et al.*, 2001; van Staveren *et al.*, 2001). To identify the cell type responsible for cGMP staining in CA1 we undertook dual labelling with antibodies against GFAP (which stains astrocytes) and cGMP. Co-localisation (yellow/orange, Figs 4.5E, F) of cGMP (green, Figs 4.5A, B) and GFAP (red, Figs 4.5C, D) staining was seen in CA1, while occasional large neurone-like cell bodies were cGMP- but not GFAP-positive (arrows Fig. 4.5F).

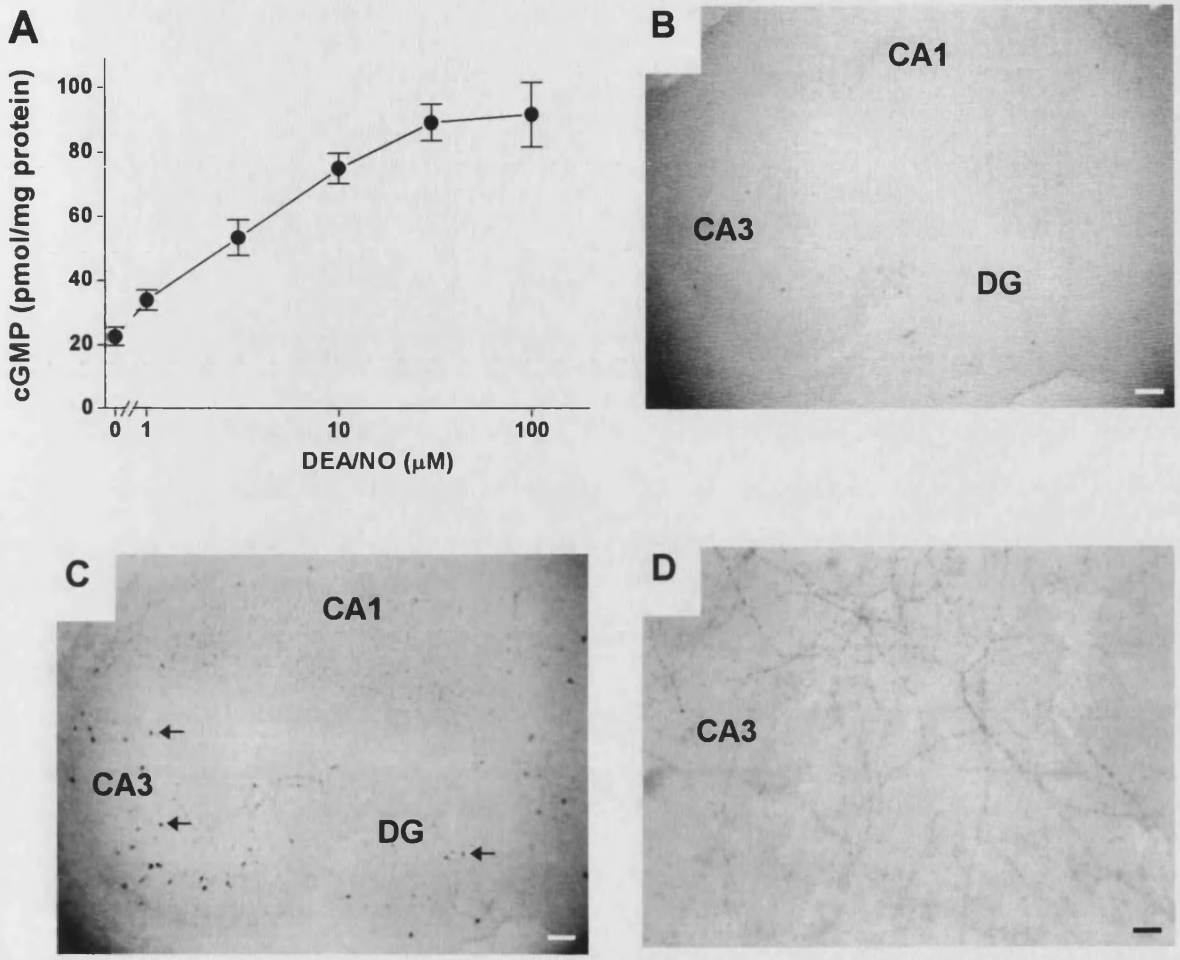


Figure 4.4 cGMP distribution and accumulation

(A) cGMP accumulation was measured 5 min following stimulation with the NO donor DEA/NO in a range of concentrations (n = 10-15 slices). **(B)** Photomicrograph of cGMP staining in a hippocampal slice culture following exposure to IBMX alone. **(C)** After a 5 min maximal (100 μM) DEA/NO stimulation, cGMP staining is evident in neuronal-like cells in CA3 and the dentate gyrus (DG, arrows) while a more diffuse staining is seen in CA1. **(D)** A clear network of cGMP positive fibers is evident throughout the slice. Scale bars = 100 μm (B,C) or 10 μm (D).

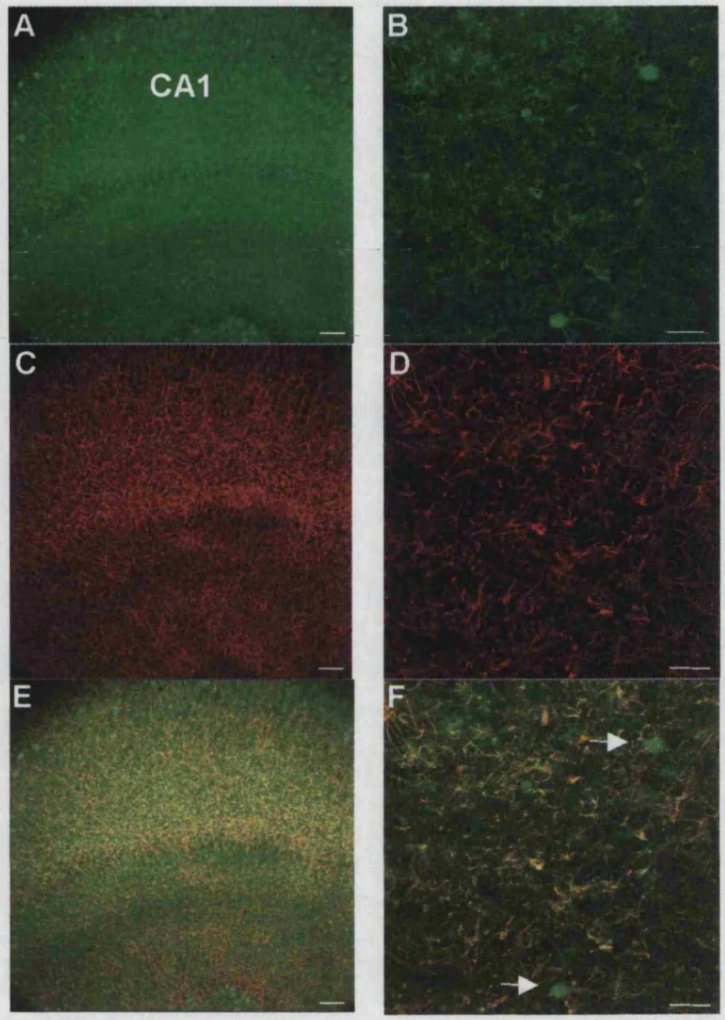


Figure 4.5 cGMP co-localises with GFAP in CA1

Following a maximal DEA/NO stimulation (100 μM, 5 min) confocal microscopy at low (A,C,E) and high (B,D,F) magnifications reveals that the staining for cGMP (green; A,B) and GFAP (red; C,D) partially co-localises in the CA1 region (yellow; E,F). Occasional neurone-like cells were cGMP-positive but GFAP-negative (arrows, F). Scale bars = 100 μm (A,C,E) or 25 μm (B,D,F).

NMDA-evoked neuronal degeneration

Hippocampal slice cultures exposed to a brief (15 min) NMDA stimulation of graded intensity (100 μM-1 mM) were recovered for up to 24 h. Neuronal viability was assessed in the separate regions of the hippocampus by PI staining. Following exposure to 100 and 300 μM NMDA, there was a regional susceptibility to damage in the order CA1>CA3>DG whereas, with 1 mM NMDA, the cell death was relatively non-selective (90% in both CA1 and CA3). Damage intensity was NMDA concentration-dependent (Figs 4.6A,B) and, at 300 μM, was delayed in onset by up to 6 h and maximal by 16 h (Fig. 4.6C). In the same experiments, addition of the non-competitive NMDA receptor antagonist MK801 (10 μM) during the 24 h recovery from NMDA afforded almost complete protection (Fig 4.6D).

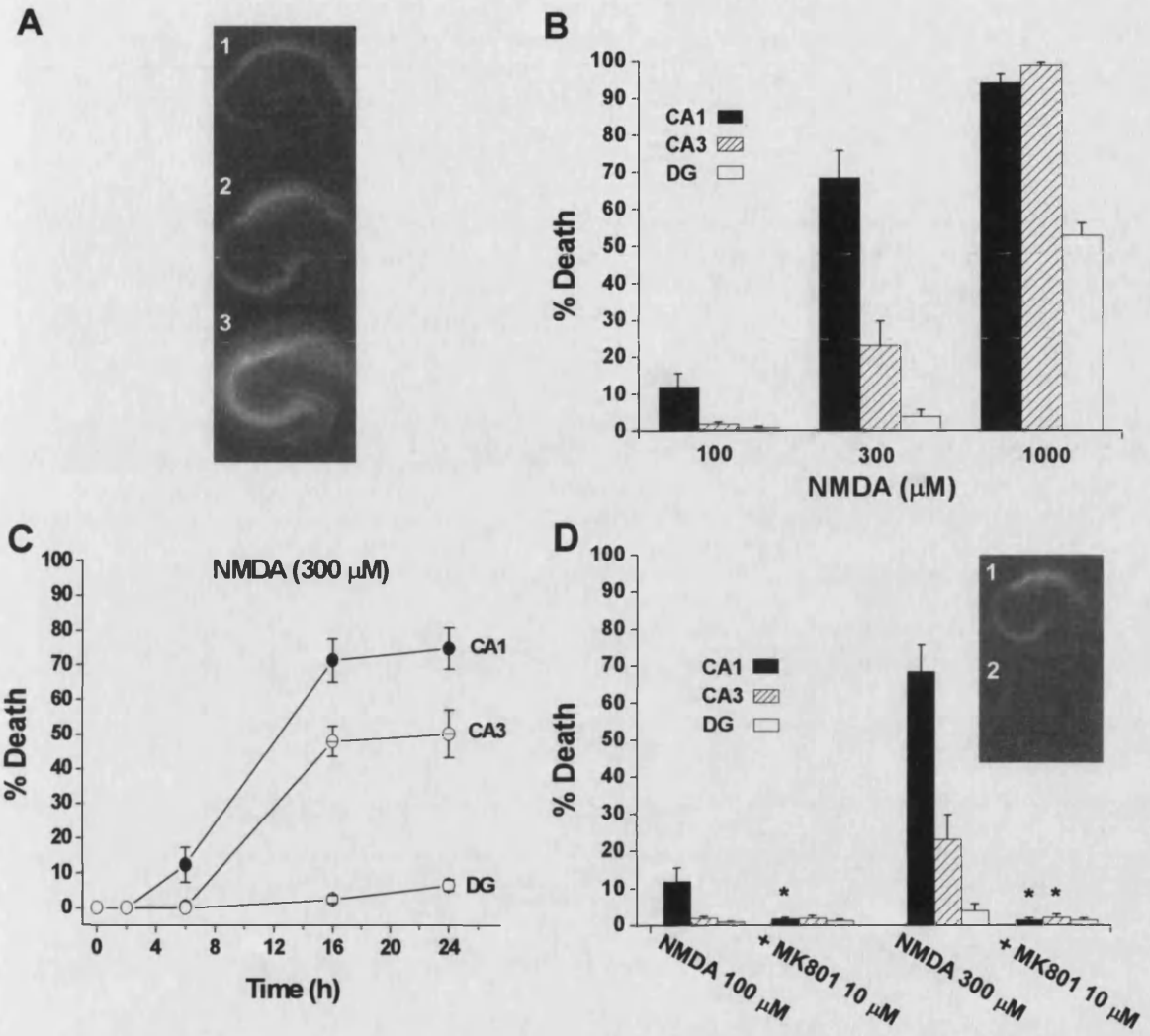


Figure 4.6 NMDA stimulations

(A) Representative photomicrographs of slices stained with PI 24 h following brief (15 min) stimulation with NMDA at concentrations of 100 μM (1), 300 μM (2) and 1 mM (3). **(B)** Summary data (means ± SEM; n = 12 slices) is expressed as percentage death in the three major hippocampal regions (CA1, CA3 and DG). **(C)** Time course of cell death in the subfields (mean ± SEM; n =12 slices) following stimulation with NMDA (300 μM, 15 min). **(D)** Summary data (mean ± SEM, n = 11-16 slices) showing percentage death in slice regions 24 h following brief (15 min) NMDA stimulation (100 or 300 μM), and protection by the NMDA antagonist MK801 (10 μM), which was added directly following NMDA stimulation.*P<0.05 versus corresponding NMDA stimulation. Insets are representative photomicrographs of slices stimulated with NMDA (300 μM) without (1) or with (2) MK801 (10 μM).

Coupling of NMDA receptors to NO formation: role in NMDA toxicity

In the hippocampus and elsewhere, NMDA receptor activation leads to NO synthesis and subsequent cGMP accumulation (East & Garthwaite, 1991; Garthwaite *et al.*, 1989). In agreement with findings using acute slices (East & Garthwaite, 1991) exposure of the slice cultures to NMDA generated a cGMP response that was maximal at 100-300 μM (Fig. 4.7A). The NOS inhibitor 7-NI (30-300 μM) concentration-dependently decreased the response back to basal levels, an effect that was replicated by another inhibitor, L-NNA (300 μM).

To examine the possible protective effect of NOS inhibition on NMDA-induced damage, L-NNA or 7-NI (both at 300 μM) was added 15 min prior to, during, and after exposure to NMDA (100 and 300 μM). No protection was seen following a 24 h recovery (Figs 4.7B, C). Surprisingly while L-NNA treated slices retained the same degree of damage as NMDA-treated controls, those treated with 7-NI exhibited a 3-fold increase in cell death in the CA3 region (to about 70%) following exposure to 300 μM NMDA. As, under the same conditions, the DMSO vehicle for 7-NI had no effect on tissue viability (data not shown) the additional toxicity of 7-NI may arise from another activity of the compound, such as monoamine oxidase inhibition (Desvignes *et al.*, 1999).

Because neuronal death *in vivo* may take several days to complete, we checked the progress of damage in the slice cultures over a recovery period longer than 24 h. With 72 h recovery, cell death induced by NMDA (100 μM) was not significantly higher than that seen at 24 h. With 300 μM NMDA, however, there was a slight increase in damage in CA1. Addition of L-NNA (300 μM) for the duration of the 72 h recovery had no effect upon the cell death induced by either 100 μM or 300 μM NMDA (Fig. 4.7D).

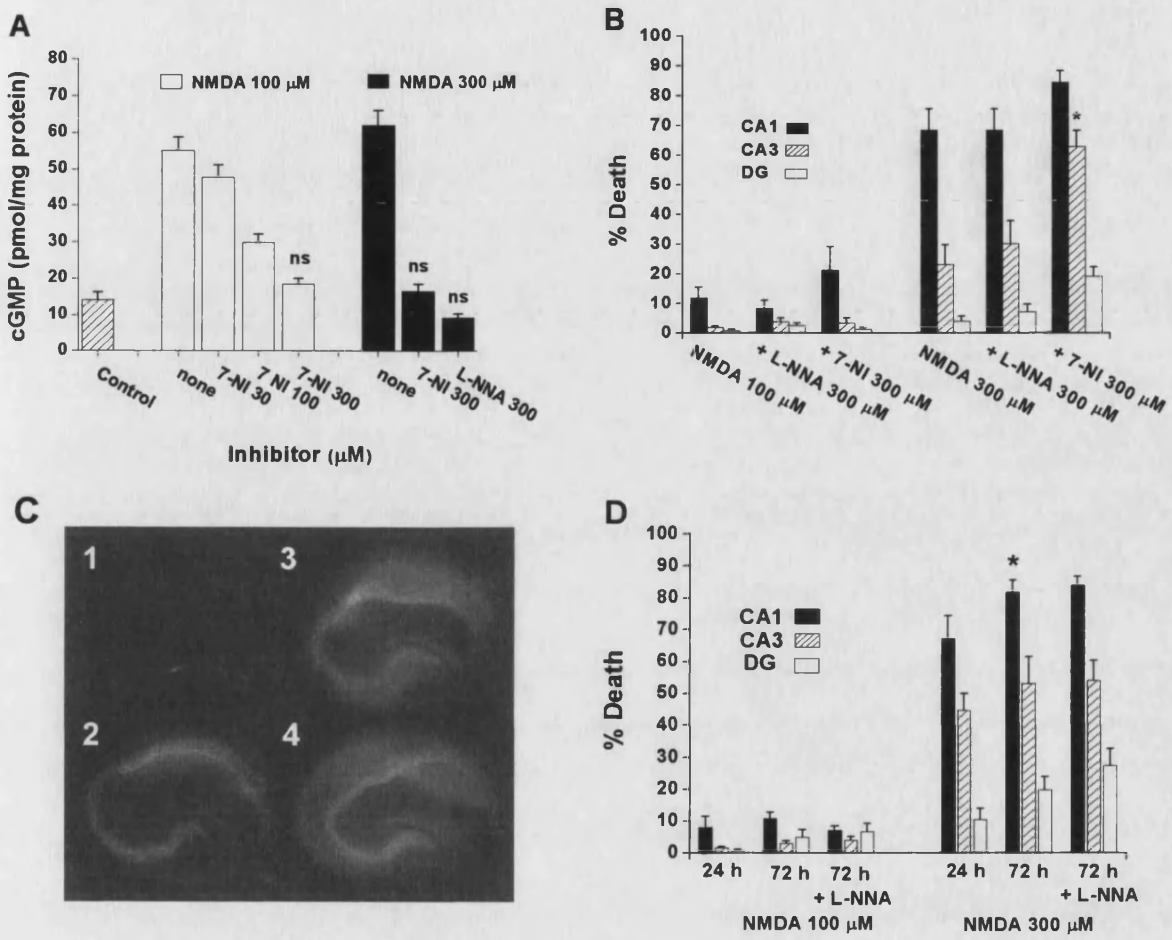


Figure 4.7 Effect of NOS inhibition

(A) The effects of NOS inhibitors L-NNA (300 μM) or 7-NI (30-300 μM) on cGMP accumulation following 15 min stimulation with NMDA (100 or 300 μM) were determined by radioimmunoassay. Results from 7-8 slices are expressed as mean ± SEM, ns = no significant difference versus IBMX control. **(B)** Summary data (mean ± SEM, n = 12 slices) showing percentage death of hippocampal regions 24 h following 15 min NMDA stimulation. L-NNA (300 μM) and 7-NI (300 μM) were added 15 min before the NMDA and remained present throughout the stimulation and recovery. **(C)** Representative photomicrographs of PI stained slices at 24 h. Treatments were as follows: (1) control slice, (2) NMDA 300 μM, (3) NMDA + L-NNA, (4) NMDA + 7-NI. **(D)** Summary data (mean ± SEM, n = 12 slices) showing the percentage death in hippocampal slice regions following recovery for 1 or 3 days after a 15 min NMDA stimulation (100 or 300 μM) in the absence or presence of L-NNA (300 μM). *P<0.05 versus corresponding NMDA stimulation with 24 h recovery.

To gauge the intensity of NO accumulation following NMDA receptor activation, comparison was made of the ensuing cGMP response relative to that produced by exogenous NO, delivered from DEA/NO. With NMDA (100 μM), the cGMP response was stable between 2 and 15 min exposure, indicating a steady-state (Fig. 4.8A). With DEA/NO at a maximal concentration (100 μM) cGMP accumulated to 2.5-fold higher levels than observed with NMDA, though the amplitude gradually declined after 2 min (Fig. 4.8B). The lower response with NMDA may be because, by increasing intracellular Ca^{2+}, NMDA receptor activation diminishes the enzymatic activity of the $NO_{GC}R$ (Kazerounian *et al.*, 2002) or, alternatively, NMDA-evoked $O_2^{\bullet-}$ formation (Lafon-Cazal *et al.*, 1993) may reduce the effective NO concentration by forming $ONOO^-$. To test these and other possibilities, DEA/NO and NMDA were co-administered for 2 min (Fig. 4.8C). Although the response to DEA/NO was reduced, cGMP levels were still twice those obtained with NMDA alone at maximal concentrations, suggesting that the peak NMDA-stimulated NO accumulation was lower than that required to maximally-activate the $NO_{GC}R$.

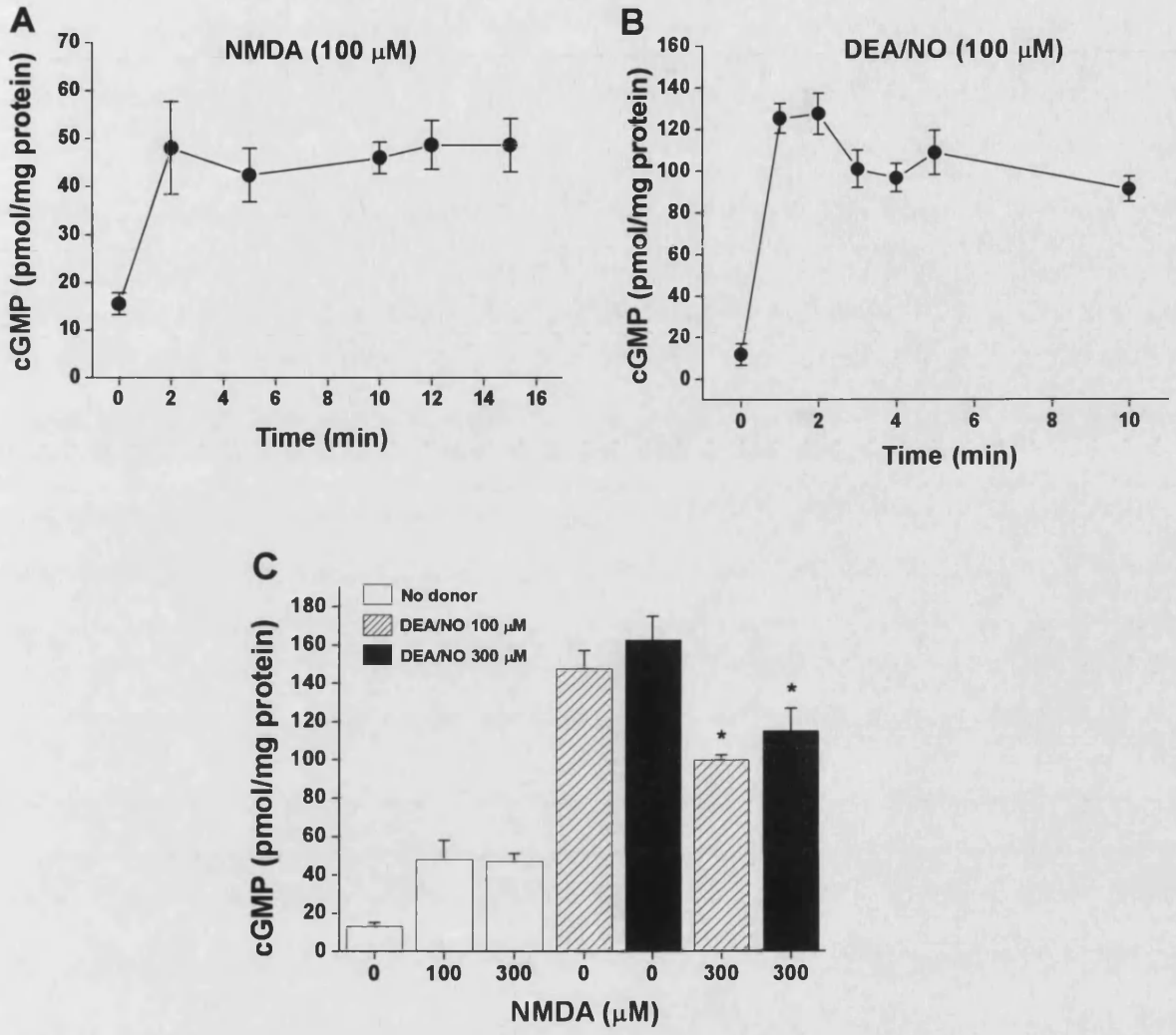


Figure 4.8 Accumulation of cGMP in response to NMDA or DEA/NO

All data are means ± SEM (n = 8-12 slices). **(A)** Time course of cGMP accumulation following stimulation of hippocampal slice cultures with NMDA (100 μM). **(B)** Time course of cGMP accumulation following exposure to DEA/NO (100 μM). **(C)** cGMP accumulation measured 2 min following stimulation with NMDA (100 and 300 μM) compared with maximal stimulation by DEA/NO (100 and 300 μM) or DEA/NO + NMDA; *P<0.05 versus respective DEA/NO concentrations alone.

4.4 DISCUSSION

The NO signalling pathway in hippocampal slice cultures

The essential first step was to characterise the preservation of the relevant pathway in organotypic hippocampal slice cultures. This had not previously been carried out despite such cultures being routinely used in physiological and pathological investigations of phenomena in which NO has been suggested to participate, such as long term potentiation (Muller *et al.*, 1993), glutamate excitotoxicity and oxygen glucose deprivation (OGD) (Lahtinen *et al.*, 2001; Pringle *et al.*, 1997), or neuronal development (Stoppini *et al.*, 1993). The results showed that the distributions of nNOS, eNOS and cGMP in slice cultures correspond well with those reported for the hippocampus *in vivo* or in acute slice preparations. This, together with functional evidence for effective coupling of NMDA receptor activation to NO formation and subsequent cGMP accumulation, commends the slice cultures for research on the NO signalling pathway.

Role of NO in NMDA neurotoxicity

In keeping with previous results using slice cultures (Adamchik & Baskys, 2000; Vornov *et al.*, 1991) brief (15 min) exposure to NMDA elicited delayed neuronal cell death (preferentially of CA1 pyramidal neurones) that could be prevented by post-application of MK801, indicating that the death involves a secondary exposure of the neurones to glutamate. The preferential susceptibility of CA1 neurones may be a function of differential receptor expression (higher NMDA receptors density), and other selective cellular changes, such as increased expression of protective genes in other cells (Lipton, 1999).

Exhaustive tests using different NO synthase inhibitors, stimuli of differing intensity, and differing recovery periods, however, failed to provide any indication that NO contributes to the ensuing damage, by respiratory inhibition or otherwise, despite clear evidence from cGMP measurements that activation of NMDA receptors couples to NO generation. Estimation of the ambient NO concentrations existing within the stimulated slice tissue provides a simple explanation for the negative results. Using the GC-coupled

NO receptor as a biosensor, the degree of receptor activation obtained following exposure to maximal concentrations of NMDA (measured as cGMP accumulation) could be augmented about 2-fold by addition of exogenous NO, suggesting that the endogenous levels were sufficient to give only half-maximal receptor stimulation. Whether based on cGMP responses to NO at steady-state in brain cells (Griffiths & Garthwaite, 2001), or on measurements of NO-evoked GC activity in cell-free preparations (Bellamy *et al.*, 2002), the half-maximally effective concentration of NO on GC-coupled receptors is about 2 nM. Hence, physiological concentrations of NO appear all that NMDA receptor stimulation is able to generate within the slices at steady-state. In an analogous approach used to explore the pathological role of NO in acute striatal slices, the peak NO concentration occurring following a period of metabolic stress (oxygen and glucose deprivation) was found to be similar, about 1 nM (Griffiths *et al.*, 2002a), indicating that the low apparent NO levels in the hippocampal slice cultures exposed to NMDA do not represent an oddity.

It should be noted that using $NO_{GC}R$ activity to estimate the NMDA stimulated rise in NO concentration in brain slices assumes that both NO and cGMP levels increase evenly across the slice. This would depend upon the distribution of both the NMDA receptors and $NO_{GC}R$ in the tissue. However, without the requisite detailed immunohistochemistry the possibility of localized microdomains of high NO concentration cannot be ruled out.

The notion that NO rises to much higher (toxic) levels in pathological conditions appears to stem from the initial measurement of NO in cerebral ischaemia/reperfusion *in vivo*, where concentrations in the low micromolar range were recorded using an electrochemical probe (Malinski *et al.*, 1993). However, it has since become evident that such probes are prone to interference from other substances such as ascorbate and tyrosine (Lin *et al.*, 1996; Stingele *et al.*, 1998). More recent measurements give the much lower value of 20 nM NO in the ischaemic penumbra *in vivo* (Lin *et al.*, 1996), and 16 nM or less following local stimulation with NMDA *in vivo* (Lin *et al.*, 1996; Wu *et al.*, 2001). Consequently, although much lower than previously assumed, the estimated maximal NMDA-induced NO concentration in the hippocampal slice cultures is compatible with recent direct measurements

made *in vivo*. Likewise, in dispersed cultures or brain slices *in vitro*, estimates for the prevailing NO concentration on exposure to NMDA or following repetitive electrical stimulation have yielded values in the low nanomolar range or below (Liu *et al.*, 1997; Shibuki & Kimura, 1997).

There is no evidence that NO in low nanomolar concentrations can be directly toxic to neurones or other cells. Nevertheless, there is evidence that nNOS can contribute to damage following both excitotoxic stimulation and cerebral ischaemia (Ayata *et al.*, 1997; Iadecola, 1997), and see chapter 1. Rather than serve as a toxin in this situation, NO might act through physiological cGMP-dependent mechanisms to potentiate synaptic transmission (Bon & Garthwaite, 2001; Huang & Hsu, 1997; Nikonenko *et al.*, 2003), thereby contributing to cell death by eliciting an additional excitotoxic load on the neurones at risk (Gao *et al.*, 1999; Miyazaki *et al.*, 1993).

Other calcium-dependent effectors

The absence of evidence that NO causes toxicity in hippocampal slice cultures suggests that other, probably Ca^{2+}-dependent, enzymes may be involved, including phospholipases, protein kinases, endonucleases and calpains. Of these, calpain is a Ca^{2+}-activated neutral cysteine protease whose activation appears very important in both focal and global ischaemia. Two calpain enzymes μ- and M-calpain are activated by micromolar and millimolar Ca^{2+} respectively. Activated caplain attacks many cytoskeletal proteins including spectrin and tubulin (Saido *et al.*, 1994), it leads to loosening of the post-synaptic density (Dosemeci & Reese, 1995) and can break down microtubules. Following global ischaemia most calpain activation can be prevented by treatment with MK801, and calpain inhibitors have attenuated cell death in both focal (Bartus *et al.*, 1994) and global models (Lee *et al.*, 1991) or in ischaemic cerebrocortical cell cultures (Wang *et al.*, 1996). Furthermore, calpain inhibition can reduce toxicity following NMDA stimulation of cultured hippocampal cells (Brorson *et al.*, 1995), and improves recovery of synaptic responses in hippocampal slice cultures (Bednarski *et al.*, 1995).

4.5 CONCLUSION

The results in this study corroborate recent *in vivo* findings that cytochrome *c* oxidase is not inhibited by nitric oxide, either in physiologic conditions or during reoxygenation after a brief anoxic period in adult rats (De Visscher *et al.*, 2002). Taken together, these studies question the concept of NO rising to toxic concentrations as a result of NMDA receptor activation.

CHAPTER 5: HIPPOCAMPAL SLICE CULTURES AND DAMAGE BY EXOGENOUS NO

5.1 INTRODUCTION

To complement the study in chapter 4, the toxicity of exogenous NO to hippocampal slice cultures was examined. The principal mechanisms by which NO may cause neuronal death, and the problems of working with older generations of NO donors, have been discussed previously in chapters 2 and 3.

Using exogenously applied NO, recent work has confirmed that NO donors may kill neurones by inhibiting respiration. One study (Brorson *et al.*, 1999) applied older NO donors (SNAP, SIN-1, SNOC) or NONOates (DEA/NO, Spermine/NO) to cultured hippocampal neurones, and found that NO concentrations of ~2 μM caused substantial ATP depletion, which correlated with up to 40% cell death. Later, experiments using PC-12 cells (Bal-Price & Brown, 2000) used steady-state levels of 1-3 μM NO released from DETA/NO or SNAP causing mitochondrial membrane potential to fall, ATP depletion and necrosis (in the absence of glucose) or, when glucose was present, apoptosis. Since these results were replicated over a similar timecourse by application of the respiratory inhibitors myxothiazol, rotenone and azide, respiratory inhibition was deemed responsible. Finally, similar methods were applied in a subsequent study. Cerebellar granule cells were susceptible to respiratory inhibition by concentrations of ~1-2 μM NO released from NO donors or NO saturated water (Bal-Price & Brown, 2001).

Aims

The principle aim of this study was to determine the concentration of exogenous NO required for substantial neuronal death in hippocampal slice cultures by means of respiratory inhibition.

5.2 METHODS

Hippocampal slice culture preparation and assessment of neuronal damage

See chapter 4.2

Exposure to NO and respiratory inhibitors

To assess the effect of a continuous application of exogenous NO on hippocampal slice viability, cultures were exposed to increasing concentrations of DETA/NO. Cultures were exposed to freshly prepared DETA/NO (3 mM) in SFM (replenished every 24 h) and viability was assessed following 24, 48 or 72 h. Control cultures were exposed in the same way to 1.2 mM DETA/NO previously left to decompose at 37°C in SFM for at least 8 half-lives (1.2 mM being the maximum concentration of decomposed DETA/NO that would be present after 24 h). Alternatively, cultures were exposed to a faster-releasing donor, NOC-12, or the appropriate concentration of decomposed donor. For comparison, cultures were exposed to the respiratory inhibitors myxothiazol (3 μM) or sodium cyanide (NaCN, 3 mM) for up to 24 h. NO release from donor compounds was measured by electrochemical probe (ISO-NOP, see chapter 2.3).

ATP measurement

Following exposure to NO from donor compounds or respiratory inhibitors as above, slices were sonicated in ice-cold trichloroacetic acid (0.5 M; 200 μl/slice). Protein was separated by centrifugation (15,000 g for 15 min), resuspended in 0.1 M KOH and quantified by the bicinchoninic acid method. ATP levels in the supernatant were determined by a D-luciferin/luciferase ATPLite™-M assay kit (Packard Bioscience, Reading, UK). Statistical

differences were analysed using one-way ANOVA with Dunnett's *post hoc* test; P values of < 0.05 were regarded as significant.

Measurement of NO consumption

Homogenates from acutely prepared hippocampus (8 and 19 day old rats), or hippocampal slice cultures (12 and 19 days old) were centrifuged at 10,000g for 5 min and resuspended at a final concentration of 0.1 mg protein/ml in tris buffer, supplemented with whole rat brain supernatant (10 %, see chapter 6.2) and further sonicated before use. NO consumption was measured in 1 ml samples following addition of DETA/NO (200 µM), using an NO electrode (ISO-NOP, see chapter 2.3).

5.3 RESULTS

Toxicity of exogenous NO

Given the apparent lack of participation of endogenous NO in NMDA toxicity, further tests were carried out to evaluate the sensitivity of the tissue to exogenous NO. To determine the concentration of NO required to elicit damage in hippocampal slice cultures, the slow-releasing NO donor DETA/NO was applied for up to 3 days. When measured using an electrochemical probe, DETA/NO (3 mM) generated a steady plateau of 4.5 ± 0.2 μM NO (n = 3) at 33°C after 3 h pre-incubation (needed to allow an equilibrium to be formed between the rate of NO release and the rate of NO consumption by autoxidation). Application of this DETA/NO concentration to the slice cultures for a prolonged interval (2 days) caused only a relatively minor degree of death (10 % or less) that was preferentially located in CA3 (Fig. 5.1A, B). After an additional day's exposure, the damage in all regions was increased with, again, that in CA3 predominating somewhat (Figs 5.1A, B). A small lowering of the DETA/NO concentration (to 2 mM) greatly reduced the damage observed after 3 days exposure (Fig. 5.1B, inset). Decomposed donor had no effect on slice viability at either of the concentrations tested (data not shown).

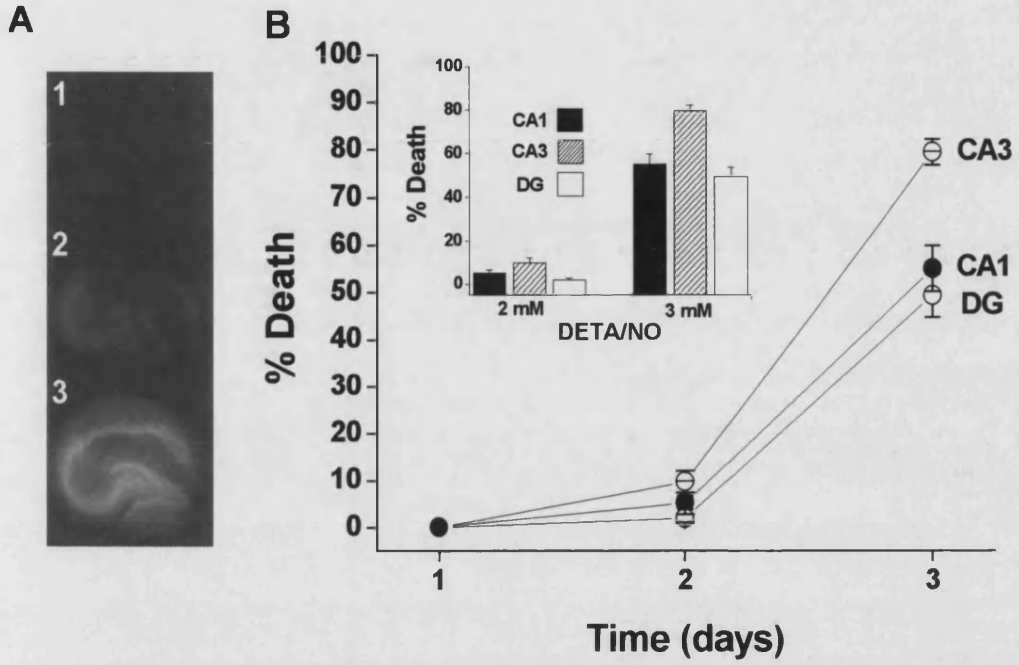


Figure 5.1 Toxicity of DETA/NO in hippocampal slice cultures

(A) Representative photomicrographs of slices continuously exposed to DETA/NO (3 mM) for 1, 2 or 3 days (labelled 1-3 respectively) and stained using PI. **(B)** Summary data (mean ± SEM; n = 12-16 slices) expressed as percentage death in the three major hippocampal regions. The effect of a lower DETA/NO concentration (2 mM) at 3 days is also shown for comparison in the inset.

Recent evidence in dispersed cultures, found that NO may elicit neuronal death by respiratory inhibition and subsequent ATP depletion (Bal-Price & Brown, 2001; Brorson *et al.*, 1999). To determine the vulnerability of the slice cultures to this intervention, they were exposed to the mitochondrial complex III inhibitor, myxothiazol (3 μM). Complete neuronal death ensued within 24 h (Figs 5.2A,B).

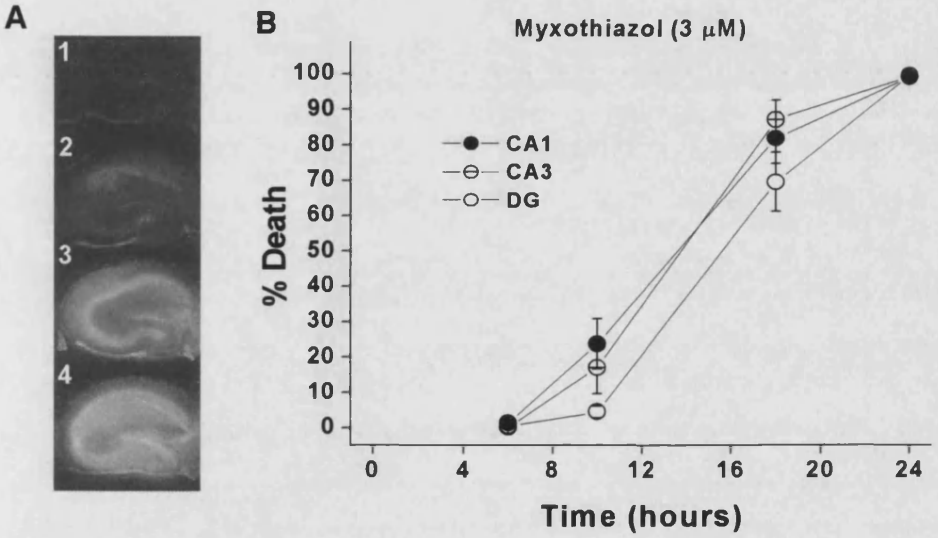


Figure 5.2 Toxicity of myxothiazol in hippocampal slice cultures

(A) Representative cell death following continuous exposure to myxothiazol (3 μM) for 6, 10, 18 and 24 h (labelled 1-4 respectively). **(B)** Summary data (mean ± SEM; n = 12) expressed as percentage death in the three major hippocampal regions.

In addition, when measured 6 h following treatment, hippocampal slice ATP levels were significantly decreased by treatment with either myxothiazol or another respiratory inhibitor, NaCN (3 mM), but were unaffected by 3 mM DETA/NO (Fig. 5.3). NaCN, like myxothiazol, caused complete cell death within 24 h (not shown).

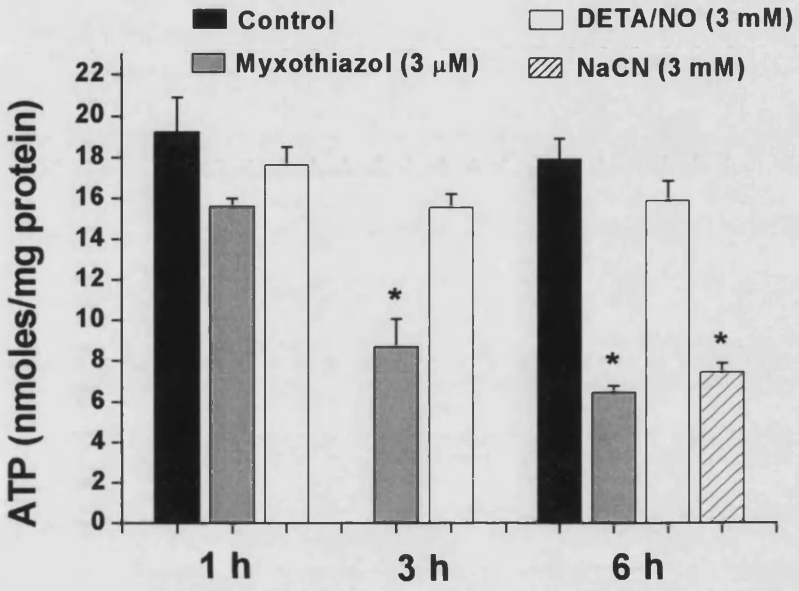


Figure 5.3 Whole slice ATP

Measurements (mean ± SEM; n = 8 slices) taken 1 to 6 h following treatments with 3 mM DETA/NO and 3 μM myxothiazol; the effect of a 6 h exposure to NaCN (3 mM) is also shown. *P<0.05 versus control ATP at 1 h.

Since high DETA/NO concentrations failed to cause toxicity, a faster NO-releaser, NOC-12 (half-life = 160 min at 33°C), was used to generate higher NO concentrations. Slices were exposed to NOC-12 (0.3-3 mM) and ATP levels were measured at 4 h, and death at 24 h. At 3 mM, NOC-12 significantly decreased ATP levels and caused a generalised 60-80 % death (Figs 5.4A – C). In contrast, lower NOC-12 concentrations (0.3 and 1 mM) caused no obvious slice toxicity and little or no change in ATP levels. From measurements of the NO concentration generated by 0.3 mM NOC-12 (2.7 ± 0.2 μM after 5 min at 33°C; *n* = 3), it can be calculated (Schmidt *et al.*, 1997) that 1 and 3 mM NOC-12 would produce peak NO concentrations of 6 and 10 μM respectively.

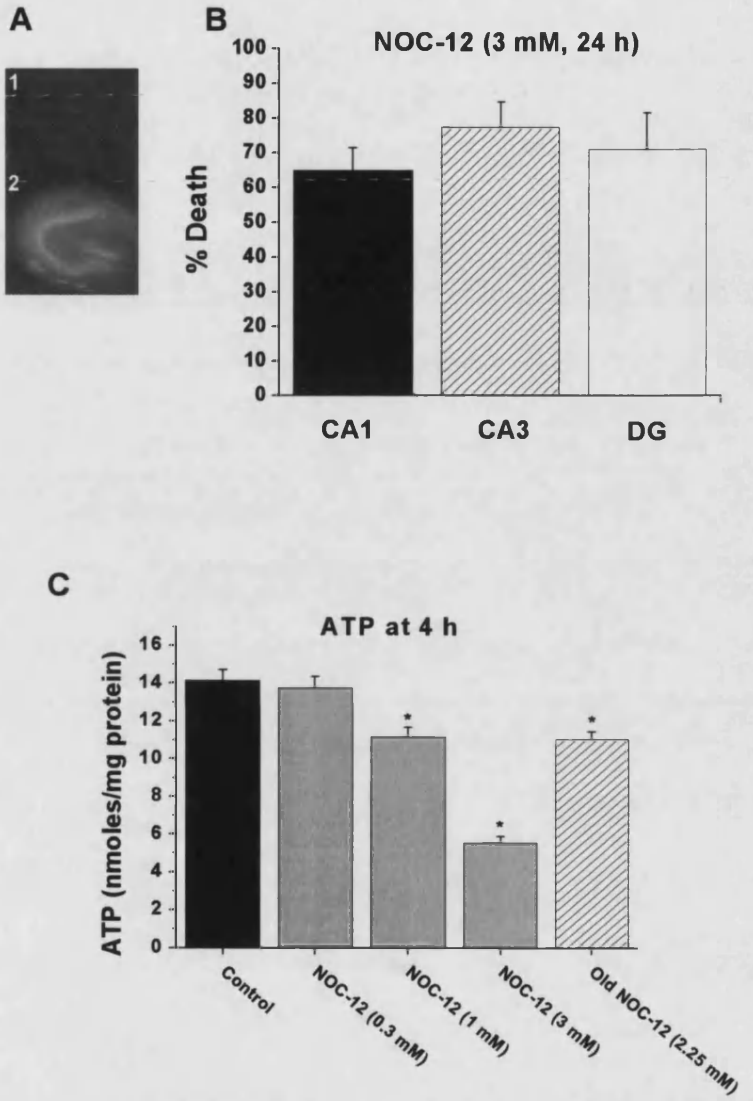


Figure 5.4 NOC-12 induced cell death in hippocampal slice cultures

(A) Representative photomicrographs of slices 24 h after exposure to 1 or 3 mM NOC-12 (labelled 1 and 2 respectively) and stained with PI. **(B)** Summary data for the toxicity of 3 mM NOC-12 (means ± SEM; n = 12 slices) expressed as percentage death in the three major hippocampal regions. **(C)** Whole slice ATP measurements (mean ± SEM; n = 8 - 16) taken 4 h following the above exposures.

NO consumption by hippocampal slices

Work in the lab has previously described the consumption of NO by both isolated cerebellar cells and whole rat brain homogenates (Griffiths & Garthwaite, 2001; Griffiths *et al.*, 2002b). Furthermore, semi-purified homogenates retained activity when the pellet and supernatant fractions were recombined (see fig 6.1). We questioned if tissue from acutely isolated hippocampal slices can consume NO in a similar manner, and whether hippocampal slice cultures differed. Upon addition of 200 μM DETA/NO to buffer, NO levels rose sharply in accordance with earlier results (fig 3.3A), reaching 150 nM within 1 min (fig 5.5A,B). In contrast, all samples from acutely isolated hippocampal slices, and hippocampal slice cultures, consumed NO in a similar manner to before (see figure 6.1 and Griffiths *et al.*, 2002b), such that NO remained below 25 nM for at least 10 min, before rising similarly to buffer. In these cases NO did not reach 150 nM until about 14 min (fig 5.5A,B)

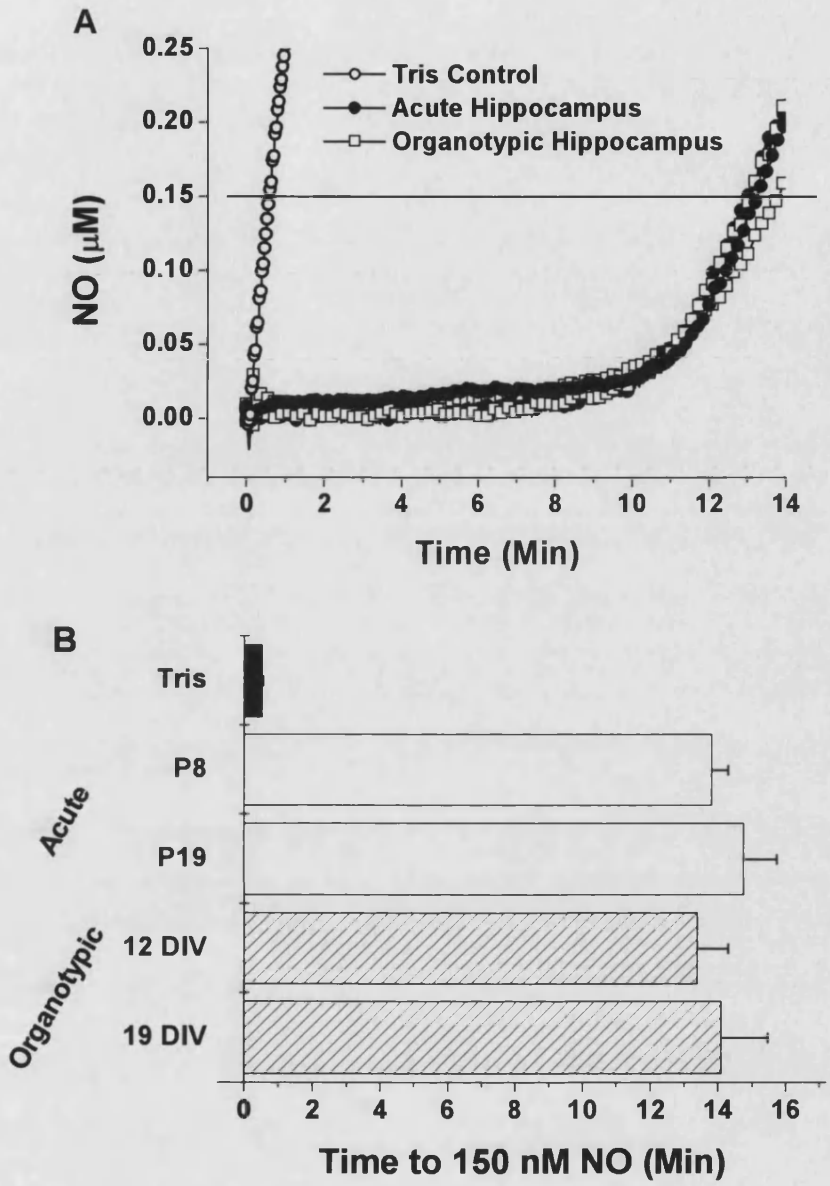


Figure 5.5 NO consumption by hippocampal slices

(A) Representative traces of NO following DETA/NO (200 μM) addition to 20 mM Tris buffer (open circles), acutely isolated hippocampus aged P8 and P19 (closed circles), or organotypic hippocampal slice cultures after 12 and 19 DIV (open boxes). **(B)** Summary data is shown as time to 150 nM NO (mean ± SEM, n = 4).

5.4 DISCUSSION

Toxicity of exogenous NO

The question addressed in this chapter concerned the vulnerability of hippocampal slice culture neurones to exogenous NO. Surprisingly, exposure to high NO concentrations (up to 4.5 μM) caused no detectable damage during a 24 h exposure period, nor were any changes in tissue ATP levels recorded. Given the positive control showing that inhibition of mitochondrial respiration with myxothiazol or NaCN depressed ATP levels and caused maximal death over an equivalent time period, these results suggest that the high NO concentrations applied were unable to inhibit cellular respiration significantly. NO competes with O_2 for binding to the terminal complex of the respiratory chain, known as complex IV or cytochrome *c* oxidase (Brown, 2001; Cooper, 2002). At O_2 concentrations found *in vivo* (20-30 μM), a steady-state NO concentration of 120 nM is required to inhibit the O_2 consumption of cerebellar cells by 50% (Bellamy *et al.*, 2002); see also (Brown & Cooper, 1994). Because of the higher O_2 concentration existing in the *in vitro* environment (about 180 μM), it would be predicted that about 0.4 μM NO should have been enough for 50% respiratory inhibition (Koivisto *et al.,* 1997). Consistent with this presumption, in dispersed hippocampal neurones (Brorson *et al.,* 1999), PC12 cells (Bal-Price & Brown, 2000), and mixed cortical cultures (Bal-Price & Brown, 2001) *in vitro*, 0.5–2 μM NO inhibited respiration and provoked neuronal cell death. In the slice cultures, however, 10 μM NO was required to have this effect.

The data show that neurones in hippocampal slice cultures are remarkably resilient to NO-induced damage, including through respiratory inhibition. The simplest explanation is that the NO concentrations within the slices are much lower than those applied in the culture medium. Supporting this interpretation was the finding that 30 μM DEA/NO was needed to maximally elevate slice cGMP levels (figure 4.4A) whereas, in dispersed cells, 30 nM DEA/NO suffices (Gibb *et al.,* 2003). A plausible reason for the poor access of exogenous NO to the slice tissue is the activity of the formidable NO inactivation mechanisms that have been found in brain and

other tissues (Griffiths *et al.*, 2002b; Liu *et al.*, 1998c). Analogous to the effect of O_2 utilization on the diffusion of O_2 into isolated tissues (Hill, 1929), and the effect of transporters on the diffusion of glutamate into brain slices (Garthwaite, 1985), avid consumption of NO by the cultured slices would result in steeply declining NO concentration gradients going from the outside to the inside of the tissue, and necessitate addition of high concentrations to the medium in order that regions near the centre be supplied. Analysis of the rate of NO inactivation by slice cultures *in situ* is technically problematic. Homogenised slice cultures, however, inactivated NO to the same extent as acute hippocampal tissue in the manner previously reported (Griffiths *et al.*, 2002b). Furthermore application of cGMP immunocytochemistry to acute cerebellar slices incubated with NO indicates that, as predicted, steep steady-state gradients of NO exist across the slice thickness as a result of NO consumption. This apparent maximal rate of consumption was 1 μM/sec (Hall *et al.,* 2003). In dispersed cells, this rate would typically be about 100-fold lower because of the effect of dilution, which would help explain why NO is more toxic to these preparations. In addition, neurones in slice culture may be inherently more resistant than dispersed cultures to oxidative stress resulting, for example, from $ONOO^-$ formation, because of the close proximity of astrocytes which can supply neurones with protective glutathione precursors (Gegg *et al.,* 2003).

Providing NO inactivation is sustained, therefore, it is difficult to envisage how NO could rise in intact hippocampal tissue to concentrations that are directly toxic. In our experiments, NO concentrations lower than 10 μM were able to cause cell death, but the concentrations still needed to be very high (about 4.5 μM) and the exposure time long (3 days for substantial cell death). In the absence of evidence that constitutive nNOS can produce global tissue increases in NO concentration of this magnitude the mechanism was not explored. Possibly, failure of NO inactivation (Griffiths & Garthwaite, 2001) may have increased the tissue NO concentrations sufficiently to achieve respiratory inhibition and/or other reactions (e.g. cumulative nitrosation of tissue thiols by products of NO autoxidation, such as N_2O_3; Augusto *et al.*, 2002; Grisham *et al.*, 1999) may have been responsible.

5.5 CONCLUSION

Intact hippocampal tissue in culture is remarkably resilient to prolonged exposure to high exogenous NO concentrations, presumably because NO consumption prevents cellular concentrations rising to those in the surrounding medium and/or because of protective cell-cell interactions. It will be important to determine if the findings are generally applicable in the brain, or if some regions are potentially more at risk, for example because of reduced NO inactivation.

CHAPTER 6: LIPID PEROXIDATION IS A COMPONENT OF NO CONSUMPTION IN VITRO

6.1 INTRODUCTION

Although our knowledge of NO synthesis by the nitric oxide synthase (NOS) enzyme family has advanced considerably (Alderton *et al.*, 2001), the mechanism by which NO is inactivated remains poorly understood. This is surprising, given that tissue NO concentrations (which reflect the balance between synthesis and breakdown) may directly influence the resultant behaviour of NO, be it as a physiological signal or toxic molecule. In aqueous solutions the major decomposition product of NO is NO_2^- (see chapter 3.1), however, in the presence of biological tissue NO_3^- is formed (Ignarro *et al.*, 1993). In tissue, various NO half-lives have been reported, ranging from a few seconds in the original cascade perfusion experiments (Palmer *et al.*, 1987) to <100 ms in perfused heart (Kelm & Schrader, 1990). Recent work, based upon the consumption of NO by isolated rat hepatocytes estimates that extravascularly the NO half-life is between 0.09 – 2 s depending upon the O_2 concentration (Thomas *et al.*, 2001). Many potential NO inactivation pathways have been examined, some of which are described below.

Accelerated autoxidation

The simple reaction of NO with O_2 (autoxidation), as described in chapter 3.1, is too slow to have much physiological relevance as an NO sink. Due to the hydrophobic nature of NO, autoxidation is accelerated in the hydrophobic interior of rat hepatocyte membranes or detergent micelles, where the reaction speed may increase up to 300 fold (Liu *et al.*, 1998c). Similarly, NO partitions into, and is consumed by, mitochondrial membranes in a non-saturable oxygen-dependent manner (Shiva *et al.*, 2001). Hydrophobic protein domains have also been proposed to accelerate NO breakdown (Nedospasov *et al.*, 2000) though their relevance is difficult to gauge.

Reaction with $O_2^{\bullet-}$

A major physiological source of $O_2^{\bullet-}$ *in vivo* is due to leakage from the electron transport chain, and it has been estimated that almost 1-2% of all electrons passing through end up as $O_2^{\bullet-}$ ions (Boveris & Cadenas, 2000). Alternatively it has been reported that under certain conditions, namely arginine or BH_4 depletion, the various NOS isoforms may produce $O_2^{\bullet-}$, at least *in vitro* (Xia *et al.*, 1996; Xia *et al.*, 1998a; Xia *et al.*, 1998b). Other enzymatic routes of synthesis include generation by xanthine oxidase (XO), which has been found co-localised with NOS in rabbit synaptosomes (Deliconstantinos & Villiotou, 1996), as follows.

$$xanthine + O_2 \xrightarrow{XO} H_2O_2 + O_2^{\bullet-} + uric\ acid$$

Non-enzymatically $O_2^{\bullet-}$ may be formed by the simple one-electron reduction of O_2 by free ferrous iron, thus.

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet-}$$

Most iron in the brain is tightly bound to proteins such as transferrin or ferritin and is unlikely to catalyse $O_2^{\bullet-}$ production, however a chelatable 'transit pool' of non-protein bound iron may exist with the potential to generate $O_2^{\bullet-}$ (Halliwell & Gutteridge, 1986).

Three endogenous superoxide dismutase (SOD) enzymes have been described. $O_2^{\bullet-}$ will not cross biological membranes and must therefore be detoxified in the compartment in which it is produced. Accordingly the enzymes have different locations. MnSOD is mitochondrial, whereas Cu,Zn-SOD is found either in the cytosolic and lysosomal fraction, or extracellularly where it is heavily glycosylated (Fridovich, 1995). SOD converts $O_2^{\bullet-}$ into H_2O_2, which is then converted to water by glutathione peroxidase and catalase. Since ~0.5% of total soluble protein in the brain is Cu,Zn-SOD, tissues have an excellent defence mechanism. NO reacts with $O_2^{\bullet-}$ with an almost diffusion-controlled rate constant of 0.7-1.9 x 10^{10} M^{-1} s^{-1} to form the strong oxidant $ONOO^-$ (Koppenol, 2001a). However, SOD consumes $O_2^{\bullet-}$

almost as quickly, at 2 x 10^9 M^{-1} s^{-1} and, being present in micromolar concentrations, is generally the major drain for removing $O_2^{\bullet-}$. Nevertheless the reaction of NO with $O_2^{\bullet-}$ may partially account for NO breakdown in various systems (Garthwaite *et al.*, 1988; Palmer *et al.*, 1987) including biological buffers (Beckman and Koppenol., 1996).

Lipid oxidation enzymes

The lipoxygenases (LOX) are a family of non-haem iron-containing enzymes that catalyse oxidation of arachidonate or linoleate to bioactive lipid hydroperoxides. 15-lipoxygenase is expressed in reticulocytes during maturation into erythrocytes, while 12/15-LOX is expressed in monocytes. Both may play a central pathogenic role in atherosclerosis. 15 and 12/15-LOX catalytically consume NO and thereby decrease $NO_{CG}R$ activity *in vitro*. Such NO consumption may be the result of reaction between the enzyme-bound lipid peroxyl radical $E_{red}LOO^{\bullet}$ with NO to form an alkyl peroxynitrite (LOONO) which will undergo hydrolysis to ultimately form lipid hydroperoxide (LOOH) (Coffey *et al.*, 2001; O'Donnell *et al.*, 1999). Often overlooked, this reactivity of NO with lipid peroxyl radicals ($LOO^{\bullet}$) is extremely rapid, and both enzymatic and radical catalysed lipid peroxidation will be terminated with the consumption of 2 molecules of NO per molecule of $LOO^{\bullet}$ (O'Donnell *et al.*, 1997).

Similarly to LOX's prostaglandin H synthase (PGHS) catalyses the initial steps of arachidonate oxidation. NO may interact with this haem enzyme in many ways, and, by acting as a reducing peroxidase substrate, NO is consumed rapidly by purified PGHS-1 plus arachidonate, or by thrombin-activated platelets. In these experiments NO consumption was fast enough to potently prevent NO-dependent activation of $NO_{GC}R$ (O'Donnell *et al.*, 2000).

Mammalian Peroxidases

NO may be catalytically consumed by multiple peroxidases including myeloperoxidase (MPO), the most abundant protein in neutrophils. MPO is present in large amounts at sites of inflammation where NO and H_2O_2 are

also likely to be present. In the presence of H_2O_2 NO serves as a substrate for MPO and the presumed intermediate, NO^+, is extremely labile and is rapidly hydrolysed to NO_2^- (Abu-Soud & Hazen, 2000).

Haemoglobin

Oxyhaemoglobin (HbO_2) and deoxyhaemoglobin scavenge NO to give either methaemoglobin and NO_3^-, or iron-nitrosyl [$Hb(Fe^{2+})$-NO] respectively. Both reaction rates are rapid (~10^7 M^{-1} S^{-1}), indeed HbO_2 is regularly used to scavenge endogenous NO *in vitro* (Palmer *et al.*, 1987). The concentration of HbO_2 in circulating blood is ~10 mM, hence NO produced by endothelial cells should have a half-life of ~1 μs and be almost entirely inactivated, preventing NO-mediated vascular relaxation. Several mechanisms have been proposed to account for the obvious discrepancy. Firstly there exists an 'erythrocyte free zone' next to the endothelium, which is created as blood flows through the vessels (Liao *et al.*, 1999), the size of the 'zone' being dependent upon the vessel diameter. Secondly, NO reacts with HbO_2 encapsulated in erythrocytes nearly three orders of magnitude slower than with free HbO_2, probably due to limited diffusion arising from the existence of an unstirred layer surrounding each cell (Liu *et al.*, 1998a; Liu *et al.*, 2002; Vaughn *et al.*, 2000). In this case the half-life of NO in the lumen is expected to be ~2 ms (Liu *et al.*, 1998a). Notably it has been suggested that Hb might not scavenge NO, but be involved in the transport and delivery of NO to tissues following S-nitrosation of a critical cysteine residue at position 93 on the β globin chain, forming SNO-Hb. The physiological relevance of such a mechanism is difficult to gauge, however, particularly since in humans basal SNO-Hb concentrations are very low (Hobbs *et al.*, 2002).

Myoglobin

Myoglobin functions as a short term O_2 reservoir in exercising skeletal muscle and in the beating heart. NO induces conversion of oxymyoglobin (MbO_2) to metmyoglobin and is converted into NO_3^-. The recent development of myoglobin knockout mice has aided the investigation of the relevance of this reaction. Hearts lacking myoglobin reacted more sensitively to both exogenous and endogenous NO (vasodilation and cardiodepressant actions

were more pronounced), while wild types were less sensitive (Flogel *et al.*, 2001). In acting like an NO oxidase the reaction of myoglobin and NO may in fact act to shield the heart from excessive NO produced from iNOS (Godecke *et al.*, 2003). The relevance of this mechanism at physiological NO concentrations has, however, been questioned (Pearce *et al.*, 2002).

Flavohaemoglobins

The bacteria *Escherichia Coli (E.Coli)* expresses an inducible, NADH-dependent, flavohaemoglobin with NO dioxygenase activity that is extremely efficient at converting toxic NO into NO_3^- under physiological conditions. The reaction rate is only ~2 times slower than that of NO with $O_2^{\bullet-}$ (Gardner *et al.*, 2000; Hausladen *et al.*, 1998). This activity is likely to be a defensive mechanism to protect bacteria from toxic levels of NO and has also been reported in other bacteria, yeast, fungi, and more recently in mammalian cell lines, most notably human colon cells (Gardner *et al.*, 2001). NO consumption by flavohaemoglobins may be inhibited by CN^- and the flavoenzyme inhibitor diphenylene iodonium (DPI), but unfortunately attempts to measure the activity in mammalian cell extracts were unsuccessful, so its functional role *in vivo* remains unknown.

Other mechanisms

Other potential mechanisms for NO inactivation include binding of NO to the protein haemopexin, which may act as a haem storage and transport system (Shipulina *et al.*, 1998) and has been identified in multiple tissues including neurones and glia (Morris *et al.*, 1993). NO also binds to the haem degradation enzyme haem oxygenase-2 (Ding *et al.*, 1999) which cleaves haem into biliverdin, iron and CO. Despite no investigation of the NO half-life being made, HO-2 has been proposed to be a potential intracellular NO sink. Accelerated NO decay has also been postulated to occur upon reaction with cytochrome *c* oxidase (Brudvig *et al.*, 1980; Torres *et al.*, 1998), and may contribute to cellular and mitochondrial NO consumption (Borutaite & Brown, 1996; Clarkson *et al.*, 1995). However, another study found no such activity (Stubauer *et al.*, 1998), and the recent observation that NO oxidation is

accelerated in phopsholipid membranes casts further doubt on the significance of this mechanism as previously described in intact cells. Finally, NO may also bind to non-haem iron proteins such as metallothionin. Metallothioneins are small, sulphur-rich metal-binding polypeptides induced in response to cytokines. Following reaction with endogenous NO, the metal moiety may be released (Pearce *et al.*, 2000), and a recent study has reported that metallothionein-III may prevent exogenous NO toxicity in cerebellar granule cell cultures (Montoliu *et al.*, 2000).

Aims

The rapid consumption of NO by suspensions of cells from rat cerebellum, an area of the brain rich in the NO-cGMP signalling pathway, has been previously described (Griffiths & Garthwaite, 2001). Functionally this sink converts constant rates of NO formation into low steady-state NO concentrations. When confronted by higher NO release rates for several minutes however, the consumption mechanism fails. Further investigation (Griffiths *et al.*, 2002b) revealed that NO inactivation is preserved in rat brain homogenates, and that NO inactivation requires O_2 and generates NO_3^- as the principal end product, as *in vivo*. NO inactivating activity in homogenate was found to be proteinase K sensitive and heat labile, strongly suggesting the involvement of a protein. Having ruled out any contribution of $O_2^{\bullet-}$ an array of candidate enzymes were tested including cytochrome *c* oxidase (Borutaite & Brown, 1996), lipoxygenases (Coffey *et al.*, 2001; O'Donnell *et al.*, 1999), peroxidases (Abu-Soud & Hazen, 2000), prostaglandin H synthase (O'Donnell *et al.*, 2000) and a flavohaemoglobin-like NO dioxygenase (Gardner *et al.*, 2001), all to no avail. This chapter continues to examine the mechanism by which NO is inactivated by acutely prepared cerebellar suspensions and whole brain homogenates.

6.2 METHODS

General methods

Unless otherwise stated, all experiments contained SOD, (1000 U/ml). Stock solutions were; ascorbate oxidase (AO, 1000 U/ml) prepared in dH_20, DTPA (10 mM) in equimolar NaOH, and Trolox (100 mM) in DMSO. The final DMSO concentration did not exceed 0.1 % in any experiment. Stock solutions of DETA/NO were made in 10 mM NaOH and kept on ice until use. Protein concentrations were measured by the bicinchoninic acid method. Measurements of NO and O_2 concentrations, used ISO-NOP and Clark type electrodes respectively (see chapter 2.2). Data are presented as means ± S.E.M., each determination (*n*) being an individually prepared and treated sample. Statistical differences were analysed using one-way ANOVA with Dunnett's *post hoc* test; *P* values of < 0.05 were regarded as significant

Cerebellar cell suspension

Cell suspensions (20 x 10^6 cells/ml; 1.25 mg protein/ml) were prepared according to published procedures (Garthwaite & Garthwaite, 1987) except that the pups were not pre-treated with hydroxyurea. The following solutions (Table 6.1) were based upon artificial cerebral spinal fluid (aCSF) which was composed in (mM): NaCl (120), KCl (2), $CaCl_2$ (2), $NaHCO_3$ (26), $MgSO_4$ (1.19) and glucose (11).

Solution	Composition
1	Ca^{2+} free aCSF
2	Solution (1) + 3 mg/ml BSA
3	Solution (2) + 0.5 mg/ml trypsin
4	Solution (2) + 0.8 mg/ml DNase, 0.52 mg/ml soybean trypsin inhibitor, 1.55 mM $MgSO_4$, 0.1 mM D-AP5
5	84 % solution (2): 16 % solution (4) (v/v)
6	Solution (2) + 1 mM $CaCl_2$, 1.24 mM $MgSO_4$
7 (Incubation buffer)	15 mM Tris-HCl, 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.2 mM Na_2HPO_4 and 11 mM glucose at pH 7.4
8	Solution (7) – Ca^{2+}
9	Solution (8) + 40 mg/ml BSA (filtered, 0.6 µm)

Table 6.1 Solutions for cerebellar granule cell preparation

Prior to use solutions (1)-(6) were gassed with 95% O_2, 5% CO_2 at room temperature. Cerebella from 8 day old Sprague-Dawley rats were removed and chopped into 0.4 mm blocks on a McIlwain tissue chopper. The blocks were triturated gently in 10 ml (2) at 10-15°C and, once they had settled, supernatant was removed and the blocks were incubated in 10 ml of (3) for 15 min at 37°C, shaking and under 95% O_2, 5% CO_2. 10 ml (5) was added and the suspension was centrifuged at 150 *g* for 5 secs. The resultant supernatant was discarded while the pellet was resuspended in 2 ml of (4) and triturated gently 20 times with a pasteur pipette. After the remaining blocks had settled the milky isolated cell supernatant was removed and added to 2 ml of (6). A further 2 ml of (4) was added to the remaining pellet

and the process repeated until no significant pellet remained (at least three times). The cell suspension was carefully underlayed with 1 ml of (9) and centrifuged at 150 *g* for 5 min. Supernatant and BSA were aspirated and the pellet resuspended in 5 ml (8), and spun at 150 *g* for 4 min. The resultant pellet was resuspended in 2 ml (7). Cell numbers were counted in a 1:1 suspension with trypan blue using a Fuchs Rosenthal counting chamber and the concentration adjusted to 20 x 10^6 cells/ml. The cell suspension was allowed to recover at 37°C, shaking for 1 h before use.

Preparing semi-purified homogenate

Homogenate of whole rat brain tissue (8 day-old Sprague-Dawley rats, ~20 mg protein/ml) was semi-purified at 4°C. Initial centrifugation was 10,000 *g* for 30 min, and then the supernatant was further spun at 100,000 *g* for 1 hr. The resultant pellet was re-suspended in Tris buffer (20 mM) at 10 mg protein/ml while the supernatant was spun overnight at 2000 *g* through 10,000 kD cut-off filters (CENTRIPLUS®, Millipore UK Ltd, Watford, England). Homogenate (0.3 or 1 mg protein/ml), supernatant (10-20 % final concentration) and pellet (0.1 mg protein/ml) components were tested for NO consumption with an ISO-NOP probe, alone or in combination (pellet + supernatant), by addition of DETA/NO (100 µM).

Haemoglobin bead assay

Haemoglobin beads (12-16 mg/ml) were triple washed in Tris buffer (20 mM) before reduction by exposure to freshly prepared sodium dithionate (10 mM) for 20 min in air. Following 2 washes in Tris, the beads were kept as a working stock at 1.2 mg/ml on ice until used. Pellet (0.1 mg/ml), supernatant (10 %) and SOD (1000 U/ml) were incubated with Tris buffer and haemoglobin beads (100 µl), in a final volume of 1 ml on a rotator at 37°C for up to 25 min. Test compounds EGTA (100 µM) and $CaCl_2$ (60-110 µM) were added where appropriate. After incubation the bead mix was pelleted by centrifugation at 10,000 *g* for 5 min and resuspended in 300 µl Tris. The degree of bead oxidation was determined by reading the absorbance ratio in nm (401-410/410).

6.3 RESULTS

NO consumption in the presence of brain tissue

Work in our lab has previously reported that the breakdown of NO is accelerated in the presence of brain tissue (Griffiths & Garthwaite, 2001; Griffiths *et al.*, 2002b). To elucidate the mechanism(s) underlying this activity at a physiological rate of NO production the NO donor DETA/NO was used. The long half-life of DETA/NO (20 h at 37°C) means that at any concentration used, the rate of NO release will effectively be constant for several hours. Following DETA/NO (100 μM) addition to Tris buffer, the NO concentration rose to a steady value of ~ 450 nM after 10 min (Fig. 6.1A). In agreement with previous studies (Griffiths & Garthwaite, 2001; Griffiths *et al.*, 2002b), following the application of DETA/NO to either cells or homogenate, a different profile was observed. Within seconds, a steady low plateau or 'clamp' was formed (~24 ± 4 and 8 ± 1 nM NO in cells and homogenate respectively). Once this mechanism became saturated a secondary rise in the NO concentration was observed (Fig. 6.1A). The clamp duration was quantified as the time taken for the NO concentration to reach 100 nM, which was 0.3 ± 0.2 min in Tris buffer, compared to 49 ± 0.4 and 13 ± 0.4 min in homogenate and cells respectively (Fig. 6.1B).

The preservation of accelerated NO inactivation in whole brain homogenate facilitated further investigation of the underlying mechanism. Previous studies had indicated the requirement of a protein, since incubation of homogenate for 1 hr with proteinase K (pK) immobilised on acrylic beads resulted in a complete loss of homogenate-accelerated NO inactivation (Griffiths *et al.*, 2002b). In order to isolate the protein species responsible classic purification methodology was undertaken (Dr C. Griffiths, unpublished data). Initially this comprised of centrifugation and filtration steps to leave crude pellet and supernatant fractions. When DETA/NO (100 μM) was applied to the pellet fraction the NO concentration was indistinguishable from that seen in buffer, rising to a steady plateau at ~ 450 nM NO. DETA/NO application to supernatant caused NO to rise to ~ 350 nM, indicating a slight retention of NO consuming activity in this fraction. In contrast, when

DETA/NO was added to recombined pellet and supernatant fractions a steady low NO plateau (6 ± 2 nM) was attained within seconds and, similarly to cells or homogenate, a secondary rise in NO concentration occurred upon this clamp exhausting (Fig. 6.1C). Clamp duration was quantified as the time taken for the NO concentration to reach 100 nM, which was 0.9 ± 0.3 min in Tris buffer compared to 0.6 ± 0.2 min in pellet, 1.6 ± 0.4 min in supernatant and 16.4 ± 2 min upon combination of pellet and supernatant (Fig. 6.1D).

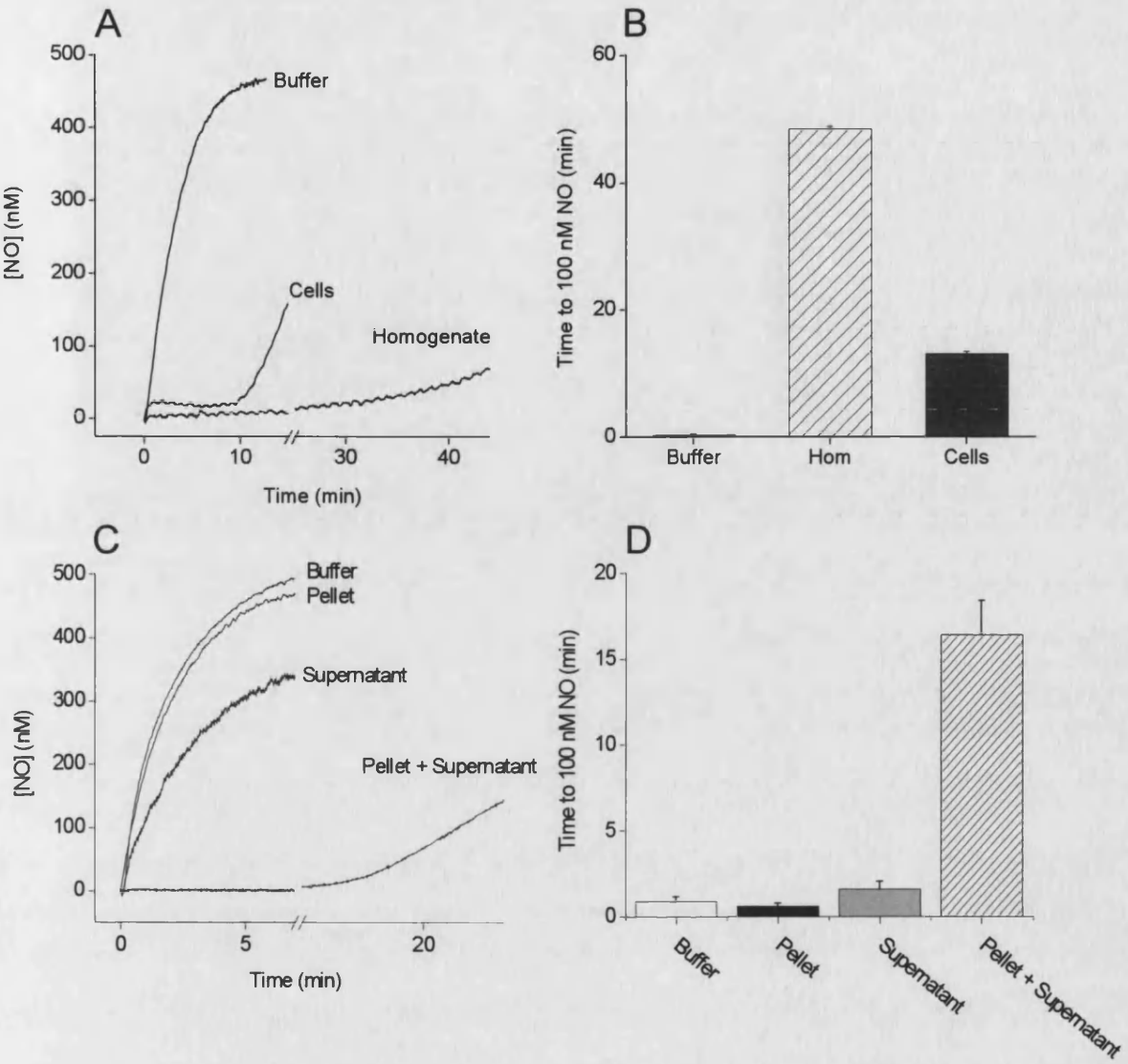


Figure 6.1 Inactivation of NO by brain tissue

(A) Representative traces following DETA/NO (100 μM) addition to either Tris buffer (20 mM), cerebellar cells (20 x 10^6 cells/ml; 1.25 mg protein/ml), or whole brain homogenate (0.3 mg protein/ml) in the presence of 1000 U/ml SOD. **(B)** The NO clamp duration was quantified as the time taken for the NO concentration to achieve 100 nM. The data are means ± S.E.M. (*n* = 3-4). **(C)** Representative traces of NO accumulation following DETA/NO addition to either Tris buffer, pellet (0.1 mg protein/ml), supernatant (10 %), or recombined pellet + supernatant. **(D)** Clamp duration (time to 100 nM NO) is summarised as mean ± S.E.M. *n* = 3-4.

Recombined fractions consume NO in an EGTA / Ca^{2+} sensitive manner

To determine whether NO consumption was dependent upon Ca^{2+} ions, the chelator EGTA (100 μM) had previously been tested on homogenate or recombined pellet + supernatant (Dr C. Griffiths, unpublished data). While having no effect upon NO accumulation in buffer (not shown), the addition of EGTA (arrow, Fig 6.2) clearly inhibited the NO 'clamp' by both homogenate (not shown) and recombined pellet + supernatant such that NO immediately rose with a similar profile to that seen in buffer. Subsequent addition of $CaCl_2$ (1 mM, arrow, Fig 6.2) restored the NO clamp within seconds.

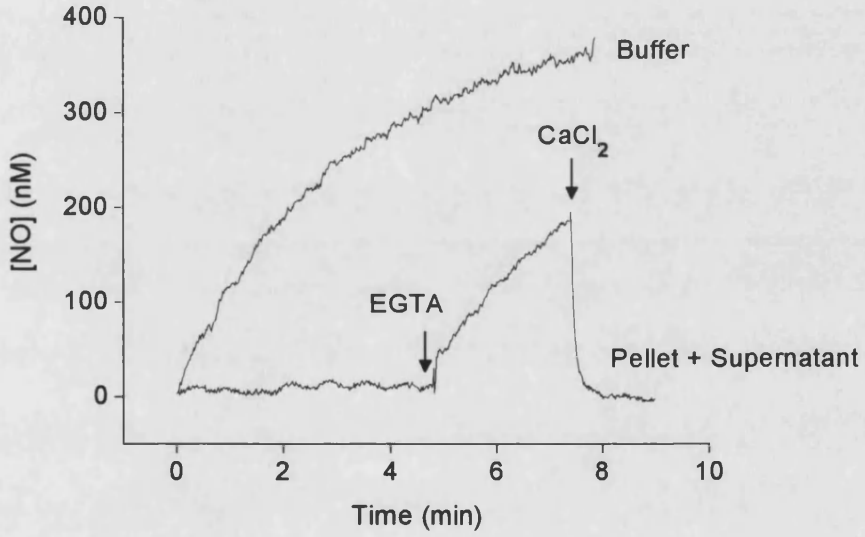


Figure 6.2 NO consumption by pellet + supernatant is EGTA / Ca^{2+} sensitive

Representative traces of NO accumulation upon the addition of DETA/NO (100 μM) to buffer, or pellet + supernatant. EGTA (100 μM) and $CaCl_2$ (1 mM) were added as indicated (arrows) but had no effect on buffer traces (not shown).

Using a haemoglobin bead assay to detect NO consumption

Although the NO electrode is a convenient and reliable means of detecting NO, it is time consuming and, therefore, not useful for high throughput experiments. To develop a means of quickly examining the effects of various compounds on NO consumption by pellet + supernatant we looked to other available methods. The use of haemoglobin to detect NO has been widely documented (Feelisch *et al.*, 1996). NO oxidises haemoglobin in the following reaction;

$$HbO_2 + NO \rightarrow metHb + NO_3^-$$

By measuring the absorbance spectrum, the NO mediated conversion of HbO_2 to metHb may be followed. For the conversion of HbO_2 to metHb the highest difference in absorbance is at 401 nm, with an isosbestic point (where no absorbance change is detected) seen at 410 nm. Calculation of the absorbance ratio (401-410 / 410) of each sample uses the isosbestic point as an internal reference for changes in volume.

As described above, the speed of NO consumption by reaction with HbO_2 is likely to be such that the haemoglobin will preferentially react with NO, discounting other, slower, NO sinks from being detected. We have utilised haemoglobin immobilized on cross-linked 4% beaded agarose to detect NO. Similarly to the slowed reaction of NO with HbO_2 in red blood cells, haemoglobin prepared in this way reacts with NO at a sufficiently slowed rate that other sinks may consume NO in competition.

Incubating haemoglobin beads with 100 µM DETA/NO for 25 min, caused the absorbance ratio to increase steadily from -0.12 to 0.13 as they became oxidised (Fig 6.3). In the presence of pellet (0.1 mg/ml) and supernatant (10%) this ratio increase was considerably lower (from -0.12 to -0.05) 25 min following DETA/NO application, indicating that NO was being consumed and prevented from reacting with the beads. In agreement with experiments on the NO probe (Fig 6.2), the addition of EGTA inhibited the NO 'clamp' by pellet + supernatant such that absorbance ratio increases were of similar magnitude to those in buffer. Likewise addition of 100 µM

EGTA inhibited the NO 'clamp' even when applied 10 min after DETA/NO (arrow Fig 6.3).

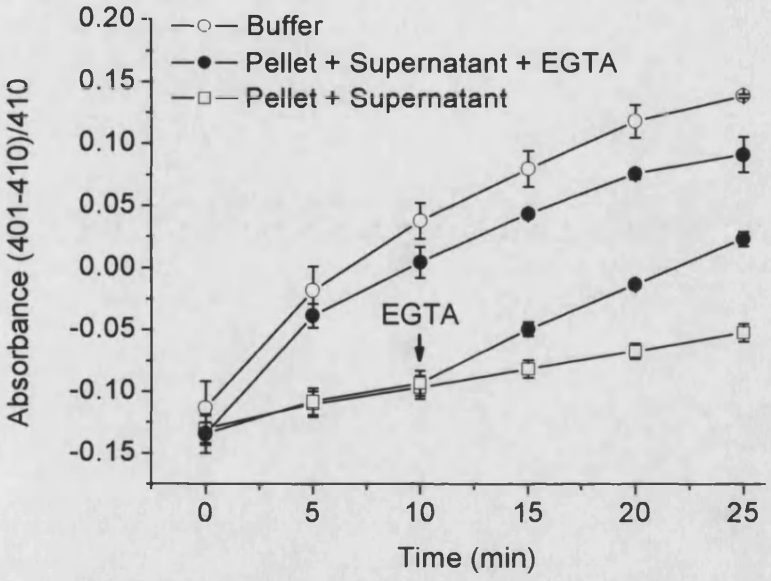


Figure 6.3 NO consumption measured using haemoglobin coated beads

DETA/NO (100 μM) was incubated with haemoglobin coated beads in Tris buffer (O), pellet + supernatant (□) or pellet + supernatant + EGTA (100 μM) (•). Where indicated (arrow) the EGTA addition was made 10 min following DETA/NO addition. Bead oxidation was determined by measuring absorbance ratios. The data are means ± S.E.M. (n = 6)

To determine the concentration of free calcium required for supernatant + pellet to resume NO consumption in the presence of EGTA, increasing concentrations of $CaCl_2$ (60-110 μM) were incubated with pellet (0.1 mg/ml) + supernatant (10%) in the presence of 100 μM EGTA, haemoglobin beads and 100 μM DETA/NO. Absorbance ratios were determined after 25 min. In the absence of EGTA the ratio was -0.1, but in its presence it was almost 0.1 (Fig 6.4), indicating that NO consumption had been inhibited by EGTA. As the concentration of $CaCl_2$ was increased this inhibition was lessened, such that by 100 μM $CaCl_2$, EGTA was no longer an effective inhibitor (Fig 6.4).

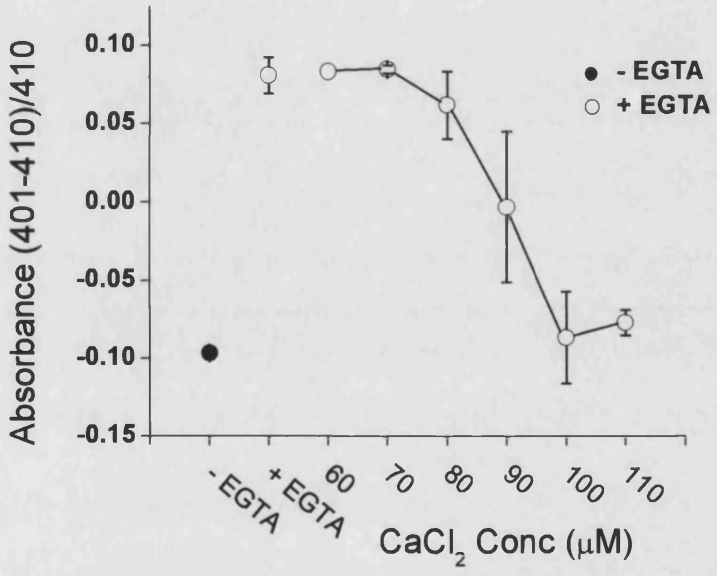


Figure 6.4 Determining Ca^{2+} requirement for NO consumption by pellet + supernatant
DETA/NO (100 μM) was incubated with haemoglobin coated beads and pellet + supernatant in the absence (•) or presence (O) of EGTA (100 μM) and increasing concentrations of $CaCl_2$ (60 – 110 μM). Bead oxidation was determined by measuring absorbance ratios after 25 min. The data are means ± S.E.M. (n = 6)

Supernatant consumes NO in a $O_2^{\bullet-}$ -dependent manner

Previous experiments investigating the NO consumption mechanism in brain tissue have contained SOD (1000 U/ml) to prevent the diffusion limited reaction of NO with $O_2^{\bullet-}$. The NO probe was used to further study the supernatant component of NO consumption in the absence or presence of SOD. Following addition of 100 µM DETA/NO to supernatant in the presence of SOD, NO levels rose, reaching a steady-state of approximately 300 nM (Fig 6.5A). In the absence of SOD, the NO released from DETA/NO was held less than 50 nM, about 20 % of the response in buffer (Fig 6.5A,B), suggesting a rapid production of $O_2^{\bullet-}$ was occurring in supernatant. As discussed in chapter 3, a possible source of $O_2^{\bullet-}$ is via metal-catalysed reactions. In the absence of SOD, but in the presence of the metal chelator DTPA (100 µM), the NO steady-state in supernatant following DETA/NO addition was similar to that seen with SOD present (300 nM). Notably, compared to buffer, some NO consuming activity was retained in SOD or DTPA treated supernatant (about 20 %, Fig 6.5A,B). DTPA had no effect upon NO levels in buffer alone (not shown).

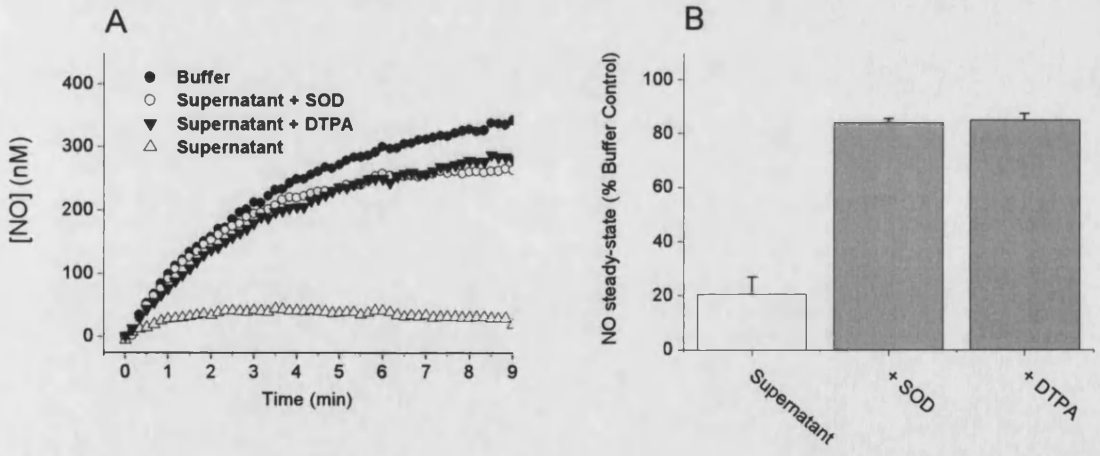


Figure 6.5 NO consumption by supernatant is $O_2^{\bullet-}$ / metal-dependent

(A) Representative traces of NO accumulation upon the addition of DETA/NO (100 µM) to buffer, supernatant alone and supernatant with SOD (1000 U/ml) or DTPA (100 µM). **(B)** Data is summarised as a percentage of the NO steady-state achieved in buffer. The data are means ± S.E.M. (n = 3)

NO consumption by supernatant is EGTA / Ca^{2+}-sensitive

In addition to chelating Ca^{2+}, EGTA can bind metal ions including Fe^{2+} and Cu^{+}. To test the possibility that EGTA could inhibit NO consumption in supernatant, 100 μM DETA/NO was added to supernatant in the absence of SOD but the presence of EGTA (100 μM). The resultant NO steady-state was similar to that seen in buffer, about 300 nM, (Fig 6.6A). Upon addition of 1 mM $CaCl_2$ to supernatant + EGTA (arrow, Fig 6.6A) the NO level immediately declined to rest at levels 20 % of those in buffer controls (Fig 6.6B). In comparison, when DETA/NO was added to supernatant in the presence of DTPA, supplementary $CaCl_2$ had no effect on NO levels, which remained ~85 % of those in buffer (Fig 6.6B, and see Fig 6.5).

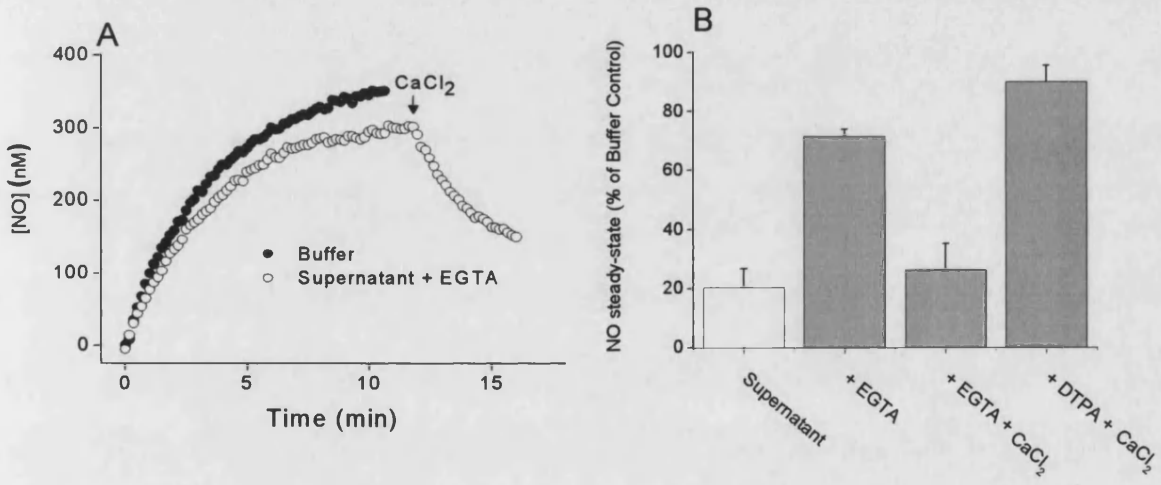


Figure 6.6 NO consumption by supernatant is EGTA / Ca^{2+} sensitive

(A) Representative traces of NO accumulation upon the addition of DETA/NO (100 μM) to buffer, or supernatant and EGTA (100 μM) in the absence of SOD. $CaCl_2$ (1 mM) was added as shown (arrow). **(B)** Data is summarised as a percentage of the NO steady-state achieved in buffer. The data are means ± S.E.M. (n = 3)

Antioxidant treatment prevents NO consumption by recombined brain fractions

In the absence of antioxidant protection, transition metal-catalysed reactions may readily peroxidise lipids, generating $LOO^{\bullet}$. In this scenario NO may serve as an antioxidant, avidly binding $LOO^{\bullet}$, and consequently be consumed (O'Donnell *et al.*, 1997). The effects of metal chelation, or antioxidant addition, on pellet and supernatant NO consumption was tested. Pre-incubation with the transition metal chelator DTPA (100 μM), or the synthetic vitamin E analogue Trolox (100 μM), almost completely abolished pellet + supernatant-dependent NO inactivation. Following either treatment, DETA/NO addition resulted in a steady NO concentration of ~200 nM (Fig. 6.7A), only slightly lower than that attained in supernatant alone ~250 nM. The addition of DTPA or Trolox to Tris buffer, or the supernatant or pellet fractions alone, had no effect (data not shown). When quantified as the time taken for the NO concentration to reach 100 nM only the untreated recombined pellet and supernatant combination (23.5 ± 0.9 min) was significantly different from buffer (0.8 ± 0.3 min; Fig. 6.7B).

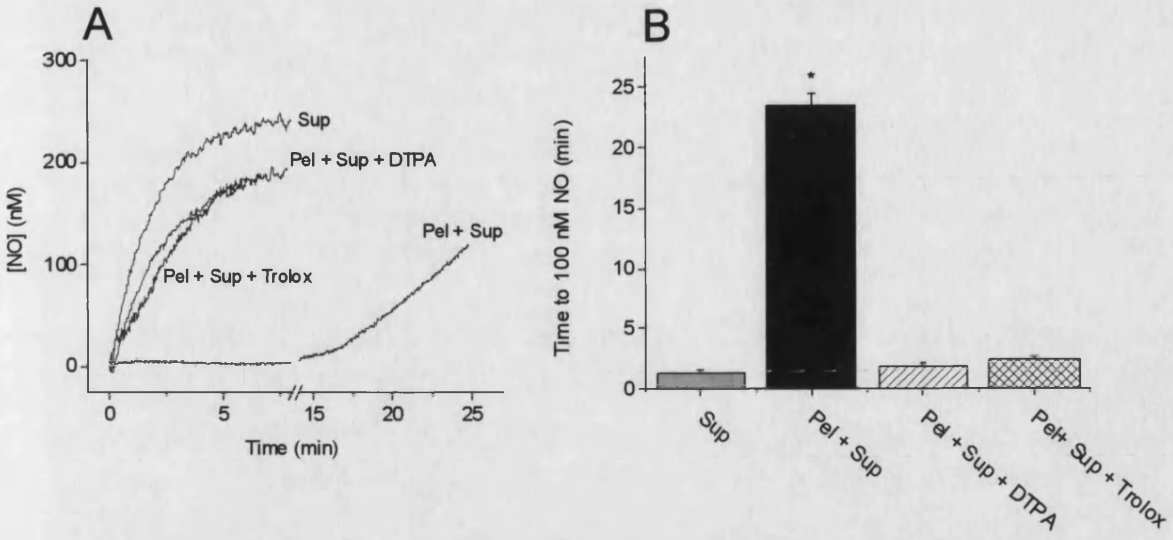


Figure 6.7 Antioxidant treatment or metal chelation inhibits NO consumption in recombined fractions

(A) 100 μM DETA/NO was added to supernatant (Sup, 20 %) or supernatant recombined with pellet (Pel, 0.1 mg protein/ml). The recombined samples were examined alone, or pre-incubated with DTPA (100 μM) or Trolox (100 μM). **(B)** Clamp duration (time to 100 nM NO) is summarised as mean ± S.E.M. n = 3-5, * $p < 0.05$ *vs* supernatant control. Neither compound affected Tris buffer, supernatant or pellet traces alone, while the vehicle for Trolox (0.1 % DMSO) affected none of these conditions (data not shown; $p < 0.05$).

Supernatant contains ascorbate required for NO consumption

The formation of lipid peroxides by transition metal catalysed redox reactions is often enhanced by addition of ascorbate. At low concentrations ascorbate is widely used as a pro-oxidant because it is an excellent reducing agent and as such may serve to redox cycle catalytic metals, for example reducing Fe^{3+} to Fe^{2+} (Buettner & Jurkiewicz, 1996). To further elucidate how lipid peroxidation may be contributing to NO consumption, the ascorbate content of supernatant was determined. The enzyme, ascorbate oxidase (AO) converts ascorbate and O_2 to water and dehydroascorbate. Thus by measuring $[O_2]$, ascorbate depletion can be monitored. AO (4 U/ml) was added to supernatant (10 % in Tris buffer) as indicated (Fig. 6.8A) and caused an immediate decline in $[O_2]$ (11.3 ± 0.8 μM) that was complete within 2 min. Further addition of AO had no additional effect on the $[O_2]$, and no decline was observed following addition of AO to Tris buffer (data not shown). To quantify the ascorbate concentration in supernatant, $[O_2]$ was

measured during addition of ascorbate standards to buffer containing AO (4 U/ml; Fig. 6.8B). The [O_2] decline was proportional to the ascorbate concentration (Fig. 6.8B, *inset*) and indicated that at 10 % supernatant, the ascorbate concentration was 25-30 μM.

The functional impact of ascorbate depletion on NO consumption was then tested. Following pre-incubation with AO for 3-5 min, recombined pellet and supernatant failed to clamp NO (Fig. 6.8C), and the time taken for NO to rise to 100 nM (1.7 ± 0.5 min) was not significantly different from Tris buffer controls (0.8 ± 0.1 min; Fig. 6.8D). AO addition had no effect upon NO concentration in Tris buffer controls (Fig. 6.8C and D). Finally the addition of ascorbate to the pellet fraction was assessed to see if it could substitute for supernatant. Increasing concentrations of ascorbate (1-30 μM) were incubated with pellet (0.1 mg protein/ml) and DETA/NO (100 μM) was added. As the ascorbate concentration was increased, the duration of the NO clamp increased, and was maximal at 10 μM ascorbate (Fig. 6.8E). The recorded NO profile was similar to that seen upon recombination of pellet and supernatant (low steady NO clamp followed by a secondary rise). As judged by the time taken to reach 100 nM NO (23.6 ± 2.8 min) the clamp maximum (10 μM ascorbate, Fig. 6.8F) is in close agreement with the above estimate of ascorbate concentration in supernatant.

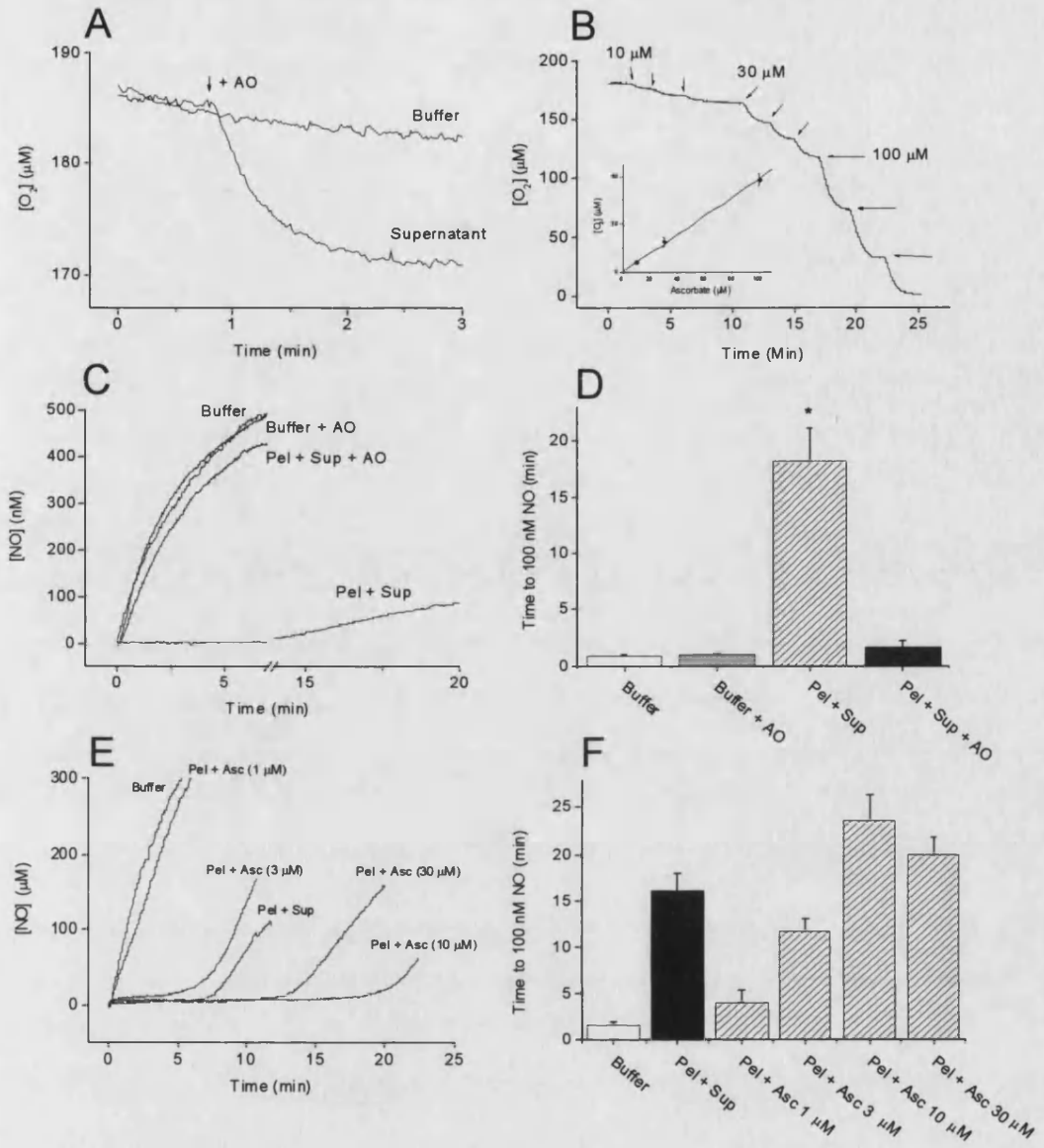


Figure 6.8 Supernatant contains ascorbate required for NO consumption

(A) Ascorbate depletion was monitored by measuring O_2 consumption upon addition of ascorbate oxidase (AO, 4 U/ml; as indicated by an arrow) to supernatant (10 %). Each trace is representative of at least three experiments. Further additions had no effect on the $[O_2]$ (not shown). **(B)** Ascorbate (10 – 100 µM; as arrows) was added to Tris buffer containing AO (4 U/ml) and $[O_2]$ monitored. The resulting linear standard curve (*inset*) allows estimation of the ascorbate concentration in pellet + supernatant. **(C)** Representative traces of NO accumulation upon addition of DETA/NO (100 µM) to Tris buffer, and pellet (0.1 mg protein/ml) + supernatant (10 %) in the presence or absence of AO (4 U/ml). **(D)** Clamp duration (time to 100 nM NO) is summarised as mean ± S.E.M., n = 3-4, * $p < 0.05$ *vs* buffer control. **(E)** Representative traces of NO accumulation upon addition of DETA/NO (100 µM) to Tris buffer, pellet (0.1 mg protein/ml) + supernatant (10 %), or pellet in the presence of ascorbate (1-30 µM). **(F)** Clamp duration (time to 100 nM NO) is summarised as mean ± S.E.M., n = 4-6.

Lipid peroxidation accounts for NO consumption in homogenate

To test the contribution of lipid peroxidation to NO consumption homogenate (0.3 mg protein/ml) was pre-incubated (3-5 min) with DTPA (100 μM), Trolox (100 μM) or AO (4 U/ml). Upon the addition of DETA/NO (100 μM) to homogenate, NO consumption proceeded as before (Fig. 6.1). The addition of AO inhibited NO consumption by homogenate with the resultant NO profile reaching a plateau of ~ 300 nM, close to that seen in buffer (Fig. 6.9A). Similar profiles (akin to buffer) were seen in the presence of both Trolox and DTPA (not shown). Measured as the time taken to reach 100 nM NO, addition of any of the three compounds inhibited the NO clamp such that none was significantly different to buffer (0.3 ± 0.2 min, Fig 6.9B). Finally, using the same method as above the ascorbate content in 3 mg protein/ml homogenate was found to be 54.2 ± 1.4 μM.

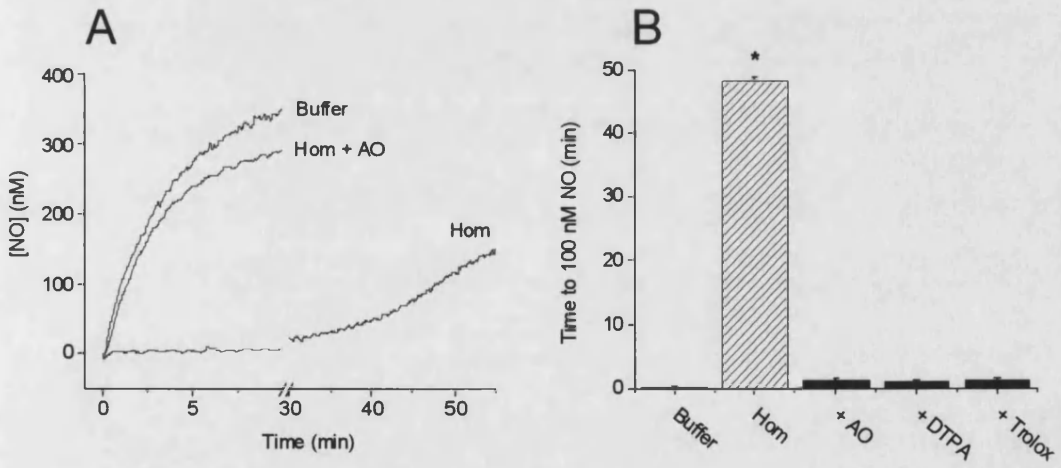


Figure 6.9 Lipid peroxidation accounts for NO consumption in homogenate

(A) Representative traces of NO accumulation upon addition of DETA/NO (100 μM) to Tris buffer, homogenate (0.3 mg protein/ml) or homogenate + AO (4 U/ml). Similar traces were recorded upon addition of DTPA (100 μM) or Trolox (100 μM), while no compounds affected Tris buffer traces (not shown). **(B)** Clamp duration (time to 100 nM NO) is summarised as mean ± S.E.M. $n = 3$, * $p < 0.05$ *vs* buffer control.

Lipid peroxidation in cerebellar cells

The efficacy of the various treatments that inhibited the homogenate NO clamp was then evaluated in the cerebellar cell suspension. In the presence of DTPA (100 μM) the 'clamp' height increased from 24 ± 4 to 44 ± 4 nM NO and the duration shortened from 13.1 ± 0.4 to 6.4 ± 0.2 min (Fig. 6.10A). Similar significant reductions in clamp duration were observed when the cells were pre-incubated (2-3 min) with either AO (4 U/ml) or Trolox (100 μM) (Fig. 6.10B) indicating that the mechanism of NO inactivation by intact cells was at least partially attributable to lipid peroxidation.

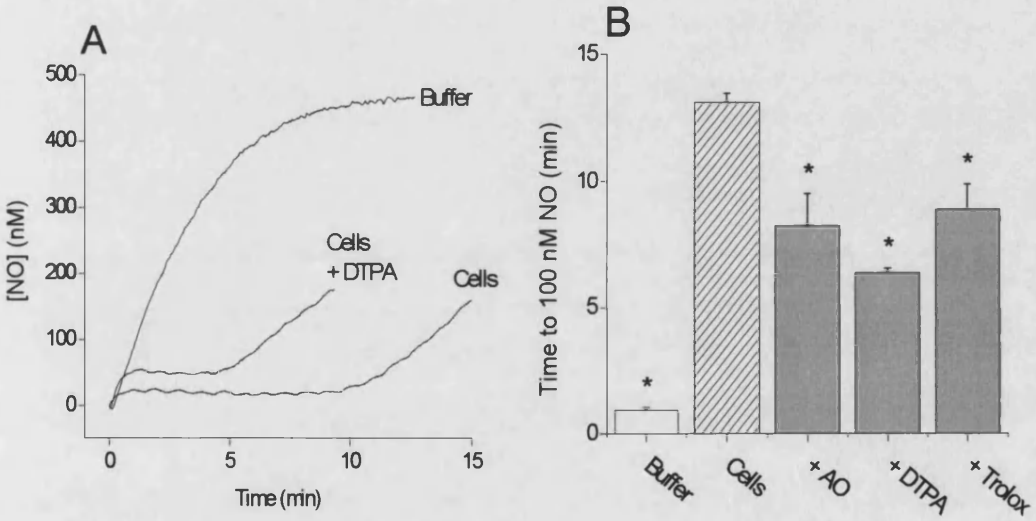


Figure 6.10 Lipid peroxidation partially accounts for cellular NO consumption

(A) Representative traces of NO accumulation upon addition of DETA/NO (100 μM) to Tris buffer, cells (1.25 mg protein/ml) or cells incubated with DTPA (100 μM). The NO profiles obtained in the cell suspension following pre-incubation with either Trolox (100 μM) or AO (4 U/ml) were indistinguishable from cells + DTPA (data not shown). **(B)** Clamp duration (time to 100 nM NO) is summarised as mean ± S.E.M., n = 3-4, * $p < 0.05$ versus cells. Controls (no cells or cells + vehicle) were not significantly different to Tris buffer or cells respectively ($p < 0.05$; not shown).

6.4 DISCUSSION

The initial observations described in this chapter, that NO consumption persists upon recombination of semi-purified pellet and supernatant fractions of brain, and that consumption was Ca^{2+}/EGTA sensitive (Dr C. Griffiths, unpublished observations), led to further investigation of the individual components. To aid a high-throughput study of compounds affecting NO consumption, a modified haemoglobin assay was developed. Utilising the slowed rate of reaction of NO with haemoglobin beads (compared to free haemoglobin), this assay enabled clear detection of brain tissue NO consumption over time by simple absorbance measurement of the bead oxidation state. Using this method up to 240 individual experiments were possible per day, compared with less than 30 using an NO electrode.

Supernatant NO consumption

In the absence of SOD, supernatant consumed NO. Since a potential source of $O_2^{\bullet-}$ is metal autoxidation, the metal chelator DTPA was tested. DTPA was found to almost fully inhibit the SOD-sensitive NO consumption. The observation that supernatant NO consumption was also EGTA sensitive, but could be reversed by Ca^{2+} (unlike the effect of DTPA), may be explained by comparing the relative affinities of DTPA or EGTA for Ca^{2+} and Fe^{2+} (Table 6.1).

	EGTA	DTPA
Ca^{2+}	Kd = 4.5 E-08 M	Kd = 3.7 E-08 M
Fe^{2+}	Kd = 4.3 E-09 M	Kd = 1.5 E-13 M

Table 6.2 EGTA and DTPA equilibrium constants for Ca^{2+} and Fe^{2+} at 37°C pH 7.4. Source, MaxChelator v2.1 (http://www.stanford.edu/~cpatton/maxc.html)

In vitro trace catalytic metals are ubiquitous in salt and buffer solutions, with iron often reaching up to 10 μM and copper reaching 0.1 μM, or they may be introduced into solution, for example from Hamilton syringes (Buettner & Jurkiewicz, 1996). Using software designed to examine the relative affinities of chelators (MaxChelator v2.1; downloadable at http://www.stanford.edu/~cpatton/maxc.html) it was determined that in the

presence of 100 μM EGTA, a contaminating concentration of 10 μM Fe^{2+} is chelated such that only 0.4 nM Fe^{2+} remains free. In the presence of 1 mM Ca^{2+}, however, free iron levels are 5 μM. In comparison, DTPA chelates 10 μM Fe^{2+} leaving only 0.02 pM free, the subsequent addition of 1 mM Ca^{2+} again increases free Fe^{2+}, but only to 0.4 nM. Clearly the EGTA/Ca^{2+} sensitivity of NO consumption by supernatant may be explained by the inability of EGTA to adequately chelate metal ions in the presence of Ca^{2+}.

EGTA/Ca^{2+} sensitivity of recombined supernatant and pellet

Similarly to the results seen in supernatant alone, both DTPA, and EGTA inhibited NO consumption by recombined supernatant and pellet. Using the haemoglobin bead assay, the exogenously applied $CaCl_2$ concentration required to maximally reverse this (100 μM) EGTA inhibition was 100 μM $CaCl_2$. Under these conditions, again assuming Fe^{2+} contamination to be 10 μM, it may be calculated that 770 nM free Fe^{2+} is available for radical reactions (taking into account that free Ca^{2+} present in tris buffer + 10 % supernatant was measured at ~70 μM, Dr C.Griffiths, personal communication). Moreover, Fe^{2+} loosely bound to membranes in the pellet fraction may make a further contribution. Similarly to the results seen in supernatant alone, the EGTA/Ca^{2+} sensitivity of NO consumption by recombined pellet and supernatant may be explained by the inability of EGTA to adequately chelate metal ions in the presence of Ca^{2+}.

NO consumption by lipid peroxidation

Since supernatant and recombined pellet and supernatant were found to consume NO in a manner dependent upon free metal ions, the nature of the NO consumption mechanism was reconsidered. Lipid peroxidation is initiated by radical species such as the hydroxyl radical ($OH^{\bullet}$), which can abstract a hydrogen atom from unsaturated lipid, thus generating a lipid radical ($L^{\bullet}$) and H_2O. The lipid radical can combine with O_2, generating the lipid 'peroxyl' radical ($LOO^{\bullet}$), which can further react with unsaturated lipid. If allowed to progress unchecked, a damaging, self-propagating cascade of peroxidation results. Ultimately peroxidation alters membrane properties, including ion-

channel activity and glucose transport, and can directly impair mitochondrial function to cause cell stress (Mattson, 1998). NO may either trigger peroxidation, following reaction with $O_2^{\bullet-}$ and formation of $ONOO^-$ (Hogg & Kalyanaraman, 1999), or may inhibit it, as has been shown in several *in vitro* systems including free fatty acids (Hiramoto *et al.*, 2003), lipoproteins (Goss *et al.*, 1997), and a cell line (Keiley *et al.*, 1999). This inhibition may occur as NO interacts with $LOO^\bullet$ at a near-diffusion limited rate, 1-3 x 10^9 M^{-1} s^{-1}; (Padmaja & Huie, 1993), thereby preventing further propagation of the signal and consuming 2 molecules of NO per molecule of $LOO^\bullet$ (O'Donnell *et al.*, 1997). Alternatively NO might inhibit peroxidation more directly by preventing $OH^\bullet$ production (Sharpe *et al.*, 2003).

The $OH^\bullet$ usually thought to initiate lipid peroxidation can be formed by either the Haber-Weiss reaction (1), though this is probably too slow at neutral pH (Koppenol, 2001b), or more rapidly by the transition metal-catalysed Fenton reaction (2):

(1) $O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^- + OH^\bullet$

(2) $Fe^{2+}/Cu^+ + H_2O_2 \rightarrow Fe^{3+}/Cu^{2+} + OH^- + OH^\bullet$

Brain tissue has high iron levels, and though mainly bound to proteins such as ferritin, $O_2^{\bullet-}$ or ascorbate may aid mobilisation of this iron to participate in $OH^\bullet$ formation (Halliwell & Gutteridge, 1986). As noted above, *in vitro* trace catalytic metals are ubiquitous in salt and buffer solutions. Addition of the transition metal chelator DTPA, or the chain breaking antioxidant Trolox to recombined pellet and supernatant fractions, or homogenate, abolished the low (nM) NO clamp in our experiments, indicating that NO consumption was indeed caused by metal catalysed lipid peroxidation reactions.

The contribution of ascorbate

Fenton / Haber-Weiss chemistry is substantially enhanced by redox cycling of the catalytic transition metal (reduction of Fe^{3+} to Fe^{2+} or Cu^{2+} to Cu^+) particularly by ascorbate (Buettner & Jurkiewicz, 1996). Brain ascorbate is an

important antioxidant, existing at an average concentration of several millimolar *in vivo* (Rice, 2000). However, lower (μM) concentrations are pro-oxidant, and this "crossover" effect is dependent upon the concentration of catalytic metals present (Buettner & Jurkiewicz, 1996). Ascorbate concentrations were measured at ~ 30 μM in supernatant and ~ 50 μM in homogenate. Depletion of this ascorbate prevented NO consumption by recombined supernatant and pellet, or homogenate preparations. Alternatively, ascorbate substituted for supernatant in accelerating NO consumption when combined with pellet. These results further imply that lipid peroxidation was responsible for NO consumption.

Notably, ascorbate is readily oxidised, and simply increasing the temperature will accelerate its degradation (Meucci *et al.*, 1985). In other experiments (Dr C.Griffiths, data not shown) it was found that following a 2 h incubation at 37°C a loss of NO consuming activity in homogenate was attributed to ascorbate depletion. This process was further accelerated in the presence of proteinase K, a misleading result that previously led to the conclusion that NO inactivation involves a novel protein (Griffiths *et al.*, 2002b). In addition, in the presence of ascorbate, a lipid mixture mimicking the fatty acid content of rat brain homogenate consumed NO identically to rat brain homogenate, and clearly underwent peroxidation as determined by the accumulation of TBARS (the major reaction product of lipid peroxidation). Moreover further purification of the pellet fraction by ion exchange and size exclusion chromatography successfully removed the majority of proteins, while NO consuming activity was always retained upon recombination with the supernatant fraction. Subsequent analysis of the purified pellet fraction by MALDI-TOF, however, revealed only the presence of a strong lipid profile (Dr C.Griffiths, data not shown). This data, combined with that presented in this chapter, provides evidence that no protein is required for the low NO clamp.

NO consumption by dispersed cells

In keeping with the results in recombined pellet and supernatant, or homogenate, NO consumption was significantly attenuated by the application of DTPA, AO or Trolox to intact cerebellar cells, thereby implicating lipid peroxidation as a consumption mechanism. Further experiments found a

steady accumulation of TBARS in cells over time. This could be inhibited by Trolox, or 100 μM DETA/NO (Dr C.Griffiths, data not shown), providing further evidence that cells are undergoing continuous lipid peroxidation *in vitro.* DTPA and AO are cell impermeable, though Trolox will access the cell membrane. Since all three compounds were similarly effective, lipid peroxidation is most likely being initiated by the metal catalysed Fenton reaction occurring extracellularly, and is enhanced by ascorbate leaking from the cells, as it can *in vitro* (Rice, 2000). To exclude the possibility that exogenous Cu,Zn-SOD was catalysing $OH^{\bullet}$ generation and subsequent peroxidation (Yim *et al.*, 1990) cellular TBARS accumulation was examined in the absence of SOD (Dr C.Griffiths, data not shown). A resultant increase in TBARS (~ 30%) demonstrated clearly that added SOD is not responsible for initiating peroxidation. Indeed allowing $O_2^{\bullet-}$ to accumulate enhances peroxidation, possibly through formation of $ONOO^-$ (Hogg & Kalyanaraman, 1999).

In previous granule cell preparations, contaminating red blood cells (RBCs) comprised 0.85 % of the total cell number, and did not account for any significant proportion of the NO consumption seen upon addition of 250 μM DETA/NO (Griffiths & Garthwaite, 2001). In the current experiments a higher level of RBC contamination (1.6 %) was found (C.Hall, personal communication) which, coupled with a lower concentration of NO donor (100 μM DETA/NO), meant the resultant NO clamp by RBCs was enhanced. This RBC clamp fully accounted for the residual NO consumption that occurred when lipid peroxidation was inhibited (see figure 6.11).

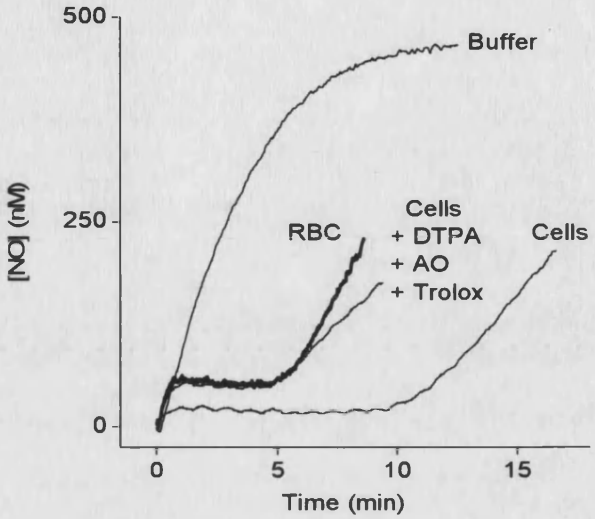


Figure 6.11 The contribution of contaminant red blood cells in the cellular NO clamp
In the presence of 1 % RBC NO reached 100 nM. Contaminant RBCs could fully account for the residual clamp activity in the cerebellar cell suspension following inhibition of lipid peroxidation (data from C.Hall).

Previously, it was reported that NO consumption was O_2-dependent in homogenate and cells (Griffiths *et al.*, 2002b), a finding in keeping with the current result, that (O_2 requiring) lipid peroxidation is involved. It was also reported that NO was ultimately degraded to form NO_3^- in homogenate. Following reaction of NO with $LOO^\bullet$ the resulting (transient) LOONO has been suggested to have two fates, rearrangement to a more stable $LONO_2$, or cleavage to give $LO^\bullet$ and $^\bullet NO_2$ (O'Donnell *et al.*, 1997). In the absence of any other targets $^\bullet NO_2$ might further react with NO to generate N_2O_3, which ultimately will hydrolyse to NO_2^-. This does not exclude the generation of NO_3^- from lipid peroxidation reactions, since NO_2^- may be further converted to NO_3^- by oxyhaemoproteins (Ignarro *et al.*, 1993).

Another important characteristic of the NO consumption described previously is its saturability. Taking homogenate as an example, and assuming that NO is consumed primarily by reaction with $LO^\bullet$ and $LOO^\bullet$, the

time taken for the clamp to saturate will be governed by both the sink of (already available) $LO^{\bullet}$ and $LOO^{\bullet}$, and their rate of formation. These parameters are, in turn, determined by the starting concentrations of metals and ascorbate. Once the sink is full, and the rate of NO production exceeds that of lipid peroxide formation, the clamp exhausts and NO levels rise. In cells the same process will occur, with the added contribution of RBCs, which, once their oxyhaemoglobin has been fully oxidised by NO, will also exhaust as an NO sink. Finally, it was previously reported that, once saturated, the cellular NO consumption mechanism regenerates over 2 hr. Lipid peroxidation in unstimulated cells was observed by measuring TBARS formation (Dr C. Griffiths, data not shown), which continued for at least 3 hr, only reaching 50 % of the maximum achievable upon stimulation with exogenous iron and ascorbate. Regeneration of the NO consumption mechanism in cells may therefore simply reflect the time taken for a sink of peroxidized lipid to be re-established. Alternatively, methaemoglobin reductase, present in RBCs, could replenish the pool of oxyhaemoglobin.

6.5 CONCLUSION

The data, together with the results discussed above (section 6.4), clearly demonstrate the ease with which freshly isolated neurones may become oxidatively stressed *in vitro*, and the profound effects this may have upon experiments with NO. Bearing this in mind it may be prudent to include a transition metal chelator or antioxidant compound during the preparation of primary neurones for work *in vitro*. Elevated lipid peroxidation is not solely a tissue preparation artefact, and is associated with cellular demise in diseases including Alzheimer's or Parkinson's disease (Moosmann & Behl, 2002). Agents that scavenge either the initiating radical or break the cycle of lipid peroxidation, by interacting with a lipid radical species, have been shown to protect brain tissue both *in vitro* and *in vivo* (Hall *et al.*, 1997; Liu *et al.*, 2003; Moosmann *et al.*, 2001). Furthermore, NO itself can also inhibit lipid peroxidation in various *in vitro* scenarios (Goss *et al.*, 1997; Hiramoto *et al.*, 2003; Kelley *et al.*, 1999; Robb *et al.*, 1999). With the exception of one (Robb *et al.*, 1999), these studies have mainly used bolus addition of large

quantities of authentic NO to demonstrate such antioxidant effects. Here it is demonstrated that physiological rates of NO production (Griffiths & Garthwaite, 2001) not only terminate lipid peroxidation, but that levels of NO itself are consequently restrained by the same process. It may be envisaged that in this way endogenous NO produced following cerebral ischaemia could limit the progression of damage *via* lipid peroxidation. NO itself would be restricted to concentrations capable of activating the NO receptor, $NO_{GC}R$ (< 20 nM), but below that which might inhibit respiration (> 100 nM; (Griffiths *et al.*, 2003)). Though no direct evidence exists for this at present, the protective effects of NO release from donors post-ischaemia correlate with a decrease in levels of reactive oxygen species (Mason *et al.*, 2000; Pluta *et al.*, 2001).

CHAPTER 7: INHIBITION OF LIPID PEROXIDATION, WHAT LIES BENEATH

7.1 INTRODUCTION

In dispersed cerebellar granule cell preparations NO is consumed by a combination of lipid peroxidation and red blood cell contamination (chapter 6). Acutely prepared cerebellar slices also consume NO avidly. However, when slice cGMP accumulation was measured in the presence of lipid-peroxidation inhibitors, or in tissue taken from perfused brains, no difference was found compared to controls. This indicates that other mechanisms may be responsible for NO consumption in slices (Catherine Hall, personal communication).

One difference between preparing *in vitro* slices and dispersed cells is the O_2 concentration required in the bathing solution. To enable all cells to respire across the thickness of a slice (400 μm), the slices are incubated in 1 mM O_2. Conversely dispersed cells are able to respire adequately in air-equilibrated solutions (~185 μM O_2).

A complication when examining NO consumption in cerebellar suspensions is that the cells consume O_2 at ~ 5 μM / min (Griffiths & Garthwaite, 2001), a rate that may cause O_2 levels to fall significantly while the experiment is performed in a sealed experimental chamber. Since NO consumption mechanisms are likely to require O_2, the existence of a further (O_2-dependent) cellular NO consumption mechanism was questioned.

Aims

The profile of NO consumption by dispersed cultures was re-examined under conditions in which lipid peroxidation was inhibited but the O_2 concentration was not limiting.

7.2 METHODS

NO consumption by cerebellar granule cells

Following addition of DETA/NO (100 and 250 μM) to a cerebellar granule cell suspension (see chapter 6.2) NO was measured in either a sealed or air-equilibrated chamber using an ISO-NOP probe (see chapter 2.2).

Cerebellar glial cultures

Large dishes (600 cm^2, Nalge Europe Limited, Hereford, UK) were coated by addition of poly-*D*-lysine (final concentration 2 $\mu g/cm^2$ in dH_2O) overnight, followed by 2 washes (15 min each) in dH_2O, and allowed to air dry. Digestion enzymes were prepared on the day of culture in Ca^{2+}/Mg^{2+} free HBSS supplemented with 10 mM Hepes, 1 mM Na pyruvate, 0.35 % Na bicarbonate and P/S (100 U/ml : 100 μg/ml). Stock concentrations were: trypsin (5 mg/ml), soybean trypsin inhibitor (6.65 mg/ml), DNase (0.88 mg/ml) and $MgCl_2$ (110 mM).

Cerebella were removed from Sprague-Dawley rats, post-natal day 7 and placed in a petri-dish containing HBSS. Using a sterile double-edged blade not more than 10 cerebella were triple chopped into 4 mm cubes. 10 ml HBSS was used to remove the cubes, which were placed into a 100 ml pot with an equal volume of trypsin (final activity 2500 U/ml) and mixed. Digestion continued for 15 min at 37°C with shaking. DNase (1 ml) and trypsin inhibitor (1 ml) were added and cells mixed before a further 10 min incubation at 37°C with shaking. Cells were transferred to a 50 ml tube, 5 ml growth media (as for hippocampal slices, chapter 4.2) was added, and a 25 ml pipette used to triturate the suspension against the bottom of the tube until no large aggregates remained. The suspension was briefly centrifuged (120 *g*, 90 sec) and the supernatant collected and maintained at 37°C. The cell pellet was washed and triturated in 10 ml growth media a further 3 times, collecting the supernatants only after the remaining blocks had settled.

Cells were counted in a 1:1 suspension with trypan blue using a Fuchs Rosenthal counting chamber, seeded at a density of 25 x 10^6 cells / plate in 60 ml growth media and maintained in a humidified incubator at 37°C. Media

was changed the following day and then every 2-3 days. Cultures were used after 6-7 days *in vitro*.

NO consumption by cerebellar glial cultures.

Glial cultures were washed twice in 50 ml calcium-free incubation buffer (chapter 6.2) before application of 25 ml trypsin/EDTA for 10 min at 37°C. To ensure the maximum number of cells detached, the plate was hit several times on the bench. Cells were collected in 20 ml incubation buffer, pelleted (120 *g*, 5 min) and resuspended in 0.5 ml incubation buffer. After counting (as above) cells were diluted and assayed for their ability to consume NO (released by DETA/NO 100 µM) at concentrations between 0.5 and 2 x 10^6/ml (2 x 10^6/ml cells was equivalent to 0.6 mg protein/ml) in an air-equilibrated chamber using the ISO-NOP probe (chapter 2.2). Glial cells (2 x 10^6/ml) were also assayed for NO consumption in the presence of DTPA, Trolox and deferoxamine (all 100 µM).

7.3 RESULTS

Lipid peroxidation-independent NO consumption

The existence of a further (O_2-dependent) cellular NO consumption mechanism was questioned by examining the effect of adding DETA/NO (100 or 250 μM) to buffer or cells in a sealed, or air-equilibrated chamber, in the presence of 100 μM Trolox (Fig. 7.1). In buffer NO rose to a steady-state level (~ 400 nM from 100 μM DETA/NO and ~ 750 nM from 250 μM DETA/NO) in the sealed chamber. These values were not significantly different when the chamber was air equilibrated (not shown). Cells exposed to 100 μM DETA/NO in the sealed chamber behaved as before (Fig. 6.1), with RBC's keeping NO clamped at a low steady-state (~ 20 nM) before exhausting after 7 min, allowing NO to rise. When the chamber was air-equilibrated, however, NO consumption continued, at least over the 20 min duration of the experiment. Upon application of 250 μM DETA/NO, the RBC clamp was shortened to about 3 min, and NO steady-state levels reached almost 100 nM. Again exhaustion of the RBC clamp allowed NO levels to rise steadily in the sealed chamber, however, in the air-equilibrated chamber a second NO steady-state was formed at about 150 nM, and remained constant for the duration of the experiment. When RBC's alone were incubated with Trolox in an air-equilibrated chamber, such O_2-dependent NO consumption was not observed (C. Hall, personal communication).

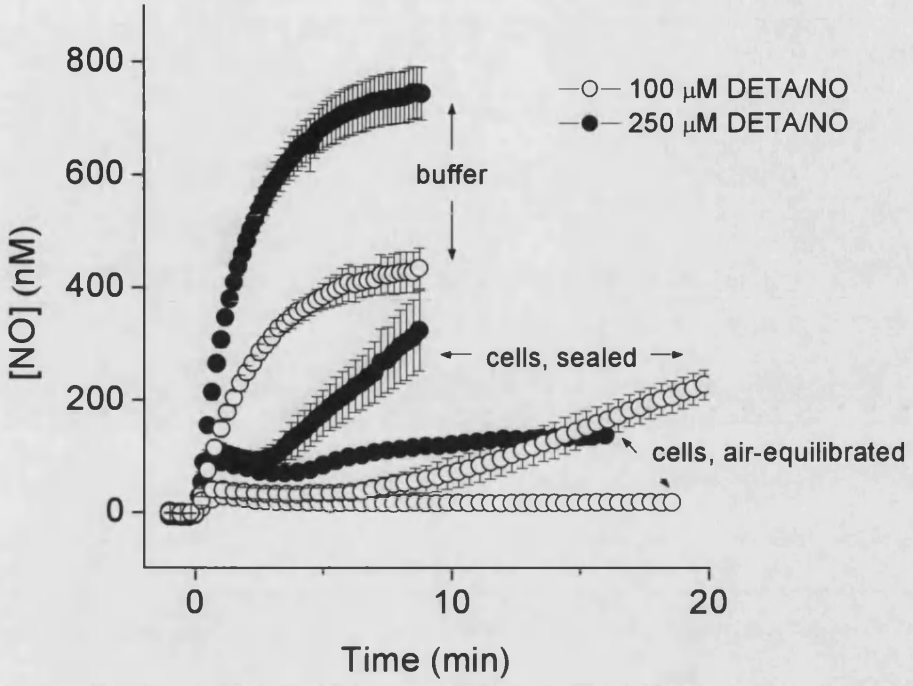


Figure 7.1 lipid peroxidation-independent NO consumption in cerebellar granule cells
Traces of NO accumulation (mean ± SEM *n* = 3) following addition of 100 or 250 μM DETA/NO to buffer in a sealed chamber, or cerebellar granule cells in either a sealed or air-equilibrated chamber. All conditions contained 100 μM Trolox.

Lipid peroxidation-independent NO consumption was then examined using cerebellar glial cultures, which have the benefit of being free from contaminating blood cells. Cultured glia close to confluence (Fig 7.2A), were suspended in an air-equilibrated chamber at differing concentrations (0.5 – $2x10^6$/ml) and challenged with 100 μM DETA/NO. Cellular NO consumption was concentration-dependent, with steady-state NO reduced to 160 ± 5 nM in $2x10^6$/ml cells compared to 360 ± 14 nM in buffer (Fig 7.2B,C). Metal chelation with DTPA, or the more cell permeable deferoxamine (both 100 μM), or antioxidant treatment (100 μM Trolox) had no significant effect upon NO consumption by $2x10^6$/ml cells (Fig 7.2D).

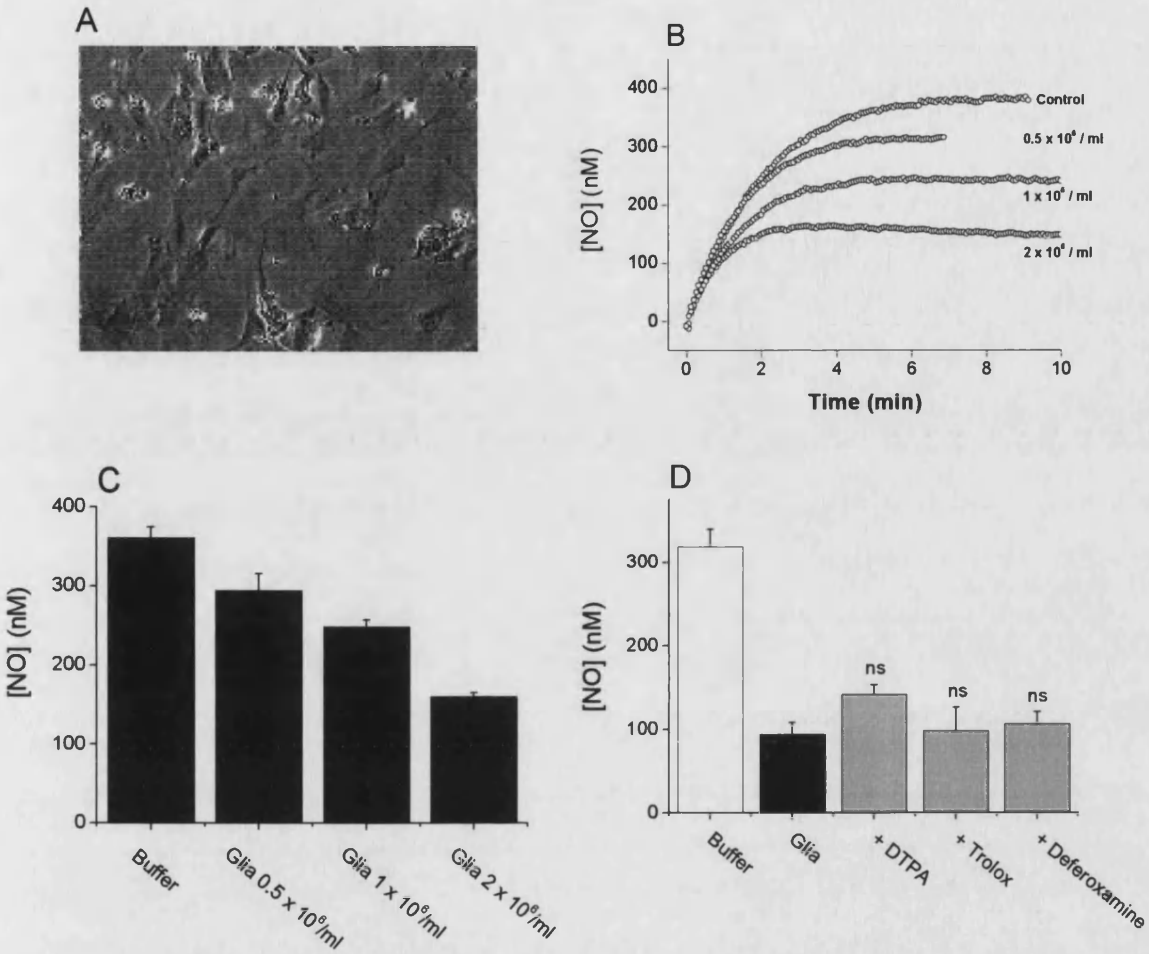


Figure 7.2 Lipid peroxidation-independent NO consumption in cerebellar glial cells **(A)** Representative photomicrograph of cerebellar glial cultures after 6 days *in vitro*. **(B)** Traces represent NO accumulation following addition of 100 µM DETA/NO to increasing concentrations of glia in air-equilibrated suspensions. **(C)** NO steady-state concentration is summarised as mean ± S.E.M. $n = 3$. **(D)** Summary of NO steady-state (mean ± S.E.M. $n = 3$) following addition of 100 µM DETA/NO to glia ($2x10^6$/ml) in the presence or absence of DTPA, Trolox or deferoxamine (all 100 µM), *ns* = no significant difference versus glia.

7.4 DISCUSSION

NO consumption persists following inhibition of lipid peroxidation

The use of compounds to prevent lipid peroxidation has unveiled another, similarly powerful, O_2-dependent, NO consumption mechanism in cerebellar cells. Further investigation of this mechanism was beyond the scope of the current study, however, its persistence (albeit 10 fold weakened) in homogenates (C.Hall, personal communication) affords good opportunity for its identification. A similar NO consumption mechanism (DTPA, Trolox and AO insensitive) also persists in mixed cerebellar glial cultures, which will allow kinetic and other investigations to be undertaken in the absence of contaminating RBCs. Initially important will be to discount the mechanisms discussed in chapter 6.1, and to this end, work has already begun.

7.5 CONCLUSIONS

The resistance of hippocampal slice cultures to high concentrations of exogenous NO (chapter 5) may involve NO consumption mechanisms, and in brain cells and homogenates a significant route for such consumption is by reaction with peroxidising tissue (chapter 6). Though this mechanism does not necessarily contribute to NO consumption by slices, the use of antioxidant compounds has uncovered another, as yet unidentified, mechanism by which NO may be consumed by cells.

SUMMARY

This study set out to question the controversial question of the role that endogenous NO plays in excitotoxic neuronal cell death. Following careful characterisation of the NO–cGMP pathway in hippocampal slice cultures, and elimination of a serious potential artifact (reaction with media components), no experimental evidence was found in support of the generation of lethal concentrations of NO during glutamate-dependent excitotoxicity. Subsequent investigation of the toxicity of exogenous NO to slice cultures suggests that a powerful inactivation mechanism exists to prevent excessive NO reaching the concentration required to inhibit neuronal respiration.

Accelerated NO consumption has been reported to occur by differing mechanisms in various experimental scenarios. In cerebellar cell suspensions and rat brain homogenates it is now evident that NO concentrations are 'clamped' by avid reaction with tissue undergoing iron/ascorbate induced lipid peroxidation, a mechanism that is of considerable significance during many disease states. The use of lipid peroxidation inhibitors has unveiled a novel, O_2-dependent NO consumption mechanism in suspensions of cerebellar granule cells and glia, the future characterisation of which may be vitally important in understanding NO physiology and pathology.

REFERENCES

Abu-Soud, H.M. & Hazen, S.L. (2000) Nitric oxide is a physiological substrate for mammalian peroxidases. *J. Biol. Chem.* 275, 37524-37532.

Adachi, N., Lei, B., Soutani, M. & Arai, T. (2000) Different roles of neuronal and endothelial nitric oxide synthases on ischemic nitric oxide production in gerbil striatum. *Neurosci. Lett.* 288, 151-154.

Adamchik, Y. & Baskys, A. (2000) Glutamate-mediated neuroprotection against N-methyl-D-aspartate toxicity: a role for metabotropic glutamate receptors. *Neuroscience* 99, 731-736.

Aizenman, E., Brimecombe, J.C., Potthoff, W.K. & Rosenberg, P.A. (1998) Why is the role of nitric oxide in NMDA receptor function and dysfunction so controversial? *Prog. Brain Res.* 118, 53-71.

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

Almeida, A. & Bolanos, J.P. (2001) A transient inhibition of mitochondrial ATP synthesis by nitric oxide synthase activation triggered apoptosis in primary cortical neurons. *J. Neurochem.* 77, 676-690.

Alonso, D., Serrano, J., Rodriguez, I., Ruiz-Cabello, J., Fernandez, A.P., Encinas, J.M., Castro-Blanco, S., Bentura, M.L., Santacana, M., Richart, A., Fernandez-Vizarra, P., Uttenthal, L.O. & Rodrigo, J. (2002) Effects of oxygen and glucose deprivation on the expression and distribution of neuronal and inducible nitric oxide synthases and on protein nitration in rat cerebral cortex. *J. Comp Neurol.* 443, 183-200.

Ando, A., Yang, A., Mori, K., Yamada, H., Yamada, E., Takahashi, K., Saikia, J., Kim, M., Melia, M., Fishman, M., Huang, P., & Campochiaro, P.A. (1996) Nitric oxide is proangiogenic in the retina and choroid. *J. Cell Physiol.* 191, 116-24.

Aoki, C., Bredt, D.S., Fenstemaker, S. & Lubin, M. (1998) The subcellular distribution of nitric oxide synthase relative to the NR1 subunit of NMDA receptors in the cerebral cortex. Prog. Brain Res. 118, 83-97.

Arnold, W.P., Mittal, C.K., Katsuki, S. & Murad, F. (1977) Nitric oxide activates guanylate cyclase and increases guanosine 3':5'-cyclic monophosphate levels in various tissue preparations. *Proc. Natl. Acad. Sci. U. S. A* 74, 3203-3207.

Atochin, D.N., Clark, J., Demchenko, I.T., Moskowitz, M.A. & Huang, P.L. (2003) Rapid cerebral ischemic preconditioning in mice deficient in endothelial and neuronal nitric oxide synthases. *Stroke* 34, 1299-1303.

Augusto, O., Bonini, M.G., Amanso, A.M., Linares, E., Santos, C.C. & De Menezes, S.L. (2002) Nitrogen dioxide and carbonate radical anion: two emerging radicals in biology. *Free Radic. Biol. Med.* 32, 841-859.

Ayata, C., Ayata, G., Hara, H., Matthews, R.T., Beal, M.F., Ferrante, R.J., Endres, M., Kim, A., Christie, R.H., Waeber, C., Huang, P.L., Hyman, B.T. & Moskowitz, M.A. (1997) Mechanisms of reduced striatal NMDA excitotoxicity in type I nitric oxide synthase knock-out mice. *J. Neurosci.* 17, 6908-6917.

Bal-Price, A. & Brown, G.C. (2000) Nitric-oxide-induced necrosis and apoptosis in PC12 cells mediated by mitochondria. *J. Neurochem.* 75, 1455-1464.

Bal-Price, A. & Brown, G.C. (2001) Inflammatory neurodegeneration mediated by nitric oxide from activated glia-inhibiting neuronal respiration, causing glutamate release and excitotoxicity. *J. Neurosci.* 21, 6480-6491.

Bal-Price, A., Matthias, A. & Brown, G.C. (2002) Stimulation of the NADPH oxidase in activated rat microglia removes nitric oxide but induces peroxynitrite production. *J. Neurochem.* 80, 73-80.

Balligand, J.L., Kelly, R.A., Marsden, P.A., Smith, T.W. & Michel, T. (1993) Control of cardiac muscle cell function by an endogenous nitric oxide signaling system. *Proc. Natl. Acad. Sci. U. S. A* 90, 347-351.

Bartus, R.T., Baker, K.L., Heiser, A.D., Sawyer, S.D., Dean, R.L., Elliott, P.J. & Straub, J.A. (1994) Postischemic administration of AK275, a calpain inhibitor, provides substantial protection against focal ischemic brain damage. *J. Cereb. Blood Flow Metab* 14, 537-544.

Bates, T.E., Loesch, A., Burnstock, G. & Clark, J.B. (1995) Immunocytochemical evidence for a mitochondrially located nitric oxide synthase in brain and liver. *Biochem. Biophys. Res. Commun.* 213, 896-900.

Beavo, J.A. (1995) Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. *Physiol Rev.* 75, 725-748.

Beckman, J.S. & Koppenol, W.H. (1996) Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am. J. Physiol* 271, C1424-C1437.

Bednarski, E., Vanderklish, P., Gall, C., Saido, T.C., Bahr, B.A. & Lynch, G. (1995) Translational suppression of calpain I reduces NMDA-induced spectrin proteolysis and pathophysiology in cultured hippocampal slices. *Brain Res.* 694, 147-157.

Bellamy, T.C. & Garthwaite, J. (2001a) "cAMP-specific" phosphodiesterase contributes to cGMP degradation in cerebellar cells exposed to nitric oxide. *Mol. Pharmacol.* 59, 54-61.

Bellamy, T.C. & Garthwaite, J. (2001b) Sub-second kinetics of the nitric oxide receptor, soluble guanylyl cyclase, in intact cerebellar cells. *J. Biol. Chem.* 276, 4287-4292.

Bellamy, T.C., Griffiths, C. & Garthwaite, J. (2002a) Differential sensitivity of guanylyl cyclase and mitochondrial respiration to nitric oxide measured using clamped concentrations. *J. Biol. Chem.* 277, 31801-31807.

Bellamy, T.C., Wood, J. & Garthwaite, J. (2002b) On the activation of soluble guanylyl cyclase by nitric oxide. *Proc. Natl. Acad. Sci. U. S. A* 99, 507-510.

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

Benveniste, H., Drejer, J., Schousboe, A. & Diemer, N.H. (1984) Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. *J. Neurochem.* 43, 1369-1374.

Bergles, D.E., Diamond, J.S. & Jahr, C.E. (1999) Clearance of glutamate inside the synapse and beyond. *Curr. Opin. Neurobiol.* 9, 293-298.

Bohme, G.A., Bon, C., Stutzmann, J.M., Doble, A. & Blanchard, J.C. (1991) Possible involvement of nitric oxide in long-term potentiation. *Eur. J. Pharmacol.* 199, 379-381.

Bon, C.L. & Garthwaite, J. (2001) Exogenous nitric oxide causes potentiation of hippocampal synaptic transmission during low-frequency stimulation via the endogenous nitric oxide-cGMP pathway. *Eur. J. Neurosci.* 14, 585-594.

Bonfoco, E., Krainc, D., Ankarcrona, M., Nicotera, P. & Lipton, S.A. (1995) Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc. Natl. Acad. Sci. U. S. A* 92, 7162-7166.

Bonmann, E., Suschek, C., Spranger, M. & Kolb-Bachofen, V. (1997) The dominant role of exogenous or endogenous interleukin-1 beta on expression and activity of inducible nitric oxide synthase in rat microvascular brain endothelial cells. *Neurosci. Lett.* 230, 109-112.

Borutaite, V. & Brown, G.C. (1996) Rapid reduction of nitric oxide by mitochondria, and reversible inhibition of mitochondrial respiration by nitric oxide. *Biochem. J.* 315 (Pt 1), 295-299.

Borutaite, V., Morkuniene, R. & Brown, G.C. (2000) Nitric oxide donors, nitrosothiols and mitochondrial respiration inhibitors induce caspase activation by different mechanisms. *FEBS Lett.* 467, 155-159.

Boulton, C.L., Southam, E. & Garthwaite, J. (1995) Nitric oxide-dependent long-term potentiation is blocked by a specific inhibitor of soluble guanylyl cyclase. *Neuroscience* 69, 699-703.

Boveris, A. & Cadenas, E. (2000) Mitochondrial production of hydrogen peroxide regulation by nitric oxide and the role of ubisemiquinone. *IUBMB. Life* 50, 245-250.

Bradley, J., Zhang, Y., Bakin, R., Lester, H.A., Ronnett, G.V. & Zinn, K. (1997) Functional expression of the heteromeric "olfactory" cyclic nucleotide-gated channel in the hippocampus: a potential effector of synaptic plasticity in brain neurons. *J. Neurosci.* 17, 1993-2005.

Bredt, D.S., Hwang, P.M., Glatt, C.E., Lowenstein, C., Reed, R.R. & Snyder, S.H. (1991) Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature* 351, 714-718.

Bredt, D.S. & Snyder, S.H. (1990) Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. U. S. A* 87, 682-685.

Brenman, J.E., Chao, D.S., Gee, S.H., McGee, A.W., Craven, S.E., Santillano, D.R., Wu, Z., Huang, F., Xia, H., Peters, M.F., Froehner, S.C. & Bredt, D.S. (1996) Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains. *Cell* 84, 757-767.

Broillet, M., Randin, O. & Chatton, J. (2001) Photoactivation and calcium sensitivity of the fluorescent NO indicator 4,5-diaminofluorescein (DAF-2): implications for cellular NO imaging. *FEBS Lett.* 491, 227-232.

Brorson, J.R., Marcuccilli, C.J. & Miller, R.J. (1995) Delayed antagonism of calpain reduces excitotoxicity in cultured neurons. *Stroke* 26, 1259-1266.

Brorson, J.R., Schumacker, P.T. & Zhang, H. (1999) Nitric oxide acutely inhibits neuronal energy production. *J. Neurosci.* 19, 147-158.

Brown, G.C. (1995) Nitric oxide regulates mitochondrial respiration and cell functions by inhibiting cytochrome oxidase. *FEBS Lett.* 369, 136-139.

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

Brown, G.C. & Borutaite, V. (2002) Nitric oxide inhibition of mitochondrial respiration and its role in cell death. *Free Radic. Biol. Med.* 33, 1440-1450.

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

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

Brudvig, G.W., Stevens, T.H. & Chan, S.I. (1980) Reactions of nitric oxide with cytochrome c oxidase. *Biochemistry* 19, 5275-5285.

Buettner, G.R. & Jurkiewicz, B.A. (1996) Catalytic metals, ascorbate and free radicals: combinations to avoid. *Radiat. Res.* 145, 532-541.

Burette, A., Zabel, U., Weinberg, R.J., Schmidt, H.H. & Valtschanoff, J.G. (2002) Synaptic localization of nitric oxide synthase and soluble guanylyl cyclase in the hippocampus. *J. Neurosci.* 22, 8961-8970.

Butler, A.R., Flitney, F.W. & Williams, D.L. (1995) NO, nitrosonium ions, nitroxide ions, nitrosothiols and iron-nitrosyls in biology: a chemist's perspective. *Trends Pharmacol. Sci.* 16, 18-22.

Buxton, I.L., Cheek, D.J., Eckman, D., Westfall, D.P., Sanders, K.M. & Keef, K.D. (1993) NG-nitro L-arginine methyl ester and other alkyl esters of arginine are muscarinic receptor antagonists. *Circ. Res.* 72, 387-395.

Cardounel, A.J. & Zweier, J.L. (2002) Endogenous methylarginines regulate neuronal nitric-oxide synthase and prevent excitotoxic injury. *J. Biol. Chem.* 277, 33995-34002.

Centeno, J.M., Orti, M., Salom, J.B., Sick, T.J. & Perez-Pinzon, M.A. (1999) Nitric oxide is involved in anoxic preconditioning neuroprotection in rat hippocampal slices. *Brain Res.* 836, 62-69.

Cherry, J.A. & Davis, R.L. (1999) Cyclic AMP phosphodiesterases are localized in regions of the mouse brain associated with reinforcement, movement, and affect. *J. Comp Neurol.* 407, 287-301.

Choi, D.W. (1992) Excitotoxic cell death. *J. Neurobiol.* 23, 1261-1276.

Christopherson, K.S., Hillier, B.J., Lim, W.A. & Bredt, D.S. (1999) PSD-95 assembles a ternary complex with the N-methyl-D-aspartic acid receptor and a bivalent neuronal NO synthase PDZ domain. *J. Biol. Chem.* 274, 27467-27473.

Ciani, E., Guidi, S., Bartesaghi, R. & Contestabile, A. (2002a) Nitric oxide regulates cGMP-dependent cAMP-responsive element binding protein phosphorylation and Bcl-2 expression in cerebellar neurons: implication for a survival role of nitric oxide. *J. Neurochem.* 82, 1282-1289.

Ciani, E., Guidi, S., Della, V.G., Perini, G., Bartesaghi, R. & Contestabile, A. (2002b) Nitric oxide protects neuroblastoma cells from apoptosis induced by serum deprivation through cAMP-response element-binding protein (CREB) activation. *J. Biol. Chem.* 277, 49896-49902.

Ciani, E., Virgili, M. & Contestabile, A. (2002c) Akt pathway mediates a cGMP-dependent survival role of nitric oxide in cerebellar granule neurones. *J. Neurochem.* 81, 218-228.

Clarkson, R.B., Norby, S.W., Smirnov, A., Boyer, S., Vahidi, N., Nims, R.W. & Wink, D.A. (1995) Direct measurement of the accumulation and mitochondrial conversion of nitric oxide within Chinese hamster ovary cells

using an intracellular electron paramagnetic resonance technique. *Biochim. Biophys. Acta* 1243, 496-502.

Clement, M.V., Long, L.H., Ramalingam, J. & Halliwell, B. (2002) The cytotoxicity of dopamine may be an artefact of cell culture. *J. Neurochem.* 81, 414-421.

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

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

Coert, B.A., Anderson, R.E. & Meyer, F.B. (2003) Is neuroprotective efficacy of nNOS inhibitor 7-NI dependent on ischemic intracellular pH? *Am. J. Physiol Heart Circ. Physiol* 284, H151-H159.

Coffey, M.J., Natarajan, R., Chumley, P.H., Coles, B., Thimmalapura, P.R., Nowell, M., Kuhn, H., Lewis, M.J., Freeman, B.A. & O'Donnell, V.B. (2001) Catalytic consumption of nitric oxide by 12/15- lipoxygenase: inhibition of monocyte soluble guanylate cyclase activation. *Proc. Natl. Acad. Sci. U. S. A* 98, 8006-8011.

Cooper, C.E. (2002) Nitric oxide and cytochrome oxidase: substrate, inhibitor or effector? *Trends Biochem. Sci.* 27, 33-39.

Culotta, E. & Koshland, D.E., Jr. (1992) NO news is good news. *Science* 258, 1862-1865.

Cummings, J.A., Nicola, S.M. & Malenka, R.C. (1994) Induction in the rat hippocampus of long-term potentiation (LTP) and long-term depression (LTD) in the presence of a nitric oxide synthase inhibitor. *Neurosci. Lett.* 176, 110-114.

Davies, K.M., Wink, D.A., Saavedra, J.E. & Keefer, L.K. (2001) Chemistry of the diazeniumdiolates. 2. Kinetics and mechanism of dissociation to nitric oxide in aqueous solution. *J. Am. Chem. Soc.* 123, 5473-5481.

Davies, M.J., Donkor, R., Dunster, C.A., Gee, C.A., Jonas, S. & Willson, R.L. (1987) Desferrioxamine (Desferal) and superoxide free radicals. Formation of an enzyme-damaging nitroxide. *Biochem. J.* 246, 725-729.

Dawson, T.M., Bredt, D.S., Fotuhi, M., Hwang, P.M. & Snyder, S.H. (1991a) Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissues. *Proc. Natl. Acad. Sci. U. S. A* 88, 7797-7801.

Dawson, V.L., Dawson, T.M., London, E.D., Bredt, D.S. & Snyder, S.H. (1991b) Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proc. Natl. Acad. Sci. U. S. A* 88, 6368-6371.

Dawson, V.L., Kizushi, V.M., Huang, P.L., Snyder, S.H. & Dawson, T.M. (1996) Resistance to neurotoxicity in cortical cultures from neuronal nitric oxide synthase-deficient mice. *J. Neurosci.* 16, 2479-2487.

de Vente, J., Asan, E., Gambaryan, S., Markerink-van Ittersum, M., Axer, H., Gallatz, K., Lohmann, S.M. & Palkovits, M. (2001) Localization of cGMP-dependent protein kinase type II in rat brain. *Neuroscience* 108, 27-49.

De Visscher, G., Springett, R., Delpy, D.T., Van Reempts, J., Borgers, M. & van Rossem, K. (2002) Nitric oxide does not inhibit cerebral cytochrome oxidase in vivo or in the reactive hyperemic phase after brief anoxia in the adult rat. *J. Cereb. Blood Flow Metab* 22, 515-519.

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

Deliconstantinos, G. & Villiotou, V. (1996) NO synthase and xanthine oxidase activities of rabbit brain synaptosomes: peroxynitrite formation as a causative factor of neurotoxicity. *Neurochem. Res.* 21, 51-56.

Demas, G.E., Kriegsfeld, L.J., Blackshaw, S., Huang, P., Gammie, S.C., Nelson, R.J. & Snyder, S.H. (1999) Elimination of aggressive behavior in male mice lacking endothelial nitric oxide synthase. *J. Neurosci.* 19, RC30.

Demerle-Pallardy, C., Gillard-Roubert, V., Marin, J.G., Auguet, M. & Chabrier, P.E. (2000) In vitro antioxidant neuroprotective activity of BN 80933, a dual inhibitor of neuronal nitric oxide synthase and lipid peroxidation. *J. Neurochem.* 74, 2079-2086.

Demerle-Pallardy, C., Lonchampt, M.O., Chabrier, P.E. & Braquet, P. (1991) Absence of implication of L-arginine/nitric oxide pathway on neuronal cell injury induced by L-glutamate or hypoxia. *Biochem. Biophys. Res. Commun.* 181, 456-464.

Denninger, J.W. & Marletta, M.A. (1999) Guanylate cyclase and the NO/cGMP signaling pathway. *Biochim. Biophys. Acta* 1411, 334-350.

Desvignes, C., Bert, L., Vinet, L., Denoroy, L., Renaud, B. & Lambas-Senas, L. (1999) Evidence that the neuronal nitric oxide synthase inhibitor 7-nitroindazole inhibits monoamine oxidase in the rat: in vivo effects on extracellular striatal dopamine and 3,4-dihydroxyphenylacetic acid. *Neurosci. Lett.* 261, 175-178.

Detre, J.A., Nairn, A.C., Aswad, D.W. & Greengard, P. (1984) Localization in mammalian brain of G-substrate, a specific substrate for guanosine 3',5'-cyclic monophosphate-dependent protein kinase. *J. Neurosci.* 4, 2843-2849.

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

Donnini, S. & Ziche, M. (2002) Constitutive and inducible nitric oxide synthase: role in angiogenesis. *Antioxid. Redox. Signal.* 4, 817-823.

Dorheim, M.A., Tracey, W.R., Pollock, J.S. & Grammas, P. (1994) Nitric oxide synthase activity is elevated in brain microvessels in Alzheimer's disease. *Biochem. Biophys. Res. Commun.* 205, 659-665.

Dosemeci, A. & Reese, T.S. (1995) Effect of calpain on the composition and structure of postsynaptic densities. *Synapse* 20, 91-97.

Dulak, J. & Jozkowicz, A. (2003) Regulation of vascular endothelial growth factor synthesis by nitric oxide: facts and controversies. *Antioxid. Redox. Signal.* 5, 123-132.

Dux, E., Oschlies, U., Wiessner, C. & Hossmann, K.A. (1992) Glutamate-induced ribosomal disaggregation and ultrastructural changes in rat cortical neuronal culture: protective effect of horse serum. *Neurosci. Lett.* 141, 173-176.

East, S.J. & Garthwaite, J. (1991) NMDA receptor activation in rat hippocampus induces cyclic GMP formation through the L-arginine-nitric oxide pathway. *Neurosci. Lett.* 123, 17-19.

Egan, T.J., Barthakur, S.R. & Aisen, P. (1992) Catalysis of the Haber-Weiss reaction by iron- diethylenetriaminepentaacetate. *J. Inorg. Biochem.* 48, 241-249.

El Husseini, A.E., Bladen, C. & Vincent, S.R. (1995) Expression of the olfactory cyclic nucleotide gated channel (CNG1) in the rat brain. *Neuroreport* 6, 1459-1463.

El Husseini, A.E., Williams, J., Reiner, P.B., Pelech, S. & Vincent, S.R. (1999) Localization of the cGMP-dependent protein kinases in relation to nitric oxide synthase in the brain. *J. Chem. Neuroanat.* 17, 45-55.

Elfering, S.L., Sarkela, T.M. & Giulivi, C. (2002) Biochemistry of mitochondrial nitric-oxide synthase. *J. Biol. Chem.* 277, 38079-38086.

Eliasson, M.J., Blackshaw, S., Schell, M.J. & Snyder, S.H. (1997a) Neuronal nitric oxide synthase alternatively spliced forms: prominent functional localizations in the brain. *Proc. Natl. Acad. Sci. U. S. A* 94, 3396-3401.

Eliasson, M.J., Sampei, K., Mandir, A.S., Hurn, P.D., Traystman, R.J., Bao, J., Pieper, A., Wang, Z.Q., Dawson, T.M., Snyder, S.H. & Dawson, V.L. (1997b) Poly(ADP-ribose) polymerase gene disruption renders mice resistant to cerebral ischemia. *Nat. Med.* 3, 1089-1095.

Fassbender, K., Fatar, M., Ragoschke, A., Picard, M., Bertsch, T., Kuehl, S. & Hennerici, M. (2000) Subacute but not acute generation of nitric oxide in focal cerebral ischemia. *Stroke* 31, 2208-2211.

Fawcett, L., Baxendale, R., Stacey, P., McGrouther, C., Harrow, I., Soderling, S., Hetman, J., Beavo, J.A. & Phillips, S.C. (2000) Molecular cloning and characterization of a distinct human phosphodiesterase gene family: PDE11A. *Proc. Natl. Acad. Sci. U. S. A* 97, 3702-3707.

Feelisch M, Kubitzek D, Werringloer J (1996) The Oxyhemoglobin Assay. In: Methods in Nitric Oxide Research (Feelisch M, Stamler JS, eds), pp 455-478. John Wiley & Sons Ltd.

Feelisch, M., Ostrowski, J. & Noack, E. (1989) On the mechanism of NO release from sydnonimines. *J. Cardiovasc. Pharmacol.* 14 Suppl 11, S13-S22.

Feil, R., Hartmann, J., Luo, C., Wolfsgruber, W., Schilling, K., Feil, S., Barski, J.J., Meyer, M., Konnerth, A., De Zeeuw, C.I. & Hofmann, F. (2003) Impairment of LTD and cerebellar learning by Purkinje cell-specific ablation of cGMP-dependent protein kinase I. *J. Cell Biol.* 163, 295-302.

Fiscus, R.R. (2002) Involvement of cyclic GMP and protein kinase G in the regulation of apoptosis and survival in neural cells. *Neurosignals.* 11, 175-190.

Flogel, U., Merx, M.W., Godecke, A., Decking, U.K. & Schrader, J. (2001) Myoglobin: A scavenger of bioactive NO. *Proc. Natl. Acad. Sci. U. S. A* 98, 735-740.

Flynn, G.E., Johnson, J.P., Jr. & Zagotta, W.N. (2001) Cyclic nucleotide-gated channels: shedding light on the opening of a channel pore. *Nat. Rev. Neurosci.* 2, 643-651.

Ford, P.C., Wink, D.A. & Stanbury, D.M. (1993) Autoxidation kinetics of aqueous nitric oxide. *FEBS Lett.* 326, 1-3.

Forman, L.J., Liu, P., Nagele, R.G., Yin, K. & Wong, P.Y. (1998) Augmentation of nitric oxide, superoxide, and peroxynitrite production during cerebral ischemia and reperfusion in the rat. *Neurochem. Res.* 23, 141-148.

Fridovich, I. (1995) Superoxide radical and superoxide dismutases. *Annu. Rev. Biochem.* 64, 97-112.

Fujisawa, H., Dawson, D., Browne, S.E., MacKay, K.B., Bullock, R. & McCulloch, J. (1993) Pharmacological modification of glutamate neurotoxicity in vivo. *Brain Res.* 629, 73-78.

Fukumura, D., Gohongi, T., Kadambi, A., Izumi, Y., Ang, J., Yun, C.O., Buerk, D.G., Huang, P.L. & Jain, R.K. (2001) Predominant role of endothelial nitric oxide synthase in vascular endothelial growth factor-induced angiogenesis and vascular permeability. *Proc. Natl. Acad. Sci. U. S. A* 98, 2604-2609.

Furchgott, R.F. & Zawadzki, J.V. (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288, 373-376.

Gahtan, E. & Overmier, J.B. (1999) Inflammatory pathogenesis in Alzheimer's disease: biological mechanisms and cognitive sequeli. *Neurosci. Biobehav. Rev.* 23, 615-633.

Gahwiler, B.H. (1981) Organotypic monolayer cultures of nervous tissue. *J. Neurosci. Methods* 4, 329-342.

Gao, T.M., Pulsinelli, W.A. & Xu, Z.C. (1999) Changes in membrane properties of CA1 pyramidal neurons after transient forebrain ischemia in vivo. *Neuroscience* 90, 771-780.

Gardner, A.M., Martin, L.A., Gardner, P.R., Dou, Y. & Olson, J.S. (2000) Steady-state and transient kinetics of Escherichia coli nitric-oxide dioxygenase (flavohemoglobin). The B10 tyrosine hydroxyl is essential for dioxygen binding and catalysis. *J. Biol. Chem.* 275, 12581-12589.

Gardner, P.R., Martin, L.A., Hall, D. & Gardner, A.M. (2001) Dioxygen-dependent metabolism of nitric oxide in mammalian cells. *Free Radic. Biol. Med.* 31, 191-204.

Garthwaite, G. & Garthwaite, J. (1994) Nitric oxide does not mediate acute glutamate neurotoxicity, nor is it neuroprotective, in rat brain slices. *Neuropharmacology* 33, 1431-1438.

Garthwaite, J. (1985) Cellular uptake disguises action of L-glutamate on N-methyl-D-aspartate receptors. With an appendix: diffusion of transported amino acids into brain slices. *Br. J. Pharmacol.* 85, 297-307.

Garthwaite, J., Charles, S.L. & Chess-Williams, R. (1988) Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature* 336, 385-388.

Garthwaite, J. & Garthwaite, G. (1987) Cellular origins of cyclic GMP responses to excitatory amino acid receptor agonists in rat cerebellum in vitro. *J. Neurochem.* 48, 29-39.

Garthwaite, J., Garthwaite, G., Palmer, R.M. & Moncada, S. (1989) NMDA receptor activation induces nitric oxide synthesis from arginine in rat brain slices. *Eur. J. Pharmacol.* 172, 413-416.

Gegelashvili, G. & Schousboe, A. (1998) Cellular distribution and kinetic properties of high-affinity glutamate transporters. *Brain Res. Bull.* 45, 233-238.

Gegg, M.E., Beltran, B., Salas-Pino, S., Bolanos, J.P., Clark, J.B., Moncada, S. & Heales, S.J. (2003) Differential effect of nitric oxide on glutathione metabolism and mitochondrial function in astrocytes and neurones:

implications for neuroprotection/neurodegeneration? *J. Neurochem.* 86, 228-237.

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

Giardino, I., Fard, A.K., Hatchell, D.L. & Brownlee, M. (1998) Aminoguanidine inhibits reactive oxygen species formation, lipid peroxidation, and oxidant-induced apoptosis. *Diabetes* 47, 1114-1120.

Gibb, B.J. & Garthwaite, J. (2001) Subunits of the nitric oxide receptor, soluble guanylyl cyclase, expressed in rat brain. *Eur. J. Neurosci.* 13, 539-544.

Gibb, B.J., Wykes, V. & Garthwaite, J. (2003) Properties of NO-activated guanylyl cyclases expressed in cells. *Br. J. Pharmacol.* 139, 1032-1040.

Gill, R., Nordholm, L. & Lodge, D. (1992) The neuroprotective actions of 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX) in a rat focal ischaemia model. *Brain Res.* 580, 35-43.

Giulivi, C. (2003) Characterization and function of mitochondrial nitric-oxide synthase. *Free Radic. Biol. Med.* 34, 397-408.

Globus, M.Y., Prado, R., Sanchez-Ramos, J., Zhao, W., Dietrich, W.D., Busto, R. & Ginsberg, M.D. (1995) A dual role for nitric oxide in NMDA-mediated toxicity in vivo. *J. Cereb. Blood Flow Metab* 15, 904-913.

Godecke, A., Molojavyi, A., Heger, J., Flogel, U., Ding, Z., Jacoby, C. & Schrader, J. (2003) Myoglobin protects the heart from inducible nitric-oxide synthase (iNOS)-mediated nitrosative stress. *J. Biol. Chem.* 278, 21761-21766.

Golde, S., Chandran, S., Brown, G.C. & Compston, A. (2002) Different pathways for iNOS-mediated toxicity in vitro dependent on neuronal maturation and NMDA receptor expression. *J. Neurochem.* 82, 269-282.

Goldstein, S. & Czapski, G. (1995) Kinetics of Nitric Oxide Autoxidation in Aqueous Solution in the Absence and Presence of Various Reductants. The Nature of the Oxidizing Intermediates. *J. Am. Chem. Soc.* 117, 12078-12084.

Gong, G.X., Weiss, H.R., Tse, J. & Scholz, P.M. (1998) Exogenous nitric oxide reduces oxygen consumption of isolated ventricular myocytes less than other forms of guanylate cyclase stimulation. *Eur. J. Pharmacol.* 344, 299-305.

Gonzalez-Zulueta, M., Feldman, A.B., Klesse, L.J., Kalb, R.G., Dillman, J.F., Parada, L.F., Dawson, T.M. & Dawson, V.L. (2000) Requirement for nitric oxide activation of p21(ras)/extracellular regulated kinase in neuronal ischemic preconditioning. *Proc. Natl. Acad. Sci. U. S. A* 97, 436-441.

Goss, S.P., Hogg, N. & Kalyanaraman, B. (1997) The effect of nitric oxide release rates on the oxidation of human low density lipoprotein. *J. Biol. Chem.* 272, 21647-21653.

Goyagi, T., Goto, S., Bhardwaj, A., Dawson, V.L., Hurn, P.D. & Kirsch, J.R. (2001) Neuroprotective effect of sigma(1)-receptor ligand 4-phenyl-1-(4-phenylbutyl) piperidine (PPBP) is linked to reduced neuronal nitric oxide production. *Stroke* 32, 1613-1620.

Grady, J.K., Chasteen, N.D. & Harris, D.C. (1988) Radicals from "Good's" buffers. *Anal. Biochem.* 173, 111-115.

Green, L.C., Ruiz, d.L., Wagner, D.A., Rand, W., Istfan, N., Young, V.R. & Tannenbaum, S.R. (1981a) Nitrate biosynthesis in man. *Proc. Natl. Acad. Sci. U. S. A* 78, 7764-7768.

Green, L.C., Tannenbaum, S.R. & Goldman, P. (1981b) Nitrate synthesis in the germfree and conventional rat. *Science* 212, 56-58.

Griffin, F.M., Ashland, G. & Capizzi, R.L. (1981) Kinetics of phototoxicity of Fischer's medium for L5178Y leukemic cells. *Cancer Res.* 41, 2241-2248.

Griffiths, C., Garthwaite, G., Goodwin, D.A. & Garthwaite, J. (2002a) Dynamics of nitric oxide during simulated ischaemia-reperfusion in rat striatal slices measured using an intrinsic biosensor, soluble guanylyl cyclase. *Eur. J. Neurosci.* 15, 962-968.

Griffiths, C. & Garthwaite, J. (2001) The shaping of nitric oxide signals by a cellular sink. *J. Physiol* 536, 855-862.

Griffiths, C., Wykes, V., Bellamy, T.C. & Garthwaite, J. (2003) A new and simple method for delivering clamped nitric oxide concentrations in the physiological range: application to activation of guanylyl cyclase-coupled nitric oxide receptors. *Mol. Pharmacol.* 64, 1349-1356.

Griffiths, C., Yamini, B., Hall, C. & Garthwaite, J. (2002b) Nitric oxide inactivation in brain by a novel O2-dependent mechanism resulting in the formation of nitrate ions. *Biochem. J.* 362, 459-464.

Grisham, M.B., Jourd'Heuil, D. & Wink, D.A. (1999) Nitric oxide. I. Physiological chemistry of nitric oxide and its metabolites:implications in inflammation. *Am. J. Physiol* 276, G315-G321.

Grzelak, A., Rychlik, B. & Bartosz, G. (2001) Light-dependent generation of reactive oxygen species in cell culture media. *Free Radic. Biol. Med.* 30, 1418-1425.

Gu, Z., Kaul, M., Yan, B., Kridel, S.J., Cui, J., Strongin, A., Smith, J.W., Liddington, R.C. & Lipton, S.A. (2002) S-nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death. *Science* 297, 1186-1190.

Gursoy-Ozdemir, Y., Bolay, H., Saribas, O. & Dalkara, T. (2000) Role of endothelial nitric oxide generation and peroxynitrite formation in reperfusion injury after focal cerebral ischemia. *Stroke* 31, 1974-1980.

Ha, K.S., Kim, K.M., Kwon, Y.G., Bai, S.K., Nam, W.D., Yoo, Y.M., Kim, P.K., Chung, H.T., Billiar, T.R. & Kim, Y.M. (2003) Nitric oxide prevents 6-hydroxydopamine-induced apoptosis in PC12 cells through cGMP-dependent PI3 kinase/Akt activation. *FASEB J.* 17, 1036-1047.

Haley, J.E., Malen, P.L. & Chapman, P.F. (1993) Nitric oxide synthase inhibitors block long-term potentiation induced by weak but not strong tetanic stimulation at physiological brain temperatures in rat hippocampal slices. *Neurosci. Lett.* 160, 85-88.

Hall, C., Griffiths, C. & Garthwaite, J. (2003) Kinetics of nitric oxide inactivation in rat brain. *J. Physiol.* 547P, 44P.

Hall, E.D., Andrus, P.K., Smith, S.L., Fleck, T.J., Scherch, H.M., Lutzke, B.S., Sawada, G.A., Althaus, J.S., Vonvoigtlander, P.F., Padbury, G.E., Larson, P.G., Palmer, J.R. & Bundy, G.L. (1997) Pyrrolopyrimidines: novel brain-penetrating antioxidants with neuroprotective activity in brain injury and ischemia models. *J. Pharmacol. Exp. Ther.* 281, 895-904.

Hall, K.U., Collins, S.P., Gamm, D.M., Massa, E., DePaoli-Roach, A.A. & Uhler, M.D. (1999) Phosphorylation-dependent inhibition of protein phosphatase-1 by G-substrate. A Purkinje cell substrate of the cyclic GMP-dependent protein kinase. *J. Biol. Chem.* 274, 3485-3495.

Halliwell, B. (1992) Reactive oxygen species and the central nervous system. *J. Neurochem.* 59, 1609-1623.

Halliwell, B. (2003) Oxidative stress in cell culture: an under-appreciated problem? *FEBS Lett.* 540, 3-6.

Halliwell, B. & Gutteridge, J.M. (1986) Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Arch. Biochem. Biophys.* 246, 501-514.

Halliwell, B., Zhao, K. & Whiteman, M. (1999) Nitric oxide and peroxynitrite. The ugly, the uglier and the not so good: a personal view of recent controversies. *Free Radic. Res.* 31, 651-669.

Han, H.S., Qiao, Y., Karabiyikoglu, M., Giffard, R.G. & Yenari, M.A. (2002) Influence of mild hypothermia on inducible nitric oxide synthase expression and reactive nitrogen production in experimental stroke and inflammation. *J. Neurosci.* 22, 3921-3928.

Hausladen, A., Gow, A.J. & Stamler, J.S. (1998) Nitrosative stress: metabolic pathway involving the flavohemoglobin. *Proc. Natl. Acad. Sci. U. S. A* 95, 14100-14105.

Hegetschweiler, K. & Saltman, P. (1986) Interaction of Copper(II) with *N*-(2-Hydroxyethyl)piperazine-*N'*-ethanesulfonic Acid (HEPES). *Inorg. Chem.* 25, 107-109.

Hewett, S.J., Corbett, J.A., McDaniel, M.L. & Choi, D.W. (1993) Inhibition of nitric oxide formation does not protect murine cortical cell cultures from N-methyl-D-aspartate neurotoxicity. *Brain Res.* 625, 337-341.

Hibbs, J.B., Jr., Taintor, R.R., Vavrin, Z. & Rachlin, E.M. (1988) Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Res. Commun.* 157, 87-94.

Hibbs, J.B., Jr., Vavrin, Z. & Taintor, R.R. (1987) L-arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. *J. Immunol.* 138, 550-565.

Hill, A.V. (1929) The diffusion of oxygen and lactic acid through tissues. *Proc. R. Soc. B.* 104, 39-96.

Hiramoto, K., Ohkawa, T., Oikawa, N. & Kikugawa, K. (2003) Is nitric oxide (NO) an antioxidant or a prooxidant for lipid peroxidation? *Chem. Pharm. Bull. (Tokyo)* 51, 1046-1050.

Hirsch, E.C. & Hunot, S. (2000) Nitric oxide, glial cells and neuronal degeneration in parkinsonism. *Trends Pharmacol. Sci.* 21, 163-165.

Hobbs, A.J. (1997) Soluble guanylate cyclase: the forgotten sibling. *Trends Pharmacol. Sci.* 18, 484-491.

Hobbs, A.J., Gladwin, M.T., Patel, R.P., Williams, D.L. & Butler, A.R. (2002) Haemoglobin: NO transporter, NO inactivator or NOne of the above? *Trends Pharmacol. Sci.* 23, 406-411.

Hogg, N. & Kalyanaraman, B. (1999) Nitric oxide and lipid peroxidation. *Biochim. Biophys. Acta* 1411, 378-384.

Holscher, C. (1999) Nitric oxide is required for expression of LTP that is induced by stimulation phase-locked with theta rhythm. *Eur. J. Neurosci.* 11, 335-343.

Holschneider, D.P., Scremin, O.U., Huynh, L., Chen, K. & Shih, J.C. (1999) Lack of protection from ischemic injury of monoamine oxidase B- deficient mice following middle cerebral artery occlusion. *Neurosci. Lett.* 259, 161-164.

Hood, J.D., Meininger, C.J., Ziche, M. & Granger, H.J. (1998) VEGF upregulates ecNOS message, protein, and NO production in human endothelial cells. *Am. J. Physiol* 274, H1054-H1058.

Huang, C.C. & Hsu, K.S. (1997) Nitric oxide signalling is required for the generation of anoxia-induced long-term potentiation in the hippocampus. *Eur. J. Neurosci.* 9, 2202-2206.

Huh, P.W., Belayev, L., Zhao, W., Clemens, J.A., Panetta, J.A., Busto, R. & Ginsberg, M.D. (2000) Neuroprotection by LY341122, a novel inhibitor of lipid peroxidation, against focal ischemic brain damage in rats. *Eur. J. Pharmacol.* 389, 79-88.

Iadecola, C. (1997) Bright and dark sides of nitric oxide in ischemic brain injury. *Trends Neurosci.* 20, 132-139.

Iadecola, C., Zhang, F., Casey, R., Clark, H.B. & Ross, M.E. (1996) Inducible nitric oxide synthase gene expression in vascular cells after transient focal cerebral ischemia. *Stroke* 27, 1373-1380.

Iadecola, C., Zhang, F. & Xu, X. (1995) Inhibition of inducible nitric oxide synthase ameliorates cerebral ischemic damage. *Am. J. Physiol* 268, R286-R292.

Ignarro, L.J. (1991) Signal transduction mechanisms involving nitric oxide. *Biochem. Pharmacol.* 41, 485-490.

Ignarro, L.J., Fukuto, J.M., Griscavage, J.M., Rogers, N.E. & Byrns, R.E. (1993) Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: comparison with enzymatically formed nitric oxide from L-arginine. *Proc. Natl. Acad. Sci. U. S. A* 90, 8103-8107.

Ivanova, S., Botchkina, G.I., Al Abed, Y., Meistrell, M., III, Batliwalla, F., Dubinsky, J.M., Iadecola, C., Wang, H., Gregersen, P.K., Eaton, J.W. & Tracey, K.J. (1998) Cerebral ischemia enhances polyamine oxidation: identification of enzymatically formed 3-aminopropanal as an endogenous mediator of neuronal and glial cell death. *J. Exp. Med.* 188, 327-340.

Izumi, Y., Benz, A.M., Clifford, D.B. & Zorumski, C.F. (1992) Nitric oxide inhibitors attenuate N-methyl-D-aspartate excitotoxicity in rat hippocampal slices. *Neurosci. Lett.* 135, 227-230.

Izumi, Y., Benz, A.M., Clifford, D.B. & Zorumski, C.F. (1993) Neurotoxic effects of sodium nitroprusside in rat hippocampal slices. *Exp. Neurol.* 121, 14-23.

Jabaudon, D., Scanziani, M., Gahwiler, B.H. & Gerber, U. (2000) Acute decrease in net glutamate uptake during energy deprivation. *Proc. Natl. Acad. Sci. U. S. A* 97, 5610-5615.

Jaffrey, S.R., Benfenati, F., Snowman, A.M., Czernik, A.J. & Snyder, S.H. (2002) Neuronal nitric-oxide synthase localization mediated by a ternary complex with synapsin and CAPON. *Proc. Natl. Acad. Sci. U. S. A* 99, 3199-3204.

Jaffrey, S.R., Snowman, A.M., Eliasson, M.J., Cohen, N.A. & Snyder, S.H. (1998) CAPON: a protein associated with neuronal nitric oxide synthase that regulates its interactions with PSD95. *Neuron* 20, 115-124.

Jin, K., Minami, M., Lan, J.Q., Mao, X.O., Batteur, S., Simon, R.P. & Greenberg, D.A. (2001) Neurogenesis in dentate subgranular zone and rostral subventricular zone after focal cerebral ischemia in the rat. *Proc. Natl. Acad. Sci. U. S. A* 98, 4710-4715.

Joshi, P.C. (1985) Comparison of the DNA-damaging property of photosensitised riboflavin via singlet oxygen ($_1O2$) and superoxide radical $O2^{\bullet-}$. mechanisms. *Toxicol. Lett.* 26, 211-217.

Kagan, V.E., Kozlov, A.V., Tyurina, Y.Y., Shvedova, A.A. & Yalowich, J.C. (2001) Antioxidant mechanisms of nitric oxide against iron-catalyzed oxidative stress in cells. *Antioxid. Redox. Signal.* 3, 189-202.

Kaku, D.A., Giffard, R.G. & Choi, D.W. (1993) Neuroprotective effects of glutamate antagonists and extracellular acidity. *Science* 260, 1516-1518.

Katsuki, S., Arnold, W., Mittal, C. & Murad, F. (1977) Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. *J. Cyclic. Nucleotide. Res.* 3, 23-35.

Kaupp, U.B. & Seifert, R. (2002) Cyclic nucleotide-gated ion channels. *Physiol Rev.* 82, 769-824.

Kazerounian, S., Pitari, G.M., Ruiz-Stewart, I., Schulz, S. & Waldman, S.A. (2002) Nitric oxide activation of soluble guanylyl cyclase reveals high and low affinity sites that mediate allosteric inhibition by calcium. *Biochemistry* 41, 3396-3404.

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

Kelley, E.E., Wagner, B.A., Buettner, G.R. & Burns, C.P. (1999) Nitric oxide inhibits iron-induced lipid peroxidation in HL-60 cells. *Arch. Biochem. Biophys.* 370, 97-104.

Kelm, M. & Schrader, J. (1990) Control of coronary vascular tone by nitric oxide. *Circ. Res.* 66, 1561-1575.

Kharitonov, V.G., Sundquist, A.R. & Sharma, V.S. (1994) Kinetics of nitric oxide autoxidation in aqueous solution. *J. Biol. Chem.* 269, 5881-5883.

Kingston, P.A., Zufall, F. & Barnstable, C.J. (1996) Rat hippocampal neurons express genes for both rod retinal and olfactory cyclic nucleotide-gated channels: novel targets for cAMP/cGMP function. *Proc. Natl. Acad. Sci. U. S. A* 93, 10440-10445.

Kingston, P.A., Zufall, F. & Barnstable, C.J. (1999) Widespread expression of olfactory cyclic nucleotide-gated channel genes in rat brain: implications for neuronal signalling. *Synapse* 32, 1-12.

Kirsch, M., Lomonosova, E.E., Korth, H.G., Sustmann, R. & de Groot, H. (1998) Hydrogen peroxide formation by reaction of peroxynitrite with HEPES and related tertiary amines. Implications for a general mechanism. *J. Biol. Chem.* 273, 12716-12724.

Kleppisch, T., Wolfsgruber, W., Feil, S., Allmann, R., Wotjak, C.T., Goebbels, S., Nave, K.A., Hofmann, F. & Feil, R. (2003) Hippocampal cGMP-dependent protein kinase I supports an age- and protein synthesis-dependent component of long-term potentiation but is not essential for spatial reference and contextual memory. *J. Neurosci.* 23, 6005-6012.

Koglin, M., Vehse, K., Budaeus, L., Scholz, H. & Behrends, S. (2001) Nitric oxide activates the beta 2 subunit of soluble guanylyl cyclase in the absence of a second subunit. *J. Biol. Chem.* 276, 30737-30743.

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

Kojima, H., Nakatsubo, N., Kikuchi, K., Kawahara, S., Kirino, Y., Nagoshi, H., Hirata, Y. & Nagano, T. (1998a) Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins. *Anal. Chem.* 70, 2446-2453.

Kojima, H., Nakatsubo, N., Kikuchi, K., Urano, Y., Higuchi, T., Tanaka, J., Kudo, Y. & Nagano, T. (1998b) Direct evidence of NO production in rat hippocampus and cortex using a new fluorescent indicator: DAF-2 DA. *Neuroreport* 9, 3345-3348.

Koppenol, W.H. (1998) The basic chemistry of nitrogen monoxide and peroxynitrite. *Free Radic. Biol. Med.* 25, 385-391.

Koppenol, W.H. (2001a) 100 years of peroxynitrite chemistry and 11 years of peroxynitrite biochemistry. *Redox. Rep.* 6, 339-341.

Koppenol, W.H. (2001b) The Haber-Weiss cycle--70 years later. *Redox. Rep.* 6, 229-234.

Kornau, H.C., Schenker, L.T., Kennedy, M.B. & Seeburg, P.H. (1995) Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. *Science* 269, 1737-1740.

Kotera, J., Fujishige, K. & Omori, K. (2000) Immunohistochemical localization of cGMP-binding cGMP-specific phosphodiesterase (PDE5) in rat tissues. *J. Histochem. Cytochem.* 48, 685-693.

Kroll, J. & Waltenberger, J. (1998) VEGF-A induces expression of eNOS and iNOS in endothelial cells via VEGF receptor-2 (KDR). *Biochem. Biophys. Res. Commun.* 252, 743-746.

Lacza, Z., Snipes, J.A., Zhang, J., Horvath, E.M., Figueroa, J.P., Szabo, C. & Busija, D.W. (2003) Mitochondrial nitric oxide synthase is not eNOS, nNOS or iNOS. *Free Radic. Biol. Med.* 35, 1217-1228.

Lafon-Cazal, M., Pietri, S., Culcasi, M. & Bockaert, J. (1993) NMDA-dependent superoxide production and neurotoxicity. *Nature* 364, 535-537.

Lahtinen, H., Autere, A.M., Paalasmaa, P., Lauri, S.E. & Kaila, K. (2001) Post-insult activity is a major cause of delayed neuronal death in organotypic hippocampal slices exposed to glutamate. *Neuroscience* 105, 131-137.

Lane, P. & Gross, S.S. (2000) The autoinhibitory control element and calmodulin conspire to provide physiological modulation of endothelial and neuronal nitric oxide synthase activity. *Acta Physiol Scand.* 168, 53-63.

Lee, K.S., Frank, S., Vanderklish, P., Arai, A. & Lynch, G. (1991) Inhibition of proteolysis protects hippocampal neurons from ischemia. *Proc. Natl. Acad. Sci. U. S. A* 88, 7233-7237.

Lei, B., Adachi, N., Nagaro, T., Arai, T. & Koehler, R.C. (1999) Nitric oxide production in the CA1 field of the gerbil hippocampus after transient forebrain ischemia : effects of 7-nitroindazole and NG- nitro-L-arginine methyl ester. *Stroke* 30, 669-677.

Leker, R.R., Teichner, A., Ovadia, H., Keshet, E., Reinherz, E. & Ben Hur, T. (2001) Expression of endothelial nitric oxide synthase in the ischemic penumbra: relationship to expression of neuronal nitric oxide synthase and vascular endothelial growth factor. *Brain Res.* 909, 1-7.

Lerner-Natoli, M., Rondouin, G., de Bock, F. & Bockaert, J. (1992) Chronic NO synthase inhibition fails to protect hippocampal neurones against NMDA toxicity. *Neuroreport* 3, 1109-1112.

Levy, L.M., Warr, O. & Attwell, D. (1998) Stoichiometry of the glial glutamate transporter GLT-1 expressed inducibly in a Chinese hamster ovary cell line selected for low endogenous Na+-dependent glutamate uptake. *J. Neurosci.* 18, 9620-9628.

Lewis, R.S. & Deen, W.M. (1994) Kinetics of the reaction of nitric oxide with oxygen in aqueous solutions. *Chem. Res. Toxicol.* 7, 568-574.

Lewis, R.S., Tamir, S., Tannenbaum, S.R. & Deen, W.M. (1995) Kinetic analysis of the fate of nitric oxide synthesized by macrophages in vitro. *J. Biol. Chem.* 270, 29350-29355.

Liao, J.C., Hein, T.W., Vaughn, M.W., Huang, K.T. & Kuo, L. (1999) Intravascular flow decreases erythrocyte consumption of nitric oxide. *Proc. Natl. Acad. Sci. U. S. A* 96, 8757-8761.

Liberatore, G.T., Jackson-Lewis, V., Vukosavic, S., Mandir, A.S., Vila, M., McAuliffe, W.G., Dawson, V.L., Dawson, T.M. & Przedborski, S. (1999) Inducible nitric oxide synthase stimulates dopaminergic neurodegeneration in the MPTP model of Parkinson disease . *Nat. Med.* 5, 1403-1409.

Limbourg, F.P., Huang, Z., Plumier, J.C., Simoncini, T., Fujioka, M., Tuckermann, J., Schutz, G., Moskowitz, M.A. & Liao, J.K. (2002) Rapid

nontranscriptional activation of endothelial nitric oxide synthase mediates increased cerebral blood flow and stroke protection by corticosteroids. *J. Clin. Invest* 110, 1729-1738.

Lin, H. & Totterdell, S. (1998) Light and electron microscopic study of neuronal nitric oxide synthase- immunoreactive neurons in the rat subiculum. *J. Comp Neurol.* 395, 195-208.

Lin, S.Z., Chiou, A.L. & Wang, Y. (1996) Ketamine antagonizes nitric oxide release from cerebral cortex after middle cerebral artery ligation in rats. *Stroke* 27, 747-752.

Lipton, P. (1999) Ischemic cell death in brain neurons. *Physiol Rev.* 79, 1431-1568.

Lipton, S.A., Choi, Y.B., Pan, Z.H., Lei, S.Z., Chen, H.S., Sucher, N.J., Loscalzo, J., Singel, D.J. & Stamler, J.S. (1993) A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature* 364, 626-632.

Lipton, S.A. & Stamler, J.S. (1994) Actions of redox-related congeners of nitric oxide at the NMDA receptor. *Neuropharmacology* 33, 1229-1233.

Liu, D.M., Wu, J.N., Chiou, A.L., Liu, J.Y. & Wang, Y. (1997) NMDA induces NO release from primary cell cultures of human fetal cerebral cortex. *Neurosci. Lett.* 223, 145-148.

Liu, J., Solway, K., Messing, R.O. & Sharp, F.R. (1998a) Increased neurogenesis in the dentate gyrus after transient global ischemia in gerbils. *J. Neurosci.* 18, 7768-7778.

Liu, R., Liu, W., Doctrow, S.R. & Baudry, M. (2003) Iron toxicity in organotypic cultures of hippocampal slices: role of reactive oxygen species. *J. Neurochem.* 85, 492-502.

Liu, X., Miller, M.J., Joshi, M.S., Sadowska-Krowicka, H., Clark, D.A. & Lancaster, J.R., Jr. (1998b) Diffusion-limited reaction of free nitric oxide with erythrocytes. *J. Biol. Chem.* 273, 18709-18713.

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

Liu, X., Samouilov, A., Lancaster, J.R., Jr. & Zweier, J.L. (2002) Nitric oxide uptake by erythrocytes is primarily limited by extracellular diffusion not membrane resistance. *J. Biol. Chem.* 277, 26194-26199.

Lo, E.H., Hara, H., Rogowska, J., Trocha, M., Pierce, A.R., Huang, P.L., Fishman, M.C., Wolf, G.L. & Moskowitz, M.A. (1996) Temporal correlation mapping analysis of the hemodynamic penumbra in mutant mice deficient in endothelial nitric oxide synthase gene expression. *Stroke* 27, 1381-1385.

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

Loke, K.E., McConnell, P.I., Tuzman, J.M., Shesely, E.G., Smith, C.J., Stackpole, C.J., Thompson, C.I., Kaley, G., Wolin, M.S. & Hintze, T.H. (1999) Endogenous endothelial nitric oxide synthase-derived nitric oxide is a physiological regulator of myocardial oxygen consumption. *Circ. Res.* 84, 840-845.

Lucas, D.R. & Newhouse, J.P. (1957) The toxic effect of sodium L-glutamate on the inner layers of the retina. *Ama. Arch. Opthalmol.* 58, 193-201.

Lucas, K.A., Pitari, G.M., Kazerounian, S., Ruiz-Stewart, I., Park, J., Schulz, S., Chepenik, K.P. & Waldman, S.A. (2000) Guanylyl cyclases and signaling by cyclic GMP. *Pharmacol. Rev.* 52, 375-414.

Malinski, T., Bailey, F., Zhang, Z.G. & Chopp, M. (1993) Nitric oxide measured by a porphyrinic microsensor in rat brain after transient middle cerebral artery occlusion. *J. Cereb. Blood Flow Metab* 13, 355-358.

Mandir, A.S., Poitras, M.F., Berliner, A.R., Herring, W.J., Guastella, D.B., Feldman, A., Poirier, G.G., Wang, Z.Q., Dawson, T.M. & Dawson, V.L. (2000) NMDA but not non-NMDA excitotoxicity is mediated by Poly(ADP-ribose) polymerase. *J. Neurosci.* 20, 8005-8011.

Martinelli, G.P., Friedrich, V.L., Jr. & Holstein, G.R. (2002) L-citrulline immunostaining identifies nitric oxide production sites within neurons. *Neuroscience* 114, 111-122.

Martinez, S.E., Wu, A.Y., Glavas, N.A., Tang, X.B., Turley, S., Hol, W.G. & Beavo, J.A. (2002) The two GAF domains in phosphodiesterase 2A have distinct roles in dimerization and in cGMP binding. *Proc. Natl. Acad. Sci. U. S. A* 99, 13260-13265.

Mason, R.B., Pluta, R.M., Walbridge, S., Wink, D.A., Oldfield, E.H. & Boock, R.J. (2000) Production of reactive oxygen species after reperfusion in vitro and in vivo: protective effect of nitric oxide. *J. Neurosurg.* 93, 99-107.

Mattson, M.P. (1998) Modification of ion homeostasis by lipid peroxidation: roles in neuronal degeneration and adaptive plasticity. *Trends Neurosci.* 21, 53-57.

Matulef, K. & Zagotta, W.N. (2003) Cyclic nucleotide-gated ion channels. *Annu. Rev. Cell Dev. Biol.* 19, 23-44.

Maulik, N. & Das, D.K. (2002) Redox signaling in vascular angiogenesis. *Free Radic. Biol. Med.* 33, 1047-1060.

Meini, A., Benocci, A., Frosini, M., Sgaragli, G.P., Garcia, J.B., Pessina, G.P., Aldinucci, C. & Palmi, M. (2003) Potentiation of intracellular Ca2+ mobilization by hypoxia-induced NO generation in rat brain striatal slices and

human astrocytoma U-373 MG cells and its involvement in tissue damage. *Eur. J. Neurosci.* 17, 692-700.

Meldrum, B. & Garthwaite, J. (1990) Excitatory amino acid neurotoxicity and neurodegenerative disease. *Trends. Pharmacol. Sci.* 11, 379-387.

Meucci, E., Martorana, G.E., Ursitti, A., Pischiutta, M.G., Miggiano, G.A. & Castelli, A. (1985) Ascorbic acid stability in aqueous solutions. *Acta Vitaminol. Enzymol.* 7, 147-153.

Mitani, A., Kadoya, F., Nakamura, Y. & Kataoka, K. (1991) Visualization of hypoxia-induced glutamate release in gerbil hippocampal slice. *Neurosci. Lett.* 122, 167-170.

Miyazaki, S., Katayama, Y., Furuichi, M., Kinoshita, K., Kawamata, T. & Tsubokawa, T. (1993) Post-ischemic potentiation of Schaffer collateral/CA1 pyramidal cell responses of the rat hippocampus in vivo: involvement of N-methyl-D-aspartate receptors. *Brain Res.* 611, 155-159.

Moncada, C., Lekieffre, D., Arvin, B. & Meldrum, B. (1992) Effect of NO synthase inhibition on NMDA- and ischemia-induced hippocampal lesions. *Neuroreport* 3, 530-532.

Moncada, S. & Erusalimsky, J.D. (2002) Does nitric oxide modulate mitochondrial energy generation and apoptosis? *Nat. Rev. Mol. Cell Biol.* 3, 214-220.

Montoliu, C., Monfort, P., Carrasco, J., Palacios, O., Capdevila, M., Hidalgo, J. & Felipo, V. (2000) Metallothionein-III prevents glutamate and nitric oxide neurotoxicity in primary cultures of cerebellar neurons. *J. Neurochem.* 75, 266-273.

Moosmann, B. & Behl, C. (2002) Antioxidants as treatment for neurodegenerative disorders. *Expert. Opin. Investig. Drugs* 11, 1407-1435.

Moosmann, B., Skutella, T., Beyer, K. & Behl, C. (2001) Protective activity of aromatic amines and imines against oxidative nerve cell death. *Biol. Chem.* 382, 1601-1612.

Morimoto, K., Murasugi, T. & Oda, T. (2002) Acute neuroinflammation exacerbates excitotoxicity in rat hippocampus in vivo. *Exp. Neurol.* 177, 95-104.

Morris, C.M., Candy, J.M., Edwardson, J.A., Bloxham, C.A. & Smith, A. (1993) Evidence for the localization of haemopexin immunoreactivity in neurones in the human brain. *Neurosci. Lett.* 149, 141-144.

Morrison, B.3., Pringle, A.K., McManus, T., Ellard, J., Bradley, M., Signorelli, F., Iannotti, F. & Sundstrom, L.E. (2002) L-arginyl-3,4-spermidine is neuroprotective in several in vitro models of neurodegeneration and in vivo ischaemia without suppressing synaptic transmission. *Br. J. Pharmacol.* 137, 1255-1268.

Muller, D., Buchs, P.A. & Stoppini, L. (1993) Time course of synaptic development in hippocampal organotypic cultures. *Brain Res. Dev. Brain Res.* 71, 93-100.

Mullershausen, F., Friebe, A., Feil, R., Thompson, W.J., Hofmann, F. & Koesling, D. (2003) Direct activation of PDE5 by cGMP: long-term effects within NO/cGMP signaling. *J. Cell Biol.* 160, 719-727.

Murphy, M.P. (1999) Nitric oxide and cell death. *Biochim. Biophys. Acta* 1411, 401-414.

Nandagopal, K., Dawson, T.M. & Dawson, V.L. (2001) Critical role for nitric oxide signaling in cardiac and neuronal ischemic preconditioning and tolerance. *J. Pharmacol. Exp. Ther.* 297, 474-478.

Naseem, I., Ahmad, M. & Hadi, S.M. (1988) Effect of alkylated and intercalated DNA on the generation of superoxide anion by riboflavin. *Biosci. Rep.* 8, 485-492.

Nedospasov, A., Rafikov, R., Beda, N. & Nudler, E. (2000) An autocatalytic mechanism of protein nitrosylation. *Proc. Natl. Acad. Sci. U. S. A* 97, 13543-13548.

Newell, D.W., Barth, A., Papermaster, V. & Malouf, A.T. (1995) Glutamate and non-glutamate receptor mediated toxicity caused by oxygen and glucose deprivation in organotypic hippocampal cultures. *J. Neurosci.* 15, 7702-7711.

Nicotera, P., Leist, M. & Manzo, L. (1999) Neuronal cell death: a demise with different shapes. *Trends Pharmacol. Sci.* 20, 46-51.

Nikonenko, I., Jourdain, P. & Muller, D. (2003) Presynaptic remodeling contributes to activity-dependent synaptogenesis. *J. Neurosci.* 23, 8498-8505.

Nurse, S. & Corbett, D. (1996) Neuroprotection after several days of mild, drug-induced hypothermia. *J. Cereb. Blood Flow Metab* 16, 474-480.

O'Dell, T.J., Hawkins, R.D., Kandel, E.R. & Arancio, O. (1991) Tests of the roles of two diffusible substances in long-term potentiation: evidence for nitric oxide as a possible early retrograde messenger. *Proc. Natl. Acad. Sci. U. S. A* 88, 11285-11289.

O'Donnell, V.B., Chumley, P.H., Hogg, N., Bloodsworth, A., Darley-Usmar, V.M. & Freeman, B.A. (1997) Nitric oxide inhibition of lipid peroxidation: kinetics of reaction with lipid peroxyl radicals and comparison with alpha-tocopherol. *Biochemistry* 36, 15216-15223.

O'Donnell, V.B., Coles, B., Lewis, M.J., Crews, B.C., Marnett, L.J. & Freeman, B.A. (2000) Catalytic consumption of nitric oxide by prostaglandin H synthase-1 regulates platelet function. *J. Biol. Chem.* 275, 38239-38244.

O'Donnell, V.B. & Freeman, B.A. (2001) Interactions between nitric oxide and lipid oxidation pathways: implications for vascular disease. *Circ. Res.* 88, 12-21.

O'Donnell, V.B., Taylor, K.B., Parthasarathy, S., Kuhn, H., Koesling, D., Friebe, A., Bloodsworth, A., Darley-Usmar, V.M. & Freeman, B.A. (1999) 15-Lipoxygenase catalytically consumes nitric oxide and impairs activation of guanylate cyclase. *J. Biol. Chem.* 274, 20083-20091.

O'Neill, M.J., Murray, T.K., McCarty, D.R., Hicks, C.A., Dell, C.P., Patrick, K.E., Ward, M.A., Osborne, D.J., Wiernicki, T.R., Roman, C.R., Lodge, D., Fleisch, J.H. & Singh, J. (2000) ARL 17477, a selective nitric oxide synthase inhibitor, with neuroprotective effects in animal models of global and focal cerebral ischaemia. *Brain Res.* 871, 234-244.

Obrenovitch, T.P., Urenjak, J., Richards, D.A., Ueda, Y., Curzon, G. & Symon, L. (1993) Extracellular neuroactive amino acids in the rat striatum during ischaemia: comparison between penumbral conditions and ischaemia with sustained anoxic depolarisation. *J. Neurochem.* 61, 178-186.

Olney, J.W. (1969) Brain lesions, obesity, and other disturbances in mice treated with monosodium glutamate. *Science* 164, 719-721.

Ou, P. & Wolff, S.P. (1993) Aminoguanidine: a drug proposed for prophylaxis in diabetes inhibits catalase and generates hydrogen peroxide in vitro. *Biochem. Pharmacol.* 46, 1139-1144.

Padmaja, S. & Huie, R.E. (1993) The reaction of nitric oxide with organic peroxyl radicals. *Biochem. Biophys. Res. Commun.* 195, 539-544.

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

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

Parmentier, S., Bohme, G.A., Lerouet, D., Damour, D., Stutzmann, J.M., Margaill, I. & Plotkine, M. (1999) Selective inhibition of inducible nitric oxide synthase prevents ischaemic brain injury. *Br. J. Pharmacol.* 127, 546-552.

Parmentier-Batteur, S., Bohme, G.A., Lerouet, D., Zhou-Ding, L., Beray, V., Margaill, I. & Plotkine, M. (2001) Antisense oligodeoxynucleotide to inducible nitric oxide synthase protects against transient focal cerebral ischemia-induced brain injury. *J. Cereb. Blood Flow Metab* 21, 15-21.

Pauwels, P.J. & Leysen, J.E. (1992) Blockade of nitric oxide formation does not prevent glutamate-induced neurotoxicity in neuronal cultures from rat hippocampus. *Neurosci. Lett.* 143, 27-30.

Pearce, L.L., Gandley, R.E., Han, W., Wasserloos, K., Stitt, M., Kanai, A.J., McLaughlin, M.K., Pitt, B.R. & Levitan, E.S. (2000) Role of metallothionein in

nitric oxide signaling as revealed by a green fluorescent fusion protein. *Proc. Natl. Acad. Sci. U. S. A* 97, 477-482.

Pearce, L.L., Kanai, A.J., Birder, L.A., Pitt, B.R. & Peterson, J. (2002) The catabolic fate of nitric oxide: the nitric oxide oxidase and peroxynitrite reductase activities of cytochrome oxidase. *J. Biol. Chem.* 277, 13556-13562.

Piantadosi, C.A. & Zhang, J. (1996) Mitochondrial generation of reactive oxygen species after brain ischemia in the rat. *Stroke* 27, 327-331.

Pieper, A.A., Verma, A., Zhang, J. & Snyder, S.H. (1999) Poly (ADP-ribose) polymerase, nitric oxide and cell death. *Trends Pharmacol. Sci.* 20, 171-181.

Plaschke, K., Kopitz, J., Weigand, M.A., Martin, E. & Bardenheuer, H.J. (2000) The neuroprotective effect of cerebral poly(ADP-ribose)polymerase inhibition in a rat model of global ischemia. *Neurosci. Lett.* 284, 109-112.

Pluta, R.M., Rak, R., Wink, D.A., Woodward, J.J., Khaldi, A., Oldfield, E.H. & Watson, J.C. (2001) Effects of nitric oxide on reactive oxygen species production and infarction size after brain reperfusion injury. *Neurosurgery* 48, 884-892.

Poderoso, J.J., Carreras, M.C., Lisdero, C., Riobo, N., Schopfer, F. & Boveris, A. (1996) Nitric oxide inhibits electron transfer and increases superoxide radical production in rat heart mitochondria and submitochondrial particles. *Arch. Biochem. Biophys.* 328, 85-92.

Poderoso, J.J., Peralta, J.G., Lisdero, C.L., Carreras, M.C., Radisic, M., Schopfer, F., Cadenas, E. & Boveris, A. (1998) Nitric oxide regulates oxygen uptake and hydrogen peroxide release by the isolated beating rat heart. *Am. J. Physiol* 274, C112-C119.

Polli, J.W. & Kincaid, R.L. (1994) Expression of a calmodulin-dependent phosphodiesterase isoform (PDE1B1) correlates with brain regions having extensive dopaminergic innervation. *J. Neurosci.* 14, 1251-1261.

Pringle, A.K., Iannotti, F., Wilde, G.J., Chad, J.E., Seeley, P.J. & Sundstrom, L.E. (1997b) Neuroprotection by both NMDA and non-NMDA receptor antagonists in in vitro ischemia. *Brain Res.* 755, 36-46.

Probert, A.W., Borosky, S., Marcoux, F.W. & Taylor, C.P. (1997) Sodium channel modulators prevent oxygen and glucose deprivation injury and glutamate release in rat neocortical cultures. *Neuropharmacology* 36, 1031-1038.

Reiter, C.D., Teng, R.J. & Beckman, J.S. (2000) Superoxide reacts with nitric oxide to nitrate tyrosine at physiological pH via peroxynitrite. *J. Biol. Chem.* 275, 32460-32466.

Repaske, D.R., Corbin, J.G., Conti, M. & Goy, M.F. (1993) A cyclic GMP-stimulated cyclic nucleotide phosphodiesterase gene is highly expressed in the limbic system of the rat brain. *Neuroscience* 56, 673-686.

Rice, M.E. (2000) Ascorbate regulation and its neuroprotective role in the brain. *Trends Neurosci.* 23, 209-216.

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

Robb, S.J., Gaspers, L.D., Wright, K.J., Thomas, A.P. & Connor, J.R. (1999) Influence of nitric oxide on cellular and mitochondrial integrity in oxidatively stressed astrocytes. *J. Neurosci. Res.* 56, 166-176.

Roettger, V. & Lipton, P. (1996) Mechanism of glutamate release from rat hippocampal slices during in vitro ischemia. *Neuroscience* 75, 677-685.

Rosenberg, P.A., Li, Y., Ali, S., Altiok, N., Back, S.A. & Volpe, J.J. (1999) Intracellular redox state determines whether nitric oxide is toxic or protective to rat oligodendrocytes in culture. *J. Neurochem.* 73, 476-484.

Rossi, D.J., Oshima, T. & Attwell, D. (2000) Glutamate release in severe brain ischaemia is mainly by reversed uptake. *Nature* 403, 316-321.

Roubaud, V., Sankarapandi, S., Kuppusamy, P., Tordo, P. & Zweier, J.L. (1997) Quantitative measurement of superoxide generation using the spin trap 5- (diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide. *Anal. Biochem.* 247, 404-411.

Roychowdhury, S., Luthe, A., Keilhoff, G., Wolf, G. & Horn, T.F. (2002) Oxidative stress in glial cultures: detection by DAF-2 fluorescence used as a tool to measure peroxynitrite rather than nitric oxide. *Glia* 38, 103-114.

Ruiz, F., Alvarez, G., Ramos, M., Hernandez, M., Bogonez, E. & Satrustegui, J. (2000) Cyclosporin A targets involved in protection against glutamate excitotoxicity. *Eur. J. Pharmacol.* 404, 29-39.

Russwurm, M., Behrends, S., Harteneck, C. & Koesling, D. (1998) Functional properties of a naturally occurring isoform of soluble guanylyl cyclase. *Biochem. J.* 335, 125-130.

Russwurm, M., Wittau, N. & Koesling, D. (2001) Guanylyl cyclase/PSD-95 interaction: targeting of the nitric oxide-sensitive alpha2beta1 guanylyl cyclase to synaptic membranes. *J. Biol. Chem.* 276, 44647-44652.

Rybalkin, S.D., Rybalkina, I.G., Shimizu-Albergine, M., Tang, X.B. & Beavo, J.A. (2003) PDE5 is converted to an activated state upon cGMP binding to the GAF A domain. *EMBO J.* 22, 469-478.

Saido, T.C., Sorimachi, H. & Suzuki, K. (1994) Calpain: new perspectives in molecular diversity and physiological-pathological involvement. *FASEB J.* 8, 814-822.

Salter, M., Duffy, C., Garthwaite, J. & Strijbos, P.J. (1995) Substantial regional and hemispheric differences in brain nitric oxide synthase (NOS)

inhibition following intracerebroventricular administration of N omega-nitro-L-arginine (L-NA) and its methyl ester (L-NAME). *Neuropharmacology* 34, 639-649.

Samdani, A.F., Dawson, T.M. & Dawson, V.L. (1997a) Nitric oxide synthase in models of focal ischemia. *Stroke* 28, 1283-1288.

Samdani, A.F., Newcamp, C., Resink, A., Facchinetti, F., Hoffman, B.E., Dawson, V.L. & Dawson, T.M. (1997b) Differential susceptibility to neurotoxicity mediated by neurotrophins and neuronal nitric oxide synthase. *J. Neurosci.* 17, 4633-4641.

Santizo, R., Baughman, V.L. & Pelligrino, D.A. (2000) Relative contributions from neuronal and endothelial nitric oxide synthases to regional cerebral blood flow changes during forebrain ischemia in rats. *Neuroreport* 11, 1549-1553.

Sattler, R., Xiong, Z., Lu, W.Y., Hafner, M., MacDonald, J.F. & Tymianski, M. (1999) Specific coupling of NMDA receptor activation to nitric oxide neurotoxicity by PSD-95 protein. *Science* 284, 1845-1848.

Schmidt, K., Desch, W., Klatt, P., Kukovetz, W.R. & Mayer, B. (1997) Release of nitric oxide from donors with known half-life: a mathematical model for calculating nitric oxide concentrations in aerobic solutions. *Naunyn Schmiedebergs Arch. Pharmacol.* 355, 457-462.

Schmidt, K., Pfeiffer, S. & Mayer, B. (1998) Reaction of peroxynitrite with HEPES or MOPS results in the formation of nitric oxide donors. *Free Radic. Biol. Med.* 24, 859-862.

Seress, L., Abraham, H., Lin, H. & Totterdell, S. (2002) Nitric oxide-containing pyramidal neurons of the subiculum innervate the CA1 area. *Exp. Brain Res.* 147, 38-44.

Sharma, V.S. & Magde, D. (1999) Activation of soluble guanylate cyclase by carbon monoxide and nitric oxide: a mechanistic model. *Methods* 19, 494-505.

Sharpe, M.A., Robb, S.J. & Clark, J.B. (2003) Nitric oxide and Fenton/Haber-Weiss chemistry: nitric oxide is a potent antioxidant at physiological concentrations. *J. Neurochem.* 87, 386-394.

Shen, W., Hintze, T.H. & Wolin, M.S. (1995) Nitric oxide. An important signaling mechanism between vascular endothelium and parenchymal cells in the regulation of oxygen consumption. *Circulation* 92, 3505-3512.

Shen, W., Xu, X., Ochoa, M., Zhao, G., Wolin, M.S. & Hintze, T.H. (1994) Role of nitric oxide in the regulation of oxygen consumption in conscious dogs. *Circ. Res.* 75, 1086-1095.

Shibuki, K. & Kimura, S. (1997) Dynamic properties of nitric oxide release from parallel fibres in rat cerebellar slices. *J. Physiol (Lond)* 498 (Pt 2), 443-452.

Shipulina, N., Hunt, R.C., Shaklai, N. & Smith, A. (1998) Coordination of nitric oxide by heme-hemopexin. *J. Protein Chem.* 17, 255-260.

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

Simonson, S.G., Zhang, J., Canada, A.T., Jr., Su, Y.F., Benveniste, H. & Piantadosi, C.A. (1993) Hydrogen peroxide production by monoamine oxidase during ischemia- reperfusion in the rat brain. *J. Cereb. Blood Flow Metab* 13, 125-134.

Simpson, J.A., Cheeseman, K.H., Smith, S.E. & Dean, R.T. (1988) Free-radical generation by copper ions and hydrogen peroxide. Stimulation by Hepes buffer. *Biochem. J.* 254, 519-523.

Smith, K.J. & Lassmann, H. (2002) The role of nitric oxide in multiple sclerosis. *Lancet Neurol.* 1, 232-241.

Smith, S.E. & Meldrum, B.S. (1995) Cerebroprotective effect of lamotrigine after focal ischemia in rats. *Stroke* 26, 117-121.

Smith, W.D. (1967) A history of nitrous oxide and oxygen anaesthesia. X. The early manufacture, storage and purity of nitrous oxide. *Br. J. Anaesth.* 39, 351-381.

Soderling, S.H. & Beavo, J.A. (2000) Regulation of cAMP and cGMP signaling: new phosphodiesterases and new functions. *Curr. Opin. Cell Biol.* 12, 174-179.

Sola, C., Casal, C., Tusell, J.M. & Serratosa, J. (2002) Astrocytes enhance lipopolysaccharide-induced nitric oxide production by microglial cells. *Eur. J. Neurosci.* 16, 1275-1283.

Southam, E. & Garthwaite, J. (1993) The nitric oxide-cyclic GMP signalling pathway in rat brain. *Neuropharmacology* 32, 1267-1277.

Spikes JD (1989) Photosensitization. In: The Science Of Photobiology (Smith KC, ed), pp 79-110. Plenum Press.

Stewart, V.C. & Heales, S.J. (2003) Nitric oxide-induced mitochondrial dysfunction: implications for neurodegeneration. *Free Radic. Biol. Med.* 34, 287-303.

Stewart, V.C., Heslegrave, A.J., Brown, G.C., Clark, J.B. & Heales, S.J. (2002) Nitric oxide-dependent damage to neuronal mitochondria involves the NMDA receptor. *Eur. J. Neurosci.* 15, 458-464.

Stewart, V.C., Sharpe, M.A., Clark, J.B. & Heales, S.J. (2000) Astrocyte-derived nitric oxide causes both reversible and irreversible damage to the neuronal mitochondrial respiratory chain. *J. Neurochem.* 75, 694-700.

Stingele, R., Wilson, D.A., Traystman, R.J. & Hanley, D.F. (1998) Tyrosine confounds oxidative electrochemical detection of nitric oxide. *Am. J. Physiol* 274, H1698-H1704.

Stoien, J.D. & Wang, R.J. (1974) Effect of near-ultraviolet and visible light on mammalian cells in culture II. Formation of toxic photoproducts in tissue culture medium by blacklight. *Proc. Natl. Acad. Sci. U. S. A* 71, 3961-3965.

Stone, J.R. & Marletta, M.A. (1996) Spectral and kinetic studies on the activation of soluble guanylate cyclase by nitric oxide. *Biochemistry* 35, 1093-1099.

Stoppini, L., Buchs, P.A. & Muller, D. (1991) A simple method for organotypic cultures of nervous tissue. *J. Neurosci. Methods* 37, 173-182.

Stoppini, L., Buchs, P.A. & Muller, D. (1993) Lesion-induced neurite sprouting and synapse formation in hippocampal organotypic cultures. *Neuroscience* 57, 985-994.

Strasser, U. & Fischer, G. (1995) Quantitative measurement of neuronal degeneration in organotypic hippocampal cultures after combined oxygen/glucose deprivation. *J. Neurosci. Methods* 57, 177-186.

Strijbos, P.J. (1998) Nitric oxide in cerebral ischemic neurodegeneration and excitotoxicity. *Crit Rev. Neurobiol.* 12, 223-243.

Strijbos, P.J., Leach, M.J. & Garthwaite, J. (1996) Vicious cycle involving Na+ channels, glutamate release, and NMDA receptors mediates delayed neurodegeneration through nitric oxide formation. *J. Neurosci.* 16, 5004-5013.

Strijbos, P.J., Pratt, G.D., Khan, S., Charles, I.G. & Garthwaite, J. (1999) Molecular characterization and in situ localization of a full-length cyclic nucleotide-gated channel in rat brain. *Eur. J. Neurosci.* 11, 4463-4467.

Stubauer, G., Giuffre, A., Brunori, M. & Sarti, P. (1998) Cytochrome c oxidase does not catalyze the anaerobic reduction of NO. *Biochem. Biophys. Res. Commun.* 245, 459-465.

Stuehr, D.J. & Marletta, M.A. (1985) Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to Escherichia coli lipopolysaccharide. *Proc. Natl. Acad. Sci. U. S. A* 82, 7738-7742.

Sugimoto, K. & Iadecola, C. (2002) Effects of aminoguanidine on cerebral ischemia in mice: comparison between mice with and without inducible nitric oxide synthase gene. *Neurosci. Lett.* 331, 25-28.

Suzuki, N., Kojima, H., Urano, Y., Kikuchi, K., Hirata, Y. & Nagano, T. (2002) Orthogonality of calcium concentration and ability of 4,5- diaminofluorescein to detect NO. *J. Biol. Chem.* 277, 47-49.

Szabo, C. (2003) Multiple pathways of peroxynitrite cytotoxicity. *Toxicol. Lett.* 140-141, 105-112.

Szabo, C., Mabley, J.G., Moeller, S.M., Shimanovich, R., Pacher, P., Virag, L., Soriano, F.G., Van Duzer, J.H., Williams, W., Salzman, A.L. & Groves, J.T. (2002) Part I: Pathogenetic Role of Peroxynitrite in the Development of Diabetes and Diabetic Vascular Complications: Studies With FP15, A Novel Potent Peroxynitrite Decomposition Catalyst. *Mol. Med.* 8, 571-580.

Szatkowski, M. & Attwell, D. (1994) Triggering and execution of neuronal death in brain ischaemia: two phases of glutamate release by different mechanisms. *Trends. Neurosci.* 17, 359-365.

Szatkowski, M., Barbour, B. & Attwell, D. (1990) Non-vesicular release of glutamate from glial cells by reversed electrogenic glutamate uptake. *Nature* 348, 443-446.

Tanaka, K., Shirai, T., Nagata, E., Dembo, T. & Fukuuchi, Y. (1997) Immunohistochemical detection of nitrotyrosine in postischemic cerebral cortex in gerbil. *Neurosci. Lett.* 235, 85-88.

Taylor, C.P. & Meldrum, B.S. (1995) Na+ channels as targets for neuroprotective drugs. *Trends Pharmacol. Sci.* 16, 309-316.

Teunissen, C., Steinbusch, H., Markerink-van Ittersum, M., Koesling, D. & de Vente, J. (2001) Presence of soluble and particulate guanylyl cyclase in the same hippocampal astrocytes. *Brain Res.* 891, 206-212.

Thomas, D.D., Liu, X., Kantrow, S.P. & Lancaster, J.R., Jr. (2001) The biological lifetime of nitric oxide: implications for the perivascular dynamics of NO and O2. *Proc. Natl. Acad. Sci. U. S. A* 98, 355-360.

Tominaga, T., Sato, S., Ohnishi, T. & Ohnishi, S.T. (1993) Potentiation of nitric oxide formation following bilateral carotid occlusion and focal cerebral ischemia in the rat: in vivo detection of the nitric oxide radical by electron paramagnetic resonance spin trapping. *Brain Res.* 614, 342-346.

Topel, I., Stanarius, A. & Wolf, G. (1998) Distribution of the endothelial constitutive nitric oxide synthase in the developing rat brain: an immunohistochemical study. *Brain Res.* 788, 43-48.

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

Toung, T.J., Bhardwaj, A., Dawson, V.L., Dawson, T.M., Traystman, R.J. & Hurn, P.D. (1999) Neuroprotective FK506 does not alter in vivo nitric oxide

production during ischemia and early reperfusion in rats. *Stroke* 30, 1279-1285.

Trabace, L. & Kendrick, K.M. (2000) Nitric oxide can differentially modulate striatal neurotransmitter concentrations via soluble guanylate cyclase and peroxynitrite formation. *J. Neurochem.* 75, 1664-1674.

Trackey, J.L., Uliasz, T.F. & Hewett, S.J. (2001) SIN-1-induced cytotoxicity in mixed cortical cell culture: peroxynitrite-dependent and -independent induction of excitotoxic cell death. *J. Neurochem.* 79, 445-455.

Valtschanoff, J.G., Weinberg, R.J., Kharazia, V.N., Nakane, M. & Schmidt, H.H. (1993) Neurons in rat hippocampus that synthesize nitric oxide. *J. Comp Neurol.* 331, 111-121.

van Staveren, W.C., Markerink-van Ittersum, M., Steinbusch, H.W. & de Vente, J. (2001) The effects of phosphodiesterase inhibition on cyclic GMP and cyclic AMP accumulation in the hippocampus of the rat. *Brain Res.* 888, 275-286.

van Staveren, W.C., Steinbusch, H.W., Markerink-van Ittersum, M., Repaske, D.R., Goy, M.F., Kotera, J., Omori, K., Beavo, J.A. & de Vente, J. (2003) mRNA expression patterns of the cGMP-hydrolyzing phosphodiesterases types 2, 5, and 9 during development of the rat brain. *J. Comp Neurol.* 467, 566-580.

Vaughn, M.W., Huang, K.T., Kuo, L. & Liao, J.C. (2000) Erythrocytes possess an intrinsic barrier to nitric oxide consumption. *J. Biol. Chem.* 275, 2342-2348.

Veltkamp, R., Rajapakse, N., Robins, G., Puskar, M., Shimizu, K. & Busija, D. (2002) Transient focal ischemia increases endothelial nitric oxide synthase in cerebral blood vessels. *Stroke* 33, 2704-2710.

Vidwans, A.S., Kim, S., Coffin, D.O., Wink, D.A. & Hewett, S.J. (1999) Analysis of the neuroprotective effects of various nitric oxide donor compounds in murine mixed cortical cell culture. *J. Neurochem.* 72, 1843-1852.

Virag, L., Szabo, E., Gergely, P. & Szabo, C. (2003) Peroxynitrite-induced cytotoxicity: mechanism and opportunities for intervention. *Toxicol. Lett.* 140-141, 113-124.

Vornov, J.J., Tasker, R.C. & Coyle, J.T. (1991) Direct observation of the agonist-specific regional vulnerability to glutamate, NMDA, and kainate neurotoxicity in organotypic hippocampal cultures. *Exp. Neurol.* 114, 11-22.

Vornov, J.J., Tasker, R.C. & Coyle, J.T. (1994) Delayed protection by MK-801 and tetrodotoxin in a rat organotypic hippocampal culture model of ischemia. *Stroke* 25, 457-464.

Wagner, D.A., Young, V.R. & Tannenbaum, S.R. (1983) Mammalian nitrate biosynthesis: incorporation of 15NH3 into nitrate is enhanced by endotoxin treatment. *Proc. Natl. Acad. Sci. U. S. A* 80, 4518-4521.

Waldman, S.A. & Murad, F. (1987) Cyclic GMP synthesis and function. *Pharmacol. Rev.* 39, 163-196.

Wang, K.K., Nath, R., Posner, A., Raser, K.J., Buroker-Kilgore, M., Hajimohammadreza, I., Probert, A.W., Jr., Marcoux, F.W., Ye, Q., Takano, E., Hatanaka, M., Maki, M., Caner, H., Collins, J.L., Fergus, A., Lee, K.S., Lunney, E.A., Hays, S.J. & Yuen, P. (1996) An alpha-mercaptoacrylic acid derivative is a selective nonpeptide cell-permeable calpain inhibitor and is neuroprotective. *Proc. Natl. Acad. Sci. U. S. A* 93, 6687-6692.

Wang, R.J. & Nixon, B.R. (1978) Identification of hydrogen peroxide as a photoproduct toxic to human cells in tissue-culture medium irradiated with "daylight" fluorescent light. *In Vitro* 14, 715-722.

Wang, X. & Robinson, P.J. (1995) Cyclic GMP-dependent protein kinase substrates in rat brain. *J. Neurochem.* 65, 595-604.

Weber, M.L. & Taylor, C.P. (1994) Damage from oxygen and glucose deprivation in hippocampal slices is prevented by tetrodotoxin, lidocaine and phenytoin without blockade of action potentials. *Brain Res.* 664, 167-177.

Wedel, B., Humbert, P., Harteneck, C., Foerster, J., Malkewitz, J., Bohme, E., Schultz, G. & Koesling, D. (1994) Mutation of His-105 in the beta 1 subunit yields a nitric oxide-insensitive form of soluble guanylyl cyclase. *Proc. Natl. Acad. Sci. U. S. A* 91, 2592-2596.

Wei, G., Dawson, V.L. & Zweier, J.L. (1999) Role of neuronal and endothelial nitric oxide synthase in nitric oxide generation in the brain following cerebral ischemia. *Biochim. Biophys. Acta* 1455, 23-34.

Wiard, R.P., Dickerson, M.C., Beek, O., Norton, R. & Cooper, B.R. (1995) Neuroprotective properties of the novel antiepileptic lamotrigine in a gerbil model of global cerebral ischemia. *Stroke* 26, 466-472.

Wink, D.A., Darbyshire, J.F., Nims, R.W., Saavedra, J.E. & Ford, P.C. (1993) Reactions of the bioregulatory agent nitric oxide in oxygenated aqueous media: determination of the kinetics for oxidation and nitrosation by intermediates generated in the NO/O2 reaction. *Chem. Res. Toxicol.* 6, 23-27.

Wink, D.A., Miranda, K.M., Espey, M.G., Pluta, R.M., Hewett, S.J., Colton, C., Vitek, M., Feelisch, M. & Grisham, M.B. (2001) Mechanisms of the antioxidant effects of nitric oxide. *Antioxid. Redox. Signal.* 3, 203-213.

Wood, A.M., Tiwari, P. & Bristow, D.R. (1997) Media composition modulates excitatory amino acid-induced death of rat cerebellar granule cells. *Hum. Exp. Toxicol.* 16, 350-355.

Wu, W.C., Wang, Y., Su, C.K. & Chai, C.Y. (2001) The nNOS/cGMP signal transducing system is involved in the cardiovascular responses induced by activation of NMDA receptors in the rostral ventrolateral medulla of cats. *Neurosci. Lett.* 310, 121-124.

Wykes, V., Bellamy, T.C. & Garthwaite, J. (2002) Kinetics of nitric oxide-cyclic GMP signalling in CNS cells and its possible regulation by cyclic GMP. *J. Neurochem.* 83, 37-47.

Xia, Y., Dawson, V.L., Dawson, T.M., Snyder, S.H. & Zweier, J.L. (1996) Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. *Proc. Natl. Acad. Sci. U. S. A* 93, 6770-6774.

Xia, Y., Roman, L.J., Masters, B.S. & Zweier, J.L. (1998a) Inducible nitric-oxide synthase generates superoxide from the reductase domain. *J. Biol. Chem.* 273, 22635-22639.

Xia, Y., Tsai, A.L., Berka, V. & Zweier, J.L. (1998b) Superoxide generation from endothelial nitric-oxide synthase. A Ca2+/calmodulin-dependent and tetrahydrobiopterin regulatory process. *J. Biol. Chem.* 273, 25804-25808.

Xu, J., He, L., Ahmed, S.H., Chen, S.W., Goldberg, M.P., Beckman, J.S. & Hsu, C.Y. (2000) Oxygen-glucose deprivation induces inducible nitric oxide synthase and nitrotyrosine expression in cerebral endothelial cells. *Stroke* 31, 1744-1751.

Yamada, M., Huang, Z., Dalkara, T., Endres, M., Laufs, U., Waeber, C., Huang, P.L., Liao, J.K. & Moskowitz, M.A. (2000) Endothelial nitric oxide synthase-dependent cerebral blood flow augmentation by L-arginine after chronic statin treatment. *J. Cereb. Blood Flow Metab* 20, 709-717.

Yildiz, G., Demiryurek, A.T., Sahin-Erdemli, I. & Kanzik, I. (1998) Comparison of antioxidant activities of aminoguanidine, methylguanidine and guanidine by luminol-enhanced chemiluminescence. *Br. J. Pharmacol.* 124, 905-910.

Yim, M.B., Chock, P.B. & Stadtman, E.R. (1990) Copper, zinc superoxide dismutase catalyzes hydroxyl radical production from hydrogen peroxide. *Proc. Natl. Acad. Sci. U. S. A* 87, 5006-5010.

Yoshida, T., Limmroth, V., Irikura, K. & Moskowitz, M.A. (1994) The NOS inhibitor, 7-nitroindazole, decreases focal infarct volume but not the response to topical acetylcholine in pial vessels. *J. Cereb. Blood Flow Metab* 14, 924-929.

Yoshioka, Y., Yamamuro, A. & Maeda, S. (2003) Nitric oxide at a low concentration protects murine macrophage RAW264 cells against nitric oxide-induced death via cGMP signaling pathway. *Br. J. Pharmacol.* 139, 28-34.

Zabel, U., Kleinschnitz, C., Oh, P., Nedvetsky, P., Smolenski, A., Muller, H., Kronich, P., Kugler, P., Walter, U., Schnitzer, J.E. & Schmidt, H.H. (2002)

Calcium-dependent membrane association sensitizes soluble guanylyl cyclase to nitric oxide. *Nat. Cell Biol.* 4, 307-311.

Zhang, F., Casey, R.M., Ross, M.E. & Iadecola, C. (1996a) Aminoguanidine ameliorates and L-arginine worsens brain damage from intraluminal middle cerebral artery occlusion. *Stroke* 27, 317-323.

Zhang, R., Wang, L., Zhang, L., Chen, J., Zhu, Z., Zhang, Z. & Chopp, M. (2003) Nitric oxide enhances angiogenesis via the synthesis of vascular endothelial growth factor and cGMP after stroke in the rat. *Circ. Res.* 92, 308-313.

Zhang, R., Wang, Y., Zhang, L., Zhang, Z., Tsang, W., Lu, M., Zhang, L. & Chopp, M. (2002a) Sildenafil (Viagra) induces neurogenesis and promotes functional recovery after stroke in rats. *Stroke* 33, 2675-2680.

Zhang, R., Zhang, L., Zhang, Z., Wang, Y., Lu, M., Lapointe, M. & Chopp, M. (2001) A nitric oxide donor induces neurogenesis and reduces functional deficits after stroke in rats. *Ann. Neurol.* 50, 602-611.

Zhang, X., Kim, W.S., Hatcher, N., Potgieter, K., Moroz, L.L., Gillette, R. & Sweedler, J.V. (2002b) Interfering with nitric oxide measurements. 4,5-diaminofluorescein reacts with dehydroascorbic acid and ascorbic acid. *J. Biol. Chem.* 277, 48472-48478.

Zhang, Z.G., Reif, D., Macdonald, J., Tang, W.X., Kamp, D.K., Gentile, R.J., Shakespeare, W.C., Murray, R.J. & Chopp, M. (1996b) ARL 17477, a potent and selective neuronal NOS inhibitor decreases infarct volume after transient middle cerebral artery occlusion in rats. *J. Cereb. Blood Flow Metab* 16, 599-604.

Zhao, X., Ross, M.E. & Iadecola, C. (2003) L-Arginine increases ischemic injury in wild-type mice but not in iNOS- deficient mice. *Brain Res.* 966, 308-311.

Zhao, Y., Brandish, P.E., Ballou, D.P. & Marletta, M.A. (1999) A molecular basis for nitric oxide sensing by soluble guanylate cyclase. *Proc. Natl. Acad. Sci. U. S. A* 96, 14753-14758.

Zhu, D.Y., Liu, S.H., Sun, H.S. & Lu, Y.M. (2003) Expression of inducible nitric oxide synthase after focal cerebral ischemia stimulates neurogenesis in the adult rodent dentate gyrus. *J. Neurosci.* 23, 223-229.

Zigler, J.S., Jr., Lepe-Zuniga, J.L., Vistica, B. & Gery, I. (1985) Analysis of the cytotoxic effects of light-exposed HEPES-containing culture medium. *In Vitro Cell Dev. Biol.* 21, 282-287.