PTERIN BIOSYNTHESIS, BINDING & MODULATION OF eNOS CATALYTIC FUNCTION

By

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Summary

Tetrahydrobiopterin (BH₄) is a limiting cofactor for nitric oxide synthase (NOS) catalysed conversion of L-arginine to nitric oxide and citrulline. Content of BH₄ in mammalian cells is regulated at many levels, but most important is de novo biosynthesis from GTP. GTP cyclohydrolase (GTPCH) is the rate-limiting enzyme for the de novo synthesis of BH₄.

While various immunostimulants, hormones and growth factors have been reported to increase GTPCH mRNA levels and intracellular biopterin (BH₄ degradation product), it is not known whether these factors act at the level of GTPCH gene transcription. To test this I utilised 1, 3 and 6 kb 5'upstream GTPCH gene sequence in a secreted alkaline phosphatase reporter vector (SEAP). These constructs were stably transfected in PC-12 cells and rat aortic smooth muscle cells, and the cells were treated with various immunostimulants and growth factors in order to determine whether these factors could enhance GTPCH gene transcription. Intracellular biopterin levels were also measured to confirm that the upregulation of the SEAP-reporter correlated with a rise in biopterin. Our investigations conclude that transcriptional regulation of the GTPCH gene is indeed a major site for control of intracellular BH₄ levels.

In further experiments, we have characterised the binding of [³H]BH₄ to endothelial NOS (eNOS) and examined influences of the substrate, arginine, on the BH₄ binding. In addition we selected tetrahydropterins (that support NOS catalysis) and dihydropterins (that are catalytically incompetent) to determine the extent to which modifications of BH₄ alter pterin binding affinity to eNOS.

Dihydropterins are unable to support NOS catalysis. Studies showed for the first time that dihydropterins, but not tetrahydropterins, support superoxide generation by eNOS. We also have determined that eNOS may be able to produce NO in the absence of BH₄ cofactor from the reaction intermediate hydroxyarginine. We have characterised this reaction and are able to provide a plausible mechanism for the NOₓ generation from eNOS in the absence of BH₄ cofactor.
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<tbody>
<tr>
<td>AAAH</td>
<td>Aromatic amino acid hydroxylases</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>Ad</td>
<td>Adrenaline</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine 5’monophosphate</td>
</tr>
<tr>
<td>Arg</td>
<td>L-arginine</td>
</tr>
<tr>
<td>AS</td>
<td>Argininosuccinate</td>
</tr>
<tr>
<td>BH₂</td>
<td>7,8-dihydrobiopterin</td>
</tr>
<tr>
<td>BH₄</td>
<td>(6R) 5,6,7,8-tetrahydrobiopterin</td>
</tr>
<tr>
<td>CA</td>
<td>Catecholamines</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine mono phosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CHX</td>
<td>Cyclohexamide</td>
</tr>
<tr>
<td>Cit</td>
<td>L-citrulline</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomeglovirus</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response element</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>CBP</td>
<td>cAMP RE binding protein (CREB) binding protein</td>
</tr>
<tr>
<td>CT</td>
<td>Cholera Toxin</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DAHP</td>
<td>2,4-Diamino-6-hydroxy-pyrimidine</td>
</tr>
<tr>
<td>DBCAMP</td>
<td>Dibutryl cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>DBCGMP</td>
<td>Dibutryl cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>DBH</td>
<td>Dopamine β-hydroxylase</td>
</tr>
<tr>
<td>DDC</td>
<td>Dopamine decarboxylase</td>
</tr>
<tr>
<td>DEPMPO</td>
<td>5-diethoxyphosphoryl-5-methyl-1-pyrroline N-oxide</td>
</tr>
<tr>
<td>Dex</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>DHFR</td>
<td>Dihydrofolate reductase</td>
</tr>
<tr>
<td>DHPR</td>
<td>Dihydropteridine reductase</td>
</tr>
<tr>
<td>DNTP</td>
<td>7,8-dihydroneopterin triphosphate</td>
</tr>
<tr>
<td>DPI</td>
<td>Diphenyleneiodonium</td>
</tr>
<tr>
<td>DPTA</td>
<td>Diethylenetriaminepentaacetic acid</td>
</tr>
<tr>
<td>DRD</td>
<td>DOPA-responsive dystonia</td>
</tr>
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</table>
DTT  Dithiothritol
EDTA  Ethylene diaminotetra-acetic acid
EDRF  Endothelium Derived Relaxing Factor
EGTA  Ethylene glycol bis (β-amino ethyl ether) NNN’N’tetra-acetic acid
EPR   Electron paramagnetic resonance
ESI-MS Electrospray ionization mass spectrometry
FAD   Flavin adenine dinucleotide
FMN   Flavin mononucleotide (Riboflavin 5’phosphate)
FSH   Follicle stimulating hormone
GC    Glucocorticoids
GFRP  GTPCH Feedback Regulatory Protein
GRE   Glucocorticoid response element
GTP   Guanosine 5’ triphosphate
GTPCH GTP cyclohydrolase
Haem  iron porphyrin IX
H₂O₂  Hydrogen peroxide
IBMX  Isobutylmethyl xanthine
IL-   Interleukin-
IFN-γ Interferon -γ
IPTG  isopropyl-D-thiogalactoside
LB    Lennox L broth
LPS   lipopolysaccharide
6MePH₄ 6-methyltetrahydropterin
MTT   3-[4,5-Dimethylthiazol-2-yl]2,5-diphenly-tetrazolium bromide
NA    Noradrenaline
NAS   N-acetylsenotomin
NADPH β-Nicotinamide adenine dinucleotide phosphate reduced form
Narg  Nitroarginine
NGF   Nerve Growth Factor
NMA   N°-methylarginine
NMR   Nuclear magnetic resonance
NO    Nitric oxide
NO₃  Combined nitrite and nitrate
NOHA  N°-hydroxy-L-arginine
NOS   Nitric oxide synthase
cNOS, Constitutively expressed NOS
iNOS  Inducible NOS (Type II),
eNOS  Endothelial NOS (Type I)
nNOS  Neuronal NOS (Type III)
O$_2^-$  Superoxide
ONOO  Peroxynitrite
OxLDL  Oxidised low density lipopolysaccharides
PAH  Phenylalanine hydroxylase
PBS  Phosphate buffered saline solution
PC-12 cells  Rat phenocytomma cells
PCD  Pterin 4a-carbinoloaminodehydratase
PH  Phenylalanine hydroxylase
PKA or C  Protein kinase A or C
PNMT  Phenylethanolamine N-methyltransferase
PNPP  p-Nitrophenyl phosphate
PTPS  6-pyruvoyl tetrahydropterin synthase
qBH$_2$  quinoid dihydrobiopterin
RASM cells  Rat aortic smooth muscle cells
RpcAMP  Rp-Adenosine 3’5’ cyclic monophosphothioate
SDS-PAGE  Sodium dodecylsulfate polyacrylamide gel electrophoresis
SEAP  Secreted alkaline phosphatase
Sep  Sepiapterin
sGC  Soluble guanylate cyclase
SOD  Superoxide dismutase
SR  Sepiapterin Reductase
TGFβ  Transforming growth factor β
TH  Tyrosine hydroxylase
TNFα  Tumour necrosis factor α
TrH  Tryptophan hydroxylase
TrK  Tyrosine kinase receptor
Tris  Tris[hydroxymethyl]aminomethane
TrH  Threonine hydroxylase
Tyr  L-Tyrosine
Chapter 1 - INTRODUCTION

1.1 NO and cell signaling: Discovery of NO

The study of nitric oxide as a mammalian cell-signaling molecule has grown into a field in its own right. The origin of this field dates to the early 1980s, when Robert Furchgott and coworkers were studying the response of isolated vascular rings to acetylcholine (ACh). ACh was observed to relax preconstricted aortic smooth muscle but only in the presence of an intact endothelium. It was proposed that acetylcholine caused the release of a factor from the endothelium that resulted in arterial vasodilatation; this factor was termed endothelium-derived relaxing factor (EDRF) (Furchgott et al., 1984; Furchgott & Zawadzki, 1980).

Soluble guanylate cyclase (sGC) converts GTP to cGMP, which can subsequently stimulate specific protein kinases and be degraded to 5’-GMP by a group of phosphodiesterases. In 1978, Murad and coworkers reported that incubation of azide with tracheal smooth muscle in an organ bath stimulated sGC, thereby increasing cGMP levels and causing muscle relaxation (Murad, 1978). Murad and colleagues similarly discovered that nitrogen oxide-containing compounds such as glycerine trinitrite, now known as nitrovasodilators, also elicit smooth muscle relaxation by increasing sGC activity (Fiscus, 1983). Increased levels of cGMP were observed in response to EDRF, apparently via stimulation of soluble guanylate cyclase (Forstermann et al., 1986; Ignarro et al., 1986).
Nitrovasodilators were therefore appreciated as mimics of the endogenous vasodilatory effects of EDRF (Ignarro, 1986).

In the early 1980s it had been reported that mammals excrete more nitrite and nitrate than they ingest (Green et al., 1981; Green et al., 1981). Stuehr and coworkers observed that macrophages, treated with lipopolysaccharide, excreted nitrite and nitrate (Stuehr & Marletta, 1985). The origin of nitrite and nitrate was ascribed to oxidation of NO (Marletta et al., 1988). L-arginine was determined as the substrate in this reaction (Palmer et al., 1988; Sakuma et al., 1988; Schmidt et al., 1988) with the guanidinium nitrogen of arginine forming NO (Rees et al., 1988). In 1987, landmark papers were published showing that citrulline was a co-product with NO (Hibbs et al., 1987; Iyengar et al., 1987).

At the same time NO, appearing to mimic the vasodilatatory effects of EDRF, was proposed to be the identity of EDRF (Ignarro et al., 1987; Palmer et al., 1987). Evidence for a CNS function of NO signaling was provided by studies in which treatment of brain slices with excitatory neurotransmitters was shown to stimulate soluble guanylate cyclase via the apparent mediation of NO (Garthwaite et al., 1988; Knowles, 1989). N°-methylarginine was identified to be a potent and selective inhibitor of the L-arginine NO pathway (Sakuma et al., 1988), providing a tool to demonstrate the role of NO in diverse and important biological systems.
1.2 NOS isoforms

Three isoforms of nitric oxide synthase (NOS) have been identified, encoded by distinct mammalian genes (Xie & Nathan, 1994). Two of the NOS isoforms are constitutively expressed and regulated on a moment-to-moment basis by calcium and calmodulin (CaM). The third isoform (iNOS; NOS2) can be induced by immunologic and inflammatory stimuli in numerous cell types. It is notable that iNOS has CaM bound with such high affinity that it remains bound even after lowering calcium to subnanomolar levels (Cho et al., 1992); accordingly iNOS is continuously active at all levels of intracellular calcium concentrations.

The first NOS to be isolated was from brain and termed neuronal NOS (nNOS) also known as (NOS1: EC 1.14.13.39; Bredt & Snyder, 1990). The deduced sequence for this enzyme revealed that there is considerable sequence homology with the C-terminus of NADPH cytochrome P450 reductases (Bredt et al., 1991; Stuehr & Ikeda-Saito, 1992). It was also discovered that both FAD and FMN were contained in this enzyme in 1:1 stoichiometry (Bredt et al., 1992), this was soon found to also be true for the other NOS isoforms (Stuehr & Ikeda-Saito, 1992; Venema et al., 1997).

nNOS is found in neurons in the brain and enteric nervous system as well as in skeletal muscle. The endothelial isoform (eNOS, NOS3) is found primarily in the endothelial lining of blood vessels, although extravascular loci are apparent. These constitutively expressed isoforms, eNOS and nNOS, are catalytically dormant until
binding of CaM, in a reaction triggered by elevated levels of intracellular Ca$^{2+}$ (Bredt & Snyder, 1990; Schmidt & Murad, 1991). The calcium-independent isoform, iNOS (Cho et al., 1992), was initially isolated from macrophages after treatment with interferon-γ (IFNγ), tumor necrosis factor-α (TNFα) and bacterial lipopolysaccharide (LPS), but has since been found to be expressed in almost all nucleated cell types following exposure to immunostimuli (Buttery, 1994; Xie et al., 1992).

1.2.1 Inducible NOS (iNOS)

Immunostimulants cause upregulation of iNOS gene expression for host defense. The inducible NOS isoform also appears to play a prominent role in wound healing (Schaffer et al., 1996), protecting intestinal (Nathan & Xie, 1994) and airway epithelium mucosa (Guo et al., 1995), and recruiting leukocytes in addition to preventing T-cell activation (Bingisser, 1998). Immunostimulant-induced increases in vascular NO levels can lead to severe hypotension, such as that caused by LPS (Kilbourn et al., 1990), TNFα (Kilbourn et al., 1990), IL-1α (Kilbourn et al., 1992) and IL-2 (Kilbourn & Griffith, 1992). Indeed, iNOS may be responsible for the darker face of NO, as the apparent mediator of septic shock (Kilbourn et al., 1990), in contrast with eNOS which mediates vasoprotective effects of NO.

The development of iNOS knock out (-/-) mice has assisted in elucidating the contribution of iNOS-derived NO to specific pathogens (MacMicking et al., 1997).
Interestingly iNOS knockout mice in non-immune challenged situations are healthy and gain weight at the same rates as heterozygous littermates (MacMicking et al., 1997). Nonetheless, knockout mice reveal that iNOS is critical for host survival following infection with bacteria such as *Mycobacterium tuberculosis* (MacMicking et al., 1997). iNOS appears to be merely beneficial, not essential to iNOS -/- mice when infected with protozoa such as the organism that causes toxoplasmosis; in some areas of the brain, wild type mice displayed the same levels of plasmodium replication as iNOS -/- mice whilst iNOS -/- mice had higher total levels of replication in brain compared to wild type (Macmicking, 1995). Paradoxically, iNOS -/- mice infected with Influenza A virus demonstrated better survival rates than wild type mice; this has been interpreted to indicate that inflammation associated with pathogen-induced iNOS caused greater damage to the host than the virus itself (Akaike, 1996). Finally iNOS appeared to be non-essential to mouse survival following exposure to the malaria causing *plasmodium* (Nathan, 1997).

Despite the inducibility of iNOS in numerous tissues, such as the intestinal epithelium and respiratory epithelium, iNOS may be constitutively present in lung and kidney (Kobzik, 1993; Nathan & Xie, 1994). It has been suggested that NO produced by iNOS in alveoli is stored in the form of S-nitrosothiols (Griscavage *et al*., 1993; Stamler *et al*., 1992). Haemoglobin (Hb) has been reported to pick up NO in lung, as S-NO-Hb and discharge it in arterioles for physiological control of blood flow (Jia, 1996). In normal
physiology, iNOS has also been suggested to play a role in uterine contractility during pregnancy (Bansal, 1997).

There is some evidence that iNOS is organised by intracellular compartmentalization; in macrophages it is contained within vesicles, possibly phagosomes, where it has been suggested to play a role in killing opsonized microorganisms (Vodovotz et al., 1995).

1.2.2 Neuronal NOS (nNOS)

It is becoming apparent that due to alternative splicing, there are several subtypes of nNOS, (α, β, γ and μ) (Brenman et al., 1996; Huang et al., 1993); the principal isoform in brain is termed nNOSα (Eliasson, 1997) and in skeletal muscle, nNOSμ (Magee et al., 1996; Silvagno et al., 1996). Both α and μ isoforms of nNOS have a unique 250 amino acid N-terminal leader sequence that is involved in binding to membrane proteins, thus locating it to distinct subcellular regions. In skeletal muscle, the N-terminal PDZ domain of nNOS (after the proteins it was first identified in: PSD-95, DLG and ZO1, for review see (Garner, et al. 2000) recognises and binds to carboxy-terminal motifs containing G(D,E)XV residues (Schepens et al., 1997). In skeletal muscle, the nNOSμ PDZ domain binds α-syntrophin, thus causing nNOS to be a component of dystrophin complexes (Chao et al., 1996). In brain, the PDZ domain targets nNOSα to PDZ domains in the postsynaptic density proteins, PSD-95 and PSD-93 (Brenman et al., 1996).
Nitric oxide is a neurotransmitter and neuromodulator in distinct cell types, where it is regulated by distinct mechanisms. In the myenteric nervous system, nNOS is activated by calcium influx through voltage-gated channels. In some brain regions, Ca\(^{2+}\) influx through NMDA receptor-associated channels mediates activation of nNOS. Notably, the same PSD-95 protein that serves to anchor nNOS also causes clustering of NMDA receptors (Kornau et al., 1997). In skeletal muscle, nNOS co-localizes with nicotinic acetylcholine receptors and is activated upon membrane depolarization (Scheller, 1998). Although nNOS has been referred to as a constitutive enzyme, it is induced in sensory neurons following tissue damage and nNOS participates in the nociceptive responses associated with this damage (Wiesenfeld-Hallin et al., 1993).

1.2.3 **Endothelial NOS (eNOS)**

Of the 3 NOS isoforms, post-translational modifications have perhaps been best characterised in eNOS. eNOS is targeted to plasmalemma, in endothelium and epicardium, where it is both palmitoylated and myristoylated (Liu et al., 1996). Dual acylation of eNOS is important for localisation to specialised membrane sites (Feron et al., 1998; Shaul et al., 1996). Activity of eNOS appears to be under the tonic influence of an autoinhibitory domain that has been recognised in the region involved in FMN binding (Salerno et al., 1997).
Within plasmalemmal and internal membranes, eNOS is predominantly associated with caveolae. Caveolae ("little caves") are membrane domains specialised for cell signaling and enriched with caveolin, glycosphingolipid and cholesterol (For review see Schlegel et al., 1998). High levels of eNOS have also been found in the non-clathrin coated "light vesicular fraction", the golgi apparatus and the endoplasmic reticulum golgi intermediate compartment (Sowa et al., 1999). Targeting of eNOS to these sites requires co-translational myristoylation on the N-terminal glycine and post-translational palmitoylation of cysteines 15 and 26 (Liu et al., 1995). Bradykinin elicits translocation of eNOS from plasmalemmal caveolae to intracellular compartments (Venema et al., 1996) and has been suggested to trigger the tyrosine phosphorylation of eNOS binding proteins as a consequence of increasing intracellular calcium levels (Prabhakar et al., 1998).

Association of eNOS with caveolin-1 inhibits NO activity (Ju et al., 1997). The Ca\(^{2+}\)/CaM dependent dissociation of eNOS from caveolae allows eNOS to become activated (Matsubara et al., 1996; Venema et al., 1997). It has been proposed that binding to caveolin-1 inhibits eNOS by preventing the binding of calmodulin in a calcium-dependent reaction (Michel et al., 1997). Recently, eNOS bound in caveolae was reported to be associated with capacitative Ca\(^{2+}\) entry channels on the plasma membrane (Lin, 2000). This group reported that eNOS bound to caveolin was more sensitive to calcium flux whilst intracellular eNOS was more sensitive to changes in intracellular Ca\(^{2+}\) levels. Activation of eNOS may be further upregulated by association with Heat Shock Protein 90.
(Hsp 90) following stimulation by the NO agonists histamine, vascular endothelial growth factor and fluid shear stress (Garcia-Cardena et al., 1998).

The serine/threonine protein kinase B (AKT) phosphorylates eNOS at serine 1179 and upregulates NO production (Fulton et al., 1999). Phosphorylation of eNOS by AKT was shown to occur in response to shear stress indicating that AKT may play a role in mediating vascular tone (Luo, Z, 2000). In addition bradykinin was demonstrated to increase AKT dependent phosphorylation (Bernier, 2000). Evidence is now emerging that phosphorylation of eNOS by AKT has effects upon VEGF mediated cell migration. While phosphorylation of eNOS tyrosine residues has been observed (Corson et al., 1996; Michel et al., 1993; Venema et al., 1996), there is still a lack of evidence to suggest how this modulates eNOS activity. PKC has been demonstrated in vitro to phosphorylate eNOS and downregulate activity (Hirata et al., 1995), and the action of PKC has been suggested to increase the affinity of eNOS for membranes (Matsubara et al., 1996).

It is no longer accurate to describe iNOS as unique amongst the NOS isoforms in its ability to be upregulated by immunostimuli. Expression of eNOS has been shown to be upregulated by some immunostimulants (Iwase, 2000; Navarro-Antolin, 2000) and eNOS activity and mRNA levels have been described to increase following administration of the cAMP agonist forskolin (Kiss, 1998). Recently, LPS was implicated in increasing eNOS mRNA levels in brain astrocytes (Iwase, 2000) and the immunosuppressant cyclosporin
was reported to induce eNOS gene transcription in bovine endothelial cells (Navarro-Antolin, 2000).

1.3 NOS catalysis

NOS subunits exhibit an overall bidomain structure with an N-terminal oxygenase domain and a C-terminal reductase domain. All NOSs are active only as dimeric enzymes, dimers are formed by interaction between oxygenase domains (Ghosh & Stuehr, 1995; Klatt et al., 1995). The NOS C-terminal reductase domain binds NADPH, FAD and FMN (Bredt et al., 1991) while the oxygenase domain binds iron porphyrin IX (haem), tetrahydrobiopterin (BH₄) and L-arginine. Studies using limited trypsinolysis and 2'5'-ADP sepharose chromatography allowed for isolation of the independent reductase and oxygenase domains, and conformation of their predicted respective high-affinity binding ligands (Sheta et al., 1994). Further information on NOS structures and function was enabled by expression of the independent domains in insect, bacterial and mammalian systems. Elucidation of the iNOS oxygenase domain crystal structure (Crane et al., 1998) and the eNOS oxygenase domain crystal structure (Raman et al., 1998) have provided molecular detail to answer many basic questions of structure and function.

In 1988, arginine was found to be a precursor for NO production and NO was shown to be derived from the guanidino nitrogen of L-arginine (Palmer et al., 1988; Rees et al., 1988; Sakuma et al., 1988). The NOS catalysed oxidation of arginine requires 2
successive monooxygenation reactions, and NO$\text{\textsuperscript{\textomega}}$-hydroxy-L-arginine (NOHA) is synthesised as an isolatable intermediate from the initial step (Fig 1.1). Formation of NOHA requires 1 mol of NADPH and 1 mol $O_2$ (Stuehr et al., 1991). The second oxidation step requires 0.5 mol NADPH and 1 mol $O_2$ to form citrulline and NO (Marletta et al., 1988; Stuehr & Griffith, 1992). All of the NOS isoforms catalyse this reaction with the same apparent mechanism.

The overall reaction catalysed by NOSs is a five-electron oxidation of one of the equivalent guanidino nitrogen atoms of arginine (see Fig 1.1). The cofactor NADPH delivers electrons through FAD to FMN in the reductase domain and then to haem in the oxygenase domain. Electron transfer between the NOS domains is variably efficient; at minimum three electrons are sequentially transferred between the reductase and oxygenase domains in a process that is the rate-limiting factor to NO synthesis (Griffith & Stuehr, 1995; Marletta, 1993; Masters, 1994). Once the haem-iron is reduced from Fe$\text{\textsuperscript{III}}$ to Fe$\text{\textsuperscript{II}}$, molecular oxygen binds (Abu-Soud et al., 1997) and arginine oxidation proceeds via the intermediate NOHA to the formation of citrulline and NO.

The flavins and NADPH in murine iNOS are contained between amino acids 532 and 1144, the reductase domain. This domain is conserved between all NOS isoforms, NADPH cytochrome P450 reductases, the flavoprotein subunit of sulphite reductase and the C-terminal half of bacterial cytochrome P450$_{BM}$. Point mutation and deletion studies of
L-Arginine $\rightarrow$ N$^\omega$-Hydroxy-L-Arginine (NOHA) $\rightarrow$ L-Citrulline $+$ Nitric Oxide

Fig 1.1: NOS - catalysed reaction
the C-terminal residues 1121 - 1122 confirmed the specific involvement of these amino acid residues in NADPH binding (Xie et al., 1994).

In each of the NOS isoforms CaM binding gates electron flux from FAD to FMN and from FMN to haem. Both nNOS and eNOS are dependent upon physiological fluxes in Ca\(^{2+}\) levels to initiate CaM binding and enzyme activation. In contrast, iNOS has CaM bound with such high affinity that it is bound over the entire physiological range of intracellular Ca\(^{2+}\) levels (Cho et al., 1992); hence iNOS is constitutively active. CaM binding may activate NOS by reorienting the haem, allowing for efficient interdomain electron flux (Abu-Soud & Stuehr, 1993). The CaM binding site of NOS shares functional homology with other enzymes and consists of a continuous sequence of 25 to 30 basic and hydrophobic residues (Newton et al., 1984). The NOS CaM site lies at the juncture between the N-terminal reductase and C-terminal oxygenase domains (Liu & Gross, 1996; Masters et al., 1996).

Analyses of comparative alignments of the reductase domain of the cNOSs revealed the insertion of a unique peptide sequence in the FMN domain that is not present in iNOS or other CaM dependent enzymes; it was suggested that this insertion is an autoinhibitory control element (Salerno et al., 1997). Consistant with this view, deletion of this 45 amino acid sequence resulted in an nNOS which maximally active with 10-fold lower calcium levels than is required for wild type nNOS (Daff, 1999).
The haem is held in position by a thiol provided by a cysteine which has been mapped to positions 184 in bovine eNOS (Chen et al., 1994), 200 in murine iNOS (Cubberley et al., 1997) and 415 in rat nNOS (McMillan & Masters, 1995). Mutation of these Cys to Ala or His residues abolished NOS catalytic activity. Studies of the oxygenase dimer of iNOS suggest that alone, it is unable to convert arginine to citrulline. However, it can convert the reaction intermediate N\textsuperscript{\textalpha}-hydroxy-L-arginine to NO and citrulline, but only in the presence of H\textsubscript{2}O\textsubscript{2} as a replacement for molecular oxygen (Ghosh et al., 1997). Thus, in the absence of the reductase domain, the oxygenase domain appears to retain partial catalytic activity.

Before the eNOS structure was determined, the only metal thought to be present in the enzyme was haem-iron. Thus, it came as a surprise when zinc was also shown to be present in the NOS oxygenase domain crystal structure (Raman et al., 1998). The zinc in eNOS is held in position through interaction with 4 cysteine residues that lie at the interface of the eNOS dimer (Cys 101 and Cys 96 of each bovine eNOS monomer). Point mutations at these sites resulted in an inability to bind zinc. In addition to the zinc at the dimer interface, there are also 85 amino acids which have interactions and hydrogen bonds holding the dimer together. In nNOS, one of the paired Cys residues on each subunit that binds Zn\textsuperscript{2+} is Cys 331 (Miller & Masters, 1999). Mutation of this residue causes a loss of Zn\textsuperscript{2+} binding and enzyme activation. The presence of Zn\textsuperscript{2+} has also been observed in the iNOS and nNOS isoforms (Fischmann et al., 1999; Raman, 2000).
1.4 Role of NO in the vasculature

NO is continually released from the endothelium and causes vasodilator “tone” which offsets the vasoconstrictor tone mediated by the sympathetic nervous system and renin-angiotensin system (Rees et al., 1989). Endogenous mediators such as acetylcholine, histamine, bradykinin, substance P, leukotrienes, ADP and ATP all cause vasodilatation by stimulating the production of NO (Kuhn, 1991; Losano et al., 1994; Moncada et al., 1989). Vasorelaxation results from NO mediated activation of guanyl cyclase, causing a rise in cyclic GMP (Murad et al., 1993; Rapoport, 1983). Administration of the NOS inhibitor, N-methylarginine (NMA), elicits vasoconstriction in animals (Togashi et al., 1992) and man.

The vascular endothelium produces constant levels of NO under basal conditions (~ 50-300 pmol/min/mg) (Buckley, 1995; Wennmalm et al., 1990). Compared to iNOS and nNOS which generate 500-1500 nmol mg⁻¹min⁻¹ NO, eNOS generates lower levels of NO 100-200 nmol mg⁻¹min⁻¹ (Nishida & Ortiz de Montellano, 1998). Endothelium-derived NO is regulated on a moment-to-moment basis by calcium and CaM binding (Bredt & Snyder, 1990; Sessa et al., 1993). G-protein coupled receptors such as the acetylcholine receptor respond to agonists with a rise in calcium (Rees et al., 1988). Binding of calcium to four EF-hand pairs in CaM causes CaM to compact permitting eNOS binding and consequent alteration in the eNOS tertiary structure such that inter- and intra-domain electron flux ensues. Binding of CaM also apparently causes a disruption in the binding of an autoinhibitory peptide within the FAD binding domain of NOS, proposed to be a key event
in eNOS activation (Salerno et al., 1997). In addition, eNOS is activated by endothelial shear-stress (Rizzo et al., 1998), this is associated with phosphorylation by AKT/PKB (Dimmeler et al., 1998). Studies of eNOS null mice have shown that they are hypertensive and that their blood vessels are refractory to the vasodilating action of agonists such as acetylcholine (Han et al., 1998).

1.4.2 Vasoprotective and anti-atherosclerotic effects of eNOS-derived Nitric Oxide

NO derived from eNOS inhibits microvascular permeability (Kubes, 1992; Nathan & Xie, 1994), platelet aggregation (Alheid, 1987), leukocyte adhesion (Akimitsu, 1995) and leukocyte migration (Hokari, 1998). NO released into the vascular lumen inhibits platelet aggregation and adhesion, protecting against thrombosis by a mechanism involving an increase in cGMP (Radomski et al., 1987). The proliferation of vascular smooth muscle cells is also attenuated by NO-induced cGMP accumulation (Nakaki et al., 1990; Nunokawa & Tanaka, 1992). Advanced glycosylation products have been demonstrated to block the anti-proliferative effects of NO (Hogan, 1992). NO inhibits the proliferation and migration of vascular smooth muscle cells stimulated by platelet-derived growth factors, thus helping to prevent the buildup of matrix molecules associated with sclerotic plaques (Murohara, 1999).

NO derived from eNOS regulates the expression of genes that are involved in atherosclerosis. NO reduces the expression of MCP-1 a chemoattractant protein (Zeitler,
surface adhesion molecules prevent the adhesion of leukocytes to the vascular endothelium. These include P-selectin (Davenpeck, 1994), CD11 and CD18 (Arndt, 1993), vascular cell adhesion molecule-1 (VCAM-1) (Tsao, 1996) and intracellular adhesion molecule (ICAM-1) (Biffl, 1996).

NO plays an important role in reducing the influx of lipoproteins to the vascular wall, thereby inhibiting low density lipoplycosaccharides (LDL) oxidation (Hogg et al., 1993). Dysregulation of eNOS has been demonstrated to generate superoxide ($O_{2}^{\cdot-}$). Rapid reaction between $O_{2}^{\cdot-}$ and NO forms peroxynitrite which increases the oxidation of LDL (oxLDL). Interestingly oxLDL has been demonstrated to increase eNOS gene expression (Pritchard et al., 1995).

1.4.3 Role of nNOS derived NO in vascular tone and blood pressure

Although eNOS null mice are hypertensive (Shesely et al., 1996) their survival indicates that eNOS derived NO is not the only mediator of blood vessel dilatation. Surprisingly, acetylcholine induces cGMP mediated vasorelaxation in eNOS -/- mice through activation of nNOS (Meng, 1998). If NOS inhibitors are given to rats following spinal cord transections, or are administered intracerebroventricularly but not intravenously, then an increase in blood pressure, sympathetic nerve activity and heart rate are observed in eNOS -/- mice. Vascular smooth muscle is innervated by nitrergic nerves that make and release NO (Forstermann et al., 1994). Nitrergic nerves are antagonistic to
sympathetic nerves and stimulation of nitrergic nerves causes vascular relaxation. It is notable that nNOS null mice do not display an increase in resting blood pressure (Huang et al., 1993).

1.5.1 Tetrahydrobiopterin (BH₄)

BH₄ is a member of the family of pteridines. In the late 1800s, Sir Frederick Hopkins isolated the yellow pigments from the wings of English butterflies (Hopkins, 1889). The structures of these wing pigments were not elucidated until the 1940s when Robert Purrman determined the structures of xanthopterin, leucopterin and isoxanthopterin. The term pterin derives from the Greek ptera meaning wing, in honour of the source from which these molecules were first isolated. The feature which all pteridines share is a [2,3-D]pyrimidine ring system.

A substitution at the 2-position by an amino group, carbonyl at the 4-position and 1,2-dihydroxypropyl group at the 6-position defines biopterin. The fully reduced biopterin is 5,6,7,8-tetrahydrobiopterin (BH₄), and partially reduced is 7,8-dihydrobiopterin (BH₂). Cleavage of the dihydroxypropyl side chain of biopterin (B) yields pterin (P).

Fig 1.2 The structure of tetrahydrobiopterin (BH₄) and numbering of atoms.
1.5.2 Functions of BH₄

In 1957, two blue fluorescent eye pigments were isolated from drosophila melanogaster (Visconti, 1957) and revealed to be pterin and biopterin. At the same time *Crithida fasiculata* was determined to require high levels of folic acid for growth, but biopterin could substitute for this need. It was later determined that pteridines were required as cofactors for the conversion of dihydro-orotic acid to orotic acid (Geary, 1985).

BH₄ has a role as a hydroxylating cofactor for the oxidative cleavage of glycol ethers; alkyl glycol ether mono-oxygenase was later shown to be dependent upon BH₄ for its activity (Kaufman *et al.*, 1990). BH₄ has since been implicated in cytokine mediated T-lymphocyte proliferation (Ziegler *et al.*, 1990), proliferation and growth regulation of erythroid cells (Tanaka *et al.*, 1989), rat PC-12 cells, rat C6 glioma cells and human fibroblast cells (Anastasiadis *et al.*, 1996).

1.5.3 BH₄ as a cofactor for catecholamine biosynthesis

In 1939 Blaschko postulated that L-tyrosine (Tyr) was the precursor for catecholamine (CA) synthesis and showed that within the adrenal medulla noradrenaline (NA) and adrenaline (Ad) were formed from Tyr (Blaschko, 1939). Tyrosine hydroxylase (TH, also known as tyrosine 3-monooxygenase) was identified as the enzyme responsible for the conversion of Tyr to L-DOPA and is the rate-limiting step in CA biosynthesis.
BH$_4$ was identified as a necessary cofactor for the conversion of phenylalanine to tyrosine by phenylalanine hydroxylase (Kaufman, 1963). Later BH$_4$ was found to be an essential cofactor for TH in the conversion of tyrosine to 3,4-dihydroxyphenylalanine (Brenneman, 1964; Nagatsu, 1964). Thus, BH$_4$ plays a pivotal role in the biosynthesis of the monoamine neurotransmitters: dopamine, epinephrine, serotonin and melatonin (Walter et al., 1998). In addition to BH$_4$, aromatic amino acid hydroxylases (AAAHS) require Fe$^{II}$ and oxygen in a reaction which will be discussed in more detail in the next section.

Levels of TH, found as both soluble and membrane bound forms can be quantitated as a marker for CA synthesis in the CNS and PNS (Nagatsu, 1964). In neuronal and neuroendocrine cells, TH is subsaturated with BH$_4$; levels of BH$_4$ in tissues are typically less than 10 µM and the $K_a$ for BH$_4$ use by TH is ~30 µM. This will be discussed in more detail later in this introduction, as well as the biological consequences of impaired BH$_4$ synthesis.

1.5.4 BH$_4$ as a neuromodulator

The literature suggests that BH$_4$ might have a greater role in adrenergic neurotransmission than simply as a cofactor for AAAHs in the support of monoamine biosynthesis. In rats, BH$_4$ was suggested to enhance dopamine release to a greater manner than could be accounted for by its potentiating effect on DA synthesis (Koshimura et al.,
This release was inhibited by 2,4-Diamino-6-hydroxy-pyrimidine (DAHP), a selective inhibitor of GTP cyclohydrolase, the first enzyme in the BH₄ synthetic pathway (Koshimura et al., 1995; Ohue, 1992). Studies using selective dopamine receptor antagonists indicated that the BH₄ effect was mediated by dopamine type 2 and 3 receptors (Tedroff, 1998). Another report demonstrated that perfusion of BH₄ can also enhance glutamate and GABA output (Mataga, 1991).

1.6 Evidence that BH₄ limits NO synthesis

Although BH₄ synthesis had been observed to increase upon immunostimulation, it was unclear why. The answers were found when extracts of cytokine-activated macrophages were observed to produce NO from L-Arg in a BH₄-dependent manner (Kwon et al., 1989; Tayeh & Marietta, 1989). Originally, it was proposed that this might be a specific requirement of iNOS since NO produced from brain extracts was not increased following BH₄ administration (Mayer et al., 1991). However, it was later realised that nNOS was isolated with BH₄ tightly bound and that all three NOS isoforms have a requirement for BH₄.

1.7 Role of BH₄ in AAAH catalysis

In AAAHs, one mole of BH₄ is able to support a single catalytic turnover (Fig 1.3); continuous BH₄-dependent catalysis occurs as a result of cofactor regeneration. In the
Fig 1.3: BH₄ as a cofactor for the catalysis of aromatic amino acid hydroxylases
Abbreviations are: DHPR, dihydropteridine reductase; PCD, pterin 4a-carbinolamine; BH₄, tetrahydrobiopterin; qBH₂, quinioid dihydropterin; PH, phenylalanine hydroxylase; TH, tyrosine hydroxylase; TrH, tryptophan hydroxylase.
process of aromatic amino acid hydroxylation BH$_4$ [A] is initially converted to an unstable BH$_4$ peroxide intermediate [B], where an oxygen briefly remains associated with the 4a-C atom (Almas et al., 1996). The peroxide intermediate rapidly rearranges to form the more stable intermediate 4a-hydroxytetrahydrobiopterin [C] (sometimes called Pterin 4a-carbinolamine) (Haavik & Flatmark, 1987). The enzyme Pterin 4a-carbinolamine dehydratase (PCD) reduces this product to quinoid dihydropterin (qBH$_2$ - [D]) and H$_2$O (Rebrin et al., 1995). In a final recycling step, qBH$_2$ is converted back to BH$_4$ by dihydropteridine reductase (DHPR) – this enzyme should not be confused with dihydrofolate reductase (DHFR) which is involved in the BH$_4$ salvage pathway. Recycling of BH$_4$ is rapid for continuous AAAH function, and all requisite enzymes reside in close proximity (Resibois et al., 1999).

1.8 Role of BH$_4$ in NOS catalysis

After the discovery that BH$_4$ is an essential cofactor for NOS activity (Kwon et al., 1989; Tayeh & Marletta, 1989), it was natural to assume that BH$_4$ would function for NOSs in the same manner as it does for the AAAHs. Arguing against this view, it was shown that catalytic quantities of BH$_4$ supports NOS activity (Giovanelli et al., 1991) despite evidence that qBH$_2$ is not a recycled intermediate. More recently, comparisons of the eNOS and iNOS oxygenase domain with the AAAH crystal structures revealed fundamental
structural differences in these BH$_4$ sites (Raman, 2000) that provide functional insights; this will be discussed in Chapter 3.

iNOS was the first NOS isoform that was demonstrated to utilise BH$_4$ as a cofactor (Kwon et al., 1989; Tayeh & Marletta, 1989). It is now appreciated that BH$_4$ is essential for activity of all NOS isoforms. BH$_4$ is unstable at physiological pH in oxygenated solutions (Gross & Levi, 1992), thus intracellular oxidants could potentially deplete BH$_4$ levels in vivo. If there is insufficient BH$_4$ present, eNOS switches from NO to O$_2^-$ generation (Vasquez-Vivar et al., 1998; Wever et al., 1997). Thus, if BH$_4$ levels are depleted, there will be a fall in NO production and a rise in oxidant accumulation that may mediate endothelial dysfunction associated with cardiovascular diseases (Cosentino & Katusic, 1995).

In the absence of BH$_4$ and arginine, the haem iron in NOS is predominantly in a six-coordinate low-spin state (Fossetta et al., 1996; Martasek et al., 1996; Rodriguez-Crespo et al., 1996; Roman et al., 1995; Wu et al., 1996). Binding of BH$_4$ or arginine has been shown to elicit a shift to a five-coordinate high-spin form of NOS haem iron. Arginine binding alone results in a shift of the haem iron spin-state from low to high, whilst BH$_4$ binding alone appears to produce a mixture of the two spin-states. In nNOS, haem is needed for subunit assembly to form high-affinity binding sites for arginine and BH$_4$; once dimers are assembled, BH$_4$ binding serves to stabilise the dimers (Klatt et al., 1996). Notably, monomeric NOSs have not been demonstrated to bind BH$_4$ or arginine, but
do retain the ability to bind haem. Assembly of two NOS monomers forms a channel into which BH$_4$ can be incorporated (Presta et al., 1998).

BH$_4$ binds exclusively within the oxygenase domain of NOS, where it has been found to interact between residues 448 and 480 in murine iNOS. Studies of G450A and A543I mutant iNOS revealed a loss of BH$_4$ binding and destabilization of the NOS dimer (Cho et al., 1995). Conceivably, these mutations could prevent BH$_4$ binding by either limiting dimerisation or contributing directly to BH$_4$ binding interactions. Residues 340 - 494 in murine iNOS were found to have sequence homology to dihydrofolate reductases, suggesting a role in BH$_4$ binding, but further mutational studies and binding experiments indicated that these residues had more of an effect on arginine/N$^\text{N}$-nitroarginine binding than pterin binding (Gachhui et al., 1997). In bovine eNOS, a point mutational study of C99A observed a reduction in BH$_4$ binding affinity (Chen et al., 1995). The crystal structure of eNOS subsequently showed this residue to be involved in Zn$^{2+}$-chelation which plays an important function in dimer stabilization (Raman et al., 1998).

BH$_4$ is needed for oxygen activation, possibly by serving as a redox-active cofactor. It has been suggested that BH$_4$ could serve this function by donating an electron to reduce the oxyferrohaem complex, Fe$^{3+}$OO to Fe$^{3+}$O-OH (Bec et al., 1998). In the absence of BH$_4$, NOS is unable to synthesize the intermediate N$^\text{N}$-hydroxy-L-arginine (Rusche et al., 1998). The redox status of BH$_4$ is also important for coupling the consumption of cofactor NADPH to NO production (Presta et al., 1998). When BH$_4$ binds to eNOS, it may donate
an electron to produce the \( \cdot \text{BH}_3 \) radical intermediate (Bec et al., 1998); which in turn might withdraw an electron from oxy-haem, thereby stabilising the oxy-haem complex. Such a mechanism could explain the ability of BH\(_4\) to prevent premature release of oxygen as superoxide anion.

Studies using arginine-based NOS inhibitors have suggested the existence of isoform differences for the substrate and cofactor binding sites. BH\(_4\) and arginine bind to the distal side of haem and influence the haem environment and each others binding (Klatt et al., 1994). The porphyrin of haem can be shielded by arginine and BH\(_4\) binding, such that inhibitory actions of alkylating agents, CN\(^-\), NO, CO and imidazole, are affected (Chabin et al., 1996; Matsuoka et al., 1994; Wang et al., 1993).

As previously mentioned, BH\(_4\) was proposed to play an important role in NOS dimer assembly (Ghosh & Stuehr, 1995), however observations that all 3 isoforms can be expressed as dimers in the absence of BH\(_4\), suggests that BH\(_4\) is not essential for dimerisation. In nNOS, absence of arginine and haem prevented dimerisation whilst BH\(_4\) absence had a partial destabilizing effect on dimers (Klatt et al., 1996). iNOS dimer stabilization by BH\(_4\) appears to require stoichiometric amounts of BH\(_4\) (Bryk & Wolff, 1998).

Studies involving titration of SDS to induce dimer dissociation were carried out to measure the extent to which BH\(_4\) contributes to NOS dimer stabilisation (Klatt et al., 1995). The nNOS dimer was found to be more stable in the presence of BH\(_4\) whilst eNOS
was less affected by BH$_4$ presence. A model for active NOS dimer formation involves the following series of events: folding of separate enzyme domains, incorporation of FAD, FMN (and for iNOS CaM) into the monomeric reductase domains, binding of haem to the oxygenase domains, NOS dimerisation through haem interaction and finally binding of BH$_4$ and arginine to stabilise the dimer.

1.8.2 BH$_4$ effects on superoxide production by NOS

In the absence of arginine, nNOS converts molecular oxygen to superoxide (O$_2^-$) (Heinzel et al., 1992; Pou et al., 1992). Superoxide generation by nNOS is markedly enhanced by binding of Ca$^{2+}$/CaM (Xia et al., 1998). In cells that are starved of arginine, nNOS converts oxygen to superoxide which reacts with NO to form peroxynitrite (ONOO$^-$) a potent oxidising agent (Hogg et al., 1994). In nNOS, the formation of O$_2^-$ occurs at both flavin and haem groups (Pou et al., 1999).

eNOS was shown to produce O$_2^-$ under pathological conditions in the vasculature (Cosentino & Katusic, 1995; Pritchard et al., 1995). Utilizing fluorescence optical and electron spin resonance spectroscopy, O$_2^-$ production of eNOS was examined (Stroes et al., 1998). In contrast to nNOS, O$_2^-$ production by eNOS appeared to occur exclusively at the haem moiety (Stroes et al., 1998). EDTA diminished O$_2^-$ production by eNOS suggesting regulation by Ca$^{2+}$/CaM (Xia et al., 1998).
If BH₄ is absent from eNOS then O₂⁻ is generated; O₂⁻ may contribute to vascular
dysfunction associated with hypertension, atherogenesis and diabetes. In contrast to
findings for iNOS and nNOS (Vasquez-Vivar et al., 1999; Xia & Zweier, 1997), when
eNOS was blocked by the NOS inhibitor L-NAME, NO production was abolished as
expected, but subsequent addition of BH₄ caused a reduction in the amount of O₂⁻ produced
(Vasquez-Vivar et al., 1998). This indicates that eNOS retains the ability to form O₂⁻ even
in the presence of an arginine analogue inhibitor of NOS. Under normal physiological
conditions eNOS apparently produces both NO and O₂⁻, thus eNOS can act as a
peroxynitrite producing enzyme. Peroxynitrite is efficient at oxidizing iron-sulphur
clusters, zinc fingers and thiols in proteins (Beckman & Koppenol, 1996), thereby
modulating protein functions.

1.9 BH₄ biosynthesis

1.9.1 De Novo synthesis pathway

In the first and rate-limiting step of the de novo BH₄ synthetic pathway (Fig 1.4)
guanosine 5’ triphosphate (GTP) is reduced to 7,8-dihydroneopterin 3’ triphosphate
(DNTP) by the enzyme GTP cyclohydrolase I (GTPCH, EC 3.5.4.16) (Burg & Brown,
1968). GTPCH should not be confused with the unrelated enzyme GTP cyclohydrolase II
that catalyzes the second step in riboflavin biosynthesis by microorganisms. In the initial
part of the GTPCH catalysed reaction, H₂O attacks GTP at C-8 resulting in an opening in
Fig 1.4 De novo BH₄ synthetic pathway
the imidazole ring of GTP to form the intermediate 2-amino-5-formylamino-6-ribofuranoside triphosphate. Subsequent protonation of the bridging oxygen in the furanose ring causes the release of C-8 as formate. An Amadori rearrangement involves the γ-phosphate of GTP and keto-enol tautomerization. The final step in the formation of DNTP is a ring closure where N-7 and C-2' are covalently joined. Crystallographic analyses of the *E. coli* GTPCH have led investigators to predict that ring closure might occur either at the surface of GTPCH, or even in solution, since spatial models of the active site pocket do not favour this step (Nar *et al.*, 1995).

GTPCH-catalysed formation of DNTP is a common initial step in the biosynthesis of unconjugated pterins, folates and riboflavin, but not molybdopterin (a cofactor of sulfite oxidase, xanthine dehydrogenase and aldehyde oxidase in man). Following the formation of DNTP, the pathway diverges between the tetrahydrofolic acid synthetic pathway in microorganisms and tetrahydrobiopterin synthetic pathway in a diverse array of organisms (Nichol *et al.*, 1985).

The conversion of DNTP to 6-pyruvoyl tetrahydropterin (PTP) is catalysed by the zinc-dependent metalloprotein, 6-pyruvoyl tetrahydropterin synthase (PTPS, EC 4.6.1.10). The reaction involves a stereospecific reduction by an internal redox transfer between atoms N-5, C-6 and C-1', oxidation of both of the side chains of the hydroxyl groups, and triphosphate elimination from the C-2'-C-3' bond in an unusual manner. Burgisser suggested that Zn^{2+} plays an important dual role in activating protons in the substrate and
stabilising the intermediates (Burgisser et al., 1995). There is stereospecific protonation of C-6 and oxidation of C1'-OH to C1'=O. In the last stage, a proton is removed from the C-2' of the carbonyl side chain, triphosphate is eliminated, and the compound undergoes a tautomerization to yield PTP. The efficiency of PTPS is 10 times greater in mouse than humans (Oppliger et al., 1995), explaining why biopterin and neopterin accumulate in these species, respectively, when GTPCH activity is induced.

The final step in the de novo synthesis of BH$_4$ involves two NADPH-dependent reductions of the 1'- and 2'-oxo groups of 6-pyruvoyl tetrahydropterin by the enzyme sepiapterin reductase (SR, EC 1.1.1.153). The first NADPH-dependent reduction of the side chain C-1'-keto forms the intermediate 1'-hydroxy-2'-oxopropyltetrahydropterin (Bracher et al., 1998). There is an internal rearrangement of the keto group to form the 1'-keto compound, 6-lactoyl tetrahydropterin. In the second NADPH dependent reduction step, 6-lactoyltetrahydropterin is reduced to BH$_4$.

The crystal structure of SR revealed that there is little homology between it and the other enzymes of de novo synthesis, apart from a conserved active site, a pterin-binding motif which also shows some similarity to the GTP binding motif in small GTP-binding proteins (Auerbach et al., 1997). Analyses of the available protein sequences for SR revealed approximately 90% homology among mammalian enzymes and high evolutionary conservation with fish and Drosophila enzymes (Thony, 2000).
1.9.2 Salvage pathway

BH$_4$ is unstable and readily undergoes non-enzymatic oxidation to form dihydropterin. Thus, a recycling pathway exists to reduce dihydropterins and restore BH$_4$ (Fig 1.5). This “salvage pathway” for dihydropterins involves 2 enzymes: sepiapterin reductase (SR) and dihydrofolate reductase (DHFR). The latter enzyme has been well characterised for its role in the production of 5,6,7,8-tetrahydrofolate from 7,8,-dihydrofolate. Sepiapterin is reduced to 7,8-dihydrobiopterin by SR, which is then reduced to BH$_4$ by DHFR (Nichol et al., 1985). Pools of sepiapterin are proposed to come from degradation of intermediates from the de novo BH$_4$ pathway, such as 6-lactoyl tetrahydropterin (Primus & Brown, 1994). It is important to note that DHFR cannot reduce the quinonoid 5,6 dihydrobiopterin (qBH$_2$) which is, however, reduced to BH$_4$ by another enzyme, dihydropteridine reductase (DHPR).

1.10 Induction of GTPCH activity and BH$_4$ synthesis

1.10.1 GTPCH structure

GTPCH, the rate-limiting enzyme for de novo synthesis of BH$_4$, was first detected in bacteria (Burg & Brown, 1968); characterisation of the enzyme suggested it was a multimeric protein (Nar et al., 1995; Nar et al., 1995; Schmid et al., 1993). The active form of GTPCH from *E. coli* is a symmetrical decamer, with 2 crab-like pentamers facing
Fig 1.5: BH₄ salvage pathway
Abbreviations are: SR, sepiapterin reductase; DHFR, dihydrofolate reductase.
inwards, with the legs (formed by N-terminal pairs of monomers) of one crab clasping the body of the other (Auerbach & Nar, 1997). The decamer is a toroid of subunits, with 10 active sites formed around the periphery of the toroid (Nar et al., 1995).

1.10.2 GTPCH gene and cDNA

Three GTPCH cDNA clones have been identified as splice variants of a single mouse gene. Type 1 GTPCH cDNA is the only variant that appears to encode an active enzyme; type 3 cDNA may be immature mRNA which still contains part of the 5th intron (Togari et al., 1992). Mouse GTPCH is encoded by a 32 kb gene containing 6 exons, the transcription start site is a guanine residue-164 that is approximately 620 base pairs upstream from the protein coding region. The mouse GTPCH gene has no TATA box in its promoter; instead, an AT rich putative promoter motif ATAAAAA is present, as is a histone binding region (H1-box; Dalton & Wells, 1988), interferon-γ stimulated region (IBP-1b; Blanar et al., 1989) and the enhancer motif (GT-2B; Xiao et al., 1987) consensus elements.

Studies of human GTPCH also revealed 3 cDNA splice variants (Togari et al., 1992) with type 1 coding for the active GTPCH (Gutlich et al., 1994). The 6 exons of the human GTPCH gene had intron/exon break points corresponding to that in mouse. The mouse/human comparison revealed differences in sequence homology in exon 6, with human having 2127 bp and mouse 296 bp, principally due to extended polyadenylation in
the human form (Ichinose et al., 1995). Chromosome mapping located human GTPCH to 14q 22.1-22.2 (Thony et al., 1995).

1.10.3 Induction of GTPCH

Monoamine-releasing neurons have abundant constitutively expressed levels of GTPCH. In contrast, in many cell types that do not contain basally detectable GTPCH, GTPCH can be induced by various cytokines. Bacterial lipopolysaccharide (LPS), interferon γ (IFNγ) (Schoedon et al., 1987), Phytohemagglutinin (Blau et al., 1985), Kit ligand-stem cell factor (Ziegler et al., 1993), tumour necrosis factor α (TNFα) interleukin-1β (IL1β) (Ziegler et al., 1993), IL-4 and IL-10 have all been demonstrated to upregulate GTPCH levels (Schoedon et al., 1993). In cardiac microvascular endothelial cells, glucocorticoids inhibit the induction of iNOS and GTPCH by LPS/IFN exposure, and thereby attenuate induced NO production (Frank et al., 1998; Simmons et al., 1996).

Various cell types have been used to study the molecular basis for GTPCH induction. IFNγ was studied in T cells and macrophages and shown to increase GTPCH activity (Schott et al., 1993; Ziegler et al., 1990). TNFα/IL-1β was shown in rat mesangial cells to increase levels of GTPCH protein (Pluss et al., 1996). GTPCH mRNA levels were increased by treatment with INFγ and kit-ligand in T cells and macrophages (Ziegler et al., 1993). LPS has been demonstrated to effect a 2-3 fold increase in GTPCH activity in a variety of rat tissues that constitutively express GTPCH, including: cerebellum, brain,
liver, spleen and the adrenal gland. Macrophages, dermal fibroblasts and tumour cell lines all demonstrate a more profound increase in GTPCH activity after treatment with IFNγ and TNFα; LPS has been observed to be a co-stimulator factor with IFNγ (Hattori & Gross, 1993). In granulosa cells IL-1α and follicle stimulating hormone (FSH) were demonstrated to increase mRNA levels of GTPCH (Tabraue et al., 1997). BH₄ levels were observed to increase following reserpine administration, cold stress and electroconvulsive shock (Abou-Donia et al., 1986; Baruchin et al., 1990) presumably via an increase in GTPCH activity.

In monoamine-containing neurons, constitutive GTPCH expression and BH₄ biosynthesis is possibly regulated by soluble protein factors. The tyrosine kinase receptor trkA is a mediator of responses to nerve growth factor (NGF). An NGF-induced increase in BH₄ levels has been observed in PC-12 cells in association with increased GTPCH mRNA expression (Hirayama & Kapatos, 1995; Suzuki et al., 1988). NGF was also observed to cause a 2-4 fold increase in GTPCH activity, GTPCH mRNA levels and BH₄ levels in superior cervical ganglia cells in culture (Hirayama & Kapatos, 1995). In the same cell line, tyrosine hydroxylase activity and mRNA levels were increased 8-13 fold. BH₄ content of sympathetic neurons is regulated by NGF receptor-mediated changes in GTPCH gene expression (Hirayama & Kapatos, 1995), perhaps by an increase in GTPCH gene transcription.
1.11 Post-translational regulation of GTPCH

1.11.1 Phosphorylation

Studies have reported the phosphorylation of GTPCH in PC-12 cells after depolarization with KCl (Imazumi et al., 1994). This phosphorylation occurs simultaneously with GTPCH and Rabphilin 3A association with synaptic vesicles. Phorbol esters (PMA), agents that activate PKC, were observed to increase neopterin/biopterin levels in a T cell line (Seidl et al., 1986). Within dopamine neurons GTPCH gene expression and BH$_4$ biosynthesis is increased by adenylate cyclase activation and alterations in the membrane potential (Zhu et al., 1994). IL-1$\alpha$, cholera toxin, theophylline, dibutyryl and 8-bromo cyclic AMP, all reagents that increase intracellular levels of cAMP, cause a rise in the GTPCH mRNA levels and activity (Pluss et al., 1996), possibly through post-translational modifications such as phosphorylation (Abou-Donia et al., 1986)

In bone marrow derived mast cells, in which phosphate pools were preloaded with [$^{32}$P]orthophosphate, it was found that antigen binding to IgE primed cells triggers a transient rise in GTPCH phosphorylation in association with increased activity and an increase in intracellular BH$_4$ (Hesslinger et al., 1998). Phosphorylation of GTPCH was observed to occur in vitro with casein kinase II (CKII) and PKC (Hesslinger et al., 1998; Lapize et al., 1998). PKC$\delta$ associates with the FcR1 $\beta$-chain and phosphorylates the FcR1
γ-chain. Hesslinger proposed that phosphorylation of the receptor may lead to increased specificity for some other substrates including GTPCH (Hesslinger et al., 1998).

1.11.2 GFRP

It had been known for many years that BH₄ can feedback to inhibit the rate-limiting enzyme GTPCH; thus de novo BH₄ synthesis is regulated by end-product inhibition (Niederwieser et al., 1986). The capacity to inhibit GTPCH is not restricted to BH₄, it is observed with many pterins, most potently with reduced pterins (Jacobson, 1989; Shen et al., 1988). It was a surprise when studies of purified recombinant rat GTPCH, derived from a recombinant bacterial expression system, revealed no inhibition of activity by BH₄ in vitro (Shen et al., 1988). This led to the identification of a novel protein in liver that bound to purified recombinant GTPCH in the presence of BH₄ and reconstituted the ability of BH₄ to inhibit purified recombinant GTPCH (Harada et al., 1993). Initially named p35, based on apparent molecular mass of 35 kDa, this protein was cloned and found to encode an 84 amino acid polypeptide, comprising only 9.5 kDa (Milstien et al., 1996). P35 was renamed GFRP for GTPCH Feedback Regulatory Protein. In the presence of bacterial-expressed rat GFRP, BH₄ was found to elicit a concentration-dependent and complete inhibition of recombinant GTPCH (Milstien et al., 1996). This finding affirmed that the 9.5 kDa GFRP was both necessary and sufficient for BH₄-mediated feedback inhibition of GTPCH. Cross-linking and sedimentation experiments revealed that in solution GFRP
appears to be a homopentamer and it is speculated that one GFRP pentamer interacts with each of the two pentameric faces of GTPCH decamer (Yoneyama et al., 1997; Yoneyama & Hatakeyama, 1998). GFRP-mediated inhibition of GTPCH is specifically reversed by phenylalanine, explaining the clinical observation that a Phe-rich meal increases circulating levels of total biopterin (Harada et al., 1993). Although phenylalanine reverses inhibition of GTPCH imposed by GFRP/BH4, it does not dissociate the protein assembly (Yoneyama & Hatakeyama, 1998). A model that summarizes this mechanism for feedback inhibition of GTPCH by pterins is depicted in Fig. 1.6.

GFRP mRNA has been detected by Northern blot and in situ hybridization and high levels were found in liver, brain and heart, organs which have a similar pattern of expression for GTPCH (Kapatos et al., 1999; Milstien et al., 1996). Given that the $K_m$ of BH$_4$ for support of NOS activity is extremely low (30 – 100 nM), by comparison with that reported for feedback inhibition (1 – 10 μM), it was unanticipated that feedback inhibition of GTPCH could influence NOS activity. Nonetheless, GFRP mRNA is detectable in vascular smooth muscle cells, inducible by immunostimulants, and GFRP mediates tonic inhibition of GTPCH (in the absence of Phe) when BH$_4$ synthesis is induced by immunostimulants (Xie and Gross, 1998). Whether this system significantly impacts on immunostimulant-induced NOS activity in vivo remains to be ascertained.
Fig 1.6: GTPCH inhibition by the GTPCH feedback regulatory protein (GFRP). The feedback inhibition by GFRP requires BH$_4$ and is reversed by phenylalanine resulting in "active" GTPCH.
1.11.3 Other tetrahydrobiopterin synthetic enzymes

6-pyruvoyl tetrahydropterin synthase (PTPS), reduces DNTP to form 6-pyruvoyl tetrahydrobiopterin in the second step of the BH₄ de novo pathway. PTPS has considerable sequence homology to aldose reductase (Steinerstauch et al., 1989; Milstien & Kaufman, 1989; Park et al., 1991). Aldose reductase is known to catalyse the reduction of carbonyl compounds to the corresponding alcohol, it is conceivable that PTPS might also have a role as a reductase.

Dogma suggests that PTPS is constitutively expressed, however in some cell types PTPS has been demonstrated to be cytokine-inducible. However, reports of GTPCH induction have described 100-fold increases in GTPCH activity compared to maximal 2-4 fold increases for PTPS (Werner et al., 1990). PTPS mRNA and protein activity in human umbilical vein endothelial cells are significantly induced (10-fold and 3-fold, respectively) by the combination of TNFα and IFN-γ, although the extent of this induction is an order of magnitude less than that for GTPCH (Linscheid et al., 1998).

Activity of PTPS is dependent on phosphorylation and post-translational modifications (Oppliger et al., 1995). Following reserpine treatment of rats, a 3-4 fold increase in PTPS mRNA was observed in the adrenal glands, and this proposed to be due to cAMP dependent phosphorylation of PTPS (Hirayama, 1995). Phosphorylation by cGMP-dependent protein kinase type II has been reported to phosphorylate human PTPS at Ser-19 (Scherer-Oppliger et al., 1999). A patient has even been identified with a defect in
BH₄ biosynthesis attributable a phosphorylation-deficient PTPS mutant (Oppliger et al., 1995). It is speculated that NO might upregulate PTPS through sGC-dependent phosphorylation. There are no known inhibitors of PTPS.

The concentration of neopterin in human plasma has served as a convenient indicator of de novo BH₄ synthesis in man (Wachter, 1979; Zeitler & Andondonskaja-Renz, 1987). Neopterin arises as a metabolite of GTPCH-produced DNTP. When GTPCH becomes markedly activated in human cells, DNTP accumulates due to a relative insufficiency of the subsequent enzyme, PTPS. Intracellular DNTP is then subject to phosphatase action, released from cells into the circulation, and non-enzymatically oxidized to yield plasma neopterin. Compared with humans, rodents possess significantly greater levels of PTPS, explaining the relative lack of neopterin in murine cells, tissues and body fluids (Werner et al., 1991).

Before any function had been ascribed, plasma neopterin levels were recognised as a sensitive indicator of immune cell activation for clinical diagnosis (Kremsner, 1989; Wachter, 1979). Since the only known function of immunologically-evoked BH₄ synthesis is to support co-induced NO synthesis, elevated neopterin levels presumably signal iNOS activation. In this regard, it is notable that neopterin concentrations correlate with the extent and the activity of disorders in which cell-mediated immune stimulation play a role. In allograft recipients, neopterin concentrations rise early during the course of rejection episodes (Muller, 1997). Increasing neopterin is among the first signs of infection by virus
(Tyor, 1992). In inflammatory conditions such as rheumatoid arthritis, neopterin levels correlate with the extent and activity of the disease (Beckham, 1992). Neopterin long been recognised as a potent marker for HIV progression (Heyes et al., 1991; Most et al., 1993). It is tempting to consider that neopterin informs us of iNOS induction in each of these clinical circumstances.

Sepiapterin reductase causes a stereospecific reduction of both carbonyl groups of the PTP to form BH$_4$ (Smith, 1987). The X-ray crystal structure of sepiapterin reductase revealed this enzyme to be a homodimer with an aspartate residue 258 that is essential for the binding of pterin substrate (Auerbach & Nar, 1997). SR is potently inhibited by the serotonin metabolite, N-acetylserotonin, presenting the possibility for a highly specific feedback regulatory mechanism between indoleamines and pterins in vivo. N-acetylserotonin (NAS) and N-acetyldopamine are potent and selective inhibitors of SR that have been used to assess the role of SR in biological systems (Katoh et al., 1982; Smith et al., 1992). SR activity is abundant in all BH$_4$-producing cells studied to date and it does not appear to be a control site for regulation of BH$_4$ synthesis. (Werner et al., 1990). Whilst mutations have been reported for the other enzymes involved in GTPCH de novo synthesis, there are no reports of SR deficiencies. Although it was reported that SR could be phosphorylated in vitro by CaM dependent protein kinase C, in vivo evidence for SR phosphorylation has not been reported (Katoh et al., 1994).
**1.11.4 Biopterin entry to cells**

BH$_4$ has been measured in the culture media of RASM cells (Gross & Levi, 1992) and endothelial cells (Walter et al., 1994), indicating that BH$_4$ can be secreted by cells. Blocking BH$_4$ synthesis prevents cytokine-induced NO production, as demonstrated in smooth muscle cells (Gross & Levi, 1992), endothelium (Gross et al., 1991) and fibroblasts (Werner-Felmayer et al., 1990). BH$_4$ supplementation leads to an increase in plasma nitrate levels and a reduction in vascular dysfunction *ex vivo* (Werner-Felmayer & Gross, 1996) and increases in NO synthesis in vascular cells (Gross & Levi, 1992). In human umbilical vein endothelial cells, levels of eNOS protein expression were decreased upon immune stimulation, however, an increase in NO synthesis was observed that correlates with an increase in BH$_4$ (Rosenkranz-Weiss et al., 1994). Inhibition of DHFR by treatment of rats with methotrexate virtually abolishes LPS induced NO-mediated vasodilation in *ex vivo* aortae, hence vasoprotection results preferentially from inhibiting BH$_4$ salvage, rather than *de novo* synthesis in vascular smooth muscle. From these observations, it is apparent that BH$_4$ availability to vascular cells is rate-limiting for NO production and BH$_4$ may be released and taken up by cells.

In brain, the synthesis of BH$_4$ is known to occur predominantly within monoamine synthesising neurons where the $K_d$ for BH$_4$ as a cofactor for NOS is approximately 1000 times lower than that for AAAHs (Giovanelli et al., 1991). Immunohistochemistry and *in situ* hybridization studies have revealed GTPCH in all dopaminergic cells, but not in nNOS.
containing cells (Hwang et al., 1998). In 1996, Tzeng et al. reported that the amounts of NO produced from iNOS transfected cells dramatically increased when co-cultured with BH$_4$ producing cells (Tzeng et al., 1996). Conceivably, NO synthesising cells may either obtain the BH$_4$ from closely situated monoamine-producing cells or they may take up BH$_4$ from circulating blood. BH$_4$ is an unstable molecule at physiological pH and therefore any cell-released BH$_4$ would be readily oxidised to BH$_2$ in the circulation (Nichol et al., 1985).

A reduced pterin transporter is presently unknown, however given the existence of a reduced folate transporter it is possible that carriers or pumps also exist for BH$_4$. The reduced folate carrier allows bidirectional transport (Goldman et al., 1968), whilst low folate selective pressure leads to the expression of cell membrane-anchored folate receptors which can endocytically translocate folates (Brigle et al., 1991). Folates may additionally be transported through active carriers (Yang et al., 1992; Saxena & Henderson, 1996). Currently, no reports have been published describing a cellular transporter for pterins.

1.12 BH$_4$ in pathology

A typical phenylketonuria is characterised phenotypically with hyperphenylalaninemia and depletion of the neurotransmitters dopamine and serotonin; the cause is a deficiency in BH$_4$ (Kaufman et al., 1978). Variants include autosomal recessive
inherited deficiencies of GTPCH and PTPS, resulting in severe neurological symptoms (Blau et al., 1996).

BH₄ in the brain is concentrated in the striatum and localised in the nigrostriatal dopaminergic terminals (Nagatsu et al., 1997). An inherited deficiency in GTPCH activity correlates with a movement disorder first described during the 1970s, in which patients respond to low doses of L-DOPA; the disease has been termed DOPA-responsive dystonia (DRD). A decrease in GTPCH activity to less than 20% of normal, causes reduced tyrosine hydroxylase (TH) activity, catecholamine biosynthesis, and a fall in dopamine levels (Nagatsu & Ichinose, 1996). Mutations in the GTPCH gene have been confirmed to be responsible for DRD (Ichinose et al., 1994; Nygaard, 1995). In contrast to DRD, juvenile Parkinson’s disease patients have normal GTPCH activity and GTPCH and TH protein levels may decrease in parallel, as secondary effects caused by cell death (Nagatsu & Ichinose, 1996).

A mouse model of BH₄ deficiency (hph-1) has a mutation that affects the steady-state levels of GTPCH mRNA (McDonald et al., 1988); the defect occurs in a presumed regulatory region of the GTPCH gene (Gutlich et al., 1994). Hph-1 mice have reduced BH₄ levels in liver, altered NO metabolism and reduced levels of phenylalanine hydroxylase (PAH) and TH (Hyland et al., 1996). Neurochemically, hph-1 mice and patients with DRD are similar and the hph-1 mouse may be a good model to investigate the pathophysiology of DRD and other diseases associated with BH₄-deficiency.
An important role of BH$_4$ in the human epidermis has been recently recognised (Schallreuter et al., 1994; Schallreuter et al., 1994). P' H, in the presence of BH$_4$ and oxygen, hydroxylates phenylalanine to tyrosine, which serves as substrate for TH in the biosynthesis of dopamine by keratinocytes and tyrosinase in the biosynthesis of melanin by melanocytes. Epidermal melanocytes and keratinocytes have a full capacity for de novo synthesis and recycling of BH$_4$, but in patients with the skin disorder vitiligo, 4a-hydroxy-BH$_4$ dehydratase levels are low, causing an accumulation of 7-BH$_4$ in the epidermis (Schallreuter et al., 1994). This non-enzymatic by-product is a potent competitive inhibitor of P' H, resulting in phenylalanine accumulation in the epidermis (Schallreuter et al., 1998). 5,6,7,8-BH$_4$ controls melanin biosynthesis in the human epidermis, but 7-BH$_4$ may disrupt tyrosinase activity via a specific binding domain (Wood et al., 1995), initiating depigmentation and vitiligo in patients (Schallreuter et al., 1994).

1.12.1 Inborn errors of BH$_4$ metabolism

Greater than 107 disease states related to BH$_4$ deficiency have been reported. These include defects with PCD and DHPR for the BH$_4$ recycling pathway, however more common are disorders of the BH$_4$ synthesis pathway involving GTPCH and PTPS. NOS has a 1000-fold higher affinity for BH$_4$ than the AAAHs, hence disorders of the BH$_4$ synthesis pathway are predominantly characterised by lowered levels of DA, NA and
serotonin rather than lower levels of NO. Thus, deficiency of CA usually is reflected in severe neurological symptoms which are unresponsive to a low phenylalanine diet.

1.12.2 NOS dysfunction associated with BH₄ deficiency

LPS-induced vascular dysfunction is mediated by the overproduction of NO. Since the NOS cofactor BH₄ is induced by LPS and required for NOS activity, blocking BH₄ synthesis was suggested as a potentially useful therapeutic intervention for conditions arising from NO excess (Gross & Levi, 1992). Gross showed that BH₄ availability also limits LPS-induced killing and vascular dysfunction in vivo (Gross, 1995). Disruption of pterin synthesis may be an important target for pharmacological interventions intended to limit NO overproduction in disease states, e.g. septic and cytokine-induced shock. NO overexpression also contributes to the cerebral ischemia and progression of infarcts. NOS activity in ischemia might be influenced by the availability of NOS cofactors such as BH₄ (Ashwal et al., 1998). In the eNOS isoform, superoxide generation is regulated by BH₄; this provides insight into potential therapy of NO/oxidant imbalance and vascular endothelial dysfunction in conditions such as hypercholesteremia, hypertension and ischemia reperfusion injury (Xia et al., 1998). The production of O₂⁻ has important pathological effects including the reaction with NO to form peroxynitrite (OONO⁻) which may mediate free radical damage in such disease states such as atherosclerosis (White et al., 1994).
Chapter 2 - TRANSCRIPTIONAL REGULATION OF THE GTPCH GENE

2.1 Introduction

Dihydroneopterin triphosphate (DNTP) is the product of the GTPCH catalysed reaction. Non-enzymatic degradation of DNTP yields plasma neopterin when the second enzyme in BH₄ de novo synthesis, PTPS, is rate-limiting (Werner et al., 1993). The earliest indication that GTPCH activity could be upregulated by pathophysiological stimuli came from the observation that patients with severe viral infections or malignant disease excreted neopterin (Wachter, 1979). Increased neopterin levels are also associated with atypical phenylketonuria (Nixon, 1980), which we now know to arise from PTPS or SR enzyme mutations.

The first direct evidence that cytokines can upregulate levels of neopterin came from studies of human macrophages in culture; when treated with IFN-γ, cells accumulated neopterin but little biopterin (Huber, 1984). Lectins were also observed to stimulate human peripheral blood mononuclear cells to accumulate neopterin and IFN-γ was identified as a principal cytokine mediator of this response (Ziegler, 1985). At the same time, phytohemagglutinin treatment of monocytes from normal patients, compared to patients with known deficiency in pterin metabolism, revealed GTPCH to be the enzyme responsive to immunostimulation (Blau et al., 1985).

Using specific monoclonal antibodies, IFN-γ was determined to directly effect
neopterin accumulation in immunostimulant-activated macrophages (Schoedon et al., 1986). The elevating action of IFN-γ was similarly observed in several other human cell lines (Werner et al., 1989). Accumulation of neopterin in media was attributed to GTPCH induction (Werner et al., 1990). Human THP-1 cells (a myelomonocytic cell line) and macrophages show higher accumulation of neopterin than fibroblasts, leading investigators to suggest that specifically PTPS is rate-limiting in human monocytes (Schoedon et al., 1986; Werner et al., 1990). It is interesting to note that in murine cells the Km of PTPS is 150 µM, levels which are never attained in mouse cells and therefore PTPS is never likely to become rate limiting in mice (Werner et al., 1990).

IFN-γ was observed to upregulate BH₄ levels in T cell lines in a synergistic manner with IL-2 (Ziegler, 1990). Other factors have also been implicated in the upregulation of GTPCH activity, including kit ligand stem cell factor (Ziegler et al., 1993) which increases GTPCH activity without influencing SR or PTPS activity. IL-4, IL-10 and TGF-β were all shown to downregulate immunostimulant-evoked GTPCH induction and biopterin accumulation in endothelial cells (Schoedon et al., 1993). The development of a human cDNA probe has enabled the demonstration that increased GTPCH mRNA levels occur following lectin treatment of human peripheral blood T lymphocytes and IFN-γ treatment of human myelomonocytic cells and a T-cell line (Schott et al., 1993). These observations show that GTPCH expression can be specifically upregulated by inflammatory stimuli and
provide an explanation for neopterin accumulation. It is notable that increases in substrate (GTP) levels can also be elicited by inflammatory stimuli, possibly contributing to observed increases in GTPCH activity (Hatakeyama et al., 1992).

Basal levels of biopterin were undetectable in rat vascular smooth muscle cells, however, biopterin and GTPCH mRNA were greatly increased following treatment with LPS and IFN-γ (Hattori & Gross, 1993). Not surprisingly, actinomycin D, an inhibitor of transcription, was observed to abolish immunostimulant-induced production of GTPCH mRNA (Hattori & Gross, 1993). The same investigators also found that the time course for GTPCH induction reflected iNOS mRNA accumulation with maximal mRNA levels seen by 4 h and maintained for 24 h (Hattori & Gross, 1993). Evidence that cytokine-stimulated GTPCH activity was mediated via an increase in GTPCH gene transcription was provided by nuclear run on assays (Katusic et al., 1998). LPS also caused a 2-3 fold increase in GTPCH activity in a variety of rat tissues in vivo, including lung, heart and kidney (Hattori et al., 1995). In vascular smooth muscle cells, LPS treatment increases both GTPCH mRNA levels and GTPCH activity (Hattori & Gross, 1993). LPS coactivates GTPCH and iNOS and BH₄ is rate-limiting for NO production in vascular smooth muscle (Gross et al., 1993; Hattori & Gross, 1993). In granulosa cells, IL-1α and follicle stimulating hormone (FSH) were demonstrated to increase GTPCH mRNA levels (Tabraue et al., 1997).

The mechanisms by which immunostimulants upregulate GTPCH transcription
have not been fully elucidated. In rat vascular smooth muscle cells, the protein synthesis inhibitor cyclohexamide (CHX) potentiated LPS and IFN-γ elicited increases in GTPCH mRNA levels, prompting the suggestion that the GTPCH mRNA stability might be regulated by a labile destabilising protein (Hattori & Gross, 1993). In murine fibroblasts, CHX was also observed to increase GTPCH activity, both alone and in combination with cytokines (Werner et al., 1991).

NF-κB is a heterodimeric complex found in the cytoplasm. When bound to the inhibitory protein I-κB, NF-κB is inactive (Novak, 1991), however, phosphorylation of I-κB results in proteolytic degradation and subsequent translocation of NF-κB to the nucleus where it binds to DNA sequences and activates transcription of specific genes (Baldwin, 1996). Increases in NF-κB activation, through cytosolic degradation of I-κB, have been observed after cell treatment with TNF-α, IL-1 and LPS (Beg, 1993). Hattori and coworkers found that pyrrolidine dithiocarbamate, a selective inhibitor of NF-κB activation, attenuated immunostimulant-induced tetrahydrobiopterin synthesis in rat aortic smooth muscle cells (Hattori et al., 1996); this finding suggests that GTPCH may be upregulated by NF-κB as was previously verified for iNOS (Chao et al., 1997).

A wealth of literature demonstrates co-induction of GTPCH and iNOS by LPS (Gross & Levi, 1992; Hattori & Gross, 1993; Hussain et al., 1997; Kapers et al., 1997; Sooranna et al., 1995; Werner-Felmayer et al., 1993). Common mediators have also been
identified which down-regulate the two enzymes; these include endothelin-1 (Hirahashi et al., 1996) and Ox-LDL (Dulak et al., 1997). In a recent report, TNFα was shown to potentiate IL-1β and IFN-γ-evoked stimulation of both NO and BH₄ synthesis, but only iNOS mRNA induction (not GTPCH mRNA) was found to be inhibited by pyrrolidine dithiocarbamate and the peptide SN-50, agents that inhibit NF-κB activation (Vann, 2000). The latter finding suggests that whilst cytokines might act in a similar manner regarding NO and BH₄ upregulation, they may do so via discrete pathways (Vann, 2000).

In accord with the view that distinct pathways control iNOS and GTPCH expression, certain cytokines have been reported to have differential actions. Notably, TGFβ attenuates GTPCH upregulation by IL-1β while having no effect on iNOS expression (Scott-Burden et al., 1993). Nonetheless, a disparity of reported cytokine actions is highlighted by the opposite findings of another group, where TGFβ was found to attenuate IFNγ-induced iNOS mRNA levels (Vodovotz et al., 1993).

In PC-12 cells, cAMP was shown to increase GTPCH activity (Kashima & Nakanishi, 1990). Similarly, in rat aortic smooth muscle cells, the adenylate cyclase activator forskolin increased GTPCH mRNA levels (Scott-Burden et al., 1994). Phorbol esters, mediate increases in PKC activity, and elevate BH₄ levels in rat liver (Hortelano, 1992). The mechanism by which cAMP and PKC may act to enhance GTPCH activity, transcriptionally or post-transcriptionally, was undefined at the start of my studies.
In cardiac microvascular endothelial cells, glucocorticoids have been demonstrated to inhibit LPS/IFN-induced iNOS and GTPCH activity, thereby downregulating NO synthesis (Frank et al., 1998; Simmons et al., 1996). However, in human umbilical vein endothelial cells, dexamethasone (Dex) did not attenuate cytokine-elicited biopterin or GTPCH mRNA levels (Hattori et al., 1997). Paradoxically, Dex was found to upregulate GTPCH mRNA levels in rat PC-12 cells (Serova et al., 1997). The specific role of glucocorticoids in GTPCH regulation awaits molecular definition.

In view of the essential role played by BH₄ in the synthesis of monoamine transmitters, it would not be surprising if these end products exerted influence on the cofactor required for their synthesis. Reserpine, an agent that depletes stored catecholamines, was reported to increase in vivo biopterin levels (Abou-Donia et al., 1986) and levels of GTPCH mRNA (Hirayama et al., 1993). Similarly, cold stress, a long established stimulant for adrenomedullary tyrosine hydroxylase activity, also elevated biopterin levels (Baruchin et al., 1990). In monaminergic neurons there have been reports of heterogeneous GTPCH mRNA levels (Lentz & Kapatos, 1996) and protein activity (Hirayama & Kapatos, 1998). Nonetheless, there is no evidence to suggest that there are distinct constitutive and immunostimulant induced isoforms of GTPCH. GTPCH mRNA and protein levels are found to be co-localised to tyrosine hydroxylase producing neurons.
(Hwang et al., 1998). There is evidence of co-induction of GTPCH and tyrosine hydroxylase in response to vasointestinal peptide (Anastasiadis et al., 1998).

In monoamine-containing neurons, constitutive GTPCH expression and BH₄ biosynthesis is apparently regulated by soluble protein factors. Nerve growth factor (NGF) has been shown to increase GTPCH expression, in addition to promoting neuronal cell differentiation (Qiu et al., 1991). NGF-induced increases in BH₄ levels have been observed in PC-12 cells in association with an increase in GTPCH mRNA (Suzuki et al., 1988). NGF was also observed to upregulate GTPCH activity, mRNA and biopterin levels in superior cervical ganglia; a concomitant increase in tyrosine hydroxylase activity and mRNA levels was noted (Hirayama & Kapatos, 1995). In PC-12 cells, NGF and epidermal growth factor induced tyrosine hydroxylase activity was associated with co-induction of GTPCH protein (Anastasiadis et al., 1996).

Despite a wealth of information on the effects of growth factors and immunostimulants as enhancers of GTPCH mRNA and enzyme activity, essentially nothing is known regarding whether these effects involve an increase in GTPCH gene transcription. Prior to the initiation of my studies into GTPCH transcription, the only report on GTPCH gene regulation was from a group who determined that 600 bp of 5'-flanking sequence was necessary to maintain basal activity (Witter et al., 1996). The GTPCH genes from mouse and human had been cloned and the sequences were reported in Genbank
(Ichinose et al., 1995). A 2.6 kb promoter linked to a luciferase reporter, was found to not respond to IFN-γ stimulation in a cell test system and the same authors identified CCAAT, TATA-like boxes, and a GC-rich region close to the transcription start site that contained putative SP-1 response elements to be necessary for basal promoter activity (Witter et al., 1996).
2.2 Aims

To examine the extent to which modulators of GTPCH act on gene transcription, I have constructed GTPCH promoter-reporter vectors that use 1000, 3000 and 6000 bp of 5'-upstream sequence from the transcriptional start site of the rat GTPCH gene, cloned upstream of a cDNA that encodes secreted human placental alkaline phosphatase (Berger, 1987). Activity of these promoter constructs was assessed in stably-transfected cells, based on heat-stable alkaline phosphatase activity secreted into the cell culture media.

Two cell types were chosen for evaluation of GTPCH gene transcription: rat aortic smooth muscle cells (RASM) and the rat pheochromocytoma cell line, PC-12. RASM have been previously characterised for iNOS expression (Durante et al., 1993; Gross & Levi, 1992; Kilbourn et al., 1992); notably, these cells do not produce detectable catecholamines and therefore BH₄ is synthesised exclusively as a cofactor for support of NOS activity. In contrast, PC-12 cells basally synthesise both tetrahydrobiopterin and catecholamines and have been frequently used as a model of sympathetic neurons. We felt it was important to study the GTPCH transcription in both cell lines since the fate of tetrahydrobiopterin and therefore the purpose of GTPCH differs markedly for each. Whereas constitutive expression of GTPCH is essential for catecholamine biosynthesis in PC-12 cells, untreated RASM are devoid of basal GTPCH activity, however, many factors have been shown to upregulate GTPCH activity in these cells, including those that trigger iNOS gene expression. Our experiments were performed to identify factors that
could act on the GTPCH promoter to upregulate gene transcription. By contrasting promoter activity elicited by three lengths of 5'-upstream GTPCH gene sequence, I sought to elucidate regions of DNA sequence that contribute to transcriptional regulation.

Numerous agents that were previously shown to modulate GTPCH activity in various cell types were evaluated for their effects on GTPCH transcription, including: LPS (Hattori & Gross, 1993), IL-1 (Scott-Burden et al., 1993), IFN-γ (Schoedon et al., 1986), IL-6, TNF-α (Katusic et al., 1998), Dex (Serova et al., 1997), CHX (Hattori & Gross, 1995) and cAMP elevating agents (Kashima & Nakanishi, 1990; Scott-Burden et al., 1994). In PC-12 cells, I also investigated the effects of NGF (Hirayama & Kapatos, 1995; Suzuki et al., 1988) and catecholamine synthesis inhibitors (Abou-Donia et al., 1986) on GTPCH gene transcription.
2.3 Methods

2.3.1 Preparation of GTPCH promoter vectors.

All 5'-genomic DNA sequence fragments in the creation of SEAP reporter constructs originated from a pBluscript KSII vector containing 8.5kb of 5'-upstream DNA sequence of the rat GTPCH gene, which had been previously cloned in our lab. The restriction enzyme Kpn I (10 U/μl) was used to create a 3' overhang in the multiple cloning site of the 8.5 kb GTPCH pBluscript vector. Subsequently, 5 μg of this DNA (0.63 pmole ends) was digested at 37°C with Exo III nuclease (2.5 U) and mercaptoethanol (10 mM) in Exo III buffer; aliquots were removed every min, heated for 15 min at 68°C, and mung bean extract (15 U) was added to terminate the digestion. The pBluscript plasmid was cleaved from the GTPCH 5'-upstream gene fragments by 1 h digestion at 37°C with BSSH II (10 U/μl). The digested GTPCH DNA was separated by electrophoresis (100 Volts, 30 min) on a 1% agarose gel containing ethidium bromide and the GTPCH digestion product bands were excised. DNA was extracted from gel pieces using a Qiaex II gel extraction kit (Qiagen, CA) and eluted in 50 mM Tris, pH 8.3.

The 5'-upstream GTPCH gene fragments were designed to be inserted into the multiple cloning site of a promoter-driven secreted alkaline phosphatase (SEAP) vector (Berger, 1987) which contained both ampicillin and geneticin resistance genes. The commercially available SEAP vector (pCMV/SEAP; Tropix, MA) contained a CMV promoter which was removed by 2 h digestion at 37°C using the restriction enzymes Hind
Ill (10 U/µl) and Bgl II (10 U/µl) in Gibco BRL restriction buffer 2.

Prior to ligation, blunt ends were created on the 5' GTPCH fragments and the SEAP vector by 1 h digestion at 37°C using the restriction enzyme Pfu (10 U/µl) in Gibco BRL restriction buffer 1. DNA T4 ligase (Life Technologies) was used to ligate the pSEAP vector and 5' GTPCH upstream gene fragments in an overnight incubation at 16°C. Ligation products were transformed into 100 µl of competent DH5α bacteria (Life Technologies). Bacteria were mixed with DNA, incubated on ice for 30 min, heat shocked at 37°C for 30 seconds, incubated on ice for a further 2 min and then Lennox broth was added (LB- 0.9 ml). Cultures were shaken for 1 h at 37°C and then streaked on ampicillin (50 µg/ml) LB agar plates and grown overnight at 37°C. Colonies were isolated and grown in 5 ml LB broth containing ampicillin (50 µg/ml) overnight. DNA from overnight cultures was purified using a Qiagen spinprep DNA extraction kit and restriction digested with BamH I (10 U/µl) to ascertain the presence of inserts. Clones showing the presence of an insert were further digested with Pvu II (10 U/µl) to determine orientation of the inserts; correctly oriented inserts were confirmed by DNA sequencing.

2.3.2 Cell culture

Rat aortic smooth muscle cells were isolated from thoracic aorta explants of Fisher rats and grown as previously described maintained in RPMI media containing penicillin
(50 U/ml), streptomycin (500 ng/ml), L-Glutamine (1 mM) and 10% newborn calf serum (Gross & Levi, 1992).

PC-12 cells were a kind gift from Dr B. Hempstead. The cells are poorly adherent and were grown in dishes that had been precoated with rat tail collagen (Kaplan, 1991) containing DMEM media supplemented with 10% newborn calf serum, 5% horse serum, penicillin (0.1U/ml)/streptomycin (100 ng/ml) and L-Glutamine (1 mM). Fresh media was replaced every 48 h. Both cell types were maintained in a humidified incubator at 37°C, charged with 5% CO₂/ 95% O₂. Cell numbers were determined using a Coulter cell counter. Stocks of RASM and PC-12 cells were stored in liquid N₂ at a density of 1 x 10⁶ cells/ml in media containing 10% DMSO.

2.3.3 Transfection of mammalian cells

RASMC at passage 8-12 were seeded at a density of 1 x 10⁴ cells/ml in 35 mm² dishes and transfected when the cells reached 30% confluence. GTPCH promoter/SEAP reporter constructs (1.5 µg) were added to 52.5 µl LipofectAMINE (Life Technologies, Inc.) and 500 µl Opti-MEM (Life Technologies, Inc.). While plasmids and LipofectAMINE were mixed by inversion for 30 min, cells were washed 3 times with 5 ml phosphate buffered saline solution (PBS). RASM cells were incubated with the DNA complexes for 6 h, followed by replacement with fresh RPMI culture media. After 48 h,
geneticin (800 µg/ml), a concentration sufficient to kill 100% of untransfected cells within 5 days, was added to cell culture media to select for resistant cells.

PC-12 cells were transfected as described for RASM cells. For this purpose PC-12 cells were grown to 50% confluence in 35 mm² dishes that had been pre-coated with rat tail collagen. Transfection used 2 µg DNA per dish and LipofectAMINE reagent.

Constructs for expression of wild type and dominant-negative mutant cAMP response element binding protein (CREB) and CREB binding protein (CBP) were a kind gift from Dr R. Goodman (Kwok et al., 1994). The genes for the expression of these proteins were constructed in pRc/RSV vectors containing Rous Sarcoma virus long terminal repeat promoter and enhancer elements and as well as G418 resistance genes. The CREB dominant-negative mutant vector was truncated in the bZIP region (Olive, 1997). These vectors were transfected to PC-12 cells as described above.

2.3.4 Secreted Alkaline Phosphatase Assay (SEAP) assay

GTPCH/SEAP expressing PC-12 or RASM cells were grown to 50% confluence in 96 well plates and treated for 24 h with test agents. Cell media (50 µl) was removed from each well and incubated at 65°C for 30 min to destroy endogenous alkaline phosphatase activity.

A chemiluminescence based assay for SEAP (Tropix, Phospha-Light™) was
performed following the manufacturer's protocol. Media (50 μl) was removed from RASM cells, combined with 75 μl Phospha-Light™ dilution buffer, incubated at 65°C for 30 min and allowed to cool to room temperature for 10 min. Phospha-Light™ assay buffer which contained alkaline phosphatase inhibitors (100 μl) was added and incubated at room temperature for 5 min. Finally Phospha-Light™ reaction Buffer (100 μl) containing 25 μM CSPD™ chemiluminescent substrate and Emerald™ luminescence enhancer was added and samples were incubated for 20 min at room temperature. Luminescence was read during a 10 sec interval in a 1450 Microbeta liquid scintillation counter (Wallac) to quantify SEAP expression.

As an alternative to chemiluminescence assay, SEAP was quantified by spectrophotometry, media (50 μl) was heat treated, as described above. SEAP buffer 50 μl [diethanolamine 2 M, magnesium chloride 1 mM and L-homoarginine 20 mM] and Sigma 104 phosphate substrate (10 mM) in SEAP buffer, was added and the increase in OD_{405} nm was measured at 30 sec intervals for 1 h, at 37°C, in a kinetic microplate spectrophotometer (Molecular Devices; Menlo Park, CA). The rate of increase in OD_{405} with untreated cells was taken as control, and the fold-increase after treatment was calculated as the ratio of treatment to the control values.
2.3.5 MTT assay

At the end of each experiment cell viability was assessed to affirm that this did not account for observed changes in SEAP expression. Media was removed from cells in 96-well plates and 200 µl fresh media was added containing 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, 0.2 mg/ml). After 60 min, at 37°C, MTT containing media was removed by suction and 100 µl DMSO was added to solubilise cells. After shaking for 2 min, OD$_{550}$ nm was determined using a spectrophotometric microplate reader (Molecular Devices; Menlo Park, CA).

2.3.6 Quantification of total biopterin content of cells

Cells were seeded in T75 flasks at a density of 1 x 10$^6$ cells/ml and grown to confluence. At the defined time, cells were harvested by treatment with 4 ml trypsin (0.05%) per 75 mm$^2$ flask for 2 min (PC-12 cells) or 4 min (RASM cells). Fresh media was added to terminate the action of the trypsin and cells were centrifuged 10,000 x g at 4°C for 15 min. Cell pellets were resuspended in 0.5 ml of lysis buffer consisting of: Tris-HCl pH 7.4 (100 mM), dithiothreitol (1 mM), phenylmethlysulfonyl fluoride (1 mM), leupeptin (5 µg/ml), pepstatin A (5 µg/ml) and chymostatin (5 µg/ml). Cells were ruptured by 3 cycles of rapid freeze-thaw using liquid N$_2$ and a 37°C waterbath. Cell supernatant was collected after centrifugation at 10,000 x g, at 4°C, for 30 min. Protein concentration was
determined using the Biorad protein assay (Macart, 1994). Biopterin in cell supernatants and culture media (50 μl) was determined after oxidation by treatment with 30 μl HCl (1 M) and 10 μl KI/I₂ (100 mM/100 mM) in the dark at 37°C for 1 h. A final concentration of ascorbic acid (10 mM) was added, samples were vortexed and pH was neutralised with 40 μl NaOH (1 M). Samples were microfuged at 10,000 x g for 2 min and transferred to HPLC vials for pterin analysis.

HPLC analysis of pterins used a C18 reverse-phase column with potassium phosphate buffer pH 6.8 (15 mM) as mobile phase, at a flow rate of 1 ml/min. Pterins were detected by fluorescence, with excitation at 350 nm and emission at 440 nm. Authentic biopterin, pterin and neopterin served as reference standards (Schircks, Jona, Switzerland).

2.3.7 Protein Assay

Biorad dye reagent (Macart, 1994) was diluted with 4 parts distilled water and filtered to remove particulates; 10 μl of sample was added to 200 μl of diluted dye reagent. Unknown protein levels were quantified by comparison with a standard curve using bovine serum albumin in the range 20 – 500 μg/ml. All samples were read at OD₆₀₀ nm using a spectrophotometric microplate reader (Molecular Devices; Menlo Park, CA).
2.3.8 Greiss assay for nitrite and nitrate determination

For nitrite assay, cell culture media (75 µl) was removed from cells in 96 well plates and added to 75 µl Greiss reagent [1:1 sulfanilamide (10%) in ortho-phosphoric acid (50%) and N-(1-naphthyl)ethylenediamine dihydrochloride (1%)]. Plates were read in the presence of sodium nitrite and nitrate standards at OD$_{550}$ nm in a spectrophotometric microplate reader (Molecular Devices; Menlo Park, CA).

To assess the combined concentrations of nitrite and nitrate (NO$_x$), 25 µl media was incubated for 45 min at 37°C with 75 µl nitrite reductase buffer [FAD (0.1 mM), Tris-HCl (200 mM), NADPH (10 mM), nitrite reductase (0.1 U/ml)]. Residual NADPH (an interference with subsequent assay) was consumed by incubation for 30 min at 37°C in the presence of 10 µl LDH (0.058 U/µl) and pyruvate (0.5 mM). Griess reagent (100 µl) was added and plates were read as described above.

2.3.9 Identification of the putative transcription factor sites

Putative consensus sites for transcription factor binding in the GTPCH gene 5’ upstream sequence were identified using MatInspector (Quandt, 1995), a transcription factor identifying computer program. GTPCH gene sequences of human, rat and mouse were obtained from the NCBI database, and aligned using the Lasergene function Megalign.
2.4 Results

2.4.1.1 Determining basal SEAP activity

Using control SEAP vectors, negative control (pSEAP – SEAP vector with the CMV promoter excised to indicate blank levels of SEAP expression) yielded $OD_{405} 0.214 \pm 0.105$ (3 experiments where $n = 4$). The positive control (pSEAP/CMV – SEAP vector with CMV promoter, which has constitutive SEAP expression) had $OD_{405} 1.818 \pm 0.472$ (3 experiments $n=4$). Throughout these experiments it has become evident that there is some variation between assay plates of the same cell type transfected with the same vector assayed under the same conditions. Importantly inactivation (heat treatment 65°C) of endogenous alkaline phosphatase activity was crucial to the success of each assay.

Following transfection of PC-12 cells with GTPCH 1, 3 and 6 kb promoter-reporter constructs, antibiotic resistant clones were identified and pooled for study. The rationale for study of pooled rather than individual clones was to reduce the potential for cell line differences that may arise from clonal diversity. Basal SEAP activity of 1, 3 and 6 kb 5' upstream GTPCH sequence inserted into pSEAP vectors was assessed in parallel with the pSEAP and pSEAP/CMV. $OD_{405}$ values were obtained for the 1, 3 and 6 kb promoter constructs respectively: $0.189 \pm 0.061$, $0.204 \pm 0.080$ and $0.222 \pm 0.077$, the values for pSEAP ($0.178 \pm 0.056$) and pSEAP/CMV $1.574 \pm 0.307$ this experiment was repeated 3 times, $n=6$. MTT values for these assays did not vary for the PC-12 cells transfected with these constructs ($OD_{550} 0.474 \pm 0.121$).
difference (ANOVA analysis coupled to Dunnett’s test) between the GTPCH promoter constructs and the empty SEAP vector, a small difference was observed.

For each of the immunostimulant treatments described in the results, cells transfected with a single construct were plated on 96 well plates and treatments applied to that plate. Since the OD_{405} values were modest and there was some variability between plates the pSEAP value was not subtracted as blank; therefore within each plate the ratio of treated to untreated was calculated to give the fold-increase.

2.4.1.2 Characterising basal PC-12 cell biopterin levels

PC-12 cells are an immortalised adrenal medullary chromaffin cell line that synthesise both BH₄ and catecholamines and have been extensively studied as a model for sympathetic neurons. The level of endogenous total biopterin (BH₄ and more oxidised species) in untreated PC-12 cell cytosol was 3.56 ± 0.39 ng biopterin/mg protein as determined by HPLC analysis using fluorescence detection (Fig. 2.1). This value was calculated by comparison with a biopterin standard curve, that was linear in the range of 0.01 – 100 ng. The biopterin standard peak eluted at 14.2 min. Fluorescence peaks at 2.5 and 5 min were also detected in cell lysates, however neither of these matched the elution profile of pterin standards, nor did they display the prototypic spectral profile of a pterin.
Fig 2.1 HPLC analysis of biopterin levels in PC-12 cells

Elution profile of biopterin standard and biopterin standard curve (Panel A). Analysis of biopterin content in an extract of untreated PC-12 cells (Panel B). Figures represent a single experiment, where n=6.
The absence of neopterin or other pterins intermediates from PC-12 lysates is consistent with GTPCH being the rate-limiting enzyme in BH₄ biosynthesis (Werner et al., 1991).

2.4.1.3 Nerve growth factor (NGF) effects

Clones were analysed for the effects of NGF on the GTPCH promoter activity during 24 h; results are summarised in Table 2.1. Ratios of SEAP expression in NGF-treated (100 ng/ml) and untreated cells were compared for each of the lengths of GTPCH promoter-reporter constructs. Additionally, stably transfected PC-12 cells with SEAP expression plasmids that were either promoterless or driven by a constitutively active cytomeglovirus promoter, were assayed to control for possible non-specific influences of NGF on SEAP activity or expression.

As shown in Table 2.1, NGF was observed to activate all GTPCH promoter lengths to elicit SEAP expression. The activation elicited by NGF on the 1 kb GTPCH promoter was very modest $1.142 \pm 0.054$ but significant, and less than observed with the 3 and 6 kb GTPCH promoters ($1.250 \pm 0.065$ and $1.418 \pm 0.128$ respectively). NGF did not elicit an increase in apparent SEAP activity in culture medium of untransfected PC-12 cells, or cells transfected with either the promoterless or the constitutively active CMV promoter vectors.
Table 2.1: Effect of NGF treatment on SEAP expression by PC-12 cell lines.

<table>
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<tr>
<th>Cell line</th>
<th>Fold increase</th>
<th>N</th>
<th>P value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-12</td>
<td>1.027 ± 0.047</td>
<td>15</td>
<td>0.377</td>
<td>NS</td>
</tr>
<tr>
<td>P(PC-12)</td>
<td>1.045 ± 0.062</td>
<td>15</td>
<td>0.407</td>
<td>NS</td>
</tr>
<tr>
<td>CMV(PC-12)</td>
<td>1.006 ± 0.041</td>
<td>15</td>
<td>0.368</td>
<td>NS</td>
</tr>
<tr>
<td>1(PC-12)</td>
<td>1.142 ± 0.054</td>
<td>15</td>
<td>0.037</td>
<td>Sig</td>
</tr>
<tr>
<td>3(PC-12)</td>
<td>1.250 ± 0.065</td>
<td>15</td>
<td>0.010</td>
<td>V. Sig</td>
</tr>
<tr>
<td>6(PC-12)</td>
<td>1.418 ± 0.128</td>
<td>15</td>
<td>0.004</td>
<td>V. sig</td>
</tr>
</tbody>
</table>

Cells were stably-transfected with GTPCH gene promoter containing or control SEAP expression vectors, as described in methods. Values are the mean ratios of NGF treated (100 ng/ml) to untreated SEAP activity, accumulated in culture media, during 24 h. Results are from three experiments (where n=5) and all 15 values were considered to determine by ANOVA coupled to Dunnett’s test the level of significance. PC-12 = untransfected cells, P(PC-12) = PC-12 cells transfected with promoterless SEAP vector, CMV(PC-12) = PC-12 cells containing CMV constitutively-driven SEAP vector, 1(PC-12) = PC-12 cells containing 1 kb GTPCH promoter in SEAP vector likewise; 3(PC-12) & 6(PC-12) refer to PC-12 cells containing 3 kb and 6 kb GTPCH promoter in the SEAP vector.

The NGF concentration-response relationship for eliciting SEAP expression was assessed for each of the stably-transfected PC-12 cell lines, after a 48 h incubation (Fig 2.2A). A maximum response was observed with 100 ng/ml NGF for each GTPCH promoter length. With 1, 3 and 6 kb GTPCH promoters NGF treatment elicited maximal 1.15, 1.4 and 1.35 -fold increases in SEAP activity, respectively. Although SEAP activity with the 1kb GTPCH promoter was modestly above the level of control, it was determined to be statistically significant at 100 ng/ml by ANOVA and Dunnett’s test (P<0.05). Cells transfected with the CMV promoter-driven SEAP vector displayed no NGF-induced increase in SEAP expression.
Fig. 2.2 Effect of NGF on GTPCH promoter-driven SEAP activity in PC-12 cells. Concentration-dependence of NGF-induced SEAP expression after 48 hour treatment (Panel A). Time course of NGF (100 ng/ml) elicited changes in SEAP expression (Panel B). SEAP activity is expressed as fold-increase over control, where control = untreated cells. In each case the depicted results are from 1 experiment, where n = 6; similar results were found in 2 other experiments.
Using the 100 ng/ml NGF, the dose which appeared to elicit maximal SEAP expression, the time course of SEAP accumulation was analysed. As shown in Fig 2.2B, a small significant response was seen by 24 h for each of the GTPCH constructs. At this time, NGF-elicited SEAP expression was greatest with the 3kb GTPCH promoter, demonstrating a 1.45 fold increase. Whereas the 3 kb promoter response fell thereafter, responses for the 1 and 6 kb GTPCH promoter continued to increase, reaching a maximum at 48 h (Fig 2.2B) and were sustained a further 48 h until 96 h. Beyond 96 h, cell viability dropped dramatically and detachment from the culture wells precluded meaningful investigation.

The influence of NGF (100 ng/ml) on endogenous levels of biopterin in PC-12 cells was studied after a 48 h treatment (Fig 2.3A). NGF (100 ng/ml) elicited a statistically significant 130% increase in biopterin levels, which was not attenuated by the cAMP antagonist RpcAMP (200 μM), but was significantly attenuated (50%) by sphingosine (5 μM), a PKC inhibitor, and abolished by the transcription inhibitor actinomycin D. RpcAMP (200 μM) also had no effect upon NGF induced 1, 3 or 6 kb GTPCH promoter-driven SEAP activity (Fig 2.3B shows lack of effect on 3 kb GTPCH promoter expression) whereas sphingosine (5 μM) attenuated both basal and NGF-induced SEAP expression. As anticipated, actinomycin D (1 μg/ml) abolished all SEAP expression, but in association with a 70% fall in cell viability.
Fig 2.3: Effect of various agents on biopterin levels and SEAP expression of PC-12 cells. RpcAMP (200 μM), actinomycin D (1 μg/ml) and sphingosine (5 mM), on basal and NGF (100 μg/ml) elicited SEAP expression by the 3 kb GTPCH promoter-reporter construct (Panel A). Values are mean ± SEM, where n=6. Similar results were observed with 1 & 6 kb GTPCH promoter-reporter constructs. HPLC analysis of PC-12 cell biopterin content (Panel B); concentrations of NGF, sphingosine, actinomycin D and RpcAMP, were as in Panel A. Dexamethasone (Dex) was applied at a concentration of 0.1 μM. Control biopterin content was 3.56 ± 0.38 ng/mg protein where n=3. * Indicates P<0.05 and ** P< 0.01, determined by ANOVA and Dunnett's test. The graph represents the mean of 3 experiments where n=2.
2.4.1.4 Influence of cytokines on NO synthesis and GTPCH promoter expression.

It is well established that immunostimulants elicit iNOS expression in numerous cell types. Following immunostimulant treatment (LPS, IFN-γ, IL-1, IL-6 and TNF-α; all over a range of concentrations) PC-12 cell culture medium was collected and analysed for nitrite accumulation during a 24 h period. Analysis with sodium nitrite standards indicated that the limit of detection is ≈ 0.5 μM by the method employed. In all cases, nitrite was undetectable and therefore PC-12 media contained less than 0.5 μM. In order to test whether NO was produced by PC-12 cells but accumulated as nitrate rather than nitrite (due to further oxidation), media was also incubated with nitrite reductase, as described in “Methods” and re-analysed. Resulting nitrite/nitrate levels were similarly found to be indistinguishable from background.

The potential for IL-1β to upregulate GTPCH gene expression was analysed in cells transfected with 1, 3 and 6 kb GTPCH promoter-SEAP reporter constructs. IL-1β was administered to cells for 24 h, over a range of concentrations (1 – 1000 ng/ml, see Fig 2.4A). With the 6 kb GTPCH promoter, IL-1β (3 ng/ml) elicited a statistically significant 25%, increase in SEAP activity. At higher IL-1β concentrations, GTPCH promoter activity fell progressively such that upregulation was abolished with 1000 ng/ml. MTT assays confirmed that reduction in promoter activity observed with these higher concentrations of IL-1β, was not associated with a loss in cell viability. Although IL-1β also stimulated
Fig 2.4: Effect of 24 h cytokine treatment on SEAP expression of PC-12 cells.
Effect of interleukin 1β (Panel A); Interferon-γ (Panel B); Tumour necrosis factor-α (Panel C); Interleukin 6 (Panel D), on SEAP expression of PC-12 cells stably transfected with 1, 3 and 6 kb GTPCH promoter-reporter constructs. IL-1 was the only cytokine to elicit a statistically significant increase in SEAP activity (* p<0.05), as measured by one way ANOVA and Dunnett's test. All SEAP activities are expressed as fold-increase over control, where control in each experiment is untransfected PC-12 cells. Panels represent results of one experiment where n=6. Values are means ± SEM and similar results were obtained in at least 2 other experiments.
SEAP expression in cells transfected with 1 and 3 kb GTPCH promoter SEAP reporter constructs, ANOVA analysis coupled to Dunnett’s test did not reveal this to be statistically significant.

GTPCH promoter activity (1, 3 and 6 kb) was not enhanced by TNF-α treatment over the concentration range of 1–1000 ng/ml, during a 24 h incubation (Fig 2.4B). Additional experiments were performed with the longer incubation period of 48 h, and still no statistically significant effect was observed with the maximal concentration of TNFα tested. IFN-γ (Fig 2.4C) and IL-6 (Fig 2.4D) over a range of concentrations also failed to elicit a change in GTPCH promoter-driven SEAP activity after either 24 or 48 h treatments.

In contrast to the above cytokines, each of the GTPCH promoter constructs in PC-12 cells responded to LPS treatment with an increase in SEAP expression (Fig 2.5A). The 3 kb promoter construct consistently showed the greatest fold-increase in response to LPS, as compared with the 1 or 6 kb constructs. The concentration of LPS necessary to elicit a maximum response was 10 ng/ml. At LPS concentrations > 100 ng/ml, SEAP activity fell towards the level of untreated cells concomitant with a loss in cell viability.
Figure 2.5: LPS, dexamethasone and cyclohexamide induced SEAP expression. LPS (Panel A), dexamethasone (Panel B) and cyclohexamide (Panel C) effects on SEAP expression by PC-12 cells, stably transfected with 1, 3 and 6 kb GTPCH promoter-SEAP reporter constructs. Control is untransfected PC-12 cells; values are means ± SEM. In each case, results are from one experiment where n=6; similar results were obtained in two other experiments.
2.4.1.5 Influence of dexamethasone & cyclohexamide on GTPCH promoter expression

PC-12 cells were treated with dexamethasone (Dex) over a range of concentrations (0.01-10 mM). It was found that the 3 kb GTPCH promoter-reporter transfected cells responded to Dex treatment (Fig 2.5B) with a dose-dependent increase in SEAP activity, whereas little (6 kb) or no (1 kb) effect was observed with other promoter constructs. For the 3 kb GTPCH promoter, a maximal 2.5-fold increase in SEAP activity was seen with 0.03 – 0.1 µM Dex; this effect diminished progressively as Dex concentration increased further. Over the entire concentration range tested, Dex manifested no detectable effects on PC-12 cell viability. Dex treatment (100 ng/ml) also elicited a 20% increase in biopterin content of untransfected PC-12 cells (Fig. 2.3B), this however was not found to be significant by ANOVA.

Cyclohexamide (CHX), an inhibitor of protein synthesis, paradoxically (0.1 mg/ml) elicited a 1.2-1.4-fold increase in SEAP activity with each of the GTPCH promoter reporter constructs tested (Fig 2.5C). In all cases, a maximum response was obtained with a CHX concentration of 1 mg/ml; above this concentration, SEAP activity and cell viability fell dramatically.
2.4.1.6 Influence of cyclic AMP on GTPCH promoter expression and BH4 accumulation

The levels of endogenous total biopterin were quantified in PC-12 cells following administration of cAMP-elevating agents. Cells were grown in 75 cm² flasks to 90% confluence ~ 1x 10⁷ cells; cell pellets from each flask were washed, lysed and supernatants assayed for biopterin content. Endogenous levels of total biopterin in untreated cells, measured by HPLC using fluorescence detection, were determined to be 3.556 ± 0.387 ng (Fig 2.6A). Following a 24 h treatment with dibutyryl cAMP (DBcAMP, 300 µM), a 4-fold increase in biopterin was observed (Fig 2.6A: P<0.01, n=4). Similarly, an approximate 4-fold increase in biopterin was observed in cells treated with cholera toxin (3 µg/ml; P<0.01, n=3, Fig 2.6A) and 1.8-fold increase in biopterin, was observed in cells after treatment with forskolin (10 µM; P<0.05, n=3, Fig 2.6A). In contrast, the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX 10 µM) alone elicited no change in cellular biopterin levels (Fig 2.6A).

Expression of SEAP activity by GTPCH promoter-reporter transfected PC-12 cells was increased following exposure to cAMP-elevating stimuli. SEAP expression was elicited for each of the 1, 3 and 6 kb GTPCH promoter reporter constructs in response to the cell-permeant cAMP analogue, DBcAMP (300 µM), forskolin (10 µM) and cholera
Fig 2.6: Effect of cAMP agonists on biopterin levels and SEAP expression of PC-12 cells. DBcAMP (300 µM), Forskolin (10 µM), cholera toxin (0.5 µg/ml), IBMX (10 µM) on basal PC-12 cell biopterin content (Panel A). SEAP expression of the 3 kb GTPCH promoter-reporter construct (Panel B), concentrations of DBcAMP, forskolin, cholera toxin and IBMX were as in Panel A, DBcGMP (300 µM). Similar results were observed for the 1 and 6 kb GTPCH promoter-reporter constructs. Values represent the means ± SEM of 3 experiments where samples were quantified in replicate. In each panel * indicates P<0.05, ** P< 0.01 and *** P<0.001, as determined by ANOVA and Dunnett's test.
toxin (0.5 mg/ml); shown in Fig 2.6B is the result obtained from PC-12 cells stably-transfected with the 3 kb GTPCH promoter-reporter construct. Maximal SEAP expression was observed with 0.3-1.0 mM DBcAMP for each of the 1, 3 and 6 kb GTPCH promoter constructs (Fig 2.7A). Forskolin also elicited a concentration-dependent increase in SEAP activity with each of the GTPCH promoters studied (1.4 - 1.6 fold, Fig 2.7B). IBMX (10 μM) failed to elicit increased SEAP activity in PC-12 cell lines stably transfected with either 1, 3 or 6 kb GTPCH promoter-reporter constructs; Fig 2.6B represents the effects exclusively with the 3 kb GTPCH promoter. A cell permeant cGMP analogue, dibutyryl cGMP, similarly failed to trigger SEAP expression of any of the GTPCH promoter-reporter constructs under investigation; whilst Fig 2.6B depicts this finding for the 3 kb promoter construct, similar results were obtained with 1 and 6 kb promoter constructs. Analysis of the GTPCH rat promoter sequence with MatInspector (Quandt, 1995), a program to identify putative transcription sites, revealed that there are at least 8 putative cAMP response elements (CRE) within the initial 6 kb of 5'-upstream GTPCH gene sequence (see table 2.2).

In order to investigate whether availability of cAMP response element binding protein (CREB) and/or CREB binding protein (CBP) could effect endogenous levels of total cellular biopterin, PC-12 cells were stably-transfected with an expression vector for wild type CREB (CREBWT), a dominant-negative mutant CREB (CREBM1) and CREB 2-34
Figure 2.7: Effect of cAMP analogues on SEAP expression by PC-12 cells. Treatment with Dibutyryl cAMP (Panel A) and forskolin (Panel B) of PC-12 cells that had been stably transfected with 1, 3 and 6 kb GTPCH promoter-SEAP reporter constructs. Results are means ± SEM from 1 experiment, where n=6. Similar results were obtained in 2 other experiments.
Table 2.2 - Transcription factor consensus sites in the 6 kb of 5'-flanking nucleotide sequence immediately upstream of the GTPCH gene

<table>
<thead>
<tr>
<th>Consensus site</th>
<th>Putative ligand</th>
<th>References</th>
<th>Site#</th>
<th>POSITION*</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP1-C</td>
<td>AP-1 binding site</td>
<td>(Rana, 1994)</td>
<td>4</td>
<td>-4189, -3294, -1091, -927</td>
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<tr>
<td>CEBP-C</td>
<td>C/EBP binding site</td>
<td>(Landschulz, 1988)</td>
<td>3</td>
<td>-4866, -3640, -14</td>
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<tr>
<td>CMYB-01</td>
<td>c-Myb</td>
<td>(Nakagoshi, 1990)</td>
<td>10</td>
<td>-5644, -4253, -4202, -3197, -2359, -2222, -2146, -1939, -961, -169</td>
</tr>
<tr>
<td>CREBP1-01</td>
<td>cAMP-responsive element (CRE) binding protein</td>
<td>(Benbrook, 1994)</td>
<td>8</td>
<td>-5232, -5191, -5189, -187, -4441, -4286, -110, -106,</td>
</tr>
<tr>
<td>GRE-C</td>
<td>Glucocorticoid response element</td>
<td>(Scheidereit, 1984)</td>
<td>1</td>
<td>-224</td>
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<td>HLF-01</td>
<td>Hepatic leukaemia factor</td>
<td>(Hunger, 1994)</td>
<td>5</td>
<td>-4082, -3637, -3390, -2479, -2364</td>
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<tr>
<td>HNF1-01</td>
<td>Hepatic nuclear factor 1</td>
<td>(Tronche, 1992)</td>
<td>2</td>
<td>-5126, -4979</td>
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<tr>
<td>IRF1-01</td>
<td>Interferon regulatory factor 1</td>
<td>(Tanaka, 1993)</td>
<td>2</td>
<td>-5372, -2463</td>
</tr>
<tr>
<td>IRF2-01</td>
<td>Interferon regulatory factor 2</td>
<td>(Tanaka, 1993)</td>
<td>1</td>
<td>-2463</td>
</tr>
<tr>
<td>MYCMAX-01</td>
<td>c-Myc/Max heterodimer</td>
<td>(Solomon, 1993)</td>
<td>2</td>
<td>-5570, -3323</td>
</tr>
<tr>
<td>MYCMAX-02</td>
<td>c-Myc/Max heterodimer</td>
<td>(Blackwell, 1993)</td>
<td>3</td>
<td>-5569, -4840, -3322</td>
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<tr>
<td>NF-IL6</td>
<td>Nuclear factor IL-6</td>
<td>(Akira, 1992)</td>
<td>1</td>
<td>-3639</td>
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<td>NFKAPPAB50-01</td>
<td>NF-kappaB (p50)</td>
<td>(Kunsch, 1992)</td>
<td>4</td>
<td>-2946, -1488, -462, -28</td>
</tr>
<tr>
<td>NFKAPPAB65-01</td>
<td>NF-kappaB (p65)</td>
<td>(Kunsch, 1992)</td>
<td>4</td>
<td>-2946, -1488, -462, -28</td>
</tr>
<tr>
<td>NFY-01</td>
<td>Nuclear factor Y (Y-box binding site)</td>
<td>(Dorn, 1987)</td>
<td>11</td>
<td>-5198, -4073, -4027, -3354, -3130, -2195, -2126, -1839, -986, -842, -93</td>
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<td>NFY-C</td>
<td>NF-Y binding site</td>
<td>(Dorn, 1987)</td>
<td>1</td>
<td>-986</td>
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<tr>
<td>NFY-Q6</td>
<td>Nuclear factor Y (Y-box binding site)</td>
<td>(Mantovani, 1992)</td>
<td>11</td>
<td>-5195, -4070, -4025, -3352, -2552, -2275, -2118, -1836, -984, -842, -91</td>
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<tr>
<td>NGFIC-01</td>
<td>Nerve growth factor-induced protein C</td>
<td>(Swirnoff, 1995)</td>
<td>1</td>
<td>-1939</td>
</tr>
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<td>RREB1-01</td>
<td>Ras-responsive element binding protein 1</td>
<td>(Thiagalingam, 1996)</td>
<td>1</td>
<td>-958</td>
</tr>
<tr>
<td>SPI-01</td>
<td>Stimulating Protein 1</td>
<td>(Schmidt, 1989)</td>
<td>4</td>
<td>-2916, -139, -134, -35</td>
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<tr>
<td>VMYB-01</td>
<td>v-Myb</td>
<td>(Ness, 1989)</td>
<td>2</td>
<td>-3880, -277</td>
</tr>
<tr>
<td>VMYB-02</td>
<td>v-Myb</td>
<td>(Grotewold, 1994)</td>
<td>2</td>
<td>-3879, -277</td>
</tr>
</tbody>
</table>
binding protein (CBP). Results of biopterin determinations are summarised in Table 2.3. Endogenous biopterin levels in pooled clones of cells harboring each of these vectors was measured by HPLC analysis. Although the CBP transfected PC-12 cells appeared to have almost twice the basal levels of biopterin in untransfected PC-12 cells, this difference was not found to be statistically significant (p>0.05). Basal biopterin levels were unchanged in PC-12 cells that overexpress either CREBWT or CREBM1 (Table 2.3). Similarly, neither CREBWT, CREBM1 nor CBP overexpression was observed to alter the ability of DBcAMP (300 µM) or cholera toxin (3 µg/ml) to elicit an increase in biopterin levels (Table 2.3).

2.4.1.7 Influence of catecholamine inhibitors on the GTPCH promoter

In PC-12 cells, BH₄ production is essential to support the synthesis of catecholamines. In this set of experiments, potential effects of catecholamine synthesis inhibitors were examined for their potential to upregulate GTPCH gene expression (Fig 2.8). Toward this end, I investigated whether p-chlorophenylalanine (PCPA), an inhibitor of both tryptophan hydroxylase and phenylalanine 4-hydroxylase (Gal, 1982), could modulate GTPCH promoter–reporter activity. Concentrations of PCPA ranging from 0.5 to 500 µM were tested, which had previously been demonstrated to inhibit catecholamine synthesis (Gal, 1982) (Fig 2.9A). No effects were observed on SEAP expression with any
Table 2.3: Effect of overexpressing CREBWT, CREBM1 and CBP in PC-12 cells on basal biopterin levels and biopterin elevation elicited by cAMP elevating agents.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Basal – untreated</th>
<th>DBcAMP (300 μM)</th>
<th>Cholera toxin (3 μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untransfected</td>
<td>3.41 ± 0.55 (4)</td>
<td>14.7 ± 1.73 (4)</td>
<td>13.2 ± 1.63 (3)</td>
</tr>
<tr>
<td>CREBM1</td>
<td>3.06 ± 0.51 (4)</td>
<td>11.2 ± 4.80 (4)</td>
<td>9.91 ± 5.67 (3)</td>
</tr>
<tr>
<td>CBP</td>
<td>6.28 ± 1.77 (4)</td>
<td>15.0 ± 1.72 (4)</td>
<td>18.6 ± 7.95 (3)</td>
</tr>
<tr>
<td>CREBWT</td>
<td>3.15 ± 1.16 (4)</td>
<td>14.1 ± 4.90 (4)</td>
<td>16.1 ± 6.81 (3)</td>
</tr>
</tbody>
</table>

Values represent means ± SEM of biopterin expressed in ng/mg protein (n is noted in parenthesis). Cells were treated for 24 h and assayed in parallel for biopterin content. Basal biopterin levels were not significantly different for transfected and untransfected PC-12 cell lines (p>0.05). Similarly, transfected and untransfected cell lines responded in a similar manner to stimulation by the cAMP elevating agents, dibutyryl cAMP and cholera toxin.
Fig. 2.8 – Enzyme targets of the inhibitors of de novo BH₄ synthesis, pterin salvage, pterin recycling and AAAH studied.

![Diagram of enzyme targets](image)

Sepiapterin reductase (SR), dihydropteridine reductase (DHPR), dihydrofolate reductase (DHFR), p-chlorophenylalanine (PCPA), α-methyl-paratyrosine (AMPT), 2,4-Diamino-6-hydroxy-pyrimidine (DAHP), N-acetylserotonin (NAS) and methotrexate (MTX), tyrosine hydroxylase (TH), phenylalanine hydroxylase (PH).
Fig 2.9: Effect of various reagents on SEAP expression of PC-12 cells. P-chlorophenylalanine (panel A), α-methyl-p-tyrosine (panel B) and methotrexate (panel C) on basal SEAP expression of PC-12 cells transfected with 1, 3 and 6 kb GTPCH promoter-reporter constructs. Control is untransfected PC-12 cells. None of the treatments elicited a statistically significant response. This represents one experiment where n=6, similar results were obtained in 2 other experiments.
of the GTPCH promoter constructs. Tyrosine hydroxylase can be selectively inhibited by \( \alpha \)-methyl-paratyrosine (AMPT) (Gronblad, 1977); the effects of this drug were similarly examined on the GTPCH promoter constructs in PC-12 cells (Fig. 2.9B). Again, no influence on SEAP expression was observed. Neither of these catecholamine synthesis inhibitors diminished cell viability at the concentrations used, as determined by measurements of cell respiration.

2.4.1.8 Influence of inhibitors of the GTPCH synthesis pathway on GTPCH promoter expression

To investigate possible transcriptional regulation of the GTPCH gene by blockers of bioppterin synthesis, several inhibitors of de novo and salvage pathways (Fig 2.8) were tested alone and in combinations. 2,4-Diamino-6-hydroxy-pyrimidine (DAHP) is a specific inhibitor of GTPCH. At 3 mM, a dose of DAHP consistently reported in the literature to inhibit GTPCH activity (Xie et al., 1998), elicited no change in basal SEAP activity by GTPCH promoter-reporter transfected PC-12 cells. Likewise NAS (Katoh et al., 1982), a potent and selective inhibitor of sepiapterin reductase (SR), the enzyme catalysing the final step in de novo BH4 synthesis, elicited no effect on SEAP expression. When both GTPCH and sepiapterin reductase inhibitors were applied in combination, still no effect was observed.
Methotrexate (MTX) is a commonly used anti-cancer drug that inhibits both dihydropteridine reductase (DHPR) and dihydrofolate reductase (DHFR) (Fig 2.8). When PC-12 cells expressing 1,3 and 6 kb GTPCH promoter-reporter constructs were treated for 48 h with MTX, over the concentration range of 0.5 μM to 50 μM, no change in SEAP expression was detected (Fig 2.9C).
2.4.2 RASM cells.

RASM cells have been intensively studied with regard to cytokine induced iNOS expression. In these cells, catecholamine synthesis is not apparent and tetrahydrobiopterin appears to be synthesised exclusively to serve as a cofactor of NOS. The biopterin content of RASM prior to cytokine treatment was below the limit of detection (0.1 ng) by HPLC analysis (Fig 2.10). Following treatment with LPS (100 µg/ml), IL-1 (100 ng/ml) and LPS (100 µg/ml)/IFN (100 U/ml), cellular biopterin levels increased to 1.28 ± 0.16, 1.7 ± 0.04 and 2.43 ± 0.08 ng/ml; respectively. DAHP (3 mM) abolished the LPS/INFγ induced response to a level below detection (Fig 2.10).

2.4.2.1 Influence of cytokines on NO synthesis, BH₄ accumulation and GTPCH promoter expression

To determine the extent to which immunostimulants increased iNOS expression, as well as GTPCH promoter expression, media from the stimulated RASM was assayed for accumulation of the stable NO oxidation product, nitrite. As anticipated, RASM nitrite levels were undetectable in media from untreated cells, whereas IL-1 and LPS (but not INFγ or TNF-α) elicited an increase in nitrite accumulation (Fig 2.11A and B).

These immunostimulants have been reported to effect a response in a synergistic manner. Whilst INFγ on its own did not cause nitrite accumulation, in combination with other
Fig 2.10 Endogenous biopterin content of untransfected RASM cells. Cells were treated for 24 h with the immunostimulants IL-1 (10 ng/ml), LPS (100 µg/ml) and IFN-γ (100 U/ml), with or without the GTPCH inhibitor 2,4-diamino-6-hydroxy-pyrimidine (DAHP 3 mM). Values represent means ± SEM, nd = none detectable i.e. < 0.1 ng biopterin/mg protein. This represents 1 experiment, similar results were obtained in 2 other experiments.
Fig 2.11: Effect of immunostimulants on nitrite production by RASM cells. Concentration-dependent effects of immunostimulants (IL-1, IFN-γ and TNFα) on nitrite accumulation in RASM culture medium after 24 h (Panel A). Effect of combinations of cytokines [LPS (10 μg/ml); IFN (100 U/ml)/IL-1 (100 ng/ml); LPS (10 μg/ml)/IFN (100 U/ml); LPS (10 μg/ml)/TNFα (100 ng/ml) and LPS (10 μg/ml)/IL-1 (100 ng/ml)] on nitrite production by RASM after 24 hours (Panel B). In both cases, values represent means ± SEM, where n=6; *denotes statistically significant synergy with combinations of cytokines. Similar results were observed in 2 additional experiments.
cytokines synergistic induction of NO synthesis was observed (Fig 2.11B). When applied alone, IFN (100 U/ml) elicited no detectable nitrite accumulation whilst IL-1 (10 ng/ml) caused 15.2 ± 1.9 μM nitrite to accumulate. In contrast, IFNγ (100 U/ml) in combination with IL-1 (10 ng/ml), elicited 18.7 ± 1.3 μM nitrite. TNF (10 ng/ml) alone elicited 1.3 ± 0.6 μM and LPS (10 μg/ml) 20.3 ± 1.6 μM; in combination TNF/LPS elicited 19.7 ± 1.0 μM nitrite. Neither TNF/LPS nor IFNγ/IL-1 elicited statistically significant synergy upon nitrite levels. In contrast, IFN (100 U/ml)/LPS (10 μg/ml) elicited 26.1 ± 2.9 μM nitrite and IL-1 (10 ng/ml)/LPS (10 μg/ml) elicited 30.3 ± 3.5 μM nitrite (Fig 2.11B); both of these combinations caused statistically significant synergism as determined by ANOVA, coupled to Dunnett’s analysis (p< 0.05). Upregulation of iNOS activity by the specified immunostimulants confirms that these agents elicited RASM activation.

 Appropriately, the untransfected RASM cells did not exhibit cytokine-induced SEAP activity. In GTPCH promoter-transfected cells, SEAP expression increased modestly following LPS treatment. Notably, at the highest concentration of LPS tested SEAP activity increased with the 6 kb GTPCH promoter transfected cells, despite 60% reduction in cell viability (Fig 2.12A). GTPCH promoter-reporter transfected cells did not exhibit increases in SEAP activity over a range of concentrations after exposure to TNF.
Fig 2.12 Effect of LPS (Panel A), TNF-α (Panel B), IFN-γ (Panel C) and IL-1 (Panel D) on SEAP expression by RASM cells transfected with 1, 3 and 6 kb GTPCH promoter-reporter constructs. Control is untransfected RASM cells and * represents a statistically significant difference as measured by ANOVA, coupled to Dunnett's test. Values represent means ± SEM of one experiment (n=6); similar results were obtained in at least 2 other experiments.
(Fig 2.12B) or IFN (Fig 2.12C). IL-1 elicited a maximal SEAP response at 10 ng/ml with 6 kb and 1 kb promoters (Fig 2.12D).

These cytokines acted synergistically to effect NO production; therefore the following combinations were tested for their effect on GTPCH promoter activity: IFN (100 U/ml)/LPS (100 μg/ml), TNF (10 ng/ml)/LPS (100 μg/ml), and IL-1 (10 ng /ml)/LPS (100 μg/ml) (Fig 2.13). In RASM cells stably transfected with 6 kb GTPCH promoter-reporter construct, the following fold increases were observed for singly applied immunostimulants: LPS (100 μg/ml), 1.35 ± 0.08; TNFα (10 ng/ml), 1.13 ± 0.15; IL-1 (100 ng/ml), 1.29 ± 0.05 and IFNγ (100 U/ml), 1.19 ± 0.09. Significant fold-increases over control were observed with the following combinations: IFN/LPS 1.44 ± 0.05, TNF/LPS 1.58 ± 0.05 and IL-1/LPS 1.70 ± 0.13 (p< 0.01;Fig 2.13). No synergism was observed between IFN/IL-1 1.28 ± 0.04, P>0.05. The untransfected cells did not respond to cytokines alone or in combination.

2.4.2.2 Influence of Dex on GTPCH promoter induced SEAP activity

In order to determine the effects of glucocorticoids upon the GTPCH promoter, RASM cells stably transfected with 1, 3 and 6 kb GTPCH promoter-reporter constructs were treated for 24 h with a range of concentrations of dexamethasone (Dex). As shown in Fig 2.14A, Dex did not elicit any changes in SEAP expression in RASM cells stably
Fig 2.13: Effect of immunostimulant treatment on SEAP expression by RASM cells stably transfected with a 6 kb GTPCH promoter SEAP reporter construct. Cell treatments were for 24 h with IFN (100 U/ml)/IL-1 (100 ng/ml), LPS (100 µg/ml)/IFN (100 U/ml), LPS (100 µg/ml)/TNFα (100 ng/ml) and LPS (100 µg/ml)/IL-1 (100 ng/ml). Values are means ± SEM, where n=6 and were reproduced in 2 other experiments. Similar results were observed for 1 & 3 kb promoter.
Fig 2.14: Effect of various reagents on SEAP expression of RASM cells. Dexamethasone (Panel A), dibutyryl cAMP (Panel B) and cyclohexamide (Panel C) on SEAP expression by RASM cells stably transfected with GTPCH promoter-reporter constructs. Values are means ± SEM, where n=6. Control for each cell type is SEAP expression by untreated cells. Similar results were obtained in 2 additional experiments. * represent p<0.05, and **p<0.01.
transfected with 1, 3 and 6 kb GTPCH promoter-reporter constructs. SEAP was also assayed after 48 h incubation with Dex, but Dex was not found to elicit any changes in the SEAP expression.

### 2.4.2.3 Influence of a cAMP analogue on the GTPCH promoter activity

GTPCH promoter activity was studied following treatment of RASM cells with increasing concentrations of a cell-permeant cAMP analogue dibutyryl cAMP (DBcAMP Fig 2.14B). Only with the 1 kb promoter was SEAP expression elicited by DBcAMP at 150 μM (p< 0.05). At 1.5 mM DBcAMP, SEAP expression was observed with all promoter-lengths studied (p <0.05 for 3 kb and 6 kb, but p < 0.01 for 1kb).

### 2.4.2.4 Influence of cyclohexamide (CHX) on the GTPCH promoter activity

Each of the GTPCH promoter-reporter transfected RASM cell lines responded to CHX with an increase in SEAP expression (Fig 2.14C). Maximal GTPCH promoter activity was observed with 100 ng/ml CHX (p<0.05 for 1 and 6 kb and p<0.01 for 3 kb). Below this concentration of CHX, the 3 and 6 kb but not 1 kb demonstrated statistically significant activity (p< 0.05). At the highest concentration of CHX tested (1 μg/ml) the cell viability fell, 1 and 3 kb GTPCH gene-flanking sequences still elicited significant increases.
in SEAP expression (p<0.01). Concentrations of CHX greater than 1 μg/ml dramatically reduced cell viability such that cells were observed to detach from the culture wells.

CHX attenuated the increase in SEAP expression elicited by LPS and LPS/INFγ in RASM cells stably transfected with a 1749 bp murine iNOS promoter SEAP reporter construct (Fig 2.15A). In contrast, low doses of CHX (0.1-1 μg/ml) potentiate SEAP expression, elicited by LPS and LPS/INFγ, with RASM cells that had been stably-transfected with the 3 kb GTPCH promoter-reporter vector (Fig 2.15B).
Fig 2.15: Effect of CHX on LPS and LPS/IFN elicited increases in 3 kb GTPCH promoter (Panel A) and 1749 bp iNOS promoter (Panel B) driven SEAP expression. Values are means ± SEM. In these experiments SEAP was determined by chemiluminescence. In both experiments LPS (10 μg/ml) and IFN (10 U/ml).
2.5 Discussion

Summary of results

1. IFNγ, TNF-α and IL-6 had little effect on GTPCH promoter.

2. IL-1β and LPS enhance GTPCH promoter activity.

3. There is synergism between the cytokines in their activation of GTPCH gene transcription. IFNγ and TNF-α alone were unable to stimulate GTPCH promoter activity, but in combination with IL-1β or LPS, further enhanced promoter activity.

4. GTPCH and iNOS are coactivated by IFNγ, TNF-α, IL-1β and LPS.

5. NGF induces GTPCH promoter activity, however the response of the 1 kb promoter is less than the 3 & 6 kb, suggesting that there is an enhancer element upstream of 1 kb. The NGF effects on biopterin levels and SEAP activity were not attenuated by the cAMP antagonist RpcAMP.

6. Dexamethasone enhanced activity of the 3 kb GTPCH promoter with a bell shaped concentration-response curve. No response to Dex was seen with the 1 kb GTPCH promoter indicating perhaps the absence of an enhancer element. A functional difference in Dex responsiveness was seen with RASM and PC-12 cells, with RASM being unresponsive.
7. Cyclic AMP analogues enhanced the GTPCH promoter-driven SEAP expression and increased biopterin levels in PC-12 cells. The effect of cAMP was found to be independent of CBP and CREB function.

8. There was no effect of catecholamine or BH₄ depletion on GTPCH gene transcription.

Basal expression of GTPCH is undetectable in most cell types, however it is abundant in adrenal medulla, hypothalamus and liver (Hirayama et al., 1993; Werner et al., 1990). Following the cloning of rat GTPCH cDNA in 1991 (Hatakeyama et al., 1991), in vivo studies showed that GTPCH mRNA expression is subject to regulation by numerous immunostimulants (Hattori & Gross, 1993; Hattori et al., 1998; Werner et al., 1990; Werner et al., 1991; Werner et al., 1993; Werner-Felmayer et al., 1993), hormones (Hattori et al., 1999; Pluss et al., 1997; Serova et al., 1997; Serova et al., 1998) growth factors (Anastasiadis et al., 1993; Anastasiadis et al., 1996) and neurotransmitters (Abou-Donia et al., 1986; Anastasiadis et al., 1993; Anastasiadis et al., 1996; Hattori et al., 1999). At the start of this study, it was unknown whether effects were modulated at the level of GTPCH gene transcription. To address this question we cloned 1, 3, and 6 kb 5'-upstream GTPCH gene flanking sequence into SEAP reporter vectors. These constructs were stably transfected into RASM and PC-12 cells in order to compare and contrast GTPCH
transcriptional regulation in a constitutively-expressed environment (PC-12 cells) and an exclusively inducible environment (RASM).

To limit the potential inter-clonal variation in GTPCH promoter-reporter construct expression, pooled clones were studied. Comparisons of SEAP expression by cells harboring GTPCH promoter-reporter constructs with cells possessing promoterless SEAP vectors and untransfected cells (PC-12 and RASM), revealed low levels of basal GTPCH promoter activity. In order to remove this variable amount of GTPCH gene expression from the analyses, the ability of factors to modulate expression of 1, 3 and 6 kb GTPCH promoter-reporter constructs was calculated as the ratio observed with treated and untreated cells, i.e. fold-increase values.

2.5.1 Immunostimulant treatment

We studied the effects of IFN-γ (10 - 1000 ng/ml) on PC-12 and RASM cells, stably-transfected with 1, 3 or 6 kb GTPCH promoter SEAP reporter constructs. In contrast to macrophages and T cells (Schoedon et al., 1986), IFN-γ had no effect on endogenous BH₄ levels or SEAP reporter gene expression in PC-12 or RASM cells. The failure of IFNγ to upregulate GTPCH promoter activity was similarly observed by another group studying PC-12 cells (Anastasiadis et al., 1996). Witter et al recently described that there was no
effect of IFN-γ on luciferase expression by mouse B cells that harbour a 2.6 kb GTPCH promoter luciferase reporter vector (Witter et al., 1996).

Despite our failure to detect upregulation of GTPCH gene activity by IFN-γ, it is notable that the GTPCH gene contains consensus elements for interferon regulatory factor-1 (IFR1) and IRF2 (Table 2.2). IFR1 functions as a transcriptional activator in various genes, whilst IFR2 serves as a repressor of IFR1 mediated transcription (Tanaka, 1993). In iNOS, deletional analyses revealed there to be an important IRF-1 site in the murine gene which mediated the synergistic effect of IFN-γ on iNOS promoter activation by immunostimulants (Kamijo et al., 1994; Martin et al., 1994; Spink & Evans, 1997).

IFN-γ can also act via STAT3 to modulate transcription, as has been shown for other acute phase response genes such as IL-6 (Narimatsu, 1997). However, we did not identify a STAT3 consensus site (Horvath, 1995) in the 6 kb GTPCH gene flanking sequence. IFNγ has also been reported to stabilise iNOS mRNA species (Vodovotz et al., 1993), although my studies do not probe for this possibility. TNFα did not increase GTPCH promoter activity, even though it had previously been reported to enhance GTPCH activity in macrophages and dermal cells (Werner et al., 1991). IL-6 administration also failed to increase GTPCH promoter activity with any of the constructs or cell lines tested.
In PC-12 cells only the 6 kb promoter construct responded to IL-1 treatment, while in RASM cells both the 3 kb and 6 kb promoters responded to IL-1. This suggests there may be a response element upstream of 1 kb, but downstream of 3 kb, that is necessary for the IL-1 mediated response. Total biopterin levels in RASM cells were increased following a 24 h treatment with either IL-1 or LPS, consistent with an increase in endogenous GTPCH gene expression. The specific involvement of GTPCH in mediating immunostimulant evoked biopterin accumulation in RASM cells was confirmed by our observation that this effect was abolished in the presence of a selective GTPCH inhibitor, DAHP.

LPS upregulated the expression of SEAP in PC-12 and RASM cells with all GTPCH promoter-SEAP reporter constructs under investigation. A maximal effect was seen at 1 μg/ml LPS for the 6 kb promoter (a dose sufficiently high to reduce cell viability, measured by MTT assay), whereas the 1 kb and 3 kb promoters were maximally activated with 100 ng/ml LPS.

Measurements of basal biopterin levels in PC-12 cells indicate that GTPCH is constitutively expressed at much higher levels than in RASM cells (where biopterin was undetectable). This is not surprising since PC-12 cells express AAAHs which require 10 - 600 μM levels of BH4 for optimal cofactor activity (Nichol et al., 1985), compared to NOSs which requires 20 - 100 nM BH4 (Klatt et al., 1994; Liu & Gross, 1996). GTPCH
probably functions in PC-12 cells exclusively for catecholamine synthesis, rather than NOS, which would be supersaturated at the basal BH₄ levels contained within these cells. There is literature to support coinduction of GTPCH and tyrosine hydroxylase in PC-12 cells (Anastasiadis et al., 1998; Hirayama & Kapatos, 1995). The NOS requirement for BH₄ will be discussed in more detail in Chapter 3. RASM cells do not contain AAAH but express iNOS following immunostimulant exposure.

GTPCH promoter response elements have some common consensus sites for transcription factor binding in common with iNOS, including NF-κB the key transcription factor that regulates murine iNOS gene expression (Table 2.2). NF-κB is a dimer containing p50 and p65 (Rel-A) subunits, which, in resting cells, is associated with inhibitory I-κBα or I-κBβ subunits (Beg, 1993). Inflammatory cytokines and mitogens activate NF-κB by increasing the phosphorylation and consequent proteolysis of I-κB (Baldwin, 1996), allowing untethered NF-κB translocation to the nucleus (Beg, 1993). In murine vascular smooth muscle cells, mutation of a specific NF-κB consensus sequence defined it to be responsible for the immunostimulant induced iNOS gene transcription (Spink, 1995).

Thirteen putative sites for NF-κB interaction were identified in the initial 6 kb of nucleotide sequence, 5'-upstream of the GTPCH gene (Table 2.2). An earlier report, showing that immunostimulant-induced GTPCH induction is inhibited by pyrroline
dithiocarbamate, NF-κB inhibitor, suggested that the GTPCH promoter activity can be enhanced by binding of NF-κB family members to one or more NF-κB response elements (Hattori et al., 1996).

In contrast pyrroline dithiocarbamate attenuated GTPCH mRNA levels in mouse osteoclasts following cytokine stimulation, only when given in combination with curcumin, an AP-1 inhibitor (Togari et al., 1998). This finding suggests that in some cell types there might be synergy action between NF-κB and AP-1 in regulating GTPCH induction. The same group found that pyrroline dithiocarbamate alone was sufficient to reduce cytokine-induced iNOS mRNA expression in osteoclasts, as had been previously reported for other cell types (Chao et al., 1997; Wong et al., 1996). This indicates that mechanisms of GTPCH and iNOS induction may differ in some cell lines. Confirmation of a role for NF-κB in GTPCH gene regulation and identification of relevant sites awaits mutagenesis and electromobility shift assay (EMSA) analysis.

There is also consensus sequence for binding of nuclear factor IL-6 (NF-IL6) within the initial 1 kb of GTPCH upstream sequence (Table 2.2). NF-IL6 has been shown to be induced in response to IL-6, IL-1 or LPS and its cognate DNA binding sequence bears homology to that for binding of C/EBP (Akira S, et al., 1992). Whilst PC-12 cells did not demonstrate GTPCH promoter upregulation by IL-6, RASM and PC-12 cells
transfected with GTPCH promoter reporter constructs elicited increased SEAP expression when challenged with IL-1 and LPS which might act through this putative consensus site.

Adrenomedullin (AM) has been demonstrated to increase IL-1 induced GTPCH expression (Hattori et al., 1999). Since AM is coupled to adenyl cyclase activation, cAMP may be involved in IL-1-induced transcriptional regulation of the GTPCH gene. Thus IL-1 may interact with a receptor which signals through the secondary messenger cAMP.

In RASM cells LPS and IFN acted synergistically to upregulate both iNOS and BH₄ levels (Gross & Levi, 1992). My results with RASM cells show that combinations of LPS/IL-1, LPS/TNFα, LPS/IFNγ each displayed a level of synergy upon GTPCH promoter-driven SEAP activity. It is notable that TNFα and IFNγ elicited no effect on SEAP expression when administered alone. Synergy was further confirmed by HPLC analyses, which demonstrated that IFN potentiates LPS-induced biopterin elevation in RASM cells although IFN alone did not influence biopterin levels.

Immunostimulants have been previously shown to co-induce iNOS and GTPCH expression. Consistent with this we found that in RASM cells, IL-1, LPS and TNFα upregulated iNOS expression, manifest as an increase in nitrite secretion into the cell culture media. This was in accord with previous findings reported for iNOS with IL-1 (Kilbourn et al., 1992), TNF (Kilbourn et al., 1990) and LPS (Kilbourn et al., 1990). In contrast, PC-12
cells did not accumulate detectable levels of NOx, even after exposure to immunostimulants.

2.5.2 Cyclohexamide

In pilot experiments with the GTPCH promoter, CHX (0.1-1.0 μg/ml) dramatically (2-6 fold) increased SEAP reporter activity, despite concurrent reduction in cell viability attributable to inhibition of protein synthesis. At high concentrations (>1.0 μg/ml) CHX prevents translation of expressed SEAP, thereby masking the detection of GTPCH promoter activity. Analysis of concentration-response relationships for CHX (0.1 - 100 μg/ml) for all three sizes of GTPCH promoter reproduced these initial findings. At low doses (0.1 - 0.3 μg/ml), CHX was also observed to significantly potentiate LPS induced GTPCH promoter activity, whereas at a higher concentration of CHX (1 μg /ml), IL-1 and LPS driven SEAP expression was inhibited. In contrast to the stimulatory effect of CHX on GTPCH promoter constructs, iNOS gene promoter-reporter activity was uniformly suppressed by CHX.

CHX is a selective inhibitor of protein synthesis. In previous studies of GTPCH upregulation by immunostimulants, CHX was shown to potentiate GTPCH mRNA expression (Hattori & Gross, 1993; Werner et al., 1991). GTPCH upregulation following administration of CHX has been similarly reported for biopterin levels and GTPCH protein
activity (Hattori et al., 1997; Katusic et al., 1998). In studies of human keratinocytes, Frank and coworkers reported that TNFα, IFNγ and IL-1 were all capable of 5-25 fold increases in GTPCH mRNA within 2 h, whereas in combination with CHX, greater than 40-fold increases were observed within 90 min of treatment (Frank et al., 1998).

Several mechanisms may be envisioned to explain CHX induced GTPCH gene expression. One possibility is that GTPCH gene transcription occurs through activation of early response genes, such as c-fos or jun b. Consensus elements for early response genes are evident within the GTPCH promoter: C/EBP, CMYB-01, c-Rel, c-Myc/Max heterodimer and v-Myb (Table 2.2). Alternatively, GTPCH gene expression may be repressed by a short-lived protein whose levels can be acutely downregulated by CHX. CHX might also act by increasing the degradation of the inhibitory subunit I-κB, which would trigger activation and translocation of NF-κB to the cell nucleus. Thirdly, CHX might stabilise GTPCH mRNA; it is notable that there are multiple destabilising AUUUA consensus sequences in the 3'-untranslated region of GTPCH mRNA (Chen, 1994).

Fold-increases in SEAP expression of the GTPCH constructs in PC-12 cells were less than in RASM cells. It has already been noted that the basal level of biopterin in PC-12 cells is considerably greater than in RASM cells and that PC-12 cells also function to synthesise catecholamines. One explanation for the disparity in fold-increase between PC-
through TrKA and pp75
receptors (Chao, 1995; Kaplan, 1991), with NGF binding causing receptor dimerisation. The TrKA receptor also triggers pathways, which affect cell cycle arrest and phosphotidylinositol kinase (PI3K) activation (For review see (Porter, 1998). One such pathway is the shc to Grb2 to SOS to p21ras which can also be activated by phospholipase C (Szeberenyi, 1992). The Ras pathway contributes to a phosphorylation cascade that signals through Raf, MEK-1 and can affect CREB, ELK-1 or pp90rsk (Meakin, 1992). In addition to triggering the MAP kinase cascade, Ras can directly affect CREB phosphorylation (Kaplan, 1994). CREB, ELK-1 and transcription factors which associate with them may be key mediators of NGF-induced neuronal cell differentiation (Meakin, 1992). The complexity of NGF-mediated cell signaling precludes a ready specification of the signaling route by which NGF elicits increased GTPCH gene transcription in PC-12 cells.

BH₄ levels were found to transiently increase in PC-12 cells after 12h treatment with NGF (Suzuki et al., 1988). In sympathetic neurons NGF receptor mediated changes were reported to regulate BH₄ content of cells, although transcriptional regulation of the GTPCH gene had not then been established (Hirayama & Kapatos, 1995). Anastasiadis et al found that BH₄ biosynthesis in PC-12 cells is increased concurrently with the activation of tyrosine hydroxylase (TH), the rate-limiting enzyme for the production of catecholamines (Anastasiadis et al., 1993). The latter group subsequently determined that
both epidermal growth factor (EGF) and NGF enhanced TH and GTPCH activity (Anastasiadis et al., 1996). There are reports that BH₄ itself may influence proliferation of PC-12 cells in response to NGF (Koshimura et al., 1999). Following EGF and NGF treatment of PC-12 cells, GTPCH inhibitors attenuated the intracellular BH₄ levels and cell proliferation (Anastasiadis et al., 1997). Induction of GTPCH in PC-12 cells was demonstrated to require DNA transcription since the effects of NGF on GTPCH induction were blocked by actinomycin D, a mRNA synthesis inhibitor. The present studies described here extend prior knowledge by demonstrating that NGF acts specifically to increase GTPCH gene transcription. NGF, through its multiple signaling modes, may utilise several mechanisms through which the GTPCH promoter activity is enhanced.

Rp-cAMP, a cAMP antagonist, was previously reported to disrupt NGF induction of GTPCH activity (Anastasiadis et al., 1993). NGF treatment has been observed to increase cAMP levels in PC-12 cells (Schubert, 1978) and cAMP has been shown to induce GTPCH gene expression in other cell lines (Abou-Donia et al., 1986). We found that when PC-12 cells were transfected with GTPCH promoter-reporter constructs and treated with a maximally effective concentration of DBcAMP, there was a greater response in NGF treated than non-NGF treated cells. Thus cAMP seemed to have an additive effect with NGF in driving the GTPCH promoter, arguing for distinct modes of cAMP and NGF action. In contrast to the Anastasiadis finding that Rp-cAMP abolished the GTPCH
response to NGF, I found that during a 48 h treatment of PC-12 cells, endogenous cell biopterin content and SEAP expression by GTPCH promoter-reporter constructs were unaffected by addition of RpcAMP. A possible explanation for this disparity is that Anastasiadis measured GTPCH activity and failed to appreciate that RpcAMP, at the concentration employed, significantly diminishes cell viability (Anastasiadis et al., 1997).

Sphingosine, an inhibitor of PKC, did not diminish the effect of NGF on GTPCH activity when administered to PC-12 cells (Anastasiadis et al., 1997). Sphingosine is phosphorylated to sphingosine-1-phosphate, which has been demonstrated to mobilise calcium and activate phospholipase D in a PKC and phosphoinositol-independent manner (Orlati, 2000). Again, in contrast to the findings of Anastasiadis and coworkers (Anastasiadis et al., 1997), I observed that sphingosine attenuated the NGF induced increase in biopterin levels by 40%. In addition, sphingosine attenuated NGF-induced SEAP expression in PC-12 cells stably expressing the 1, 3 and 6 kb GTPCH promoter-reporter constructs.

Analysis of the 5'-upstream GTPCH promoter sequence revealed a consensus site for nerve growth factor induced protein C (NGFIC - Table 2.2) at -1939. NGFIC is associated with the binding of immediate early gene encoded transcription factors from the family of Egr3 and Krox20, which have three Cys2 – His2 zinc finger binding motifs (Swirnoff, 1995). This NGFIC site is absent from the 1kb GTPCH promoter, perhaps
explaining why NGF-induced promoter activity was less with this construct than the 3 and 6 kb GTPCH promoter-reporter constructs. Mutation of the -1939 consensus sequence would be important to ascertain whether NGF specifically acts at this site to upregulate GTPCH gene expression in PC-12 cells.

Since the 1 kb GTPCH promoter displayed a modest response to NGF stimulation, NGF might signal through cAMP induced secondary messenger pathways as well as interacting through the putative NGFIC site. The GTPCH promoter contains consensus sites for Ras response element binding protein, CREB, AP-1, NF-Y and SP-1 all of which might be effected by NGF binding to the TrKA receptor and consequently effect signal transduction (Meakin, 1992). Mutation of these putative consensus sites would determine whether they effect the NGF-elicited increases in GTPCH promoter activity and intracellular biopterin levels.

2.5.4 Dexamethasone

Dex treatment consistently increased SEAP expression by PC-12 cells that had been transfected with the GTPCH promoter constructs, a maximal effect was observed with 1 µM Dex. This dose of Dex also increased endogenous biopterin levels in PC-12 cells. The various GTPCH promoter lengths elicited varying responses to Dex, with the 1 kb having lost the response to Dex. This would suggest that there is an enhancer element
that is activated by Dex upstream of the initial 1 kb 5'-to the GTPCH gene. Computer identified consensus sequences for transcriptional regulation suggests a putative glucocorticoid response element (GRE) site at −224, which is insufficient to explain the differences between the 1 kb and 3 kb promoter.

In support of my findings, immobilization stress of rats, a procedure that releases the glucocorticoid corticosterone was shown to increase levels of GTPCH mRNA (Serova et al. 1998) and it was observed that this effect could be abolished by hypophysectomy (Serova et al. 1998). The same group treated PC-12 cells with the plasma from immobilized rats, or Dex, and observed a rise in GTPCH mRNA after 12 h which remained elevated for 48 h (Serova et al. 1998). While GTPCH mRNA levels were increased 2-fold after only 30 min following immobilization stress in vivo, the response seen in cell culture experiments was much slower in onset.

Available literature about the effects of glucocorticoids on GTPCH regulation provides much contradictory information. Although Dex increases GTPCH mRNA expression in PC-12 cells (Serova et al., 1997), in macrophages Dex was reported to cause a fall in GTPCH levels (Schoedon et al., 1993). In human umbilical vein cells which had been stimulated TNF/IL-1β and IFNγ, Dex had no effect upon cellular biopterin levels or upon levels of GTPCH mRNA (Hattori et al., 1997).
Dex binds with nanomolar affinity to the glucocorticoid receptor, which then translocates to the cell nucleus. Once within the nucleus this complex acts as a transcription factor binding through GRE on DNA. A putative hemi-GRE was identified in the 6 kb GTPCH 5’-upstream gene sequence (Table 2.2). Dex also upregulates transcription of I-κB (Scheinman, 1995). TNFα causes degradation of I-κB, therefore, TNFα effects are downregulated by cotreatment with Dex (Scheinman, 1995). In rat hepatocytes, immunostimulant-induced iNOS is inhibited by Dex-mediated protection against I-κBα degradation; accordingly Dex inhibits NF-κB-activated gene transcription (De Vera et al., 1997).

CHX did not attenuate Dex activation of the GTPCH promoter in PC-12 cells, suggesting that de novo protein synthesis is unnecessary for Dex action. Dex suppresses the activity of genes modulated by the transcription factor AP-1 by a mechanism that does not require protein synthesis (Kerppola, 1993; Schule, 1990). This action of Dex may be mediated by post-translational down regulation of AP-1, through a mechanism which may involve a protein-protein interaction between the Dex-bound to the glucocorticoid receptor and the AP-1 hormone receptor (Jonat, 1990). AP-1 consists of 2 subunits coded for by the nuclear oncogenes c-jun and c-fos (Chiu, 1988). There are 8 putative AP-1 sites within the GTPCH promoter (Table 2.2).
In cardiac microvascular endothelial cells, Dex caused a fall in the cytosolic GTPCH mRNA levels; half-life of the mRNA fell 10-fold relative to control (Simmons et al., 1996). Following IL-1β induction in rat renal mesangial cells, nanomolar Dex attenuated the induction of GTPCH mRNA levels, protein expression and the protein activity (Pluss et al., 1997). However, GTPCH induction by DBcAMP (μM) was unaffected by Dex while low nM Dex, potentiated the effects of DBcAMP (Pluss et al., 1997). Another group reported an increase in tyrosine hydroxylase activity following Dex/cAMP treatment which, unaccounted for by increased TH mRNA levels, indicated increased BH4 levels (Fossom, 1992). In a rat model of endotoxic shock, Hattori & coworkers reported that while Dex attenuated LPS-induced biopterin levels, it did not affect the expression of GTPCH mRNA (Hattori et al., 1997). Dex attenuates iNOS induction by cytokines, however, following TNFα, IL-1 and IFNγ stimulation of human endothelial cells, micromolar amounts of Dex had no effect on the cytokine induced biopterin levels or GTPCH mRNA (Hattori, 1997).

In contrast to the observed activation by Dex of GTPCH promoter activity in PC-12 cells, Dex did not significantly influence GTPCH transcriptional activity in RASM cells. Glucocorticoids inhibit inflammation; although the means by which this happens is not fully understood, Dex-mediated inhibition of iNOS induction may be key (Kleinert et al., 1996). In RASM cells, where NF-κB is known to mediate IL-1 and LPS responses (Spink,
1995), GTPCH promoter activity in response immunostimulants may be effected by Dex elicited transcription of the I-κB regulatory subunit (Saldeen, 1994). Future experiments using the GTPCH promoter driven SEAP expression, in RASM cells, could examine whether Dex abolishes immunostimulation of the GTPCH promoter; Dex has been previously reported to attenuate IL-1 stimulated GTPCH mRNA levels and activity (Pluss et al., 1997).

Based on observations of other investigators and findings presented here, it is concluded that the GTPCH promoter activity can be differentially regulated, depending upon the cell type. In addition, elements may reside upstream of the rat 1 kb promoter which contribute to a greater response to Dex by more extended promoter sequences. Analyses suggest that activity of the 3 kb GTPCH promoter in response to increasing Dex concentrations is subject to a bell shaped relationship, whilst the 6 kb promoter consistently showed increasing reporter activity over the full range of Dex concentrations tested. My findings do not preclude the possibility of a Dex inhibitory response element upstream of the 6 kb promoter which might explain why I did not observe a statistically significant elevation in total biopterin levels following Dex treatment of PC-12 cells.
2.5.5 cAMP

In both PC-12 and RASM cells, DBcAMP, a cell permeant cAMP analogue, was found to elicit a statistically significant increase in GTPCH promoter activity. Forskolin, cholera toxin and isobutylmethylxanthine (IBMX) were also observed to increase SEAP expression and GTPCH promoter activity in PC-12 cells. Upregulation of GTPCH activity was additionally demonstrated by measurement of increased biopterin levels in PC-12 cells, following the cAMP-elevating stimuli; all but IBMX resulted in statistically significant increases in total biopterin levels. In contrast, a cell-permeant analogue of cGMP failed to upregulate the GTPCH promoter.

The second messenger cAMP acts indirectly through DNA sequences termed cAMP response elements (CRE). cAMP responsive element binding protein (CREB) and CBP (CREB binding protein) are transcriptional activators which form complexes with CRE (Kwok et al., 1994). Competition for limited amounts of CBP, may determine cellular responses to specific signals (Kamei et al., 1996). Phosphorylation of CREB by PKA is necessary for binding and activation of CBP (Kwok et al., 1994). CBP interacts with TFIIB and TBP (TATA binding protein) contributing to the basal initiation complex. The CREB/CBP complexes can also interact with NF-κB, AP-1, MAPK and PKA sites and thereby influence transcription (for review see (Andrisani, 1999; Della Fazia, 1997). It
is notable that the 5'-flanking region of the GTPCH gene contains 15 apparent consensus sites for binding cAMP response element binding protein (Table 2.2).

I studied the effects of the cAMP-elevating agents on PC-12 cells that stably overexpress CREB, CBP and a dominant negative mutant form of CREB. The cells overexpressing these proteins did not respond in a significantly manner from untransfected PC-12 cells. Whereas, CBP overexpressing PC-12 cells had slightly higher levels of biopterin than control cells, consistent with the idea that CBP can be limiting for protein expression this effect was not statistically different. Surprisingly, the dominant negative CREB did not attenuate the DBcAMP elevated increase in biopterin levels. This finding suggests that cAMP is signaling predominantly through an alternate site, such as Sp1 or NF-Y; precedents for such alternative signaling pathways are well documented (Matsubasa et al., 1994; Tansey, 1993).

Kapatos and coworkers determined by mutagenesis that in the 5'-upstream gene sequence of the GTPCH gene a cAMP response element (-104 relative to the transcription start site) and a CCAAT-box cassette (-84) could independently contribute to the 8-Br-cAMP response that had been observed. This group also found that AFT-4 overexpression in the PC-12 cells transactivated the promoter response to cAMP. The CCAAT-box, ATF-4, CCAAT enhancer binding protein, nuclear factor Y as well as the multiple CREB sites,
are located throughout the GTPCH gene 5'-upstream sequence and each may contribute to cAMP activation of the GTPCH promoter.

### 2.5.6 Inhibitors of BH₄, NO and catecholamine synthesis.

There is a precedent for GTPCH stimulation following catecholamine-depleting drugs such as reserpine, tetrabenazine and brocresine (Abou-Donia et al., 1986; Hirayama et al., 1993). Patients with a PH disorder express increased levels of serum biopterin (Thony et al., 1994). Since BH₄ in PC-12 cells serves predominantly as a cofactor for catecholamine synthesis, I looked at the possible upregulation of GTPCH promoter activity by α-methyl-p-tyrosine (AMPT - a specific inhibitor of tyrosine hydroxylase) and p-chloromethylphenylalanine, (PCPA -a specific inhibitor for phenylalanine hydroxylase). I found that neither of these CA inhibitors had an effect on GTPCH promoter activity. Thus if there is a feedback response of GTPCH caused by inhibition of catecholamines it does not occur at the level of transcription.

Several enzymes are involved in the maintenance of intracellular BH₄ levels. GTPCH is the rate-limiting enzyme for de novo BH₄ synthesis and it can be specifically blocked by 2,4-diamino-6-hydroxypyrimidine (DAHP). Sepiapterin reductase is also involved in the de novo synthesis and it is specifically inhibited by N-acetylserotonin (NAS). The enzymes dihydropteridine reductase (DHPR) and dihydrofolate reductase
DHFR, are involved in salvage of dihydropterins (qBH₂ and 7,8-BH₂ respectively) and can be inhibited by the commonly used anti-cancer drug methotrexate (MTX, Fig 2.8). NAS, MTX, and DAHP applied alone or in combination to cells transfected with GTPCH/SEAP promoter constructs did not elicit changes in SEAP activity. DAHP, at the concentration tested had been demonstrated to abolish the LPS induced biopterin levels in RASM cells (Fig 2.10). From this I conclude that blocking BH₄ synthesis and attenuating CA synthesis does not result in a feedback upregulation of GTPCH gene transcription.

2.5.7 GTPCH transcription start site

Our lab had previously cloned the rat GTPCH gene and determined the transcriptional start site by primer extension. A single gene was confirmed by southern blotting (Smith 1996 unpublished). The rat GTPCH gene transcription start site was observed to be 127 bp upstream of the ATG start codon CCTAG'ACT. This compares to a finding by Kapatos et. al. (Kapatos et al., 1999) who found CCT'AGACT to be the transcriptional start site (129 bases upstream of ATG) using rat liver nuclear extract.

Since my work commenced, another group studied truncated GTPCH promoter constructs in a luciferase reporter vector to determine transcriptional modification by cAMP response elements (Kapatos et al., 1999). A TATA-like sequence, is conserved in mouse and human genes, may help to position the pre-initiation complex (Kapatos et al.,
The TATA-like sequence in GTPCH is located further from the transcription start site than has been observed in other promoters (Reiter, 1990). The cAMP sensitivity of the GTPCH promoter was suggested to depend upon a cAMP response element (CRE) and CCAAT-box (Kapatos et al., 1999) whereas basal transcription may be inhibited by an SP1/GC box sequence.

ATF-4 is a transcriptional activator which interacts with TBP, TFIIB, TFIIF and CBP to mediate transcription (Liang, 1997). ATF-4 also forms heterodimers with C/EBP which can further complex with NF-Y, thus mobilizing histone acetyltransferase and resulting in chromatin modification (Liang, 1997). In PC-12, cells ATF-4 and C/EBP are constitutively expressed, perhaps explaining why there is a higher level of basal GTPCH gene expression than in RASM cells (Kapatos et al., 1999).
Chapter 3 - PTERIN BINDING TO ENOS

3.1 Introduction

BH₄ is a required cofactor for NOS activation (Kwon et al., 1989; Tayeh & Marletta, 1989). BH₄ had been theorised to function in NOS catalysis as it does for the aromatic amino acid hydroxylases (AAAH) (Witteveen et al., 1996). However, while pure AAAHs use stoichiometric quantities of BH₄ in catalysis, a single BH₄ molecule supports the synthesis of multiple molecules of NO by NOS (Giovanelli et al., 1991). Several features of the NOS oxygenase domain crystal structure have provided insights into functional differences between NOS and AAAHs. Firstly, BH₄ is tightly bound within NOS at the dimer interface where it is protected from the surrounding solvent (Crane et al., 1998; Raman et al., 1998). In contrast, the BH₄ site for the AAAHs is closer to the surface of the enzyme and there is little homology with the pterin site of NOSs. Secondly there is a non-haem iron which interacts with BH₄ in AAAHs, whereas in NOS BH₄ interacts with a haem iron (Crane et al., 1998). Interestingly, the distance between BH₄ and the haem iron in NOS is more than twice the distance between BH₄ and non-haem iron in AAAH, suggesting that electron transfer would be very different for the two enzymes (Raman et al., 1998). Thus, crystallographic analyses provide evidence that the role of pterin in NOS catalysis differs from that in AAAH catalysis.

BH₄ has been implicated in NOS catalysis providing for effectively coupled oxygen consumption for NO production. In the absence of BH₄, NOSs produce H₂O₂ (Heinzel et
and superoxide (Saura et al., 1996; Vasquez-Vivar et al., 1998); this will be discussed in more detail in the next chapter.

One of the initial proposals for the role of BH₄ in NOS catalysis was to assist NOS dimerisation (Baek et al., 1993). Purification of NOS from bacterial expression systems (in which BH₄ is not synthesised), revealed the presence of dimers, providing evidence against a requirement for BH₄ in NOS dimerisation (Klatt et al., 1995; Rodriguez-Crespo et al., 1996; Roman et al., 1995). In addition, crystal structures of pterin-free eNOS and iNOS oxygenase domains clearly reveal that these species are exclusively dimeric (Crane et al., 1998; Raman et al., 1998). It is now widely accepted that the haem moiety is necessary for dimerisation, not BH₄ (Klatt et al., 1996; Venema et al., 1997). Nonetheless BH₄ may confer NOS dimer stability (Stuehr, 1997). Dissociation experiments in the presence of SDS have suggested that iNOS and nNOS may have a greater dependency for BH₄ in dimer stabilisation than eNOS (Klatt et al., 1995).

Competition with the haem binding ligands, imidazole and 7-nitroindazole, inhibited BH₄ binding to NOS (McMillan & Masters, 1993; Wolff & Gribin, 1994), suggesting that BH₄ interacted with the haem moiety of NOS (Klatt et al., 1994). A high incorporation of haem to NOS is necessary for BH₄ binding, but even with stoichiometric amounts of haem in nNOS monomers, only 0.5 BH₄ was observed to be bound per nNOS monomer (Harteneck et al., 1994). The production of NO increased when BH₄ was added to NOS with 1 BH₄ bound per dimer, indicating the presence of more BH₄ binding sites.
(Mayer et al., 1991). BH₄ has been suggested to bind with high affinity to a first site and low affinity to a second site, therefore the sites may interact with negative cooperativity (Hevel & Marletta, 1992).

From studies of nNOS haem domains (Boyhan et al., 1997), BH₄ and arginine bound to a region between amino acid residues 221 and 350. There appears to be homology at the core of the pterin site between the NOS isoforms (Boyhan et al., 1997). In human iNOS cysteine 200 was deemed necessary for dimerisation since the C200A mutant was found to be monomeric and inactive (Cubberley et al., 1997). The iNOS oxygenase domain binds BH₄ with high affinity and a 49 amino acid region is critical for binding. Mutation of iNOS Cys 106 reduced BH₄ binding affinity without affecting dimerisation, mutation of D379 affected arginine binding but not BH₄ binding, whilst mutation of D454 affected both BH₄ and Arg binding (Gachhui et al., 1997). These mutation experiments further suggest that dimerisation does not require BH₄ binding and that allosteric interactions may occur between the BH₄ and arginine binding sites.

While the role of BH₄ in NOS catalysis remains undefined, BH₄ has been shown to convert haem iron from low spin to high spin (Gorren et al., 1996; Wang et al., 1995). In the absence of BH₄, the iNOS oxygenase dimer was shown to bind haem iron in a penta-co ordinate structure, whereas the BH₄-bound iNOS oxygenase domain binds haem in a hexa-co ordinate state (Crane et al., 1998). EPR studies have demonstrated that BH₄ affects the occupancy of the arginine site and geometry of the haem (Salerno et al., 1997).
In nNOS, mutation of Cys 331 resulted in protein which could not bind to BH₄, however BH₄ binding could be reinstated by prolonged incubation with a large concentration of arginine (Martasek et al., 1998). Whereas arginine binding to wild type nNOS causes a shift from low spin to high spin of the haem iron, a mutation of Cys 331 resulted in haem iron which was predominantly low spin and could not bind to either Narg or BH₄, unless an excess of arginine was present (Martasek et al., 1998). It is interesting that whilst this mutation disrupted BH₄ binding, it had no impact upon nNOS ability to bind CaM, imidazole or cytochrome C (Martasek et al., 1998).

In studies comparing the activity of dihydropterins versus tetrahydropterins for support of iNOS activity, the oxidation state of the pterin was shown to affect NO synthesis and haem dependent NADPH oxidation (for the structures of select tetra- and dihydropterins see Fig 3.1) (Hevel & Marietta, 1992). While the biopterin side chain has an effect upon the pterin binding affinity to NOS, the status of haem iron is effected by the oxidation state of the pterin ring. Dihydropterins were reported to support haem iron reduction and promote dimer stability in a similar manner to tetrahydropterins (Presta et al., 1998). The pterin analogue 4-aminoBH₄ had a higher affinity for the NOS BH₄ binding site than BH₄, but 4-aminoBH₄ only caused a partial shift from low spin to high spin of haem; addition of arginine was able to complete the shift to high spin of haem (Mayer et al., 1997). Recent crystal structures of NOS bound to pterin analogues suggested that the
Fig 3.1: Structures of BH$_4$ and pterin analogues
positive charge of the amino group of 4-aminoBH$_4$ might prevent the formation of a pterin radical (Crane, 2000).

Elucidation of the oxygenase domain crystal structures has defined the BH$_4$ binding sites in iNOS and eNOS (Crane et al., 1998; Raman et al., 1998). A schematic representation of BH$_4$ bound to eNOS and the interaction of the pterin ring and side chain with the amino acid residues is shown in Fig 3.2 (Raman, 2000). BH$_4$ binds NOS at the fold of N- and C-terminal loops and helices in a central region which forms the dimer interface (Crane et al., 1998; Crane et al., 1999). The N3 and O4 of BH$_4$ both form H-bonds with haem propionate. BH$_4$ directly forms H-bonds with arginine residue 375 (iNOS), and 367 (eNOS), through O4 and through N5 via H$_2$O (Crane et al., 1998; Raman, 2000). Consequences of BH$_4$ binding, based on the iNOS crystal structure, included an alteration of the active center channel, increased sequestration of the haem ligand Cys194 and extension of a negative haem A propionate from the distal plane of haem (Crane et al., 1998). An increase in basicity of Cys194 arising from the sequestration in the hydrophobic environment was suggested to promote O$_2$ activation and pterin-induced increase in autooxidation of the ferrous haem-dioxy complex (Crane et al., 1998).

The eNOS crystal structure revealed the presence of a zinc which appeared to stabilise BH$_4$ binding site and facilitate BH$_4$ recognition by eNOS (Raman et al., 1998). Raman et. al. compared the BH$_4$-bound and the pterin-free eNOS structures and concluded
**Fig 3.2: Schematic representation of BH₄ interaction with eNOS.**

This diagram is based upon the analysis of the eNOS oxygenase domain crystal structure. Dotted lines represent hydrogen bonds and "wat" represents a water molecule. A and B illustrate that BH₄ binds at the dimer interface and interacts with amino acid residues from both subunits.

Diagram is adapted from (Raman et al 2000)
that BH$_4$ did not elicit conformational changes in the BH$_4$ site or assist in the creation of an active site channel. The investigators argued that because there were no identifiable differences between the two BH$_4$ binding sites there could not be anti-cooperativity in BH$_4$ binding. The eNOS crystal structure did not suggest that BH$_4$ affected the L-arginine site (Raman et al., 1998).

The quaternary structure, tertiary topology and substrate cofactor sites of eNOS and iNOS are highly conserved (Li et al., 1999). Zinc sulphate was demonstrated to stabilise the subunits and maintain the integrity of the BH$_4$ cofactor binding site of iNOS (Li et al., 1999). An N-terminal $\beta$ hairpin hook was found to interact between the subunits of the NOS dimer and the Zn$^{2+}$, mutations in the hook affected the dimer interface and subunit assembly (Crane et al., 1999). The N-terminal hook appears necessary for catalysis, and mutations in this have been reported to reduce the affinity of BH$_4$ binding (Ghosh et al., 1999).

Comparison of eNOS and iNOS crystals complexed with arginine or S-ethylthiourea determined that association of arginine and BH$_4$ created a cavity near the dimer interface which was large enough to enable the diffusion of citrulline and arginine (Fischmann et al., 1999). The paper further described how haem was found deep within the NOS monomers with the propionate groups orientated towards the dimer interface and when BH$_4$ was bound a haem 5 coordinate high spin state resulted (Fischmann et al., 1999). Since BH$_4$ was tightly bound with H-bonds to the haem, it was protected from bulk
solvent. The interaction of BH$_4$ and eNOS included ring stacking with Trp 477 and Trp 463 (Fischmann et al., 1999).

Alignments of nNOS amino acid sequence with the eNOS and iNOS crystal structure backbone revealed conservation of aromatic amino acid residues (Sagami, 2000). Mutation of Tyr$^{706}$, which interacts with haem propionate, increased the EC50 for BH$_4$ by 30-40 fold, whilst reducing the activity to 13–29% of wild type nNOS. In the iNOS oxygenase domain, mutations revealed that Arg 375, Trp 455, Trp 457 and Phe 470 were important for BH$_4$ binding (Ghosh et al., 1999).
3.2 Aims

BH₄ binding has been less well characterised for eNOS than the other NOS isoforms. I developed a microplate filtration assay and set out to characterise the binding of a custom synthesised [³H]BH₄ to pterin-free bacterially-expressed eNOS. Using pterin analogues that had altered side chains or oxidised rings, I also sought to learn how pterin structure impacted on binding to eNOS and support of catalytic activities.
3.3 Method

3.3.1 Purification of NOS:

Rat neuronal NOS (nNOS) and bovine endothelial NOS (eNOS) were purified from BL21 cells harboring pGroELS and pCW vector expression systems for eNOS and nNOS (McMillan et al., 1992 and Martesek et al., 1996). *Escherichia coli* containing these vectors were grown in terrific broth containing ampicillin (50 μg/ml) and chloramphenicol (35 μg/ml) in 2.6 l Fernbach flasks (0.5 l/flask). Flasks were shaken at 250 rpm at 37°C. When OD$_{600}$ reached 0.8-1.2, δ-aminolevulinic acid (450 μM) was added and, 1 h later, isopropyl β-D-thiogalactoside (0.5 mM), riboflavin (3 μM) and ATP (1 mM) were additionally added. Growth was continued at 23°C in the dark for 40-48 h. Bacteria were harvested by centrifugation at 2500 x g for 20 min at 4°C. Pellets were stored at -70°C for up to 3 weeks prior to NOS purification.

For NOS purification, cell pellets were resuspended in 30 ml lysis buffer [Tris-HCl pH 7.4 (100 mM), glycerol (10%), dithiothreitol (1 mM), EDTA (1 mM), phenylmethylsulfonyl fluoride (1 mM), leupeptin (5 μg/ml), pepstatin A (5 μg/ml), chymostatin (5 μg/ml)] and subjected to pulse sonication (4 min, 80% power, using a Fisher Scientific sonicator Model 550). The lysate was centrifuged at 150,000 x g for 70 min at 4°C and the supernatant was applied to a 2',5'-ADP-sepharose 4B column (8 ml, Pharmacia) that had been pre-equilibrated with Buffer A [Tris-HCl pH 7.6 (50 mM), Glycerol (10%), dithiothreitol (0.1 mM), EDTA (0.1 mM), NaCl (100 mM)]. The column
was washed with 10 volumes of Buffer A, followed by 10 volumes of Buffer A containing NaCl (500 mM), but excluding EDTA, and then eluted with 5 ml of buffer A containing NADPH (10 mM), CaCl₂ (100 μM) and excluding EDTA.

The sample was purified further on a CaM-sepharose column (Sigma) washed with 10 volumes of Buffer B [Tris-HCl pH 7.6 (50 mM), Glycerol (10%), dithiothreitol (0.1 mM), CaCl₂ (100 μM), NaCl (100 mM)]. This was followed with a second wash with 10 volumes of Buffer B containing 500 mM NaCl and NOS was eluted with 15 ml Buffer B containing 10 mM EGTA, without CaCl₂. Protein-containing fractions (Biorad assay) were dialysed to remove EGTA and concentrated using a Centriprep 30 filtration unit. Purified NOS was assayed for enzyme activity based on NO₂ accumulation (Greiss assay). The concentration of NOS was determined by haem absorption. Toward this end, sodium metabisulphite was added to NOS in a saturated solution of carbon monoxide, and the resulting change in absorption at 444 and 500 nm was used to determine NOS concentration, based on an extinction coefficient ΔE₄₄₄₅₀₀ = 74 mm⁻¹cm⁻¹ (Martasek et al., 1996; Roman et al., 1995).

3.3.2 Protein Assay

Biorad dye reagent was diluted with 4 parts distilled water and filtered to remove particulates. Diluted dye reagent was added in 20-fold excess over sample (10 μl). A standard curve based upon bovine serum albumin in the range 20 – 500 μg/ml was
prepared and the plate read at OD₆₀₀ nm in a microplate spectrophotometer.

3.3.2 Preparation of 5,6[³H]BH₄ and calculation of specific activity

5,6[³H]BH₄ was custom synthesised (NEN) by complete reduction of 7,8-dihydrobiopterin with sodium borotritide. Since tritium at the 5 and 6-positions is readily lost upon BH₄ oxidation, binding experiments with this ligand inform specifically about BH₄ interactions, and not that of more oxidised species that might accumulate. 5,6[³H]BH₄ was stored as a 1 mM stock solution in equimolar HCl at -70°C. Aliquots were purified by HPLC using a Partisyl 10 SCX column and gradient flow from 100% Buffer A [Ammonium acetate, pH 3.8 (100 mM), DTE (1 mM)] to 100% Buffer B [Ammonium acetate pH 3.8 (100 mM), DTE (1 mM), Methanol (5%)] over a 30-min period at a flow rate of 1.0 ml/min. Products were detected by fluorescence (excitation at 350 nm, emission at 440 nm) and photodiode array spectrophotometry. Biopterin, BH₂, 6S-BH₄ and 6R-BH₄ were used as standards. The first run with [³H]BH₄ was performed with the detector lamps on. In subsequent runs fractions were collected based on elution times determined by the initial run with lamps off; this was necessary to prevent [³H]BH₄ photolysis. All fractions were frozen in liquid N₂ after elution, reserving an aliquot for scintillation counting. Based on the elution profile of standards, 6R-[³H]BH₄ peak fractions were pooled and stored at -70°C for use in radioligand binding experiments.
3.3.4 \(^{3}\text H\)BH\(_4\) binding to NOS

\(^{3}\text H\)BH\(_4\) binding assays were performed using PVDF (hydrophobic) membrane-bottom 96 well filtration plates (Millipore). Prior to the assay, filtration membranes were washed under vacuum with 100 \(\mu\)l ethanol (50%), then twice with 200 \(\mu\)l Tris (50 mM) pH 7.6. All binding reactions contained Tris pH 7.6 (50 mM), DTT (1 mM), NOS (10 pmoles - unless otherwise stated), \(^{3}\text H\)BH\(_4\) (22 pmole - unless otherwise stated) and desired additions in a final volume of 100 \(\mu\)l. Binding reactions were terminated by rapid filtration and three washes with iced Tris buffer (50 mM), pH 7.6. Plates were air-dried for 30 min, 25 \(\mu\)l scintillation cocktail was added (Optiphase, Wallac) and plates were counted in a Microbeta scintillation counter (Wallac).

Equilibrium binding assays were carried out at 23°C with a 15 min incubation period. Binding reactions were initiated by the addition of NOS for equilibrium binding experiments. Association binding experiments were initiated by the addition of \(^{3}\text H\)BH\(_4\); all wells were filtered together to terminate binding. In dissociation experiments, eNOS (10 \(\mu\)l) was added to a 90 \(\mu\)l volume containing other reactants including \(^{3}\text H\)BH\(_4\); after a 15 min incubation period, dissociation was initiated by addition of 10 \(\mu\)l of excess unlabelled BH\(_4\) or BH\(_2\) (detailed in results section). Competition assays following pre-incubation of \(^{3}\text H\)BH\(_4\) and eNOS were preformed in the same manner as the dissociation experiments in a 90 \(\mu\)l volume, with competing pterin added after 15 min, (the time needed to add the 7-10 concentrations of competing pterin was less than a min). Samples were
incubated for a further 30 min, before quantification of remaining [3H]BH₄/NOS complexes.

3.3.5 Handling of pterins

Pterins used in the binding assays were dissolved in equimolar HCl; stock solutions were quickly aliquoted, frozen in liquid N₂ and then stored at -70°C. Just prior to each experiment, aliquots were thawed and diluted in 10 mM Tris pH 7.6 and 0.1 mM DTT. All reactants were kept on ice and, whenever possible, the diluted pterins were the last constituents to be added to the assay mix. No pterins were used after they had been thawed for more than 3 h.

3.3.4 Analysis of Radioligand Binding Data

Equilibrium binding:

Equilibrium binding parameters were assessed by computer-assisted non-linear least squares regression analysis, using the Prism program (GraphPad Software Inc.) to fit the equation:

\[ B = \frac{(B_{\text{max}} + T + K_d)}{2} - \sqrt{\left(\frac{(B_{\text{max}} + T + K_d)}{2}\right)^2 - B_{\text{max}} T} \]

B is the concentration of bound BH₄, B_{max} is the total concentration of NOS, T is the total concentration of BH₄ and K_d is the concentration of free BH₄ that results in half-maximal
binding. This equation derives from the basic equilibrium formula \([L][B_{max}]/[B] = K_d\), under the specialized condition where free ligand concentration \((L)\) is significantly less than \(T\), due to formation of complexes, \(B\). Analysis of association and dissociation kinetics of CaM binding and comparison of one-site vs. two-site binding models was performed using the *Prism* program.

**Association binding kinetics:**

Total bound = maximal bound*\(e^{(K*X)}\)

\(Y\) (total bound) starts at zero and ascends to maximal bound, with a rate constant \(K\). Half time is \(0.69/K\).

**Dissociation binding:**

\(Y = \text{Span} \times e^{(K*X)} + \text{Plateau}\)

\(Y\) (Specific bound) starts at maximal bound and decays to a plateau (minimal amount radioligand bound) with a rate constant \(K\), the span is defined maximal bound – plateau i.e the amount of displaced \(^3\text{H}\)BH\(_4\). The half life is \(0.69/K\).
**One site competition:**

EC50 values for pterin competition with \([\text{^3}H]BH_4\) for eNOS were calculated from sigmoidal concentration-response curves which were fitted to the equation:

\[
\text{Specific } [\text{^3}H]BH_4 \text{ bound} = \text{Bound min} + (\text{Bound max} - \text{Bound min}) \cdot (1+10^{\text{LogEC50} \cdot X})
\]

Where \(X\) is the logarithm of pterin concentration, \(Y\) is the specific bound, \(Y\) has minimal response in the absence of \([\text{^3}H]BH_4\) or eNOS (\([\text{^3}H]BH_4\) bound min) and with a maximal \([\text{^3}H]BH_4\) bound (\([\text{^3}H]BH_4\) – bound max - \([\text{^3}H]BH_4\) bound min).
3.4 Results

3.4.1 Determination of $[^3]H\text{BH}_4$ specific activity

$[^3]H\text{BH}_4$ specific activity and concentration was determined by isotope dilution. For this purpose, increasing concentrations of unlabelled BH$_4$ were added to a fixed amount of $[^3]H\text{BH}_4$ and eNOS. The concentration of $[^3]H\text{BH}_4$ was defined by the unlabelled BH$_4$ concentration that diminished binding to NOS by 50%; the specific activity for the $[^3]H\text{BH}_4$ was calculated to be 633 cpm nM$^{-1}$.

3.4.2 Determination of the stability of purified $[^3]H\text{BH}_4$ in 30 min eNOS binding assay.

In order to determine the fraction of BH$_4$ which could bind to NOS, $[^3]H\text{BH}_4$ concentration was fixed and eNOS concentration was increased until saturation binding was obtained at room temperature during a 30 min incubation. These experiments revealed the concentration of our $[^3]H\text{BH}_4$ stock (40 nM – Figure 3.3). Results indicated that by 30 min, only 25% of added radioactivity was able to bind eNOS; this was despite a careful purification of the $[^3]H\text{BH}_4$ and inclusion of 0.1 mM DTT in the binding assay buffer. The apparent Kd of BH$_4$ for binding to eNOS in this experiment was determined to be 17.1 nM. A complicating variable is that since only 25% of radioactivity appeared to bind eNOS, degradation of $[^3]H\text{BH}_4$ must have occurred during the assay period.
Fig 3.3: Saturation binding of $[^3]H]BH_4$ to eNOS.
All samples were incubated with 50 nM $[^3]H]BH_4$ for 30 min. This represents a single experiment, similar results were obtained in 2 other experiments. Points are means ± SEM of triplicate determination.
3.4.3 What is the Kd for BH₄ binding to eNOS and nNOS?

Saturation binding was measured after a 15 min incubation with [³H]BH₄ at room temperature, with a fixed concentration of eNOS or nNOS. Saturation reached a plateau and fit to a single site model for binding to both eNOS and nNOS. The apparent Kd for binding of [³H]BH₄ to eNOS and nNOS was similar, 82.1 ± 31.1 (Fig 3.4A) and 67.8 ± 2.65 nM (Fig 3.4B), respectively. All experiments were performed in the presence of 0.1 mM DTT to minimise the oxidation of [³H]BH₄.

The binding of [³H]BH₄ to nNOS (50 nM) had a Bmax 29.16 ± 2.911 nM with a stoichiometry of ~1 BH₄ per NOS dimer. Stoichiometry for BH₄ binding to eNOS was lower ranging from 0.35 –0.5; this variation probably reflects differences in the relative incorporation of haem into eNOS. Indeed, BH₄ interacts with haem propionate and the stoichiometry of BH₄ has been demonstrated to depend upon NOS haem content (Berka & Tsai, 2000; Raman et al., 1998).

3.4.4 What are the kinetics for [³H]BH₄ binding to eNOS and nNOS?

To determine the kinetic constant, Koff, dissociation experiments were performed. NOS and [³H]BH₄ were preincubated for 15 min before addition of 100 µM unlabelled BH₄ to prevent the rebinding of [³H]BH₄ after dissociation. The dissociation constant for eNOS, Koff was found to be 0.0267 ± 0.00442 min (n=3, Fig 3.5A). This dissociation was relatively slow and appeared to be monophasic, with T₁/₂ = 28.09 ± 5.67. For nNOS
Fig. 3.4: Saturation binding of $[^3]$H$BH_4$ to NOS isoforms.
Binding isotherms are shown for eNOS (10 pmol; Panel A) and nNOS (5 pmol; Panel B). Points are means ± SEM of triplicate determinations. Apparent Kd values are eNOS 82.1 ± 31.1 nM, nNOS 67.8 ± 2.6 nM. Similar results were obtained in 2 other experiments.
Fig 3.5: Kinetics of $[^3]H$BH$_4$ dissociation from NOS isoforms. $[^3]H$BH$_4$ complexes were formed with eNOS (Panel A) and nNOS (Panel B). $[^3]H$BH$_4$ (50 nM) was pre-incubated with NOS (10 pmole) for 15 min and dissociation was initiated by the addition of 100 μM unlabelled BH$_4$. Points are means ± SEM of triplicate determinations. This represents a single experiment, similar results were obtained in 2 other experiments.
dissociation was slower and monophasic, with $T_{1/2} = 49.24$ min and $K_{off} = 0.0141 \pm 0.0017$ (Fig 3.5B).

Association binding experiments were performed with eNOS and nNOS. With both isoforms binding was monophasic, achieving >95% of maximum at 30 min with 50 nM $[^3]$H]BH$_4$ (Fig 3.6). In the case of eNOS, half-maximal association was 5.33 ± 3.19 min (n=5) and the observed association constant ($K_{ob}$) was calculated to be 0.169 ± 0.087 min (n=5). For nNOS, $K_{ob}$ was 0.111 ± 0.021 and $T_{1/2} = 6.22$ min (Fig 3.6).

### 3.4.5 Do NOS cofactors effect the binding of $[^3]$H]BH$_4$ to eNOS?

The effects of calcium/calmodulin ($Ca^{2+}$/CaM; 1μM/100 nM), NADPH (0.1 mM) and arginine (Arg, 0.1 mM) were examined for their potential impact on $[^3]$H]BH$_4$ binding to eNOS (Fig 3.7). Notably, these concentrations of ligands/substrates were chosen to elicit > 90% occupancy of NOS (Gachhui et al., 1996). Neither the presence of $Ca^{2+}$/CaM nor NADPH alone appeared to effect $[^3]$H]BH$_4$ equilibrium binding to eNOS. In contrast when $Ca^{2+}$/CaM and NADPH were added together, $[^3]$H]BH$_4$ binding was enhanced by 50 ± 20%; this increase was significantly different from control (p<0.05). Arg dramatically increased the binding of $[^3]$H]BH$_4$ to eNOS, by 100 ± 10%, (p<0.001). Binding of $[^3]$H]BH$_4$ to eNOS was not further enhanced, over that with Arg alone, when Arg was combined with other cofactors.
Fig 3.6: Kinetics of $[^3H]BH_4$ binding to NOS isoforms.

Association binding of 50 nM $[^3H]BH_4$ to 10 pmole eNOS or 10 pmole nNOS. Points are means ± SEM of triplicate determinations. This is one experiment; similar findings were found in 3 other experiments (nNOS) and 5 other experiments (eNOS). eNOS $T_{1/2} = 5.33 \pm 3.19$ min whilst nNOS $T_{1/2} = 6.22 \pm 0.59$ min.
Fig 3.7: Effect of NOS cofactors and substrate upon eNOS (10 pmole) equilibrium binding to $[^3H]BH_4$ (50nM). Control represents eNOS/$[^3H]BH_4$ binding in binding buffer (Tris 50 mM pH 7.6, DTT 0.1 mM) in the absence of ligands / cofactors, see text for substrate/cofactor concentrations. Bars are mean values ± SEM where n=3. ** represents p< 0.001 and * p<0.05.
3.4.6 What concentration of arginine produces maximal $[^3H]BH_4$ binding to eNOS?

A concentration-response experiment was performed to assess the maximal concentration of arginine necessary to elicit maximal $[^3H]BH_4$ binding to eNOS. Incubation with Arg for 30 min was found to elicit a maximum effect at 100 μM and a half-maximal effect at 22.4 ± 2.8 nM (Fig 3.8A). Note that in this experiment, 30 min incubation resulted in only a 50% maximal increase in $[^3H]BH_4$ binding, in contrast to the 100% increase in binding elicited by Arg after 60 min incubation.

3.4.7 Can the competitive Arg-based inhibitor of NOS, nitroarginine (Narg), also promote $[^3H]BH_4$ binding to eNOS?

Nitroarginine, an Arg analogue used frequently as a selective probe to study the arginine binding site on NOS, had no effect on the $[^3H]BH_4$ binding to eNOS over a concentration range of 1-100 μM (Fig 3.8B). The Kd for Narg binding to nNOS is 25 nM (Alderton et al., 1998; Liu & Gross, 1996); this finding indicates that eNOS occupancy by Narg does not mimic the Arg elicited increase in $[^3H]BH_4$ binding.

3.4.8 Does arginine effect the kinetics of $[^3H]BH_4$ binding to eNOS?

To determine whether arginine could effect the kinetics of $[^3H]BH_4$ binding to eNOS, BH$_4$ association was compared in the absence and presence of Arg (Fig. 3.9). In the presence of Arg (100 μM), Kob was 0.044 ± 0.004, compared to 0.169 ± 0.087 min (n=5)
Specific $[^{3}\text{H}]\text{BH}_4$ bound (% control)

Fig 3.8: Influence of arginine and an arginine analog on BH$_4$ binding to eNOS. Arginine (Panel A) and nitroarginine (Panel B) were incubated with eNOS (10 pmoles) / $[^{3}\text{H}]\text{BH}_4$ (50 nM) at 23°C in binding buffer (Tris 50 mM pH7.5, DTT 0.1 mM). In both figures control represents eNOS binding to $[^{3}\text{H}]\text{BH}_4$ alone. Points are shown as mean values ± SEM where n=3.
Fig 3.9: Effect of arginine on kinetics of $[^3\text{H}]\text{BH}_4$ binding to eNOS isoforms. Association binding of 50 nM $[^3\text{H}]\text{BH}_4$ to 10 pmole eNOS was compared with and without 100 µM Arg. Points are means ± SEM of triplicate determinations. This represents one experiment; similar findings were found in 2 other experiments. The $T_{1/2}$ values were found to be 15.42 ± 1.82 and 5.33 ± 3.19 min in the presence and absence of arginine, respectively.
in the absence of Arg. Conversely, T_{1/2} for association was slowed in the presence of Arg to 15.42 ± 2.82 min (n=3), compared to 5.33 ± 3.19 min (n=5). Association in the presence of 0, 1, 10 and 100 μM Arg progressively diminished the Kob.

3.4.9 To what extent do other pterins compete with [³H]BH₄ for binding to eNOS and nNOS?

The relative ability of pterin analogues to compete for [³H]BH₄ binding to both eNOS (Fig 3.10) and nNOS (Fig 3.11) was investigated, (structures of the pterins studied are shown Fig 3.1). Competition of unlabelled BH₄ was identical for eNOS and nNOS with EC₅₀ values of 59.26 ± 18.99 nM and 58.65 ± 34.78 nM respectively (n=3 in both cases). That both NOS isoforms would have similar affinity for unlabelled BH₄ binding was predicted from the similar Kₐ values that had been obtained in [³H]BH₄ binding. BH₂, which cannot support NOS catalysis, competed for BH₄ binding to eNOS with EC₅₀ = 67.38 ± 1.14 nM and nNOS with EC₅₀ = 28.67 ± 6.372 nM (n=3 in both cases). Sepiapterin competed for BH₄ binding to eNOS with EC₅₀ = 2.26 ± 0.51 μM and nNOS with EC₅₀ = 30.99 ± 17.80μM (n=3 in both cases). The 6-methyl analogue of tetrahydropterin (6MePH₄) competed for BH₄ binding to eNOS with EC₅₀ = 26.95 ± 23 μM and nNOS with EC₅₀ = 47.78 ± 6.74 μM (n=2). Reduced pterins alone are able to support eNOS activity but 6MePH₄ had EC₅₀ values for eNOS and nNOS several orders of magnitude higher than for BH₂. To further examine the phenomenon that
Fig 3.10: Competition by pterins for $[^3\text{H}]\text{BH}_4$ binding to eNOS.
$[^3\text{H}]\text{BH}_4$ (50nM) and eNOS (10 pmole) were incubated 15 min, 23°C with one of the following pterins: $\text{BH}_4$, $\text{BH}_2$, sepiapterin, $6\text{MePH}_4$, $\text{PH}_4$, $\text{DMPH}_4$. Values are means ± SEM of triplicate determinations. This represents one experiment, similar results were obtained in 2 other experiments.
Fig 3.11 Competition by pterins for $[^3\text{H}]\text{BH}_4$ binding to nNOS. 
$[^3\text{H}]\text{BH}_4$ (50nM) and nNOS (10 pmole), were incubated 15 min, 23°C with either: BH$_4$, BH$_2$, sepiapterin or 6MePH$_4$. Points are mean values ± SEM of triplicate determinations. This represents one experiment, similar results were obtained in 2 other experiments.
tetrahydropterins, which can support NOS activity, bind with lower affinity than catalytically incompetent pterins, two other tetrahydropterins, tetrahydropterin (PH₄) and 6-dimethyltetrahydropterin (DMPH₄) were studied for their ability to compete for [³H]BH₄ binding to eNOS (Fig 3.10). These tetrahydropterins had lower affinities for eNOS than 6MePH₄ with EC₅₀ values of 112.3 μM and 308.1 μM respectively. These results indicate that the oxidation state of the pterin ring has little effect upon NOS binding whereas alteration of the 6-position side chain has a profound effect. Moreover, distinct differences, exceeding an order of magnitude, were observed in the binding profile of pterins to the BH₄ site of eNOS versus nNOS.

3.4.10 Can pterins cause displacement of prebound [³H]BH₄ from eNOS?

Since the eNOS affinity for BH₂ appeared to be equal to BH₄, this may have significant consequences for vascular pathophysiology that occurs secondarily to oxidative stress (see discussion). To examine this possibility, experiments were performed to assess the extent to which 7,8-BH₂ and other pterins can effectively displace bound [³H]BH₄ from eNOS. Preincubation of [³H]BH₄ and eNOS was carried out for 15 min to allow complex formation, then various concentrations of pterins were added to the [³H]BH₄-eNOS complexes (Fig 3.12). It is notable that the dihydropterins tested (sepiapterin and dihydropterin), diminished [³H]BH₄ binding to eNOS by 80% after a 30 min incubation, whereas tetrahydropterins (BH₄ and 6MePh₄), diminished [³H]BH₄ binding to eNOS by
Fig 3.12: Competition of pterins for $[^3H]BH_4$ pre-bound to eNOS.

$[^3H]BH_4$ (50nM) and eNOS (10 pmole) were incubated 15 min, 23°C, after 15 min one of the following pterins was added BH$_4$, BH$_2$, sepiapterin or 6MePH$_4$ and incubated a further 30 min. Values are means ± SEM of triplicate determinations. This represents one experiment, similar results were obtained in 2 other experiments.
only 60% under identical conditions. This disparity suggests that dihydropterins can actively promote $[^3H]BH_4$ efflux from eNOS, rather than merely occupying a site made available by $[^3H]BH_4$ dissociation. In this regard, dihydropterins may differ fundamentally from tetrahydropterins.

3.4.11 Is there a difference in rate between BH$_2$ and BH$_4$ elicited dissociation of $[^3H]BH_4$ from eNOS?

The possibility that dihydropterins can promote $[^3H]BH_4$ dissociation from eNOS was tested directly. Towards this end, $[^3H]BH_4$ was incubated with eNOS for 15 min to form complexes, then either BH$_2$ or BH$_4$ (each 100 μM) was added and the loss of $[^3H]BH_4$/eNOS complexes was monitored over time (Fig 3.13). BH$_2$ was found elicit a modestly more rapid $K_{off}$ than BH$_4$ (0.031 ± 0.009 min and 0.027 ± 0.004 min respectively). The measured dissociation half times were 22.34 ± 3.26 min and 28.09 ± 5.67 min, respectively. Student’s unpaired t-test did not reveal a statistically significant difference between the slopes for dissociation elicited in the presence of BH$_2$ versus BH$_4$. 
Fig 3.13: Kinetics of $[^3\text{H}]\text{BH}_4$ dissociation from eNOS elicited by BH$_4$ or BH$_2$.

$[^3\text{H}]\text{BH}_4$ (50 nM) was pre-incubated with eNOS (10 pmole) for 15 min and dissociation was initiated by the addition of either 100 $\mu$M unlabelled BH$_4$ or BH$_2$. Values are means ± SEM where n=4. This represents a single experiment, similar results were obtained in 2 other experiments.
3.5 Discussion

A summary of results is as follows:

1. Kd for $[^3H]BH_4$ binding to eNOS and nNOS were not very different. Binding stoichiometry was 0.5 BH$_4$ per NOS monomer. The kinetics of dissociation and association were also very similar for eNOS and nNOS.

2. Arginine occupancy was found to double the total $[^3H]BH_4$ bound to eNOS (stoichiometry ~1:1). A maximal enhancement with arginine was seen at 100 μM, whereas nitroarginine did not increase $[^3H]BH_4$ binding to eNOS.

3. The observed association rate of $[^3H]BH_4$ binding to eNOS decreased as Arg concentration increased.

4. BH$_2$ and BH$_4$ competed with $[^3H]BH_4$ for binding to eNOS and nNOS with near identical affinity.

5. Differences (> 10-fold) were observed between eNOS and nNOS in their relative affinity for sepiapterin and 6MePH$_4$ binding.

6. Tetrahydropterins with truncated or deleted 6'-side chain had greatly reduced affinity for both eNOS and nNOS, compared to BH$_4$.

7. Competition of pterins with $[^3H]BH_4$-bound eNOS revealed that the dihydropterins could reduce $[^3H]BH_4$ binding by >80% under conditions where tetrahydropterins only displaced ~60%. Furthermore BH$_2$ elicited a slightly faster $K_{off}$ for the dissociation of $[^3H]BH_4$-bound eNOS than observed with BH$_4$. 

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Studies were performed to characterise pterin binding to eNOS and elucidate functional implications. The BH$_4$ binding site of eNOS had been less well characterised than the BH$_4$ sites in the other NOS isoforms, perhaps due to the extensive eNOS post-translational modifications that complicate eNOS purification (List et al., 1997). Previously in our laboratory, the effects of BH$_4$ upon nNOS were studied and there are several published reports describing BH$_4$ binding to nNOS (Boyhan et al., 1997; Gorren et al., 1998; Martasek et al., 1998; Roman et al., 1995). Companion experiments using nNOS were performed in parallel, to identify the similarities and differences with the eNOS pterin binding site.

Interpretation of the results of the earliest [$^3$H]BH$_4$ binding assays to NOS isoforms were complicated because endogenous BH$_4$ remained bound to the purified enzymes. The development of a system to purify recombinant NOS from E. coli has made the rigorous characterisation of BH$_4$ binding sites on NOS feasible. E. coli lack PTPS, the second enzyme in the de novo BH$_4$ synthesis pathway (Martasek et al., 1996; Roman et al., 1995) and hence are unable to synthesise BH$_4$. Thus, prokaryotic expressed NOS is BH$_4$-free and can be produced in large quantities. It is notable that E. coli do synthesise some pterins, such as monapterin (Klein et al., 1994), but these do not appear to interact with NOS-BH$_4$ binding sites (Klatt et al., 1992; Raman, 2000).
BH₄ is readily oxidised to qBH₂ which can be further oxidised to 7,8-dihydrobiopterin (BH₂) and 7,8-dihydropterin (PH₂) (Kaufman, 1963). [³H]BH₄ was custom synthesised, labeled with [³H] at the 5,6 position by exhaustive tritiation of 7,8-BH₂. If oxidation of [³H]BH₄ were to occur during binding incubation reactions, (beyond formation of qBH₂) tritium at these positions would be lost, and the resulting unlabelled degradation products would be undetectable in our binding assay. All BH₄ binding assays were performed in buffer that contained DTT (0.5 mM) to minimise BH₄ oxidation in solution. Furthermore in the presence of DTT, qBH₂ is rapidly reduced to BH₄. Binding reactions were performed using Tris buffer, pH 7.6, at 25°C where BH₄ oxidation was reportedly slower than with other buffers tested (Davis et al., 1988).

With increasing amounts of eNOS added to a fixed amount of BH₄, a maximal of only 25% of added radiolabel was found associated with eNOS. The [³H] which did not bind might have arisen from the 5-position [³H] of BH₄ which is predicted to be more readily exchangeable than 6-[³H]. Unbound radiolabel may be in water or a BH₄ degradation product that does not bind NOS with high affinity.

The Kₐ determined for [³H]BH₄ by saturation binding was 82.1 ± 31.1 nM and 67.8 ± 2.65 nM for eNOS and nNOS, respectively. These values were not significantly different from one another. For both enzymes binding was saturable at a stoichiometry of 0.5 per NOS monomer and was best fit by a one-site model; this result is consistent with a single
class of high affinity binding sites. Another group reported eNOS to bind \[^{3}H\]BH\(_4\) with a 
K\(_d\) of 147 nM (List \textit{et al.}, 1997) and binding was shown to depend upon the haem content of NOS (List \textit{et al.}, 1996). As shown in Fig 3.2 the crystal structure of the eNOS oxygenase domain revealed that eNOS-bound BH\(_4\) makes H-bonded interactions with a haem propionate group at the eNOS dimer interface (Raman \textit{et al.}, 1998), explaining why haem is required for tight binding of BH\(_4\).

Using a scintillation proximity assay and the recombinant haem binding domain of nNOS, another group reported a K\(_d\) value of 20 nM for BH\(_4\) binding, which is in reasonable agreement with my determination of 67.8 ± 2.65 nM (Alderton & Lowe, 1999) In contrast, the K\(_d\) reported for BH\(_4\) binding to porcine holo nNOS was 250 nM (Klatt \textit{et al.}, 1994), but interpretation of this result is complicated by the fact that the nNOS studied was largely replete with BH\(_4\) prior to study; this already bound BH\(_4\) was bound with much higher affinity than that at sites that were amenable to study (Klatt \textit{et al.}, 1994). In aromatic amino acid hydroxylases the K\(_d\) value for BH\(_4\) is 100 – 600 µM (Nichol \textit{et al.}, 1985) approximately 1000 times the values observed for BH\(_4\) binding to NOS and consistent with the functional differences of BH\(_4\) usage between the two enzymes.

The binding of 50 nM nNOS had a Bmax 29.16 ± 2.911 nM, indicating a stoichiometry of ~ 0.5 BH\(_4\) per NOS monomer. The stoichiometry for BH\(_4\) binding to eNOS ranged from 0.35 - 0.5 this variation is probably due to variable incorporation of
haem in eNOS. The NOS expression system we used allows for the optimal incorporation of 1 haem per eNOS monomer (Berka & Tsai, 2000). Recombinant nNOS was also reported to bind to BH$_4$ with a 65% maximal occupancy (Roman et al., 1995). Additionally a stoichiometry of 0.5 BH$_4$ per NOS monomer was observed for nNOS (Boyhan et al., 1997) in keeping of the 0.06 - 0.48 BH$_4$ per NOS monomer previously reported (Gorren et al., 1998; Harteneck et al., 1994; Hevel & Marletta, 1992; Mayer et al., 1991).

In the nNOS oxygenase domain, BH$_4$ bound to NOS with Kd = 20 nM and binding could be inhibited by 7-nitroindazole and S-ethylisothiourea (Alderton et al., 1998). Association binding was triphasic but BH$_4$ dissociated from nNOS with a single off rate (Alderton et al., 1998). The stoichiometry was measured with 0.4 mol BH$_4$ per NOS monomer and dissociation was best fit to a single site (Alderton et al., 1998). In iNOS binding experiments looking at [${}^{14}$C]BH$_4$ binding to crude cytosol, the Kd was 31.89 ± 5.12 nM (Liu & Gross, 1996).

The crystal structures of iNOS and eNOS reveal clearly that there are two equivalent BH$_4$ binding sites on each NOS dimer. Paradoxically, we agree with other groups that only 1 BH$_4$ is bound with high affinity per eNOS or nNOS dimer (Berka & Tsai, 2000; Boyhan et al., 1997; Harteneck et al., 1994; Hevel & Marletta, 1992; Mayer et al., 1991). Reconciliation of these two seemingly discrepant conclusions is needed. An early observation that BH$_4$-bound NOS produced increased quantities of NO when extra
BH₄ was added, led Hevel and coworkers to propose that there are 2 BH₄ sites on NOS which act with negative co-operativity (Hevel & Marletta, 1992). With such a model, NOSs would have a high affinity BH₄ binding site (nM) and a low affinity site (µM). There is no reason to believe that the two BH₄ binding sites on eNOS are not equal prior to BH₄ binding, a most reasonable explanation is that once one site is occupied, this bound BH₄ may hinder the binding of BH₄ to a second site.

It has been argued from comparisons of pterin-free and pterin-bound eNOS, that the BH₄ sites are equivalent and that BH₄ does not effect conformational changes at the BH₄ site or elsewhere in the enzyme (Raman et al., 1998). Negative co-operativity cannot be adequately addressed by crystallography, since the essence of this technique entails addition of millimolar quantities of BH₄ to eNOS, concentrations which could support full occupancy of sites, despite negative co-operativity, Due to the constraints imposed by the specific activity of our [³H]BH₄, it is not possible in my assay system to elevate [³H]BH₄ concentrations above 400 nM. Therefore if BH₄ binds with negative co-operativity and the Kd for the first site in eNOS is ~ 80 nM, a span of several orders of magnitude might be necessary to evaluate whether reinforcing BH₄ levels can overcome negative co-operativity, by binding to the second BH₄ site.

The association rate with 50 nM [³H]BH₄ was calculated to be 0.169 ± 0.087 and 0.111 ± 0.021 min⁻¹ for eNOS and nNOS, respectively. The association for both isoforms
appeared to reach a maximum by 30 min. For both isoforms BH$_4$ binding fit to a single-site model under these conditions. A single class of binding sites was further supported by dissociation experiments. Koff was found to be fit by a single-site model; 0.0267 ± 0.004 and 0.0141 ± 0.0017 min$^{-1}$ for eNOS and nNOS, respectively. Another group has reported similar findings for nNOS that dissociation was slow and 1$^{st}$ order, they reported Koff at 37°C to be 0.32 min$^{-1}$ (Klatt et al., 1994). A recent paper about the eNOS crystal structure suggested that BH$_4$ is protected from bulk solvent once bound to eNOS (Fischmann et al., 1999), perhaps explaining our observed slow dissociation of BH$_4$.

The analysis of [${}^3$H]BH$_4$ binding to NOS that I have described so far was performed in the absence of NOS cofactors. Additional studies investigated potential effects of Arg, Ca$^{2+}$/CaM and NADPH. Neither Ca$^{2+}$/CaM nor NADPH alone had an effect upon the BH$_4$ binding but, when applied together, they significantly enhanced the binding by 50 ± 20%. Neither Ca$^{2+}$/CaM nor NADPH were anticipated to effect the binding of BH$_4$ by eNOS. Indeed, NADPH is incorporated into the reductase domain, and CaM lies at the juncture between the oxidase and reductase domains, not in proximity to the BH$_4$ binding site (Crane et al., 1998; Raman et al., 1998).

Crystal structures (Crane et al., 1998; Raman et al., 1998), mutational studies (Klatt et al., 1996) and haem inhibitors (Alderton et al., 1998; Klatt et al., 1994; Wolff & Gribin, 1994) have all demonstrated the necessity of haem for BH$_4$ binding to NOS. NADPH and
Ca\textsuperscript{2+}/CaM together increased BH\textsubscript{4} binding to eNOS; one reason might be a change in the electron environment surrounding the haem, resulting in either increased binding affinity of BH\textsubscript{4} to eNOS or protection of eNOS-bound BH\textsubscript{4} from dissociation.

Arginine increased the observed number of \[^{3}H\]BH\textsubscript{4} binding sites on eNOS by 100\%, suggesting that arginine is an allosteric effector of the BH\textsubscript{4} binding site. Other groups previously have reported that arginine increases the apparent Kd for BH\textsubscript{4} binding for nNOS (Alderton & Lowe, 1999; Klatt \textit{et al.}, 1994) and eNOS (List \textit{et al.}, 1997). The enhanced eNOS-BH\textsubscript{4} binding in the presence of arginine was observed only after a prolonged incubation (i.e. 30 min). Arginine has also been reported to reduce dissociation of \[^{3}H\]BH\textsubscript{4} from iNOS and to complete the shift from low-spin to high-spin with analogues of BH\textsubscript{4} (i.e. 4-aminoBH\textsubscript{4}) that alone elicit only a partial shift in spin-state (Mayer \textit{et al.}, 1997).

Having determined that the additional binding observed in the presence of Arg required a longer incubation, the influence of arginine concentration was examined for its ability to increase eNOS/\[^{3}H\]BH\textsubscript{4} complex formation. We observed a maximal effect of arginine at 0.1 mM, a concentration that exceeds the Km of arginine for NO generation by 10-fold (Frey \textit{et al.}, 1994; Olken \textit{et al.}, 1991; Pollock \textit{et al.}, 1991). Values for intracellular concentrations of Arg in endothelial cells have been described to range from 0.1 – 3 mM (Billiar \textit{et al.}, 1992; McDonald \textit{et al.}, 1997). There is debate about the amount of cellular
arginine which is actually available to NOS, which appears to be substantially less than this level; this phenomenon is unresolved and has been termed the "arginine paradox".

For analysis of association experiments in the presence of 1, 10 and 100 μM Arginine, we defined 100% occupancy as maximal [³H]BH₄ binding observed with zero arginine. As the concentration of Arg increased, the Kₒbinding decreased and the T½ and Bmax for BH₄ binding was found to increase. A 100% increase in eNOS-bound BH₄ was observed in the presence of maximal arginine, improving the observed stoichiometry to 1 BH₄/eNOS monomer. The r² for curve fitting did not improve when data was fit to a 2-site model. Another study of eNOS reported in the presence of arginine that a second class of BH₄ binding sites appears and a 2-fold increase in [³H]BH₄ bound to eNOS was observed, therefore the stoichiometry approached 1 BH₄/eNOS monomer (List et al., 1997). Since at the maximal levels of [³H]BH₄, saturation binding studies revealed only 1 BH₄ site per NOS dimer in the absence of additional arginine, Arg enables [³H]BH₄ to bind to a second site. This result is best reconciled by arginine-reversible negative co-operativity between the 2 BH₄ binding sites.

NOS has multiple binding ligands. In a recent study, the small haem ligands NO, imidazole and CN were used to probe for relations between the haem, arginine and BH₄ in eNOS (Berka & Tsai, 2000). BH₄ was bound to eNOS at 0.5 mM for 2 hours and then the enzyme was stringently washed to unbound BH₄. This enabled BH₄ to be studied
exclusively as a cofactor, not as a free ligand. The investigators found 0.7 BH$_4$ per eNOS monomer. Using stopped-flow experiments and rapid-scan diode array spectrometry to assess the effects of arginine and BH$_4$ (as cofactor) upon the haem, the investigators determined that the haem centers in the eNOS dimer were functionally distinct and that the BH$_4$ and arginine interacted differently at each. A conclusion of this study is that there may be negative co-operativity between the BH$_4$ sites on eNOS dimers (Berka & Tsai, 2000).

Nitroarginine (Narg) did not effect eNOS-BH$_4$ binding, Narg has ~ 10-fold higher affinity for eNOS than Arg and [$^3$H]Narg has been frequently used to study the arginine binding site of NOS. Our lab has previously reported that BH$_4$ increased the affinity of [$^3$H]Narg binding to eNOS (Liu & Gross, 1996). Like NMA, Narg binds to the Arg site of NOS, however it is not significantly metabolised and has a much slower rate of dissociation. Narg has also been reported to additionally interact with haem (Salerno et al., 1997). One explanation for the reduction in eNOS-BH$_4$ binding observed in the presence of Narg might be the Narg interaction with haem; Narg can disturb BH$_4$ interaction with the proximal face of haem involving H-bonds between haem propionate and BH$_4$ at N3, and the amine at C2 and through water to O4 (Fig 3.2)(Klatt et al., 1994; Raman, 2000).

Competition binding of pterins to NOSs is important, since variation in pterin binding between the isoforms would enable development of isoform-selective inhibitors. Based upon the Kd values I had earlier measured, the EC50 for BH$_4$ competition binding to
nNOS and eNOS was very similar: 58.65 and 59.26 nM respectively. The tetrahydropterin 6MePH₄, which has been reported to support NOS activity (Hevel & Marletta, 1992), bound with low affinity to both eNOS and nNOS (EC50 values were 26.95 and 47.78 μM, respectively), almost 3 orders of magnitude lower than BH₄. Similarly in [³H]BH₄ competition binding experiments with eNOS, PH₄ and DMPH₄ pterins which support NOS activity, bound with almost 4 orders of magnitude lower affinity than BH₄ (112.3 and 308.1 μM, respectively). These data suggest that binding to the BH₄ site alone is not sufficient to support catalysis. PH₄ lacks a propyl side chain, while 6MePH₄ and DMPH₄ have methyl substitutions for the propyl group. Truncation or deletion of the propyl side-chain appears to reduce the affinity of tetrahydropterins for the eNOS-BH₄ binding site. Binding studies with iNOS also revealed that the side-chain was important for the affinity of pterins for the BH₄ binding site (Presta et al., 1998). Another tetrahydropterin with intact 6'-side chain, 4-aminoBH₄, has a high affinity for the BH₄ binding site of iNOS (Mayer et al., 1997) although it failed to support NO synthesis. The pterin inhibitor 2-amino-4,6-dioxo-3,4,5,6,8,8a,9,10-octahydro-oxazolo[1,2f]-pteridine (PHS-32) which has an altered C6 side chain has also been demonstrated to displace BH₄ bound to nNOS and have high affinity for the BH₄ binding site (Reif, 1999). The pterin side-chain interacts with residues from both subunits of the NOS dimer through direct hydrogen binding of the
hydroxyl groups as well as through interactions with water in the BH$_4$ binding site (Raman et al., 1998).

The effects of dihydropterins and tetrahydropterins for iNOS were examined and the oxidation state of the pterin ring was found to effect NO synthesis and haem dependent NADPH oxidation (Presta et al., 1998). Sepiapterin, a naturally occurring product of the *de novo* BH$_4$ synthetic pathway, bound to eNOS with EC$_{50}$ of 2.255 µM but with lower affinity to nNOS 30.99 µM, therfore displaying a significant difference in affinity between the 2 constitutive NOS isoforms.

Surprisingly BH$_2$ had a very high affinity for the BH$_4$ binding site for both eNOS, 67.38 nM and nNOS, 28.67 nM. Since BH$_4$ can be readily oxidised to BH$_2$, my finding that this reduced biopterin can bind to constitutive NOS with similar affinity as the NOS cofactor BH$_4$ is of potential pathophysiological importance. Indeed, under conditions of oxidative stress BH$_4$ levels become depleted. Endothelial dysfunction arising from oxidative stress involves eNOS dysregulation. The addition of BH$_4$ has been reported to improve endothelium dysfunction in patients with hypercholestremia (Stroes et al., 1997) and also endothelium-derived vasodilation in chronic smokers (Heitzer, 2000).

Since BH$_4$ is limiting in endothelial dysfunction, the effects of pterins were observed upon BH$_4$-bound eNOS. eNOS was pre-bound to [$^3$H]BH$_4$ during a 15 min incubation, in the absence of cofactors and competition for the pterins was over a
subsequent 30 min incubation. The dissociation elicited by high levels of unlabeled BH4 was only 40% complete at the end of the 30 min competition period. Similarly the other tetrahydropterin tested, 6MePH4, was only able to displace 40% pre-bound eNOS/[3H]BH4. In contrast, both the dihydropterins, sepiapterin and BH2, were able to reduce levels of bound BH4 to <20%, after 30 min.

The findings from the pterin competition binding to pre-bound eNOS was surprising and suggested that not only did BH2 have a similar affinity to BH4 for the binding of eNOS but also that BH2 might actively displace pre-bound BH4 from eNOS. In order to determine whether BH2 could displace [3H]BH4 from eNOS, a dissociation experiment was performed where the dissociation was elicited by BH2 or BH4. The Koff elicited by BH2 was 0.031 compared to 0.027 for BH4. The T1/2 was slightly lower for BH2 (22.34 min) compared to BH4 induced dissociation (28 min). This modest difference between the BH2 and BH4 elicited dissociation indicates that BH2 may either displace or substitute for BH4 bound to eNOS (Fig 3.14).

To summarise I have found that Arg increases the amount of eNOS bound BH4 and only 1 BH4 site on eNOS dimers is occupied in the absence of Arg but when Arg is present the maximal BH4 bound doubles (Fig 3.14). This indicates that there is negative co-operativity which is overcome by arginine. The single class of BH4 binding sites I have described for eNOS and nNOS appear to have equal affinity for the dihydropterin BH2 as
the NOS catalytic cofactor BH₄. In eNOS, BH₂ displaces or substitutes eNOS bound-BH₄ from this site (Fig 3.14). In conditions of oxidative stress when BH₂ levels are elevated, this might contribute to eNOS dysregulation and potentially endothelial dysfunction. This hypothesis will be examined more closely in chapter 4.
Fig 3.14: A cartoon model for the sequence of pterin binding to eNOS.

1. A single BH$_4$ binds eNOS dimer
   $K_d \sim 80$ nM

2. One or two Arg bind, making a second BH$_4$ site available for binding

3. BH$_2$ binds with equal affinity as BH$_4$ and may substitute for, or displace, BH$_4$ on eNOS
Chapter 4 - PTERIN CONTROL OF eNOS CATALYTIC ACTIVITIES

4.1 Introduction

NOSs convert arginine to citrulline and NO in a reaction which requires BH₄ as a cofactor (Kwon et al., 1989; Tayeh & Marletta, 1989). Hydroxyarginine (NOHA) is the intermediate in the NOS reaction (Marletta, 1993; Stuehr et al., 1991). Hydroxylation of a guanidino nitrogen of arginine, to form NOHA, requires 1 mol NADPH and 1 mol O₂ (Fig 1.4). This first step in NOS catalysis is thermodynamically more difficult than the second step, involving the formation of an N-O bond via a carbanion-like transition state. Formation of NOHA requires that 2 electrons are transferred from NADPH to haem in order to consume O₂ for hydroxylation of arginine to NOHA (Kwon et al., 1990; Leone et al., 1991). BH₄ and the reductase domain of NOS have been proposed to facilitate this first step by reducing the activation energy required for the formation and stabilisation of the carbanion-like transition state (Adhikari, 2000).

The second step in NOS-mediated catalysis involves C-O bond formation, conversion of NOHA to citrulline and NO, and consumption of 0.5 mol NADPH and 1 mol O₂; this requires a three electron oxidation of nitrogen. The oxygens incorporated into NO and citrulline are derived from different molecules, this was demonstrated utilising [¹⁸O₂] (Kwon et al., 1990; Leone et al., 1991).

Analysis of the iNOS crystal structure led to the suggestion that bound BH₄ contributes to the active site channel (Crane et al., 1998), although this was disputed by the
authors of a paper describing the eNOS oxygenase domain crystal structure (Raman et al., 1998). In the previous chapter, I provided evidence that the affinity of BH₄ for binding to eNOS was indistinguishable from the partially reduced pterin, BH₂. While dihydropterins are sufficient to elicit increases in Arg-analogue affinity, and stabilise NOS dimers (Presta et al., 1998), only tetrahydropterins have been shown to support NOS catalysis (Hevel & Marletta, 1992; Milstien & Katusic, 1999).

BH₄ is required for both NOHA formation and a putative spectroscopically detectable intermediate in the formation of NOHA (Bec et al., 1998). A model was proposed in which BH₄ donated an electron to form this intermediate, a postulated pterin radical (*BH₃) (Bec et al., 1998). Elucidation of an eNOS oxygenase domain crystal structure with Arg occupying the pterin binding site led to the suggestion that the pterin site of eNOS can accommodate a positively charged pterin radical (Raman et al., 1998).

At the start of the NOS reaction, in an aerobic environment, CaM and Ca²⁺ enable the rapid formation of ferrous-dioxyhaem (Fe²⁺OO) (Abu-Soud et al., 1997); in the reaction with arginine this is the only detectable haem intermediate. In the reaction with NOHA, a transient ferric NO (Fe³⁺NO) complex is also detectable before it degrades to ferric NOS (Fe³⁺) (Boggs, 2000). The first measurements of what appears to be *BH₃ came from studies of the iNOS haem domain; superfine coupling of exchangeable protons suggested that BH₄ forms *BH₃ at a rate of 15-20 s⁻¹ and the radical is quite stable, decaying at a rate of 0.12-0.7 s⁻¹ (Hurshman, 1999). The group described how within 127 ms, 80% of the
BH₄ had become •BH₃ in the presence of arginine, but only 2-8% formed •BH₃ with
NOHA. Therefore NOHA was suggested to prevent formation of the putative •BH₃ radical
(Hurshman, 1999). The rate of formation of •BH₃ was consistent with the rate of
degradation of the Fe⁶⁰OO complex. Interestingly, in the absence of BH₄, and presence of
BH₂, Fe⁶⁰OO is formed and decays slowly, but is unable to hydroxylate arginine. This
provides a potential molecular explanation for the uncoupled oxidation of NADPH
observed with NOS (Wei, 2000).

In the past 3 years, evidence has accumulated that all of the NOS isoforms are
capable of superoxide (O₂⁻) production (Miller et al., 1997; Vasquez-Vivar et al., 1998;
Vasquez-Vivar et al., 1999; Xia et al., 1998; Xia & Zweier, 1997). In contrast to iNOS and
nNOS, eNOS production of O₂⁻ is not triggered by arginine deficiency alone. Indeed, BH₄
deficiency was shown to trigger O₂⁻ production by eNOS (Brandes, 1997; Wever et al.,
1997). Subsequent addition of BH₄ was found to diminish O₂⁻ production by substrate-free
eNOS (Vasquez-Vivar et al., 1998) and more recently nNOS (Kotsonis, 2000). BH₄
allosterically modulates nNOS and stabilises dimers, whereas a pterin inhibitor, 2-amino-
4,6-dioxo-3,4,5,6,8,8a,9, 10-octahydro-oxazolo[1,2f]-pteridine (PHS-32), has been
demonstrated to displace BH₄ and mimic the stabilising effect of BH₄ on nNOS dimers
(Reif, 1999).

Superoxide is generated by eNOS in conditions of diabetes, hypertension and aging
(Wever et al., 1998). NO derived from eNOS contributes to the modulation of blood
pressure in response to receptor-mediated stimuli (ACh, ATP, Substance P, thrombin and bradykinin) and endothelial shear-stress (For review, see (Balligand, 1999; Heusch, 1997)). Endothelial dysfunction occurs in a variety of disease states, which include atherosclerosis, hypercholestremia, hypertension, diabetes and heart failure; the extent of endothelial dysfunction is also dependent upon age and gender (for review, see (Dhalla, 2000)). In atherosclerosis, endothelial dysfunction is localised to plaques, with normal NO synthesis in adjacent areas of the blood vessel (for review, see (Busse & Fleming, 1996)).

Blood vessels that have been depleted of BH\textsubscript{4} have also been reported to produce H\textsubscript{2}O\textsubscript{2} (Stroes \textit{et al.}, 1997). In the absence of BH\textsubscript{4} and arginine, nNOS produces H\textsubscript{2}O\textsubscript{2} in a CaM/Ca\textsuperscript{2+}-dependent manner (Heinzel \textit{et al.}, 1992). NOHA addition has been reported to block H\textsubscript{2}O\textsubscript{2} production (Klatt \textit{et al.}, 1993). Reduced NO levels have been observed in a hypertensive rat model (Panza, 1993). Notably, while levels of eNOS mRNA were found to increase in hypertensive compared to wild type rats, O\textsubscript{2}\textsuperscript{=} levels were substantially higher in the hypertensive animals (Kerr, 1999). The investigators concluded that O\textsubscript{2}\textsuperscript{=} generated in the blood vessels of hypertensive rats was derived from eNOS.

The reaction between NO and O\textsubscript{2}\textsuperscript{=} to form the potent oxidising agent peroxynitrite (ONOO-) is rapid, far exceeding the ability of SOD to scavenge O\textsubscript{2}\textsuperscript{=} (Hogg \textit{et al.}, 1994). ONOO- can oxidise BH\textsubscript{4} to qBH\textsubscript{2} and 7,8PH\textsubscript{2} (Milstien & Katusic, 1999) which may further contribute to oxidative stress by promoting eNOS-derived O\textsubscript{2}\textsuperscript{=} production (Vasquez-Vivar \textit{et al.}, 1999). Additionally, ONOO- can react with low density
lipopolysaccharide (LDL) to form oxidised LDL (oxLDL) (Violi, 1999). oxLDL has been implicated in inhibiting acetylcholine (ACh) induced vasodilatation, increasing the release of $O_2^+$ by eNOS, and enhancing levels of oxidative damage in the blood vessel (Pritchard et al., 1995). In hypercholesterolemia, small increases in cholesterol levels have been demonstrated to increase the vasoconstrictor response to serotonin (Merkel, 1990); L-arginine administration has been reported to improve vasodilatation in atherosclerotic humans (Creager, 1992) and hypercholesterolemic rabbits (Girerd, 1990), indicative of reinstated NO production.

$BH_2$ levels were increased and $BH_4$ levels were depleted in insulin-resistant hyperglycemic rats (Pieper, 1997). This biopterin imbalance was pin-pointed as a cause of increased formation of $O_2^+$ and ONOO- (Shinozaki, 1999). $BH_4$ administration has also been shown to reverse vascular dysfunction in hypercholestremia (Stroes et al., 1997) and diabetes (Pieper, 1997). In addition to restoring eNOS catalytic function, $BH_4$ and tetrahydroneopterin have been reported to scavenge $O_2^+$ (Heitzer, 2000).

Endothelial dysfunction also occurs in smokers, where vascular responses to calcium ionophore (A23187) and effects of NMA are diminished, compared to vessels from non-smokers (Higman et al., 1996). Whilst arginine and superoxide dismutase (SOD) do not restore vascular relaxant action in vessels of smokers, administration of $BH_4$ increased endothelium-dependent vasodilatation, in response to ACh, and increased levels of nitrite and cGMP accumulation (Higman et al., 1996).
4.2 Aims

In the previous chapter, I described the similar affinities of BH$_2$ and BH$_4$ for the pterin binding site on eNOS. Accordingly, under conditions that promote BH$_4$ oxidation, BH$_2$-bound eNOS is likely to accumulate. Unlike tetrahydropterins, dihydropterins are unable to support NO or NOHA generation from arginine, presumably due to an inability to generate a $^*$BH$_3$ radical intermediate. There is evidence from the literature that depletion of BH$_4$ leads to uncoupled oxidation of NADPH and formation of O$_2^-$ by eNOS. In this chapter, using EPR, we examined the effects of dihydro- versus tetrahydropterins on O$_2^-$ generation by eNOS. In the absence of BH$_4$ and presence of $H_2O_2$, NOS, as well as cytochrome P450s, have been suggested to generate NO from NOHA and hydroxyguanidines (Clement et al., 1999; Clement et al., 1994; Renodon-Corniere et al., 1999). In the following experiments, I sought to determine whether BH$_2$-bound eNOS (which is likely to occur during oxidative stress in vivo), like BH$_4$-bound eNOS, is able to metabolise NOHA to NO or a related species.
4.3 Methods

4.3.1 EPR Measurements.

Electron paramagnetic resonance (EPR) spectra were recorded at room temperature on a Varian E-109 spectrometer operating at 9.03 GHz and 100KHz field modulation equipped with a loop-gap resonator. Reactions were initiated by the addition of eNOS to incubation mixtures [HEPES (50 mM pH 7.4) calcium (0.2 mM), CaM (1.2 μM), NADPH (0.1 mM), and DPTA (0.1 mM)] containing 5-diethoxyphosphoryl-5-methyl-1-pyrroline N-oxide (DEPMPO). After 1 min incubation samples were scanned 10 times and the resultant mean EPR spectrum quantified. Concentration of the radical adducts was calculated by double integration of the computer based simulation spectra from the experimental data. 3-Carbamoyl-2,2,5,5,-tetramethylpyrrolidine-N-oxyl was used as standard. Computer simulation was performed using simulation written by D. Duling (Duling, 1994).

4.3.2 Assay of NOS activity by NADPH Consumption

NADPH consumption by NOS was assessed at 37°C in 96 well microtiter plates, as previously described (Gross, 1996). Incubation mixtures contained: Tris-HCl pH 7.6 (50 mM), CaCl₂ (100 μM), BH₄ or BH₂ (10 μM), NADPH (500 μM), DTT (1 mM), calmodulin, (100 nM) and either NOHA or L-arginine, in a final volume of 100 μl. Reactions were initiated by the addition of eNOS (100 nM) and the rate of decrease in A₃₄₀
nm was measured at 30-sec intervals, for a period of 30 min, in a kinetic microplate spectrophotometer (Molecular Devices; Menlo Park, CA). The rate of decline in $A_{340}$ nm measured when eNOS was omitted from incubates was subtracted from all values, total NADPH consumed was determined from an NADPH concentration curve ($A_{340}$ nm).

**4.3.3 Greiss assay**

Reaction mixtures contained: Tris - HCl (50 mM) pH 7.6, CaCl$_2$ (100 μM), CaM (100 nM), DTT (1 mM), NADPH (0.5 mM), either NOHA or L-arginine (0.3 mM), and either BH$_4$ or BH$_2$ (100 μM), in a final volume of 100 μl. The reaction was initiated by the addition of NOS (100 nM) and samples were incubated at 37°C. After 30 min, 25 μl of reaction mixture was removed, added to 75 μl nitrite reductase buffer [FAD (0.1 mM), Tris-HCl (200 mM), NADPH (10 mM), nitrite reductase (0.1 U/ml)], and incubated for 45 min at 37°C. Residual NADPH was consumed by incubation at 37°C, for 30 min, in the presence of 10 μl lactic dehydrogenase (0.058 U/μl) and pyruvate (0.5 mM). Greiss reagent [1:1 sulfanilamide (10%) in ortho-phosphoric acid (50%) and N-(1-naphthyl)ethylenediamine dihydrochloride (1%)] was added as 100 μl volume to all samples and the plate was read at 550 nm and analysed in relation to sodium nitrite and nitrate standards.
4.3.4 Analysis of concentration-response relationships

Data were analysed using the Graphpad Prism and EC50 values for substrate conversion to NO\textsubscript{x} were calculated from sigmoidal concentration-response curves which were fitted to the equation:

\[
\text{NO}_x = \text{NO}_x \text{ min} + (\text{NO}_x \text{ max} - \text{NO}_x \text{ min}) (1+10^{(\log(\text{EC50} \times X)})
\]

Where X is the logarithm of substrate concentration, Y is the response (NO\textsubscript{x}). Y has minimal response in the absence of substrate/eNOS (NO\textsubscript{x} min) and with a maximal response in NO\textsubscript{x} generation (NO\textsubscript{x} max- NO\textsubscript{x} min).

4.3.5 Electrospray Ionization (ESI) Mass Spectrometry (MS/MS) of NOHA products formed upon reaction with eNOS

Incubation mixtures contained ammonium acetate (50 mM) pH 7.6, eNOS (10 nM) CaCl\textsubscript{2} (100 μM), BH\textsubscript{4} or BH\textsubscript{2} (10 μM), NADPH (500 μM), L-arginine or NOHA (300 μM), DTT (1 mM) and calmodulin (100 nM). Spectra were acquired on a Quattro II extended mass range (1-8000 Da) triple quadrupole mass spectrometer (Micromass UK Ltd, Manchester, UK), interfaced to a windows NT workstation utilising MassLynx version 2.3 software (Micromass). The mass spectrometer was scanned in positive ionization mode with a source temperature of 80°C and a 3.5 kV capillary potential. For MS/MS, collision-induced dissociation energy of 17 volts was applied.

Samples were incubated for 1 h at 37°C and 100 μl reaction mix was diluted with
900 µl of [50:50:2 H₂O: Methanol: acetic acid] and continuously infused into the mass spectrometer’s co-axial probe at a rate of 0.3 ml/h. The prominent products were subjected to argon collision gas bombardment of the parent ion and fragmented to daughter ions.
4.4 Results

4.4.1 What are the effects of pterins upon $O_2^-$ generation by eNOS?

EPR experiments utilised DEPMPO, a specific superoxide scavenger that binds to superoxide and forms the adduct DEPMPO-OOH which is stable at ambient temperature. DEPMPO has been reported to trap 60-70% of the $O_2^-$ available (Roubaud, 1997).

Superoxide was produced by eNOS (7 pmole) in the complete NOS assay buffer containing: HEPES (50 mM pH 7.4) calcium (0.2 mM), CaM (1.2 μM), NADPH (0.1 mM), DEPMPO (50 mM) and DPTA (0.1 mM) (Fig 4.1A). The addition of BH$_4$ to this reaction (10 μM) markedly attenuated $O_2^-$ production in the absence (Fig 4.1C) or presence (Fig 4.1E) of arginine (0.1 mM); arginine alone was observed to cause only a modest ~10% reduction in $O_2^-$ production. In contrast, BH$_2$ (100 μM) did not attenuate $O_2^-$ in either the absence (Fig 4.1B) or presence (Fig 4.1D) of arginine.

The dihydropterin, sepiapterin (100 μM) had no effect on the eNOS $O_2^-$ production in the absence or presence of arginine (Fig 4.2B and C). Surprisingly, the ability of BH$_4$ to attenuate $O_2^-$ was mimicked by a pterin with low eNOS binding affinity, 6MePH$_4$ (10 μM; Fig 4.2D), with and without arginine (0.1 mM; Fig 4.2E). These findings suggest a fundamental difference between tetra- and dihydro- pterins in their ability to determine $O_2^-$ production by eNOS.
Fig 4.1: Effect of 7,8-BH$_2$ and BH$_4$ on the generation of superoxide by eNOS.

All reactions contained eNOS (7 pmoles) in complete assay buffer: calcium (0.2 mM), calmodulin (1.2 μM), NADPH (0.1 mM), DEPMPO (50 mM), DTPA (0.1 mM) in HEPES (50 mM) pH 7.4). A] No further addition; B] 7,8BH$_2$ (100 μM); C] BH$_4$ 10 μM; D] 7,8BH$_2$ (100 μM) and arginine (0.1 mM); and E] BH$_4$ 10 μM and arginine (0.1 mM).

Each panel is the mean of 10 scans.
Fig 4.2: Effect of sepiaperin and 6MePH₄ on the generation of superoxide by eNOS.
All reactions contained eNOS (7 pmoles) in complete assay buffer: (calcium (0.2 mM), calmodulin (1.2 μM), NADPH (0.1 mM), DEPMPO (50 mM), DTPA (0.1 mM) in HEPES (50 mM) pH 7.4). A] No further addition; B] sepiaperin (100 μM); C] sepiaperin (100 μM) and arginine (0.1 mM); D] 6MePH₄ (10 μM) and E] 6MePH₄ (10 μM) with arginine (0.1 mM). Each panel is the mean of 10 scans.
4.4.2 Can dihydropterins reinstate O$_2^·$ production by eNOS after attenuation by BH$_4$?

In the previous chapter, I described how BH$_2$ appeared to displace [³H]BH$_4$ from eNOS (Fig 3.12 and 3.13). Following up on observations from these binding assays (Chapter 3), we set out to determine if BH$_2$ could compete with BH$_4$ to reinstate O$_2^·$ production. BH$_4$ (10 µM) and eNOS (7 pmoles) were preincubated in complete assay buffer and levels of O$_2^·$ were found to be undetectable (Fig 4.3A). Subsequent addition of sepiapterin (50 µM) reinstated O$_2^·$ production ~ 60% (Fig 4.3B) and BH$_2$ (1 mM) completely reinstated O$_2^·$ production (Fig 4.3C). This result is in accord with the finding of Chapter 3, that BH$_2$ could initiate [³H]BH$_4$ dissociation from eNOS.

4.4.3 Can BH$_2$ support NO$_2$ production from Arg or NOHA?

BH$_4$ supports production of NO from arginine which can be detected in the Griess assay as NO$_x$. In contrast, BH$_2$ (10 µM) is unable to support the NOS-mediated NO$_x$ production from arginine (300 µM; Fig 4.4). However when BH$_2$ was incubated with eNOS and NOHA (300 µM), nitrite was detected (Fig 4.4). The BH$_4$-bound eNOS generated approximately 3 times the amount of NO$_x$ with NOHA as substrate, compared to Arg as substrate.
Fig 4.3: Sepiapterin and BH₄ increase superoxide formation from eNOS.
All reactions contained eNOS (7 pmoles) and arginine (0.1 mM) in complete assay buffer:
calcium (0.2 mM), calmodulin (1.2 μM), NADPH (0.1 mM), DEPMPO (50 mM), DTPA (0.1 mM)
in HEPES (50 mM) pH 7.4) A] BH₄ (10 μM), B] BH₄ (10 μM) and sepiapterin (50 μM) and
C] BH₄ (10 μM) and BH₂ (1 mM). Each panel is the mean of 10 scans.
Fig 4.4: Catalysis of arginine and hydroxyarginine (NOHA) by eNOS. In the presence of BH$_4$ (10 μM) but not BH$_2$ (10 μM), eNOS (10 pmole) can catalyse the production of NO from arginine, measured by NO$_x$ generation. In contrast eNOS can produce NO$_x$ from hydroxyarginine (NOHA) in the presence of both BH$_4$ and BH$_2$. Values are means ± SEM where n = 4.
4.4.4 What is the EC50 requirement for eNOS conversion of NOHA to NOx?

NOHA dose-response curves were constructed for eNOS in the presence of BH₂ or BH₄ (Fig 4.5). BH₄ appeared to be a more efficient cofactor for this reaction than BH₂, producing more NOₓ for each concentration of NOHA tested. EC50 values for BH₄-bound and BH₂-bound eNOS were 62.25 ± 1.21 and 245.9 ± 1.68 μM, respectively (where n=4). Computer generated concentration-response curve fits of the data suggested that the same maxima would be reached with eNOS bound BH₄ and BH₂ (119.6 ± 6.2 and 105.0 ± 26.19 μM NOₓ respectively). In the absence of eNOS, less than 5 μM NOₓ was measurable, indicating that eNOS is a prerequisite for the observed NOₓ accumulation.

4.4.5 What is the effect of increasing the concentration of eNOS on the NOHA reaction with BH₂ or BH₄ as cofactor?

In a parallel experiment, where NOHA concentration was fixed (300 μM, Fig 4.6A) and eNOS concentration was increased in the presence of 10 μM BH₄, 1 pmol of eNOS was sufficient to convert all NOHA to NOₓ. However with BH₂ as cofactor several-fold more eNOS was required to produce an equivalent quantity of NOₓ. This suggests that NOₓ generated from NOHA by eNOS occurs more rapidly with BH₄ as cofactor than with BH₂ as cofactor (Fig 4.6A). However there appears to be no significant difference in NADPH consumption with increasing concentrations of eNOS comparing BH₂ and BH₄ as cofactors.
Fig 4.5: Hydroxyarginine concentration-response relationships for eNOS production of NO\textsubscript{x}, in the presence of BH\textsubscript{2} or BH\textsubscript{4} (10 \mu M).

In the absence of eNOS (10 pmole), less than 5\% of hydroxyarginine was converted to NO\textsubscript{x}. Values are means SEM where n=4, similar results were observed in two other experiments.
Fig 4.6 Effect of increasing [eNOS] on NOHA (300 μM) in the presence of BH2 or BH4 on A] NOx generation, B] NADPH consumption. Values are means ± SEM where = 4, similar results were obtained in 2 other experiments.
This suggests that BH$_4$-binding to eNOS increases the efficiency of NO$_x$ formation per mol of NADPH, relative to the BH$_2$-driven reaction.

4.4.6 Is the eNOS catalysed NOHA reaction, with BH$_2$ as cofactor, dependent upon calmodulin?

As anticipated, in the absence of CaM, BH$_4$-bound eNOS could not convert Arg to citrulline and NO (Fig 4.7A) consistent with the plethora of reports in the literature (Bredt & Snyder, 1990; Mayer et al., 1990). Interestingly, the eNOS NOHA reaction with BH$_4$ as cofactor was not completely dependent upon the addition of CaM, since removal of CaM from the assay buffer caused only a ~60% reduction in NO$_x$ generated (Fig 4.7C). The effect of removing Ca$^{2+}$/CaM upon the eNOS bound BH$_2$ catalysed NOHA reaction was even less marked, with ~ 20% reduction in NO$_x$ production (Fig 4.7D).

4.4.7 Do arginine-based NOS inhibitors block the production of NO$_x$ from NOHA by eNOS?

NOS-catalysed production of NO from arginine is inhibited by N$^{	ext{m}}$-methyl arginine (NMA) and Nitroarginine (Narg), agents which compete for Arg binding to the substrate site of NOS. Narg has additionally been reported to interact with the haem site of NOS (Salerno et al., 1997). The effects of Narg (1 mM) and NMA (1 mM) on eNOS (10 pmole) catalysed conversion of NOHA to NO$_x$ was analysed in the presence of either BH$_2$ or BH$_4$. 

4-20
Fig 4.7: Effects of Ca\(^{2+}\)/CaM on BH\(_4\)- and BH\(_2\)-bound eNOS catalysis.
A] BH\(_4\) (10 \(\mu\)M), eNOS (10 pmole) and arginine (100 \(\mu\)M) B] As in [A] but BH\(_2\) (10 \(\mu\)M), C] BH\(_4\) (10 \(\mu\)M), eNOS (10 pmole) and hydroxyarginine (NOHA - 100 \(\mu\)M) and D] As in [C] but with BH\(_2\). Values are means ± SEM where n=4, similar results were observed in two other experiments.
(both 10 μM). As expected, both Narg and NMA inhibited the BH₄ bound-eNOS arginine reaction, manifest by an increase in apparent Arg EC₅₀ from 62.25 ± 1.21 μM to 815 ± 4.27 μM and 369.2 ± 1.75 μM in the presence of Narg (1 mM) and NMA (1 mM) respectively (Fig 4.8A). Paradoxically, in the BH₂ catalysed reaction, NMA and Narg significantly enhanced NOₓ generation from NOHA (Fig 4.8B). The failure of arginine analogue inhibitors to attenuate eNOS catalysed NOₓ formation from NOHA suggests that metabolism by BH₂-eNOS does not require binding to the arginine site.

4.4.8 Is the haem domain of eNOS necessary for NOₓ production from NOHA?

To further examine the role of haem in eNOS mediated NOHA metabolism, the haem binding ligand, imidazole (100 nM to 100 mM) was used as a competitor. No effects were seen on NOₓ production from NOHA with either BH₂ or BH₄ catalysed eNOS reactions (Fig 4.9A). Similarly, imidazole had little effect on NADPH consumption by eNOS with either BH₂ or BH₄ as cofactor (Fig 4.9B). Combined with observations that NOₓ production by BH₂-eNOS is not inhibited by Narg, which also interacts with haem, these results indicate that unliganded haem is not necessary for NOₓ formation by BH₂-bound eNOS.
Fig 4.8: Effect of NMA and nitroarginine (Narg) on eNOS catalysis of NOHA. Where NMA (1 mM), Narg (1 mM) and eNOS (10 pmoles) in the presence of A] BH4 (10 µM) and B] BH2 (10 µM). Values are means ± SEM where n=4, similar results were observed in two other experiments.
Fig 4.9: Effect of imidazole on eNOS (5mole) catalysis of NOHA (300 μM) in the presence of either BH2 (10 μM) or BH4 (10 μM). Panel A] Effect on NADPH consumption, Panel B] Effect on nitrate/ nitrite production. Values represent means ± SEM of quadruplicate determinations, similar results were observed in 2 other experiments.
4.4.9 Are the FAD/FMN domains of eNOS required for NO\textsubscript{x} production from NOHA?

To investigate whether the FAD/FMN domains of eNOS are involved in the production of NO\textsubscript{x} from NOHA, we studied the influence of the flavoprotein inhibitor, diphenyleneiodonium (DPI). DPI inhibited the formation of NO\textsubscript{x} (Fig 4.10A) and blocked NADPH consumption (Fig 4.10B). The EC\textsubscript{50} for DPI inhibition of NO\textsubscript{x} generated from NOHA was found to be 295.3 ± 4.3 and 567.2 ± 12.6 nM for the BH\textsubscript{2}- and BH\textsubscript{4}-bound eNOS reactions, respectively.

4.4.10 What is the source of electrons for the eNOS NOHA reaction?

The observed inhibition by DPI indicates that electron flux through the flavins of eNOS is necessary for metabolism of NOHA to NO\textsubscript{x}. NOHA concentration-response curves for NO\textsubscript{x} generation by eNOS were compared with complete reaction buffer [Tris-HCl pH 7.6 (50 mM), CaCl\textsubscript{2} (100 μM), BH\textsubscript{4} or BH\textsubscript{2} (10 μM), NADPH (500 μM), DTT (1 mM), calmodulin, (100 nM) and eNOS (10 pmoles)], and identical buffer, devoid of either NADPH or pterin. Omission of either NADPH or pterin elicited a small rightward shift in the curves describing NOHA-derived NO\textsubscript{x} formation (Fig 4.11A).

In all of the NOS reactions, DTT was present. To test whether DTT or NADPH was obligate to supply electrons for conversion of NOHA to NO\textsubscript{x}, we investigated the influence of omitting DTT and/or NADPH, in the presence of BH\textsubscript{2} and BH\textsubscript{4} (Fig 4.11B).
Fig 4.10: Effect of Diphenyleneiodonium on eNOS (10 pmole), NOHA (300 μM) catalysis in the presence of 10 μM BH₂ or BH₄. Panel A] effect on NADPH consumption, Panel B] Effect on NOₓ generation. Values are means ± SEM of quadruplicate determinations, similar results were observed in 2 other experiments.
Fig 4.11: Effect of reductants on NOHA metabolism to $\text{NO}_x$ by eNOS.

Panel A] NOHA concentration-response curve for eNOS (10 pmole) in complete assay buffer (complete, squares) [Tris-HCl pH 7.6 (50 mM), CaCl$_2$ (0.1 mM), BH$_4$ (10 $\mu$M), NADPH (0.5 mM), DTT (1 mM), and CaM (100 nM)]; without pterin (diamonds), without NADPH (triangles) and without eNOS (circles). Panel B] Effect of DTT and/or NADPH removal upon eNOS NOHA catalysis in complete assay buffer in the presence of BH$_2$ and BH$_4$. Values are means ± SEM where n=4, similar results were observed in two other experiments.
Without NADPH, BH₄-bound eNOS metabolism of NOHA to NOₓ was substantially reduced (~70%, Fig 4.11B). Removal of DTT alone from the reaction had less of an effect (~15% reduction), whereas in the absence of both DTT and NADPH NOₓ accumulation was diminished >80%. In contrast, the BH₂-bound eNOS metabolism of NOHA to NOₓ was unaffected by removal of DTT, diminished ~50% in the absence of NADPH but diminished >75% in the absence of both DTT and NADPH.

4.4.11 Can O₂⁻ or H₂O₂ generate NOₓ from NOHA?

In the absence of BH₄, eNOS generates superoxide. Superoxide can be chemically synthesised by incubating xanthine with xanthine oxidase (McCord, 1969). In an effort to determine whether O₂⁻ may mediate the ability of BH₂-eNOS to produce NOₓ from NOHA, xanthine/xanthine oxidase was incubated with NOHA and NOₓ accumulation was measured. The O₂⁻ generating system was validated using a standard spectroscopic method that evaluates Fe³⁺ reduction to Fe²⁺ in ferricytochrome c and a ratio of xanthine to xanthine oxidase was employed that was predicted to produce 10 nmole/ml/min O₂⁻ (McCord, 1969). NOHA was observed to generate NOₓ in the presence of O₂⁻ and this was significantly attenuated by the addition of superoxide dismutase (SOD, 2000 U/ml) (Fig 4.12A).

Similarly, incubation of NOHA (300 µM) with H₂O₂ was observed to generate NOₓ from NOHA, with maximal NOₓ observed in the presence of 1 mM H₂O₂ (Fig 4.12A).
Fig 4.12: Determination of whether H$_2$O$_2$ and O$_2^\cdot$ can generate NO$_x$ from NOHA.

Panel A] Effect of H$_2$O$_2$ (0.1 - 10 mM) and O$_2^\cdot$ (predicted ~10 nmole/ml/min for more than 20 minutes from xanthine/xanthine oxidase) on NO$_x$ generated from NOHA (300 μM), catalase (2000 U/ml) and superoxide dismutase (2000 U/ml) were respectively able to abolish NO$_x$ generation from hydroxyarginine. Panel B] Effect of SOD (2000 U/ml) and catalase (2000 U/ml) upon BH$_2$-bound eNOS NO$_x$ generation from NOHA (300 μM) in complete assay buffer (control). Values for both panels are means ± SEM where n=4, similar results were observed in 2 other experiments.
Paradoxically, accumulation of NOx was diminished in the presence of a greater concentration of H2O2 (10 mM). Catalase (2000 U/ml) abolished the H2O2-mediated production of NOx from NOHA (Fig 4.12A).

To test whether in the BH2-bound eNOS reaction H2O2 or O2⁻ was enabling the production of NOx from NOHA, the influence of catalase (2000 U/ml) and SOD on this reaction was assayed (Fig 4.12B). While catalase did not prevent the formation of NOx, SOD (2000 U/ml) attenuated NOx generation by ~50% (Fig 4.12B).

4.4.12 Can eNOS catalyse the conversion of hydroxyguanidine to NOx?

NOx generated by eNOS from NOHA originates from the hydroxyguanidine group. To determine whether hydroxyguanidine itself can be converted to NOx by eNOS, various concentrations of hydroxyguanidine were incubated with eNOS in the presence of BH4 or BH2. As shown in Fig 4.13, NOx was undetectable after incubation with BH2- or BH4-bound eNOS at all concentrations of hydroxyguanidine tested (Fig 4.13).

4.4.13 What are the products of the eNOS NOHA reaction?

Citrulline is a co-product with NO in the NOS reaction. In order to determine whether citrulline was also a product of NOHA metabolism by BH2-eNOS, ESI mass spectrometry was utilised. It was determined from sample standards, in complete assay buffer [ammonium acetate (50 mM) pH 7.6, eNOS (10 nM) CaCl2 (100 μM), BH4 or BH2
Unlike NOHA, eNOS can not produce NOx from hydroxyguanidine. This experiment was performed in complete assay buffer, eNOS (10 pmoles) and either BH$_2$ or BH$_4$ (10μM). Values are means ± SEM where n=4, similar results were observed in 3 other experiments. As a positive control in this experiment eNOS was observed to produce ~ 125 μM NOx from 300 μM NOHA with BH$_4$ as cofactor and 60 μM NOx with BH$_2$ as cofactor.
(10 μM), NADPH (500 μM), L-arginine or NOHA (300 μM), DTT (1 mM) and calmodulin (100 nM)], that dominant peaks could be obtained at 175, 176, and 191 Da, for arginine, citrulline and NOHA, respectively (the mass of the compound +1 for addition of a single proton). The citrulline peak of these standards was much smaller than those observed for Arg and NOHA, indicating relatively poor ionization in the mass spectrometer.

A small peak was observed at 176 Da, for the reaction containing NOHA, BH₂ and eNOS (Fig 4.14A). In contrast NOHA and BH₂ in the absence of eNOS did not create a distinct peak at 176 (Fig 4.14B). Other control samples were analysed by mass spectrometry (Arg, BH₄ and eNOS) which generated a similar 176 Da peak and (Arg, BH₂ and eNOS) which did not have a peak at 176, this latter control provided evidence that the 176 peak was not derived from eNOS per se. Griess assay was used to determine whether NOₓ had been generated in the incubates as anticipated. The peak at 190.0 Da (Fig 4.14A and B) was identified as NOHA and the 175 peak as arginine (derived from NOHA degradation in the MS probe) which might have arisen from NOHA degradation. Incubates which contained BH₂, NOHA and eNOS also had peaks below 170 Da which were not seen in the absence of eNOS or the presence of arginine. Future identification of these peaks utilising MS/MS is anticipated.
Fig 4.14: Electrospray mass spectrometry analysis of products of NOHA catalysis in complete reaction buffer and BH₂ with eNOS (Panel A) and without eNOS (Panel B). Samples were diluted in 50:50:2 (H₂O: methanol: acetic acid) and infused at 0.3 ml/h, cone=20, and average of 8 scans. This represents 1 experiment where n< 10.
To determine whether the 176 Da peak was citrulline, MS/MS was performed using argon as a collision gas. A citrulline standard generated dominant daughter ions of 176 Da (Fig 4.15A) at 159.1, 158.1, 142, 141, 133, 130, 115.9, 115.1, 113.4, 113.2, and 112.7 Da. Below 70 Da fragments from the solvent are prominent and therefore not analysed. In the presence of BH$_2$-eNOS, daughter ions of 176 Da (Fig 4.15B) were found at 158.8, 157.6, 129.8, 116, 115.4, and 113.2. This pattern provided a sufficiently detailed match to that found with citrulline standard to confirm citrulline as a product. Conduction of this same reaction in absence of eNOS (Fig 4.15C) failed to reveal a fragmentation pattern consistent with citrulline. Most notable is the 159.1 and 158.1 peaks which were very prominent with citrulline standard and clearly missing when the reaction was performed in the absence of eNOS.
Fig 4.15: MS/MS of 176 peak A] Citrulline standard, B] NOHA, BH$_2$ and eNOS and C] NOHA, BH$_2$ without eNOS. Instrument parameters as for Fig 4.14 except collision energy =17
4.5 Discussion

Summary of results

1. EPR experiments have taught us that 6MePH$_4$ and BH$_4$ attenuate $O_2^-$ production whilst BH$_2$ and sepiapterin do not.

2. BH$_2$ and sepiapterin are able to reinstate the production of $O_2^-$ from BH$_4$-bound eNOS.

3. While BH$_2$ cannot support NO$_x$ generation from arginine, it can generate NO$_x$ from NOHA but at several-fold reduced rate compared to the reaction supported by BH$_4$.

4. NO$_x$ generation from NOHA by BH$_2$-bound eNOS is CaM-independent and not inhibited by arginine-based NOS inhibitors or the haem binding ligand imidazole.

5. Flavins are necessary for NO$_x$ generation from NOHA by BH$_2$-bound eNOS, indicated by the finding that DPI abolishes both NADPH consumption and NO$_x$ generation.

6. Removal of NADPH from the reaction mixture incompletely attenuates NO$_x$ generation by BH$_2$-bound eNOS.

7. NO$_x$ generation from NOHA is catalysed by eNOS in the absence of any pterin.

8. Both H$_2$O$_2$ and $O_2^-$ produce NO$_x$ from NOHA in the absence of eNOS.

9. SOD but not catalase attenuates NO$_x$ generation by BH$_2$-bound eNOS.

10. Citrulline is an apparent co-product with NO$_x$ in BH$_2$-bound eNOS mediated NOHA metabolism.
EPR studies revealed that BH₄ or 6MePH₄ attenuate O₂⁻ production by eNOS, whether or not arginine is present. On the other hand, dihydropterins, BH₂ and sepiapterin, did not reduce O₂⁻ production. As described in chapter 3, the affinity of 6MePH₄ for eNOS (IC₅₀ = 27.0 ± 23 µM) was 500-fold lower than BH₄ (59.3 ± 19.0 nM) for the inhibition of binding of [³H]BH₄. In contrast, BH₂ and sepiapterin had IC₅₀ values of 67.4 ± 1.1 nM and 2.3 ± 0.5 µM, for inhibition of BH₄ binding to eNOS, respectively. The fully reduced pterin, 6MePH₄, attenuated eNOS O₂⁻ production, although not as potently as BH₄ (predicted from binding affinity). We conclude that high affinity for eNOS is not itself a determining factor for O₂⁻ abolition. Indeed, BH₂ and sepiapterin have intact side chains but oxidised pterin rings, yet neither were able to attenuate eNOS O₂⁻ production. Loss of the biopterin side chain appears to be less important than the redox status of the pterin in the ability of a pterin to attenuate eNOS O₂⁻ formation. Protection against O₂⁻ production (i.e., effective coupling of NADPH consumption to NO synthesis) may be unique to fully reduced pterins.

BH₄ has been previously reported to scavenge O₂⁻ (Heitzer, 2000). In our experiments using DEPMPO to scavenge O₂⁻, BH₄ continuously attenuated O₂⁻ whereas scavenging O₂⁻ would only temporarily deplete O₂⁻ levels, until all BH₄ was oxidised. Recently, a controversial paper was published which suggested that the O₂⁻ might be generated from NOS cofactors rather than NOS itself (Xu, 2000). The findings of this group were criticised by two independent groups citing capture efficiency of the probe and
inadequate controls as reasons for apparent $O_2^-$ generation by NOS cofactors (Mayer, 2000; Vasquez-Vivar, 2000).

The tetrahydropterin, 6MePH$_4$, substitutes for BH$_4$ in supporting catalysis by eNOS (Hevel & Marletta, 1992). Although dihydropterins and fully oxidised pterins bind to NOS and stabilise dimers (Presta et al., 1998), they have never been demonstrated to support NOS catalysis (Hevel & Marletta, 1992; Milstien & Katusic, 1999). BH$_4$ was reported to attenuate uncoupled NADPH oxidation and superoxide production by eNOS via an induced alteration in the haem environment (Kotsonis, 2000; Vasquez-Vivar et al., 1999; Vasquez-Vivar et al., 1998; Wever et al., 1997). Consistent with this view, a cation pterin radical intermediate was suggested, based upon consideration of the electroenvironment within the BH$_4$ binding site of eNOS (Raman et al., 1998). Using rapid freeze-quench EPR, a $^\cdot$BH$_3$ radical was putatively trapped in the iNOS haem domain (Hurshman, 1999). Decay of the Fe$^{II}$OO complex occurs at the same rate as pterin radical formation consistent with a precursor-product relationship. In full length NOS, the $^\cdot$BH$_3$ radical is likely to be reduced to BH$_4$ by NADPH derived electrons from the flavins in the reductase domain (Hurshman, 1999).

In Chapter 3, I described how BH$_2$ elicited the dissociation of prebound BH$_4$ from eNOS. Results of Chapter 4 demonstrate that upon addition of either BH$_2$ or sepiapterin, $O_2^-$ production is initiated in eNOS-BH$_4$ reaction mixtures that were previously not producing $O_2^-$. Observations from the binding studies, taken with the EPR findings,
suggest that dihydropterins effectively displace BH$_4$ from eNOS converting NO production to O$_2^·$ production (Fig. 4.3).

At physiological pH, BH$_4$ is rapidly oxidised in aerobic buffers to qBH$_2$ (Fisher, 1973), which is then further degraded to yield 7,8-dihydrobiopterin (BH$_2$), 7,8-dihydropterin (PH$_2$), and dihydroxanthopterin. BH$_4$ autooxidation has an accelerating time course, which may in part be due to BH$_4$-derived reduced oxygen species and their reaction products such as OONO- (Milstien & Katusic, 1999). Thus, once BH$_4$ oxidation commences, a feed-forward process may ensue that converts NOS to an O$_2^·$ generator, from an NO generator.

In contrast to reports about nNOS and iNOS (Heinzel et al., 1992; Pou et al., 1992), arginine alone did not suppress O$_2^·$ production by eNOS (Vasquez-Vivar et al., 1998). Like nNOS (Miller et al., 1997), production of O$_2^·$ by eNOS is partially under the control of Ca$^{2+}$/CaM, and reduced in the presence of the Ca$^{2+}$-chelator EDTA (Xia et al., 1998). The haem binding ligands cyanide and imidazole were demonstrated to abolish O$_2^·$ production by eNOS (Stroes et al., 1998; Vasquez-Vivar et al., 1998), indicating ferrous dioxygen to be the source of O$_2^·$. The flavins within the reductase domain are necessary for eNOS O$_2^·$ generation, with DPI attenuating O$_2^·$ production by eNOS (Vasquez-Vivar et al., 1998).

In complete reaction buffer containing BH$_4$, eNOS produced 3-fold higher levels of NO$_x$ with NOHA (300 µM) than with Arg (300 µM) as substrate (Fig 4.4). As previously
described, BH₂ (10 μM) was unable to support eNOS production of NO from the substrate Arg. Nonetheless, when NOHA was the substrate, BH₂-eNOS produced significant levels of NOₓ. We set out to characterise the metabolism of NOHA to NOₓ by eNOS, and the pterin requirement for this reaction.

NOHA concentration curves were determined for eNOS in the presence of BH₂ and BH₄ (Fig 4.5). BH₄ was more efficient cofactor for this reaction than BH₂, producing more NOₓ for each concentration of NOHA tested. It is important to note that NOₓ production from NOHA showed a strict dependence on the presence of eNOS. In an experiment where eNOS concentration was progressively increased, in the presence of a fixed concentration of NOHA (300 μM, Fig 4.6), BH₄-bound eNOS produced NOₓ at several-fold faster rate than BH₂-bound eNOS.

In the absence of pterin, eNOS also appears to generate NOₓ (Fig 4.11). However, based upon the high affinity of BH₂ for binding to eNOS, and evidence that BH₄ is readily oxidised to BH₂ (Milstien & Katusic, 1999), performing experiments with BH₂-bound eNOS represents a state of eNOS that is likely to exist in vascular cells exposed to oxidative stresses.

During hydroxylation of Arg, ferrous dioxygen (Fe^{II}-OO) is formed. Formation of Fe^{II}-OO is not completely dependent upon BH₄, but forms 40 times slower in the presence of BH₂ (Wei CC, et al. 2008). Since BH₂ cannot donate an electron to form the pterin
radical, enhanced degradation of Fe$^{II}$–OO is likely to contribute to uncoupled oxidation of NADPH and generation of O$_2^\cdot$.

Interestingly the formation of •BH$_3$ appears to be significantly influenced by the presence of arginine or NOHA; when Arg was present, >80% of BH$_4$ formed •BH$_3$, whereas only 2-8% formed •BH$_3$ when NOHA was present (Hurshman, 1999). If NOHA prevents the formation of the pterin radical, it might lead to the increased production of O$_2^\cdot$. However in the NOS catalysis of Arg, •BH$_3$ is likely to be formed prior to NOHA.

NMA and nitroarginine (Narg) are potent inhibitors of NOS, both are arginine analogues that occupy the Arg binding site of NOS (Gross et al., 1990). These inhibitors caused a rightward shift in the BH$_4$-bound eNOS NOHA concentration-response curve, indicating that NOHA interacts with BH$_4$-bound eNOS at the Arg site (Fig 4.8A). NOHA has previously been reported to cause vasorelaxation of bovine pulmonary artery in a manner which was inhibitable by NMA, N-Arg and aminoarginine (Wallace, 1991). In addition, LPS-treated macrophages produce increased levels of NO following NOHA administration, and this NO synthesis is blocked by NMA (Vetrovsky et al., 1997).

From analyses of iNOS oxygenase (iNOSox) domain complexed with NOHA, Arg or citrulline, it was found that NOHA and Arg bind in the same conformation, adjacent to haem iron, but without direct interaction with either the iron or BH$_4$ (Crane, 2000). The major difference between NOHA and Arg, when bound to iNOS, was found to be that the NOHA hydroxyl extended into the base of the haem pocket (Crane, 2000). There is also a
significant difference in haem iron redox potential, when arginine or NOHA is bound to NOS (Hurshman, 1999).

While NMA and Narg inhibited NOx generation by BH4-eNOS, these NOS inhibitors potentiated NOx generation by BH2-eNOS. This finding indicates that NOHA is not interacting at the Arg binding site in the reaction catalysed by BH2-eNOS (Fig 4.8B). L-NOHA in bovine endothelial cells potentiates the relaxation due to NO elicited by ADP and bradykinin and releases a vasodilator which was not inhibited by Narg or NMA (Zembowicz et al., 1991). If NOHA is not interacting at the Arg-binding site on NOS, the only method by which naturally occurring methylarginines could only regulate NOHA oxidation by BH2-eNOS would be through competition of the Y+ transporter (Azuma, 1995; Bogle, 1995).

In the conversion of Arg to Cit and NO, it is well established that eNOS requires both calcium and calmodulin (Fig. 4.7). However, when eNOS was incubated with BH4 and NOHA in the absence of calmodulin, there were still significant levels of NOx produced (40% of that with calmodulin, Fig 4.7 A). In contrast to the reaction catalysed by BH4-bound eNOS, BH2-bound eNOS catabolism of NOHA was inhibited only 20% in the absence of CaM (Fig 4.7B). The formation of O2•− by eNOS, is only partially dependent upon Ca2+/CaM (Xia et al., 1998), and it was considered that the fall in NOx generation observed in the absence of Ca2+/CaM is attributable to reduced O2•− production, support for this idea was obtained as described below.
Formation of $\text{H}_2\text{O}_2$ by nNOS was also shown to be dependent upon $\text{Ca}^{2+}/\text{CaM}$ (Heinzel et al., 1992). This latter finding is rationalised by a requirement for $\text{Ca}^{2+}/\text{CaM}$ to form $\text{Fe}^{2+}$-OO, the apparent precursor of $\text{H}_2\text{O}_2$ (Abu-Soud & Stuehr, 1993).

In the normal NOS reaction (i.e. in the presence of $\text{BH}_4$), conversion of arginine to citrulline and NO requires 1.5 mol NADPH overall. One mol of NADPH is required for Arg oxidation to NOHA and 0.5 mol of NADPH are needed for NOHA oxidation to NO and citrulline. In this process, electrons flux from NADPH through FAD and FMN in the reductase domain, to haem in the oxygenase domain; this reaction is comparable to the cytochrome P450 reductase/monooxygenase system. Using CO as an inhibitor, haem was determined to be essential for both steps in the NO reaction (Marletta, 1993). Haem ligands, cyanide and imidazole, have also been demonstrated to inhibit $\text{O}_2^-$ production by eNOS (Xia et al., 1998). In our experiments, concentrations of imidazole as high as 10 mM elicited no effect on either the $\text{BH}_4$- or $\text{BH}_2$- bound eNOS conversion of NOHA to NO$_x$ (Fig 4.10). Imidazole also failed to inhibit NADPH consumption by $\text{BH}_2$-bound or $\text{BH}_4$-bound eNOS when NOHA was provided as substrate. It is notable that Narg, which interacts with both haem (Cubberley et al., 1997) and Arg binding sites on NOS, also had little ability to inhibit NO$_x$ generation from NOHA. These findings suggest that the flavins, rather than haem, may be the critical sites allowing NOHA oxidation by $\text{BH}_2$- eNOS.

Electron flux through FAD and FMN, in the reductase domain, is essential for NOS mediated NO production. DPI is a potent and irreversible inhibitor of flavoproteins; it has
been shown to bind covalently to the NOS protein preventing NADPH-utilisation, by inhibiting FAD and FMN function in NOS catalysis (Stuehr et al., 1991). Using chemiluminescence, DPI was demonstrated to abolish the generation of $O_2^\cdot$ by eNOS (Wever et al., 1997). When added to BH$_4^-$ or BH$_2^-$ bound eNOS, DPI elicited a concentration dependent inhibition of both NADPH consumption (Fig 4.11 A) and NO$_x$ production (Fig 4.11B). This finding underlines the importance of the flavins in the oxidation of NOHA, DPI also prevented NADPH consumption in both the BH$_2^-$ and BH$_4^-$ bound eNOS reactions. Uncoupled oxidation requires that electrons are fluxed from NADPH, DPI is likely to be preventing the transfer of electrons to the eNOS flavins, therefore preventing $O_2^\cdot$ generation. We suggest that $O_2^\cdot$ is responsible for eNOS-BH$_2$ production of NO$_x$ from NOHA and provide additional evidence below that it is because of flavin dependent $O_2^\cdot$ generation that NO$_x$ can be measured from NOHA.

Our elucidation of the electron flux involved in the NMA non-inhibitable reaction I have described is far from complete. It appears that the haem domain is not essential for the production of NO$_x$ from NOHA while the flavins appear to be vital for NOHA oxidation since DPI abolished all measurable NADPH consumption and NO$_x$ generation.

I measured NO$_x$ in the absence of NADPH (Fig 4.11) however NOS electrons must be fluxed in order for DPI dependent FAD/FMN inhibition. Marletta's group found that nNOS could catalyse an NADPH independent reaction between H$_2$O$_2$ and NOHA which resulted in the formation of citrulline, N-$\delta$-cyanoornithine and either nitrite or nitrate as
products (Clague et al., 1997). The rate for this nNOS catalysed reaction was found to be 10 fold slower that the production of NO catalysed by NADPH/O₂ and hydroxyarginine.

We measured NOₓ after H₂O₂ and xanthine/xanthine oxidase (O₂⁻ generating system) were incubated with NOHA, we found that both reagents could generate NOₓ. Marletta previously reported that H₂O₂ could form citrulline from NOHA but not NOₓ. We incubated BH₂-bound eNOS in complete reaction buffer with catalase and with SOD. The BH₂-bound NOₓ production from NOHA was decreased in the presence of SOD (Fig. 4.12), suggesting that O₂⁻ itself, or a derived species, is involved in the oxidation of NOHA. The generation of NOₓ was not attenuated in the presence of catalase, arguing against the possibility that H₂O₂ is involved in this process.

NO is produced from the guanidinium nitrogen of arginine (McMillan et al., 1992). While NOS has high substrate specificity, there are reports of NOS producing NO from other compounds: L-homoarginine; L-hydroxyhomoarginine as well as NOHA (Abu-Soud et al., 1999). Additionally it was reported that iNOS can convert N-(4-chlorophenyl)-N'hydroxyguanidines to NO and urea in a BH₄ dependent manner (Renodon-Corniere et al., 1999). Having established that eNOS could convert the intermediate NOHA to NOₓ, we were curious as to whether it could also produce NOₓ from other substrates containing hydroxyguanidino groups. Hydroxyguanididine, a compound that differs from NOHA by having only the functional group devoid of the carbon backbone and α-carboxy and α-amino groups of Arg, was incubated with BH₄ and BH₂-bound eNOS and NOₓ formation.
was assessed. Neither the presence of BH\textsubscript{2} nor BH\textsubscript{4} endowed eNOS with the capacity to produce NO\textsubscript{x} from hydroxyguanidine (Fig. 4.13).

The crystal structure of the iNOS oxygenase domain with NOHA, citrulline and Arg has been elucidated and the conservation between citrulline, NOHA and Arg in the binding site of NOS was remarkable (Crane, 2000). If catalysis of NOHA was dependent upon the ability of hydroxyguanididine to interact at the active site, the absence of an amino acid backbone would effect the binding affinity for the active site. Since NOHA catalysis occurs in the presence of the Arg-based inhibitors and haem ligands it is likely that the oxidation is not at the active site but rather in solution or within the reductase domain.

Cytochrome P450 has been reported to catalyse the formation of urea from hydroxyguanidine in a reaction which involves H\textsubscript{2}O\textsubscript{2} and O\textsuperscript{2+} (Clement, 1993). An engineered cavity of haem peroxidase was also reported to oxidise hydroxyguanidine (Hirst, 2000).

Measurement of NO\textsubscript{x} is a standard technique for the measurement of NO production but products other than NO such as NO- might be formed; formation of NO- would require an additional electron, and degradation of NO- would generate N\textsubscript{2}O (Fukuto \textit{et al.}, 1993). Without the results of NO electrode analysis NO cannot be proved to be the product of the eNOS catalysed NOHA reaction. NOS might catalyse the NOHA reaction to form HNO-, nitrosylarginine or cyanoornithine as potential products in addition to, or instead of, NO and citrulline, in a manner similar to that for haem peroxidase (Hirst, 2000).
Technically, any tetravalent iron (Fe^{IV}) can potentially add electrons or protons or alter the oxygen of a substrate, however the structure of NOS active site controls the reactions at the haem moiety and restricts product formation (Hirst, 2000).

In order to identify additional products of the reaction we used electrospray mass spectrometry (ESI). Reactions were carried out in complete reaction buffer using the volatile buffer ammonium acetate pH 7.6 to enable direct injection of samples into the mass spectrometer without complex extractions. Although the reaction mixture was a heterogeneous mix, we were able to detect differences between the samples containing BH$_2$ and Arg, compared to BH$_2$ and NOHA. In the presence of BH$_2$-eNOS and NOHA a small peak at 176 Da (citrulline$^-$) was observed which was in much lower abundance in the absence of eNOS (Fig 4.14). Also, eNOS reduced the intensity of a 190.9 Da peak (NOHA$^-$). MS/MS of this 176 Da peak, associated with BH$_2$-eNOS revealed a fragmentation pattern that largely matched that of citrulline standard. In contrast, peaks arising from citrulline fragmentation could not be observed when 176 Da ions were introduced into the collision cell from the identical reaction, but without eNOS (Fig 4.15). Thus ESI MS/MS results support the view that citrulline is a product of the BH$_2$-eNOS –mediated metabolism of NOHA.

Amino acids do not always ionize with mass spectrometry, as evidenced by the poor peak at 176 Da observed for citrulline. Analyses are also complicated by multiple charge states of products and formation of adduct ions with salts in the reaction buffer. We
were unable to detect hydroxylamine (HNO) or cyanoornithine, candidate products suggested in the literature as NOHA reaction products from eNOS catalysed reactions (Clague et al., 1997; Rusche et al., 1998). There were many other peaks that may derive from NOHA which appeared to differ between reactions conducted with and without eNOS. MS/MS analysis of these peaks may reveal other products of BH₄-eNOS mediated catalysis of NOHA.

NOHA is unequivocally the intermediate product in the reaction catalysed by NOS (Abu-Soud et al., 1997; Boucher et al., 1994; Campos et al., 1995). Horseradish peroxidase and cytochrome P450 can also convert NOHA to citrulline and nitrite utilising H₂O₂, this reaction is abolished by haem binding ligands cyanide (HRP) and miconazole (Cytochrome P450) (Boucher, 1992; Boucher et al., 1992). Marletta’s group found that a reaction between H₂O₂ and NOHA formed citrulline, N-deltacyanornathine and either nitrite or nitrate from nNOS (Clague et al., 1997). Other researchers reported that NADPH oxidase-derived O₂⁻ could convert NOHA to NO during the respiratory burst of phagocytic cells (Modolell et al., 1997). H₂O₂ is produced from NOS and NADPH oxidase (Nunoshiba et al., 1993; Tsai et al., 1998) and H₂O₂ has been proposed as an intermediate that can be released from NOS haem following BH₄ reduction of Fe²⁺OO (Benson, 1997).

The formation of H₂O₂ is diminished in the absence of arginine (Abu-Soud et al., 1997; Hurshman, 1999). Whereas NOHA promotes the formation of various nNOS iron haem states, including Fe³⁺OO and Fe³⁺NO, Arg only promotes formation of Fe²⁺OO
Fe^{II}OO nNOS has been reported to react stoichiometrically with NOHA to form NO, while the NO produced from Arg-bound nNOS was substoichiometric (Boggs, 2000). Therefore in the presence of BH$_4$, NOHA catalysis is very efficient.

NOHA can be hydrolysed directly to form NO or indirectly via hydroxylamine in the presence of O$_2^-$ (Vetrovsky et al., 1996). It was previously speculated that O$_2^-$ might provide eNOS with an additional mechanism to oxidise NOHA to form NO (Everett et al., 1996). The reaction of NOHA with O$_2^-$ to form NO is predictably pH dependent (NOHA, pK$_A$ = 8), since the hydroxyguanidine group is more reactive when it is deprotonated (Everett et al., 1996). The degradation of NOHA by O$_2^-$ has been measured at 200 – 500 M$^{-1}$s$^{-1}$, a rate too low to compete with NO or SOD reactions under normal physiological conditions.

Physiological levels of arginine compounds have been measured in plasma by HPLC and found to be: NOHA (9.1 µM), Arg (91µM), monomethylarginine (NMA, 0.1 µM) and dimethylarginine (NDA, 0.4 µM) (Meyer, 1997). In patients with rheumatoid arthritis and systemic lupus erythematosus, levels of serum hydroxyarginine are reported to be increased (Wigand et al., 1997). Thus, levels of circulating NOHA may be sufficient to serve as substrate for BH$_2$-eNOS, particularly under pathophysiological conditions where oxidative stress is prominent.

In chapter 3 I reported that BH$_2$ and BH$_4$ had virtually the same affinity for binding to eNOS. In EPR experiments it was revealed that BH$_4$ prevents eNOS derived O$_2^-$
production but BH₄ can be displaced by BH₂ triggering O₂⁻ generation. Numerous recent reports have demonstrated that addition of BH₄ to hypertensive atherosclerotic and hypercholesterolemic animals acutely restores NO production and improves vasodilatation (Heitzer, 2000; Stroes et al., 1997; Tiefenbacher et al., 1996; Van Amsterdam & Werner, 1992; Verma et al., 1998). Endothelial dysfunction is a complex problem and addition of BH₄ to redress pterin depletion acutely might indirectly exasperate the situation long-term.

Under conditions of oxidative stress, involving oxLDL or advanced glycosylation end-products, exogenously administered BH₄ can become oxidised to qBH₂ or BH₂. Thus, feed-forward endothelial dysfunction may ensue from an initial triggering event in which BH₄ becomes oxidised to BH₂. This situation would foster BH₂-eNOS generated O₂⁻ production, ONOO⁻ generation via the reaction between NO and O₂⁻, and consequently further oxidation of BH₄ (Milstien & Katusic, 1999).

Although BH₂-eNOS cannot produce NO from Arg, our findings show that this dysfunctional enzyme can oxidise NOHA to form NOₓ and citrulline. Accordingly, we envision that under conditions of oxidative stress, administration of NOHA could conceivably generate NO and restore vasodilatation to otherwise inappropriately constricted blood vessels. This possibility awaits future in vivo testing.

In this chapter, we demonstrated that addition of NOHA to BH₂-bound eNOS enables the formation of NOₓ. The reaction does not appear to occur at the eNOS Arg site, since neither NMA nor Narg could prevent NOₓ formation, nor does the reaction appear to
rely completely upon $\text{Ca}^{2+}/\text{CaM}$. The haem binding ligand imidazole did not inhibit the $\text{NO}_x$ generation, but the flavoprotein inhibitor DPI abolished NOHA metabolism. We showed that $\text{NO}_x$ can be produced from chemically generated $\text{H}_2\text{O}_2$ and $\text{O}_2^\cdot$, and that $\text{NO}_x$ derived from BH$_2$-eNOS metabolism of NOHA could be diminished by SOD, but not catalase. Taken together, these results implicate eNOS derived $\text{O}_2^\cdot$ as a key mediator of $\text{NO}_x$ formation from NOHA by BH$_2$-eNOS. We conclude that eNOS can metabolise NOHA by two distinct reaction mechanisms:

1) In the presence of BH$_4$, NOHA binds to the active site of eNOS and is converted to NO and citrulline via an NMA inhibitable haem-dependent reaction.

2) In the presence of BH$_2$, $\text{O}_2^\cdot$ derived from eNOS can elicit the oxidation of NOHA to $\text{NO}_x$ and citrulline in a haem-independent reaction that depends upon electron flux through flavins. The site of $\text{O}_2^\cdot$ formation would appear to be reductase domain flavins (FAD and/or FMN), rather than the oxygenase domain haem.

4.6 Future work

Further work is needed to ascertain whether $\text{NO}_x$ is formed from NO, or an NO related intermediate, such as HNO (NO-). Additional experiments using MS/MS ESI are required to determine whether products, other than citrulline, may derive from this reaction.
Chapter 5 - SUMMARY AND FUTURE DIRECTIONS

GTPCH GENE TRANSCRIPTION

At the start of this study we knew that GTPCH is the rate-limiting enzyme for de novo synthesis of BH₄. Although an array of immunostimulants and growth factors had been implicated in upregulating GTPCH activity prior to our initiation of these studies, the transcriptional regulation of GTPCH was unknown. Utilising 5’-upstream GTPCH gene sequence in SEAP reporter vectors, effects of a panel of immunostimulants, growth factors and hormones were examined upon these GTPCH promoter-reporter vectors stably transfected in PC-12 cells and rat aortic smooth muscle cells.

I found that analogues of cyclic adenosine monophosphate and the immunostimulants IL-1 and LPS enhance GTPCH promoter driven-reporter gene expression. Synergistic GTPCH gene upregulation was observed with IFN-γ and TNF-α when given in combination with LPS or IL-1, but not alone, and enhanced cell secretion of NO derived products (NOₓ) was also observed with these immunostimulants. These factors have been previously implicated in upregulating iNOS gene expression; while it had been known previously that iNOS and GTPCH could be co-induced, we can now state unequivocally that GTPCH induction is associated with an increase in GTPCH gene transcription.
In contrast to reports about iNOS gene transcription, cyclohexamide stimulated GTPCH promoter activity. The mechanistic basis for this phenomenon needs to be specified. Dexamethasone in PC-12 cells, but not RASM cells, enhanced GTPCH promoter driven-reporter SEAP expression. This latter observation indicates that regulation of the GTPCH gene may be cell type specific. The basis for cell-specific regulation of the GTPCH gene awaits identification.

While I have identified some of the factors which can elicit a response from the GTPCH promoter and elevate intracellular biopterin levels, the mechanisms of action await elucidation. It will be important to determine the signal transduction pathways through which the cognate transcription factors act, as well as the key DNA sequences that are germane to transcriptional control. Indeed, we have identified multiple putative transcription factor consensus sites within the 5’-upstream GTPCH gene sequence; mutagenesis followed by DNA footprinting, promoter-reporter gene assays and gel shift/supershift assays will be used in future to assess the relevance of these sites to modulation of GTPCH gene regulation. We have hypothesised that NFkB is involved in the LPS and IL-1 upregulation of GTPCH. Functional analysis and mutagenesis of the 13 putative NFkB sites which we have identified may be necessary to characterise the potential role of NFkB in the regulation of GTPCH gene transcription.
PTERIN BINDING TO eNOS

We have characterised the binding of [3H]BH₄ to eNOS and determined the effects of the substrate, arginine, on BH₄ binding. In addition, we have looked at selected tetrahydropterins and dihydropterins to determine which modifications of BH₄ alter the binding affinity to eNOS. During this research, NOS oxygenase domain crystal structures have been determined and further work using pterin analogues as probes for interactions with eNOS have established the nature of the interactions at the molecular level. Nonetheless, the static information provided by X-ray diffraction fails to illuminate the sequence or importance of specific molecular interactions or the basis for negative co-operativity in pterin binding. Resolution of these important issues awaits further experimentation.

The competition binding assays I have described were in the absence of arginine, and under these conditions it was clear that BH₂ and BH₄ had equal affinity for binding eNOS. It is important to re-examine the competition binding in the presence of arginine to gain insight into whether arginine reverses the apparent negative co-operativity in pterin binding to eNOS and, if so, how.

PTERIN CONTROL OF eNOS CATALYTIC ACTIVITIES

The generation of superoxide by eNOS contributes to oxidative stress in the endothelium. Here we have demonstrated that tetrahydropterins, but not dihydropterins, can attenuate superoxide production from eNOS. Uncoupled oxidation of NOS isoforms
leads to $O_2^\cdot$ generation; this dysregulation of eNOS may be a key pathogenetic mechanism for endothelial dysfunction that has been implicated in the disease states of atherosclerosis, diabetes and hypercholesteremia.

Combined observations, from pterin binding studies and electron paramagnetic resonance detection of superoxide, indicate that dihydropterins displace BH$_4$ from eNOS and trigger superoxide production. The dihydropterin, BH$_2$, cannot support NO generation from the NOS substrate arginine, but can generate NO$_x$ from the reaction intermediate hydroxyarginine. We have characterised this reaction and suggest a plausible mechanism for NO$_x$ generation that involves $O_2^\cdot$ as an intermediate.

Further work is needed to identify by electrospray mass spectrometry what other products may be formed with citrulline, potentially $\delta$-cyanoornithine. In addition, it is essential to determine whether the NO$_x$ product generated by BH$_2$-eNOS derives from NO itself, or a related species. Towards this end, it will be important to ascertain whether BH$_2$-eNOS makes a product that can mimic the bioactivity of NO (i.e. vasodilation, inhibition of platelet aggregation and adhesion, activation of soluble guanylate cyclase). If NO bioactivity is affirmed, NOHA might then be administered to individuals with dysfunctional endothelium to reinstate vasodilation by BH$_2$-eNOS in oxidatively-stressed endothelial cells. This is a potential therapeutic approach with perceived advantages over treating patients with endogenous BH$_4$. 

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Chapter 6 - REFERENCES


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