## REVIEW ARTICLE

# Nitric oxide synthases: structure, function and inhibition

Wendy K. ALDERTON*, Chris E. COOPER† and Richard G. KNOWLES*[1]

*In Vitro Pharmacology Department, GlaxoSmithKline Research and Development, Medicines Research Centre, Gunnels Wood Road, Stevenage SG1 2NY, U.K., and †Department of Biological Sciences, Central Campus, University of Essex, Wivenhoe Park, Colchester CO4 3SQ, U.K.

This review concentrates on advances in nitric oxide synthase (NOS) structure, function and inhibition made in the last seven years, during which time substantial advances have been made in our understanding of this enzyme family. There is now information on the enzyme structure at all levels from primary (amino acid sequence) to quaternary (dimerization, association with other proteins) structure. The crystal structures of the oxygenase domains of inducible NOS (iNOS) and vascular endothelial NOS (eNOS) allow us to interpret other information in the context of this important part of the enzyme, with its binding sites for iron protoporphyrin IX (haem), biopterin, L-arginine, and the many inhibitors which interact with them. The exact nature of the NOS reaction, its mechanism and its products continue to be sources of controversy. The role of the biopterin cofactor is now becoming clearer, with emerging data implicating one-electron redox cycling as well as the multiple allosteric effects on enzyme activity. Regulation of the NOSs has been described at all levels from gene transcription to covalent modification and allosteric regulation of the enzyme itself. A wide range of NOS inhibitors have been discussed, interacting with the enzyme in diverse ways in terms of site and mechanism of inhibition, time-dependence and selectivity for individual isoforms, although there are many pitfalls and misunderstandings of these aspects. Highly selective inhibitors of iNOS versus eNOS and neuronal NOS have been identified and some of these have potential in the treatment of a range of inflammatory and other conditions in which iNOS has been implicated.

Key words: cofactor, free radical, haem enzyme, inhibitor, pterin.

## INTRODUCTION

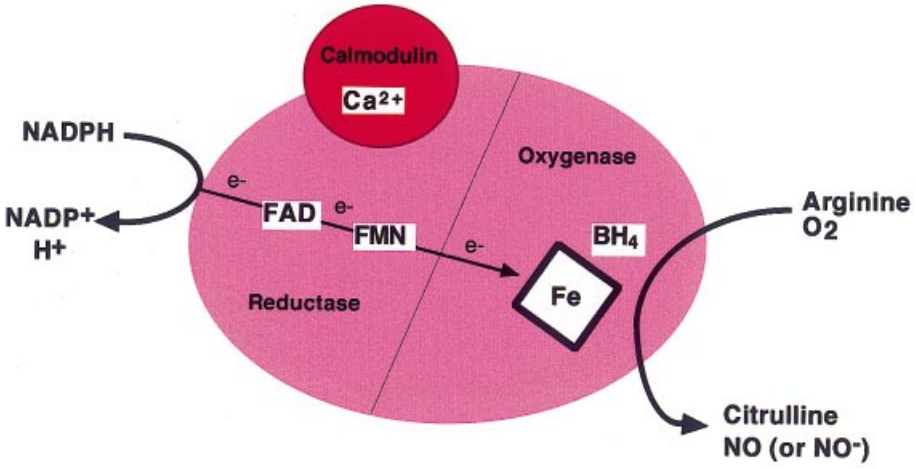
In 2001 it is timely to review what is now known about the nitric oxide synthases (NOSs, EC 1.14.13.39) for several reasons. Firstly, the NOSs were first identified and described in 1989, the three major isoforms were cloned and purified between 1991 and 1994, and in 1994 there were several reviews of the work on these enzymes to that time; in the 7 years since then the field of research has matured, with something over 16000 papers being published on NOS, including a great deal on the structure, function and inhibition of the enzymes. Second, as can be seen from a search on Medline, the rate of publication on nitric oxide synthase appears to be approaching a plateau at approx. 2800 papers per year. Third, the first X-ray crystal structures of NOS domains have been presented and published in 1998 and 1999, allowing us to put together information, such as that obtained by site-directed mutagenesis, as well as the large amount of indirect and functional information, into the context of the actual 3-dimensional structure. Fourth, the importance of the field of nitric oxide research was recognised in 1998 by the award of the Nobel Prize to R. Furchgott, L. Ignarro and F. Murad for the work that led to the discovery of NO as a biological mediator produced by mammalian cells. Finally, in 1999 it was reported that the most highly cited U.K. biomedical scientist of that decade was S. Moncada, because of the seminal work of his group on NO [1].

The known NOS enzymes are usually referred to as 'dimeric' in their active form, ignoring the required calmodulins (CaMs) which, strictly speaking, mean they are tetramers (of two NOS monomers associated with two CaMs). They contain relatively tightly-bound cofactors (6*R*)-5,6,7,8-tetrahydrobiopterin ($BH_4$), FAD, FMN and iron protoporphyrin IX (haem) and probably (although see below) catalyse a reaction of L-arginine, NADPH, and oxygen to the free radical NO, citrulline and NADP [2–4] (Scheme 1). Three quite distinct isoforms of NOS have been identified, products of different genes, with different localization, regulation, catalytic properties and inhibitor sensitivity, and with 51–57% homology between the human isoforms. These isoforms will be referred to by the most common nomenclature: nNOS (also known as Type I, NOS-I and NOS-1) being the isoform first found (and predominating) in neuronal tissue, iNOS (also known as Type II, NOS-II and NOS-2) being the isoform which is inducible in a wide range of cells and tissues and eNOS (also known as Type III, NOS-III and NOS-3) being the isoform first found in vascular endothelial cells. These isoforms have in the past been also differentiated on the basis of their constitutive (eNOS and nNOS) versus inducible (iNOS) expression, and their calcium-dependence (eNOS and nNOS) or -independence (iNOS), but as discussed below, such things now appear to be by no means that straightforward! As also detailed below, splice variants of these isoforms are starting to be identified, and it is not yet certain that other gene products will not be found, extending the family of NOSs further.

This review is intended to cover the advances which have occurred in the last 7 years, building on what was known and reviewed in 1994 [2–4] and in particular on the review in this

Abbreviations used: NOS, nitric oxide synthase; iNOS, inducible NOS isoform; eNOS, endothelial NOS isoform; nNOS, neuronal NOS isoform; $NO^-$, nitroxyl anion; $BH_4$, (6*R*)-5,6,7,8-tetrahydrobiopterin; NHA, $N^{\omega}$-hydroxy-L-arginine; haem, iron protoporphyrin IX; RNS, reactive nitrogen species; SOD, superoxide dismutase; L-NMMA, $N^G$-monomethyl-L-arginine; CaM, calmodulin; PIN, protein inhibitor of NOS; Hsp90, heat-shock protein 90; PSD-95 and -93, post synaptic density proteins 95 and 93; PDZ, **P**SD-95 **d**iscs large/**Z**O-1 homology; NMDA, *N*-methyl-D-aspartate; L-NNA, $N^G$-nitro-L-arginine; 7-NI, 7-nitroindazole; L-NIO, $N^5$-iminoethyl-L-ornithine; L-NIL, $N^6$-iminoethyl-L-lysine; 1400W, *N*-[3-(aminomethyl)benzyl]acetamidine; ARL 17477, *N*-[4-(2-{[(3-chlorophenyl)methyl]amino}ethyl)phenyl]-2-thiophenecarboximide dihydrochloride; GW273629, *S*-[2-[(1-iminoethyl)amino]ethyl]-4,4-dioxo-L-cysteine; GW274150, *S*-[2-[(1-iminoethyl)amino]ethyl]-L-homocysteine.

[1] To whom correspondence should be addressed (e-mail rgk30535@gsk.com).




**Scheme 1 Overall reaction catalysed and cofactors of NOS**

Electrons ($e^-$) are donated by NADPH to the reductase domain of the enzyme and proceed via FAD and FMN redox carriers to the oxygenase domain. There they interact with the haem iron and $BH_4$ at the active site to catalyse the reaction of oxygen with L-arginine, generating citrulline and NO as products. In some circumstances (see text) $NO^-$ may be a product instead of NO. Electron flow through the reductase domain requires the presence of bound $Ca^{2+}$/CaM.

journal [2]. It will address the structure, function and inhibition of the NOSs. It concentrates on the mammalian, and wherever possible the human NOSs (although brief mention will be made of NOSs in non-mammalian species); wherever there are differences between isoforms or between species these will be highlighted. It will not address detailed methodologies [5–8], the physiological and pathophysiological roles of the NOSs (the reader is directed to some excellent recent reviews on this area [9–12]), nor will it discuss other enzymes which may cause release of NO other than from L-arginine, e.g. enzymes acting on NO donors (e.g. *S*-nitrosoglutathione and nitroglycerine, reviewed in [13]), bacterial nitrite reductases [14,15] or xanthine oxidase [16]. We apologise in advance for any omissions we may inadvertently make; with approx. 50 papers per week mentioning NOS in their title or abstract, 100 % coverage would take most of our working lives!

## STRUCTURE

The three distinct genes for the human neuronal, inducible and endothelial NOS isoforms exist, with a single copy of each in the haploid human genome [17–23] (Table 1). The NOS genes have a similar genomic structure, suggesting a common ancestral NOS gene. NOSs exhibit a bidomain structure, shown in Figure 1, in which an N-terminal oxygenase domain containing binding sites for haem, $BH_4$ and L-arginine is linked by a CaM-recognition site to a C-terminal reductase domain that contains binding sites for FAD, FMN and NADPH [24–26]. The oxygenase and reductase domains have been defined by limited proteolysis [27,28] permitting the high-level expression in a variety of expression systems, permitting structural and biophysical studies of the NOS domains. The separate domains are catalytically active and reconstitution of the second step of NO synthesis has been achieved by combining the reductase and oxygenase domains of human eNOS and murine iNOS [29,30].

### NOS oxygenase domain structure

The last four years have seen significant advances in structural studies of the NOS isoforms, with crystal structures being solved for truncated oxygenase domains of murine iNOS [31,32], human iNOS [33,34], bovine eNOS [35] and human eNOS [35] with a variety of ligands bound. A structure of the nNOS oxygenase domain has not been published to date. The human iNOS oxygenase domain (amino acids 82–508) is an elongated shape with a novel $\alpha$–$\beta$ fold described as resembling a baseball catcher's mitt with the haem group in the palm of the mitt [31]. The NOS distal pocket, primarily constructed from $\beta$-sheet structure differs considerably from the distal pockets of other haem-based oxygenases such as cytochrome P450s, and peroxidases and catalases, which are all largely $\alpha$-helical. The two enzyme families appear to have achieved similar catalytic activity through convergent evolution.

In the human iNOS oxygenase domain the haem iron is pentacoordinate and axially co-ordinated to the proximal $Cys^{200}$, consistent with site-directed mutagenesis studies [36]. The haem is buried in the protein interior and makes extensive van der

**Table 1 Genes for human NOS isoforms**

| Human NOS isoform | Gene structure and size | Chromosomal location | Number of amino acids (aa) in predominant form, protein size | Genbank accession numbers | References |
|---|---|---|---|---|---|
| nNOS (NOS-1) | 29 exons, 28 introns, complex structural organization, locus over region of > 200 kbp | 12q24.2–12q24.3 of chromosome 12 | 1434 aa, 161 kDa | L02881, U11422 | [17,18] |
| iNOS (NOS-2) | 26 exons, 25 introns, 37 kbp | 17cen–q11.2 of chromosome 17 | 1153 aa, 131 kDa | L09210, L24553, X73029 | [19–21] |
| eNOS (NOS-3) | 26 exons, 25 introns, 21–22 kbp | 7q35–7q36 of chromosome 7 | 1203 aa, 133 kDa | M93718, M95296 | [22,23] |

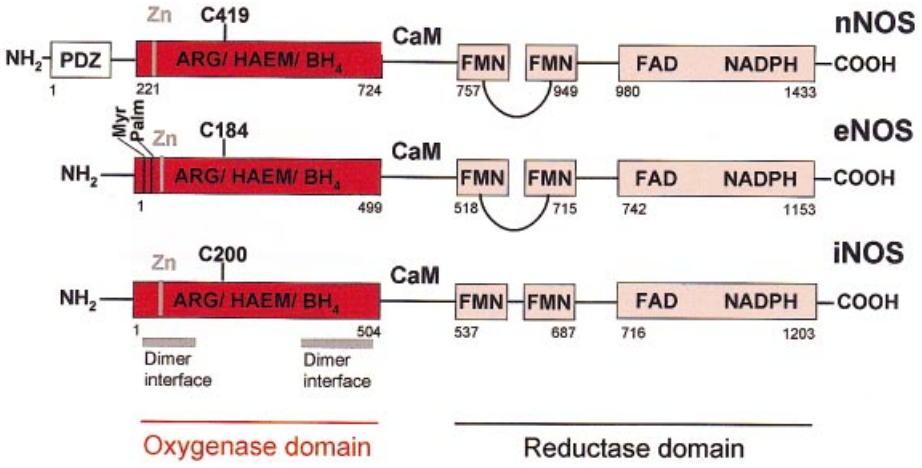



**Figure 1 Domain structure of human nNOS, eNOS and iNOS**

Oxygenase, reductase and PDZ domains are denoted by solid boxes and the amino acid residue number at the start/end of each domain is shown. The cysteine residue which ligates the haem and the CaM-binding site is indicated for each isoform, myristoylation (Myr) and palmitoylation (Palm) sites on eNOS are shown, as is the location of the zinc-ligating cysteines (Zn in grey). The autoinhibitory loop within the FMN regions of nNOS and eNOS are also shown and grey bars indicate the dimer interface in the oxygenase domain.

Waals interactions with hydrophobic and aliphatic side chains. Two aromatic residues, $Trp^{188}$ and $Phe^{363}$ (of murine iNOS, corresponding to $Trp^{194}$ and $Phe^{369}$ in human iNOS), sandwich the porphyrin ring (Figure 2). Dimerization creates an $\approx 30$ Å deep, funnel-shaped active-centre channel formed primarily from the residues of one subunit. The larger cavity opening is $\approx 10$ Å $\times 15$ Å in cross section, large enough to allow diffusion of both arginine and citrulline and is near the dimerization interface. Arginine binds with the side-chain terminus fitted tightly into the narrow part of this active-site cavity and with the guanidino group lying coplanar to the haem. In the structure of the murine iNOS oxygenase domain (amino acid residues 66–498) the L-arginine guanidinium group makes two hydrogen bonds to both carboxy oxygens of $Glu^{371}$ (corresponding to $Glu^{377}$ in human iNOS) localizing the substrate over the haem, and one hydrogen bond to the carbonyl of $Trp^{366}$ [32]. $Glu^{371}$ has been found to be critical for substrate binding to murine iNOS [37,38]. Additional hydrogen bonds are formed between the L-arginine carboxy group and $Tyr^{367}$ and the carboxy of $Asp^{376}$, which is replaced by an Asn in eNOS. The binding of $N^{\omega}$-hydroxy-L-arginine (NHA) to murine iNOS oxygenase domain shows an identical hydrogen-bonding pattern and orientation to arginine [39]. Located near the dimer interface, but buried within the protein and removed from bulk solvent is the $BH_4$ cavity. The primary interaction is the parallel stacking of indole aromatic rings of human eNOS $Trp^{447}$ (human iNOS $Trp^{463}$; Figure 2) and the $BH_4$ pterin, and in addition a number of hydrogen-bonding interactions occur between heteroatoms of the pterin and in human eNOS $Arg^{381}$ and $Ile^{462}$, and $Arg^{365}$, $His^{461}$ and $Ala^{446}$ in human iNOS. However, mutational analysis of these residues in iNOS showed that preserving residue identities or aromatic character is not essential for $BH_4$ binding or activity [40].

An unexpected finding in the crystal structure was a zinc tetrathiolate centre located at the bottom of the dimer interface, since the presence of zinc in NOS had not previously been detected. The zinc iron is tetrahedrally coordinated to two cysteines ($Cys^{110}$ and $Cys^{115}$) from each subunit. The Cys-($Xaa_4$)-Cys motif is strictly conserved across all NOS sequences known to date, suggesting that the zinc centre is expected to be a common feature of all NOSs [35]. Site-directed mutagenesis has shown that iNOS $Cys^{115}$ and the corresponding residue in eNOS, $Cys^{99}$, are essential for dimer stability [41]. Furthermore, a comparison between the zinc-free and zinc-bound human iNOS oxygenase domain structures demonstrated a net gain of eight hydrogen bonds with zinc binding, which would favour dimer stabilization [34]. The zinc is positioned equidistant from each haem with one of its ligands, $Cys^{115}$, separated by only four residues from $Ser^{119}$, which hydrogen-bonds directly to $BH_4$, suggesting that the zinc centre acts in a structural capacity by helping to maintain the integrity of the $BH_4$-binding site. Studies on nNOS also reported a zinc playing a role in dimer stabilization but not affecting enzyme activity [42]. A structural zinc was not reported in the structure of murine iNOS oxygenase (amino acids 66–498); instead it was proposed that $Cys^{109}$ in each of the murine iNOS monomers make an interchain disulphide bridge. The $ZnS_4$ centre in both iNOS and eNOS oxygenase structures is surrounded by a large, solvent-filled inter-subunit cavity of $\approx 750$ Å$^3$ molecular surface, which may be a binding site for a so far unidentified ligand [34].

A comparison of human eNOS and iNOS oxygenase structures reveals them to be very similar in overall molecular shape, relative orientation of cofactors and stereochemistry within the catalytic centre [33]. The only significant difference in the arginine binding site is at iNOS $Asp^{382}$, which is an Asn in eNOS and lies some 9 Å from the substrate guanidinium. The $BH_4$ cavity is also similar between isoforms, with eNOS $Ala^{446}$ being Ile in iNOS and eNOS $Val^{104}$ being Met in iNOS. As the catalytic sites of iNOS and eNOS are so similar it is not obvious from a structural starting point how selective inhibitors may be designed [43].

The structure of the human iNOS oxygenase domain with *S*-ethylisothiourea [33] has provided a structural basis for interpreting the structure–activity relationship of this inhibitor with iNOS [44]. The ethyl group of *S*-ethylisothiourea is packed near the haem and $Phe^{369}$ side chain in a relatively small and narrow cavity, consistent with ethyl and propyl compounds being optimal for NOS inhibition, while compounds with larger aliphatic substituents bind with lower affinities. The crystal structures of NOS isoforms with the more selective inhibitors described below are awaited with interest.

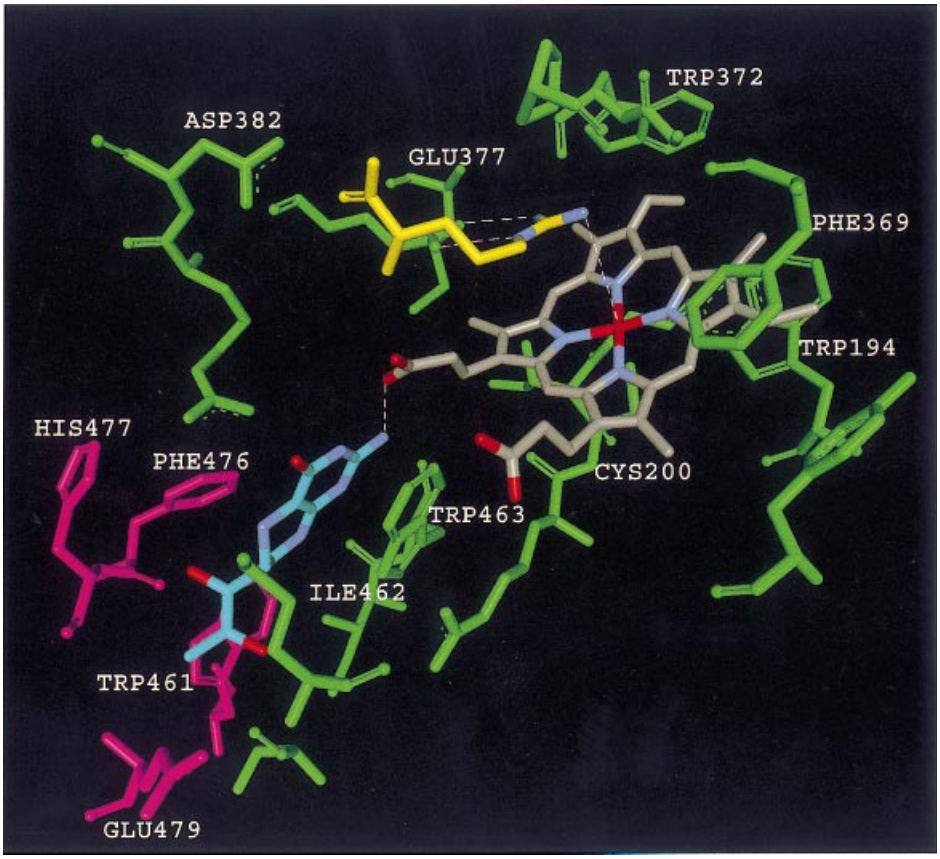



**Figure 2 Catalytic site of human iNOS domain**

The catalytic site of the human iNOS domain is shown with ball and stick representations of the haem (grey), L-arginine (yellow) and $BH_4$ (cyan). Amino acid residues from each NOS monomer are labelled and coloured in a different colour, either green or magenta, illustrating that $BH_4$ binding is at the dimer interface. Some of the key hydrogen bonds between L-arginine and $Glu^{377}$ (corresponds to $Glu^{371}$ in murine iNOS), L-arginine and the haem, and $BH_4$ and the haem are represented by white dashed lines. The Protein Data Bank file 1NS1.PDB (from [34]) was used for visualization of the structure of human iNOS domain in this picture.

## NOS dimerization

As mentioned above, the association of the NOS into active dimers involves a large interface in the oxygenase domain involving two sections of the primary structure of NOS (Figure 1) [32,33,45]. This interface includes the binding site for $BH_4$ and helps to structure the active-site pocket containing the haem and the L-arginine binding site; it also has the two cysteine residues per monomer, which either form a disulphide bridge between the monomers or else ligate a zinc ion between the monomers [35,45]. Furthermore, there is an 'N-terminal hook' domain which swaps between the two monomers as the dimer is formed, helping to stabilize it [45].

Although no crystal structure has yet been published for the reductase domains of NOSs, other methods have investigated the possible involvement of these in the formation of dimeric full-length enzyme, as well as other factors involved, revealing some interesting differences between the isoforms. Yeast two-hybrid studies with oxygenase and reductase domains [46] suggest that, for iNOS, only the oxygenase domain is involved in dimer formation, whereas for nNOS and eNOS, there are interactions between the reductase domains and between the reductase and oxygenase domains across the dimer which may also play a role. This may explain why iNOS dimer formation is more critically dependent on the presence of $BH_4$ than is the case for nNOS or eNOS (reviewed in [47,48]), if nNOS and eNOS dimerization involves other regions of the enzyme away from the $BH_4$-binding site. For example, substantial proportions of nNOS or eNOS expressed as $BH_4$-free enzyme in *Escherichia coli* are in dimeric form, unlike similarly expressed iNOS [47,49,50].

Nevertheless, $BH_4$ as well as haem and L-arginine all promote and/or stabilize the active dimeric form of all three isoforms. The presence of haem appears to be mandatory, with $BH_4$ and L-arginine promoting dimer formation and stabilizing the dimer once formed. In the case of nNOS, addition of $BH_4$ and L-arginine to nNOS that was already dimeric induced conversion into a more stable dimer which was unusually resistant to SDS [51]; it is not clear whether this also occurs with the other isoforms. Recent evidence with very potent inhibitors of dimerization ([52]; see the section below on inhibition) suggests that, for iNOS in intact cells, once the dimer is formed there is little or no significant return to the monomer; again it is unclear whether this will be true also for the other isoforms in the physiological context.

Thus at present it is not possible to be sure of the physiological or pathophysiological relevance of the distribution of NOS between monomeric and dimeric forms, although it is of considerable biochemical and possibly therapeutic interest.

## REACTIONS CATALYSED BY NOSs

The physiological biochemist is interested in NOS, as it is part of the first mammalian pathway known to synthesize a gas as a signalling molecule. However, the enzyme is at least as interesting to the inorganic biochemist/enzymologist as it catalyses a novel reaction for a haem protein, and involves a unique role for a $BH_4$ cofactor.

Scheme 1 shows the NO synthesis reaction by NOS, as understood in 1994 [2]. While many aspects of this scheme have been questioned in the interim, it nonetheless provides a basis for discussion and probably still represents the broad consensus of the 'normal' functional reaction of NOS in the cell.

### What does NOS synthesize?

It may be easiest to mention first which elements of this scheme are *not* controversial, i.e. that L-arginine, NADPH and $O_2$ are substrates and that NADP and citrulline are products. The observant may have noticed that we do not specify L-citrulline, despite the propensity of many authors to state that this is the product. This is because as far as we are aware it has not been published as to whether the product is L- or D/L-citrulline. There is precedent from other amino acid-metabolizing enzymes for reactions to result in a racemic D/L mix of products being formed from a single substrate isomer. Finally it is well accepted that NHA is an intermediate in the reaction [53].

### NO versus nitroxyl

Perhaps most surprisingly for those not familiar with the field is that it remains a matter of much debate as to whether NOS directly synthesizes NO or not! This issue is important in understanding the stoichiometry and mechanism of the NOS reaction, as well as in understanding the physiological and pathophysiological consequences of NOS catalysis (see below). Whilst it is clear that cells and tissues containing NOS, and extracts from them, can make NO, this does not necessarily mean that it is the initial reactive nitrogen species (RNS) formed, and 'NO' synthesis is often inferred from the accumulation of breakdown products, such as nitrite and nitrate, or from reactions with haem proteins, such as the oxidation of oxyhaemoglobin to methaemoglobin, or the stimulation of guanylate cyclase. Other potential RNS [e.g. nitroxyl ($NO^-$ ion or the protonated species HNO), peroxynitrite ($ONOO^-$ ion or the protonated species ONOOH) and nitrosothiols] could in principle be formed first, with the same products and reactions occurring either directly or indirectly after subsequent reactions to form NO.

Schmidt et al. [54] in a provocatively titled paper 'No NO from NO synthase' showed in studies of purified nNOS that unless they added superoxide dismutase (SOD), they were unable to detect authentic NO formation with a specific NO electrode or by an NO chemiluminescence assay. Similarly others [55] have shown enhanced NO synthesis from iNOS or nNOS on addition of SOD. The authors concluded that nitroxyl anion ($NO^-$) was being formed and oxidized to NO in a reaction catalysed by SOD [56]. Moreover, sub-stoichiometric amounts of other nitrogen-centred species (nitrous oxide and hydroxylamine), which are not plausible products of NO but rather of nitroxyl, were formed by purified nNOS [54]. However, SOD can also enhance NO formation by removing superoxide, that would otherwise rapidly convert the NO into peroxynitrite [57]. Superoxide can be readily produced in NOS assay mixtures, from the flavin cofactors in the reductase domain [58] or from flavins added in the reaction mixture [59]; also biopterin-depleted nNOS [60] and eNOS [61] readily produce superoxide at the oxygenase (haem) domain. Therefore the requirement of SOD for NO detection cannot uniquely be assigned to $NO^-$ formation. The NO spin trap *N*-methyl-D-glucamine-dithiocarbamate *did* detect NO under these conditions, leading some authors [59] to conclude that NO is the major product of 'NO' synthase. However, a 1:1 stoichiometry with citrulline was not obtained, suggesting the possibility that $NO^-$ may be a side product. Recently it has been suggested that *N*-methyl-D-glucamine-dithiocarbamate may not be a specific spin trap for NO, but can also trap $NO^-$, re-opening the debate [62]. Although it is difficult to detect $NO^-$ specifically in the aerobic conditions under which NOS operates, there is evidence for its formation in murine iNOS from NHA, either with $NADPH/O_2$ as co-substrates in the absence of $BH_4$ [49] or with hydrogen peroxide as substrate to the $BH_4$-replete enzyme [63]. However, in the presence of $BH_4$, NO formation seemed favoured when arginine was the substrate [49]; presumably under conditions of sub-optimal $BH_4$ availability and the presence of L-arginine and NHA, both NO and $NO^-$ might be synthesized *in vivo*, the ratio of these products depending on the conditions. We conclude that it is still an open question as to whether NO or $NO^-$ is the immediate product of NOS; it seems likely that under different conditions *in vitro* it is possible to get either product.

### NO versus peroxynitrite

As mentioned above, NOS can under some circumstances catalyse an 'uncoupled' NADPH oxidation (uncoupled from NO formation), forming superoxide. It appears that nNOS has a particular propensity to catalyse this uncoupled reaction, at least in the test tube, e.g. at sub-saturating arginine or $BH_4$ concentrations (below the $K_m$) or in the presence of some NOS inhibitors, such as $N^G$-monomethyl-L-arginine (L-NMMA). At the other extreme, eNOS appears to synthesize detectable but much lower amounts of superoxide in the absence of arginine or $BH_4$, although equivalent amounts ($\approx$ 500 nmol/min per mg) were synthesized in the presence of L-NMMA [64,65]. iNOS has also been shown to produce detectable amounts of superoxide, although this was lower ($\approx$ 100 nmol/min per mg) under the conditions tested than production by nNOS or eNOS and was only suppressed by very high (mM) concentrations of arginine [66]. Thus all three isoforms can make superoxide, depending on the conditions, although it is at present unclear whether this occurs physiologically. Because of the very rapid reaction of superoxide with NO [67], synthesis of both species by the same enzyme is likely to result in peroxynitrite formation, either in the bulk solution following independent release of the two species, or conceivably within the active site.

Another possibility, if nitroxyl is a product of NOS, is the reaction of this species with oxygen to form peroxynitrite [68]. This reaction has a much slower rate constant than that of NO with superoxide, but this is offset by the much higher concentrations of oxygen available. Thus, independently of whether NO or nitroxyl is the initial product of NOS, peroxynitrite could be formed subsequently.

### NO or not, does it matter?

Yes! Quite aside from the point about understanding the enzyme mechanism mentioned at the start of this section, if NOSs can make a range of RNS, depending on the isoform and the circumstances, then this could be of crucial importance in understanding the physiological and pathophysiological implications of NOSs and their inhibition. Many NO donors specifically

release NO; if the RNS produced endogenously *in vivo* is, at least in part, not NO, then this can explain the differences sometimes observed between activation of NOS and addition of exogenous NO. It could for example explain the apparent paradox that NO donors have many anti-inflammatory and cytoprotective effects, whereas NOS inhibition also appears to be anti-inflammatory and cytoprotective [11,12,69–71], if the key effect of NOS inhibition is to prevent nitroxyl and/or peroxynitrite synthesis. It could also have a part to play in understanding the differences in the roles played by eNOS compared with iNOS or nNOS (e.g. in ischaemic brain injury, reviewed in [11,12,72]) if, as seems to be the case, eNOS is less inclined than nNOS or iNOS to produce RNS other than NO. Moreover it opens up the possibility that different NOS inhibitors may have very different effects on biological systems depending on whether they inhibit the synthesis of *both* NO and other species [e.g. $N^G$-nitro-L-arginine (L-NNA) on nNOS] or not (e.g. L-NMMA on nNOS) [73].

So it would seem that for the time being we need to think of the NOS enzymes as 'nitrogen oxide synthases' rather than nitric oxide synthases, and to be aware of the possible consequences of this.

## NOS reaction stoichiometry

Coupled to this controversy over the reactive nitrogen product(s) is that over the stoichiometry of the reaction. Clearly the stoichiometry for the production of NO:

$$R{=}NH + 1.5NADPH + 1.5H^+ + 2O_2 \rightarrow R{=}O + 1.5NADP^+ + 2H_2O + {}^{\bullet}NO$$

would need to be adjusted if the product were nitroxyl, to:

$$R{=}NH + 2NADPH + 2H^+ + 2O_2 \rightarrow R{=}O + 2NADP^+ + 2H_2O + HNO$$

Since the first step (monooxygenase I) in both cases is fairly confidently stated as:

$$R{=}NH + NADPH + H^+ + O_2 \rightarrow R{=}NOH + NADP^+ + H_2O$$

Then the second step (monooxygenase II) for each should be:

$$R{=}NOH + 0.5NADPH + 0.5H^+ + O_2 \rightarrow R{=}O + 0.5NADP^+ + H_2O + {}^{\bullet}NO$$

or,

$$R{=}NOH + NADPH + H^+ + O_2 \rightarrow R{=}O + NADP^+ + H_2O + HNO$$

where R=NH is arginine, R=NOH is $N^{\omega}$-hydroxy-L-arginine and R=O is citrulline.

While in principle this should provide an experimental test for whether NO or nitroxyl is the prime product, in practice this is made difficult by some of the technical challenges. Values in the range 1–2 have been reported for the NADPH utilization: citrulline formation stoichiometry, but these have to be interpreted with care. In particular, there is usually an 'uncoupled' rate of NADPH oxidation in the absence of amino acid substrate (or presence of inhibitors such as L-NMMA), which may or may not be redirected to support the reaction during NO/nitroxyl synthesis, so that it is unclear whether or not this uncoupled rate should be subtracted or not. If this rate is subtracted, it may constitute a substantial under-estimation of the NADPH stoichiometry. Moreover, since both NO or nitroxyl may form peroxynitrite (in the presence of superoxide from NOS or its cofactor, or oxygen from the atmosphere respectively), and since this species will oxidize NADPH, more NADPH may be oxidized than is required for the direct reaction to form NO/nitroxyl, over-estimating the NADPH utilization [54]. Combining these caveats it is not possible at present to say whether the true stoichiometry is 1, 1.5 or 2 NADPH consumed for each arginine consumed/citrulline produced.

## Roles of the flavin domain in NOS

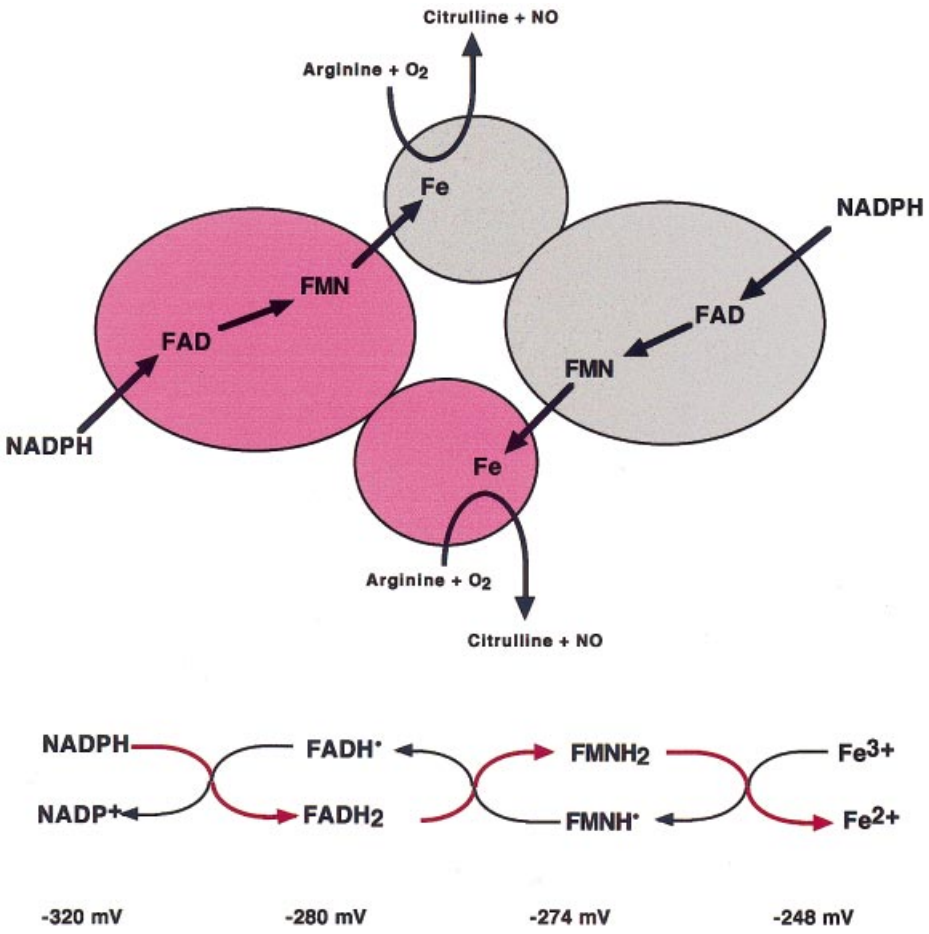
Whilst much of the chemistry of NOS is novel, it is occasionally reassuring to come across a part of the enzyme that behaves 'as expected'. The FAD and FMN in the reductase domain accept electrons from NADPH and pass them on to the haem domain. The role of the reductase domain can therefore be likened to that of the cytochrome P450 reductases, which donates electrons to the cytochrome P450 enzyme family. The essential role of the flavin cofactors is to allow a two-electron donor (NADPH) to donate electrons to a one-electron acceptor (haem), by forming stable semiquinone radical intermediates. Characterization of FMN-deficient mutants [74] of nNOS reveals that the electron flow is in the direction NADPH → FAD → FMN. Electron flow between the two flavins has been suggested to be the control point where CaM acts, electron transfer from FAD to FMN being slow in the absence of CaM–$Ca^{2+}$ [75]; the effect of CaM is kinetic, rather than thermodynamic, since CaM–$Ca^{2+}$ binding has little effect on the redox potentials of the flavins [76]. A comparison of the relevant flavin and haem redox potentials is given in Scheme 2, along with the suggested pathway of electron flow. Intriguingly the pathway of electron flow appears to 'cross over' between different subunits of the dimer, i.e. the flavin domain of one polypeptide chain donates its electron to the haem domain of the other [77]. The physiological reason for this is unclear, but it is clearly a major reason why the NOS monomer is inactive.

## Role of the haem in NOS

Just as it possible to liken the NOS flavin domain to cytochrome P450 reductase, the NOS haem domain has apparent similarities to cytochrome P450 enzymes. Both haems have a thiolate (cysteine) ligand and the haem redox potentials are similar. Both enzymes catalyse oxidations of substrate molecules bound close, but not directly ligated, to the haem iron. However, there are fundamental structural and functional differences between the haems in the two enzymes. Structurally in NOS a tryptophan is hydrogen bonded to the haem thiolate ligand, unlike in P450s [32]. Functionally NOS catalyses a multi-electron, rather than a two-electron, oxidation and therefore performs two separate oxidation cycles, one to form NHA (monooxygenase I) and the other (monooxygenase II) to convert NHA to NO (or $NO^-$). No such stable intermediate is formed in P450 reactions, where a single cycle is all that is required. However, the most striking structural and functional difference is undoubtedly the presence of a pterin co-factor in NOS.

## Roles of the pterin cofactor in NOS

It has been appreciated for over 10 years that $BH_4$ is required for the expression of NOS activity (see [2] for references), but it has been a source of considerable controversy as to what roles it might be playing, and in particular, whether it plays a redox role in the reaction mechanism [32,35,51,60,73,78–92]. The monooxygenase I reaction has an absolute requirement for a redox-active $BH_4$ [93] but it is unclear whether or not it is required for the monooxygenase II reaction [49]. Table 2 shows the rather startlingly diverse range of functions proposed for $BH_4$ in NOS.




**Scheme 2 Redox potentials and direction of electron flow in nNOS**

The electron flow in the NOS dimer goes via NADPH → FAD → FMN in the reductase of one monomer to the haem iron in the oxygenase domain of a separate monomer. The redox potentials are poised thermodynamically to make this occur. The potentials for the two-electron oxidation of NADPH and the one-electron oxidations of $FADH_2$, $FMNH_2$ and ferric haem are illustrated at the bottom of the Scheme, with the red arrows indicating the direction of electron flow. Note that given the variety of redox couples and the closeness of the $FADH_2$ and $FMNH_2$ potentials the detailed picture is more complex than that illustrated. For example the $FMNH^{\bullet}$/FMN couple has a redox potential of −49 mV and is likely to donate electrons to the high-potential ferric superoxide species to form the ferryl intermediate (see Scheme 4). Reduction of the FMN back to $FMNH^{\bullet}$ would then require an electron from $FADH^{\bullet}$. For more details of electron flow in nNOS, see Noble et al. [76]. Redox potentials for the flavins in the other NOS isomers are not known as yet.

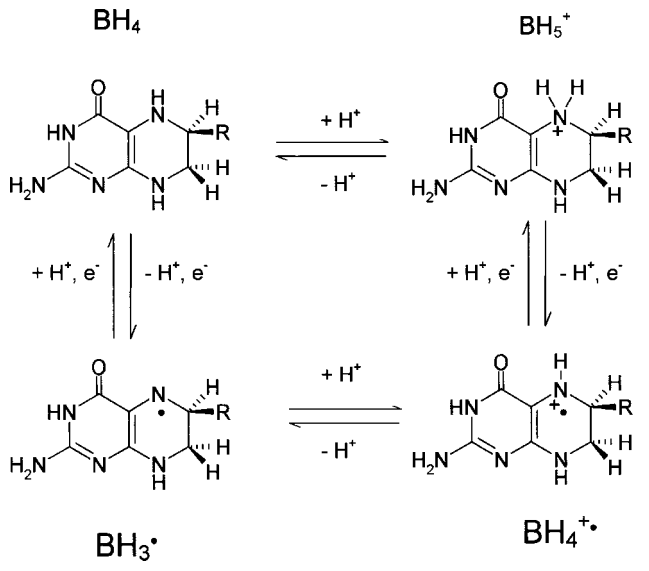
**Table 2 Suggested functional roles of $BH_4$ in NOS**

| Mechanism | $BH_4$ function/observation | References |
|---|---|---|
| 1 | Promotes coupling of NADPH oxidation to NO synthesis and inhibits superoxide and hydrogen peroxide formation | [60,73,78] |
| 2 | Promotes dimer formation and increases tightness/stability of dimer (e.g. to SDS) | [51,79–82] |
| 3 | Allosteric binding effects: on subsequent arginine binding and on the binding of a second molecule of $BH_4$ | [83–85] |
| 4 | Modifies haem environment (low spin to high spin) | [86–88] |
| 5 | Protects against inactivation, autoinactivation | [32,89] |
| 6 | Redox active in catalysis | [35,90–92] |

Intriguingly none of these involve the two-electron $BH_2/BH_4$ cycle that is the 'classical' role for $BH_4$ in aromatic amino acid hydroxylases, e.g. phenylalanine hydroxylase [94].

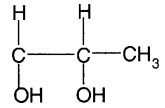
Studies using limited proteolysis, site-directed mutagenesis, expression of truncated domains of NOS and X-ray crystallography of such domains have provided a structural basis for many of these observations. $BH_4$ binds to the haem-containing domain of NOS, interacting with the haem propionate and thus binding immediately adjacent to the active site where L-arginine is bound, and also forming part of the NOS dimer interface (Figure 2) [35,45]. These insights do not, however, provide compelling evidence for which of roles 1–6 in Table 2 are the most important, and whether $BH_4$ plays a direct catalytic role in the NOS reaction.

Further insights have come from studies with a wide variety of pterin analogues. Collectively, these have shown that many such




**Scheme 3 Structures of the biopterin cofactors involved in NO synthesis**

Shown are both the protonated and unprotonated forms of $BH_4$ and of the $BH_3^{\bullet}$ radical, where R represents:



It is hypothesized that $BH_4$ plays a redox role in NOS catalysis in which bound $BH_5^+$ donates one electron to the haem, forming $BH_4^{\bullet+}$ which is then returned to the reduced state by accepting an electron from a flavin in the reductase domain.

analogues are able to bind to NOS and have some of the effects listed in Table 2, favouring the dimeric form and shifting the haem to a high-spin state [95–97]. However, most of these analogues are nonetheless ineffective in supporting NO synthesis, and in fact many are inhibitors, showing that these effects are insufficient to explain the critical role of $BH_4$.

Although there are data to support a redox role for $BH_4$ in the NOS reaction, little has been published to support a classical $BH_4$–quinonoid $BH_2$ cycle such as that which occurs between aromatic amino acid hydroxylases and dihydropteridine reductase. Recently, however, several lines of evidence have pointed to an alternative. The possibility of the involvement of a pterin radical was initially proposed on the basis of rapid-reaction kinetics [90]. This was then supported by structural information suggesting that a pterin species could be involved [35]. This hypothesis has been confirmed by direct observation of $BH_3^{\bullet}$ by rapid freeze-quench EPR, formed by the haem domain of iNOS during haem oxidation with oxygen [91]. This technique cannot distinguish between the neutral radical and the cationic form, but Raman et al. [35] proposed that both $BH_3^{\bullet}$ and $BH_4$ might be in their protonated cationic forms ($BH_4^{\bullet+}$ and $BH_5^+$ respectively, Scheme 3), based on the ability of the pterin-binding site of their eNOS haem domain to bind L-arginine via its cationic groups, when the arginine site was occupied by *S*-ethylisothiourea.

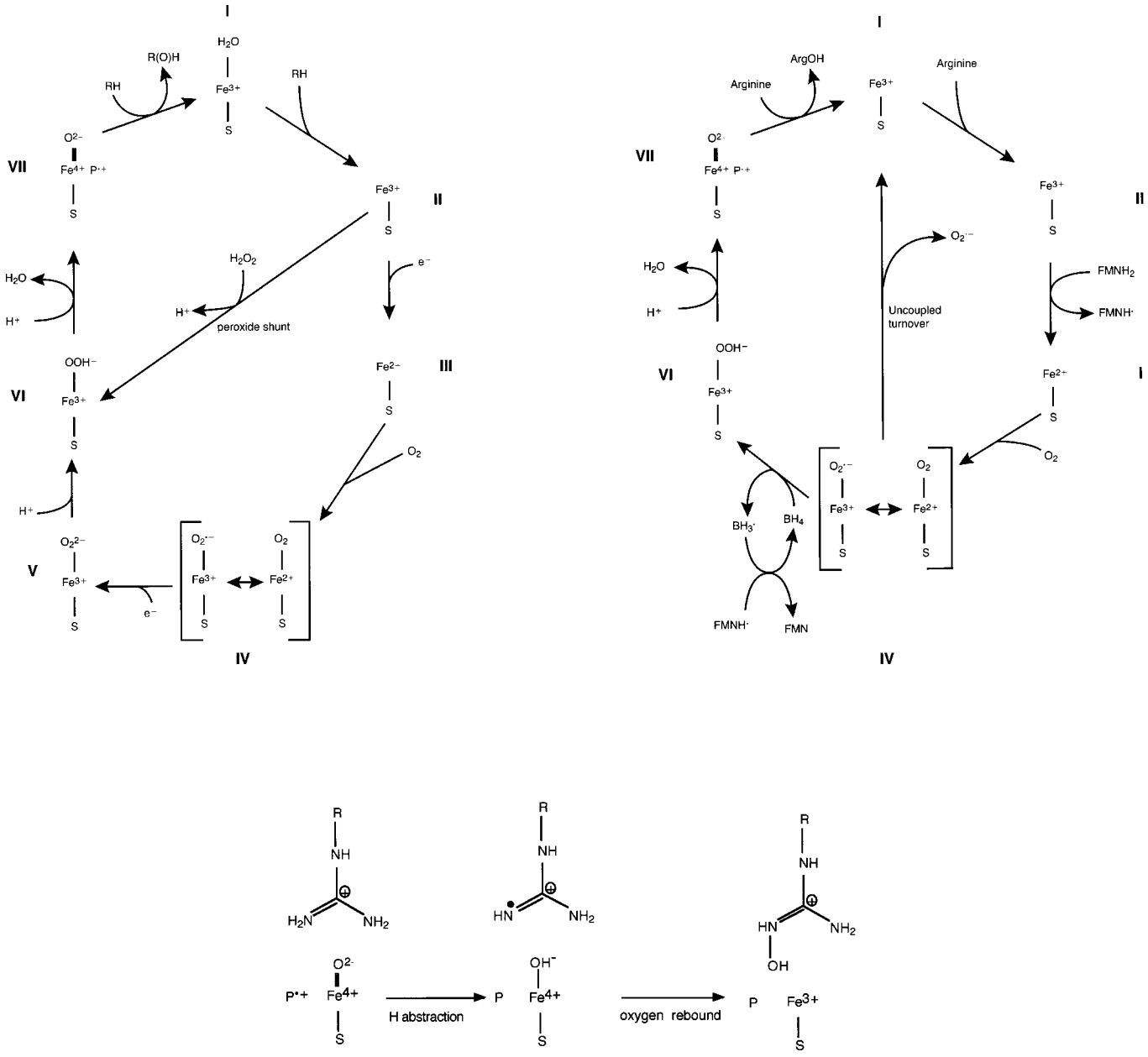
It therefore seems most likely that $BH_4$ does play a redox role in NOS catalysis in which bound $BH_5^+$ donates one electron to the haem, forming $BH_4^{\bullet+}$ which is then returned to the reduced state by accepting an electron from a flavin in the reductase domain (Scheme 3). The other effects of $BH_4$ may play important ancillary roles in promoting NO synthesis, in addition to this key catalytic role.

## DETAILED NOS REACTION MECHANISMS

In order to understand the NOS mechanism it is helpful first to understand how the haem iron in P450 catalyses the oxidation of substrate molecules [98] (Scheme 4, upper left panel). In its resting state the haem iron is low spin with cysteine and water (or hydroxide) ligands. This form of the enzyme has a low redox potential (−330 mV) and cannot be reduced by electrons from P450. Therefore uncoupled turnover is minimized; this prevents 'wastage' of electrons and decreases the possibility of forming reactive intermediates ($Fe^{4+}{=}O_2^-$, $Fe^{3+}{=}O_2^{\bullet-}$) which, in the absence of the intended substrate, can damage the protein and/or biomolecules in its vicinity. Substrate binding displaces the distal ligand and increases the redox potential (−170 mV), such that electrons can now flow from the reductase to reduce the haem iron. The ferrous iron then binds dioxygen-initially the same reaction as in haemoglobin, forming the [$Fe^{2+}{=}O_2 \leftrightarrow Fe^{3+}{=}O_2^{\bullet-}$] intermediate. This is immediately reduced by another electron to form a ferric peroxide complex [$Fe^{3+}{=}O_2^{2-}$] which then undergoes oxygen–oxygen bond scission to form the reactive oxygenating intermediate. This species formally contains iron in the 5+ oxidation state, but it is likely, as in peroxidase and catalases, that one of the oxidizing equivalents is held on the porphyrin ring as a cation radical [$Fe^{4+}{=}O_2^-]\cdot P^{\bullet+}$. The exact nature of this species is controversial as it is only formed in the presence of the substrate and rapidly reacts with the substrate to form the product and re-form the low-spin ferric form of the enzyme. In the absence of the reductase it is possible to add hydrogen peroxide to the enzyme and directly oxidize the ferric form to the oxygenating [$Fe^{4+}{=}O_2^-]\cdot P^{\bullet+}$ species; this peroxide shunt is directly analogous to the reaction mechanism of peroxidases.

Superficially the NOS monooxygenation I reaction (the two-electron oxidation of arginine to NHA) might be expected to be similar to that of P450. Although there is not a consensus mechanism for NOS, Scheme 4 (upper right panel) illustrates a mechanism that is increasingly favoured by a number of groups [48,99–102]. The enzyme starts off in the ferric state. Here the first difference from P450 is observed, since purified iNOS is low spin with a redox potential of −347 mV [103]. Substrate (arginine) binding increases this potential to −235 mV. Although superficially this increase is similar to the effect of substrate binding in cytochrome P450, this arginine-bound form of the enzyme is inactive as there is no bound $BH_4$. Adding the $BH_4$ to the isolated enzyme makes it high spin with a redox potential of −295 mV, and the subsequent addition of arginine raises the potential to −263 mV. In nNOS the $BH_4$-bound enzyme has an even more positive potential of −257 mV, which only changes to −248 mV following subsequent arginine binding. Therefore in the absence of arginine the NOS haem potential is not significantly lower than that of the relevant flavins in the reductase domain (−274 mV for the FMN/$FMN^{\bullet}$). NOS is less tightly coupled than P450 and it is possible to reduce the iron in the absence of substrate binding. The consequences of this are that superoxide production from the haem is possible. The finding that *in vitro* nNOS produces more superoxide than iNOS is consistent with the higher potential of the nNOS haem in the presence and absence of $BH_4$.

The first step in the mechanism is the addition of an electron to reduce the ferric haem. The ferrous form then binds oxygen [104] to form the stable oxy complex [$Fe^{2+}{=}O_2^- \leftrightarrow Fe^{3+}{=}O_2^{\bullet-}$]. It is the dissociation of this species that is likely to be a source of superoxide ion formation. Intriguingly, both arginine and $BH_4$ decrease the rate of superoxide formation, but arginine decreases the rate of decay of the oxy complex, whereas $BH_4$ increases this rate [104]. These apparently contradictory findings can be

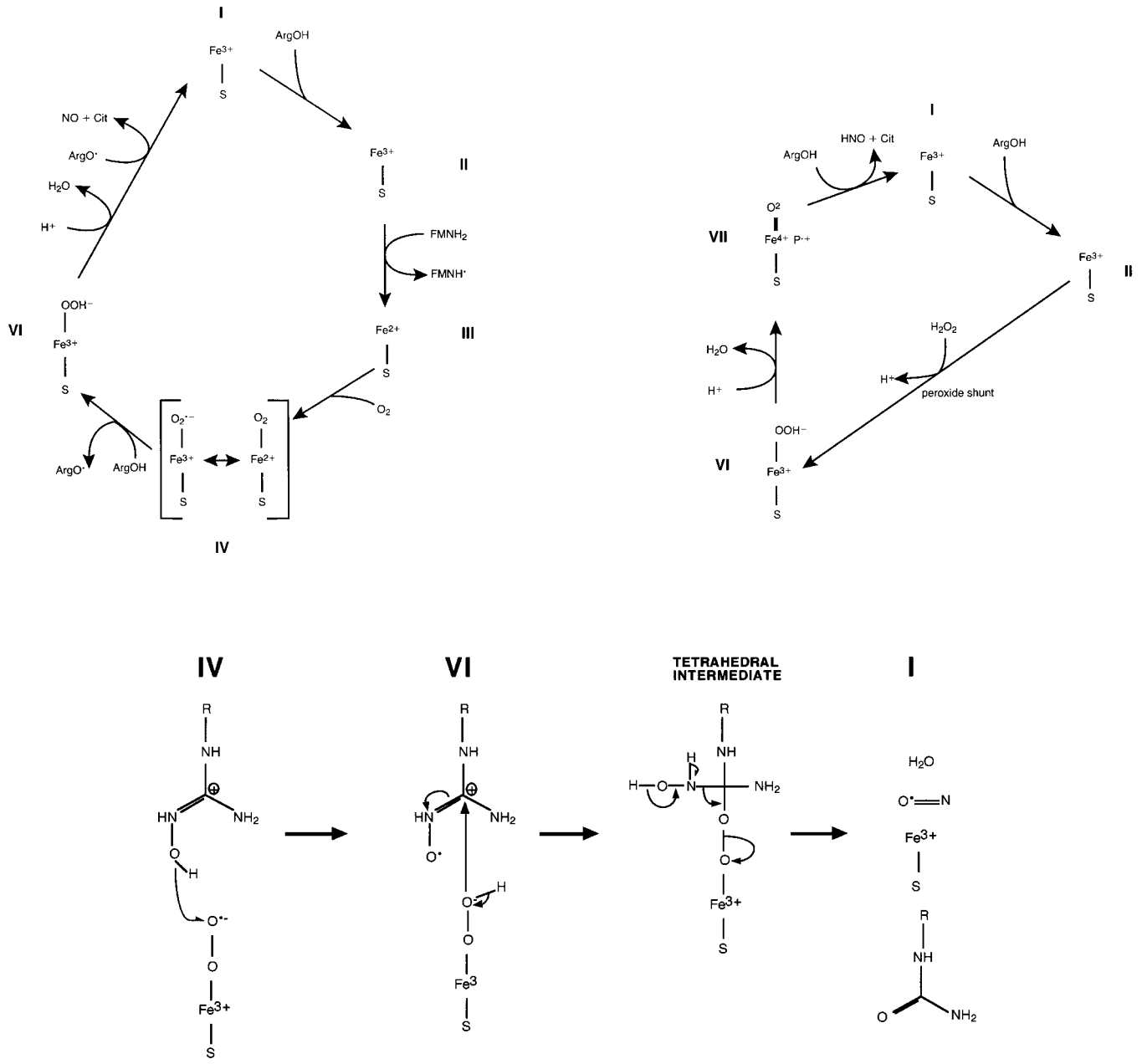






**Scheme 4 Comparison of cytochrome P450 and NOS monooxygenation I reaction**

Upper left panel: in P450 the haem in the resting state is in the low-spin ferric form (I). Substrate (RH) binding displaces the distal ligand and shifts the haem to a high-spin state with a raised redox potential (II). Electrons, from P450 reductase, can then reduce the iron to the ferrous state, species III. Oxygen then binds resulting in the ferrous–oxy complex (IV). This species is formally equivalent to a ferric–superoxide complex. Addition of another electron reduces the bound superoxide to peroxide (V) which is then protonated to the hydroperoxide (VI). A further protonation induces the irreversible breaking of the oxygen–oxygen bond, resulting in species VII, a ferryl iron with a protein-bound cation radical ($P^{\bullet+}$). This highly oxidizing species rapidly oxygenates the nearby substrate molecule, regenerating the resting state (I). The reaction can be short-circuited by the addition of peroxide to the high-spin ferric species (II), generating the hydroperoxide (species VI). Thus in P450 enzymes, peroxide can frequently drive the oxygenation reaction in the absence of a supply of electrons from a reductase (the peroxide shunt). Upper right panel: in NOS in the presence of biopterin the haem is already high spin (I) and substrate binding has a relatively small effect on the redox potential (II). An electron from $FMNH_2$ in the reductase reduces the iron to the ferrous state (species III). Oxygen then binds, resulting in the ferrous–oxy complex (IV). This species is formally equivalent to a ferric–superoxide complex. Simultaneous addition of an electron and a proton from $BH_4$ reduces the bound superoxide to hydroperoxide (VI). Subsequent protonation induces the irreversible breaking of the oxygen–oxygen bond, resulting in species VII, a ferryl iron with a protein-bound cation radical ($P^{\bullet+}$). This highly oxidizing species rapidly oxygenates arginine to NHA (ArgOH), regenerating the resting state (I). The peroxide shunt is inoperative in NOS, but there is a significant rate of uncoupled turnover, generating superoxide, especially in the absence of $BH_4$. [Note that in the two schemes in the upper panels the substrate once bound, remains attached to the protein throughout the reaction cycle but is omitted in the intermediate structures for clarity.] Lower panel: suggested details of the arginine monooxygenation. The ferryl–radical intermediate (VII) is capable of abstracting a hydrogen from the arginine, generating a substrate radical. This then reacts with the oxygen bound to the iron in a radical rebound mechanism, generating the ferric resting state and NHA (ArgOH).




**Scheme 5 NOS monooxygenation II reaction**

Upper left panel: the same roman numerals are used in Scheme 5 to illustrate the same intermediates described in Scheme 4. Following NHA (ArgOH) binding, (II) a single electron from the reductase domain reduces the haem iron (III). Subsequent oxygen binding generates the oxy complex (IV). NHA oxidation is suggested to be catalysed via a haem–peroxy (VI), rather than a ferryl, intermediate. This occurs as NHA reacts with the oxy intermediate to generate the hydroperoxy complex (VI) and an NHA radical (ArgO$^{\cdot}$), which then rebound to react with each other, generating NO, citrulline (Cit), water and the ferric haem protein again (I). A possible detailed mechanism for this reaction is illustrated in the lower panel. Upper right panel: it is possible to oxidize NHA using hydrogen peroxide as an artificial oxidant. This mechanism probably occurs via a direct conversion of the bound NHA–ferric-haem complex (II) into a hydroperoxy (VI) and then ferryl (VII) intermediate. The final product is $NO^{-}$, not NO.

rationalized if in the presence of arginine the dissociation of superoxide from the oxy complex is inhibited, whereas in the case of $BH_4$ the reduction of this complex is favoured. The transient formation of a $BH_3$ radical following oxidation of the ferrous haem by oxygen (see above) also points to a direct role for $BH_4$ in reducing the oxy complex [91]. The peroxy complex is then protonated (possibly by L-arginine itself [32]) and the oxygen–oxygen bond broken to form the same $[Fe^{4+}{=}O_2^{-}]\cdot P^{\cdot+}$ intermediate as seen in P450. This intermediate can then perform the two-electron oxidation of arginine to NHA, re-forming the ferric enzyme (Scheme 4, lower panel).

The second oxidation cycle is unique to NOS (Scheme 5). There is less agreement on reaction mechanisms here (or even reaction products, see earlier), but it is thought that a high-valent iron intermediate is not involved. Instead the oxidizing species is suggested to be a ferric–peroxy intermediate. Ferric iron is reduced to ferrous and oxygen binds. This form of the enzyme then reacts directly with NHA to form an NHA radical and a

haem–peroxy complex. The NHA radical and the peroxy complex then react with each other in a 'radical rebound' mechanism to generate citrulline and NO, and re-generate the ferric haem iron. The presence of a $Fe^{3+}$—NO intermediate has recently been detected [105]. As ferric NO complexes are generally less stable than the $Fe^{2+}$—NO species, this makes thermodynamic sense as a reaction intermediate. Several possible chemical mechanisms of NO product formation during the monooxygenase II reaction are discussed in detail by Adams et al. [100]. An adapted version of the model suggested by Korth et al. [106] is illustrated in Scheme 5 (upper right panel); however, the experimental evidence at present is not sufficient to discriminate between different mechanisms (especially given the argument as to whether NO or $NO^-$ is the ultimate product).

As the haem group is responsible for both monooxygenation cycles in NOS, there are opportunities to 'short-circuit' the reaction pathway that must be avoided. Both cycles have an oxy complex and a peroxy complex. Therefore in the second monooxygenation cycle the enzyme must favour NHA reduction of the oxy complex over $BH_4$ reduction. Given the similar proximity of the arginine- and $BH_4$-binding sites to the haem iron, one would expect this difference to be due to a larger redox-potential difference favouring NHA reduction. Similarly the NHA radical rebound has to be favoured over the O—O bond scission of the peroxy complex. Perhaps the protonation that occurs prior to O—O bond breaking is easier for bound arginine than bound NHA?

In P450 chemistry, peroxide shunt reactions have led to many useful insights [98]. By analogy with P450, one would expect the NOS monooxygenation I to allow peroxide as an electron donor. In fact this is another difference between the two enzymes. NHA formation from arginine has an absolute requirement for electrons from NADPH. However, NHA can be oxidized to citrulline by peroxide, a reaction that occurs in the purified, isolated haem domain. However, in the case of NOS it seems unlikely that the 'normal' monooxygenation II process is occurring. There is evidence for the formation of a ferryl iron species [107], the product is predominantly $NO^-$ [63] and, as well as citrulline, significant amounts of $N^-$-cyanoornithine are formed [107].

### Feedback inhibition of NOS by NO

Due to the stability of ferrous nitrosyl ($Fe^{2+}$—NO) complexes, any enzyme that forms a reduced-ferrous-haem intermediate has the potential to be inhibited by NO [108]. NOS is no exception. For example, the reduction of the suggested $Fe^{3+}$—NO intermediate would generate $Fe^{2+}$—NO, as would the reaction of $NO^-$ with the ferric enzyme.

Both iNOS and nNOS can form inhibitory nitrosyl species during turnover. In the case of iNOS [109], NO inhibition appears weak and is partly due to build-up of the $Fe^{3+}$—NO complex, but in nNOS up to 95% of the enzyme in the steady state can be in the tighter $Fe^{2+}$—NO form [110]. Unlike iNOS, nNOS also seems able to react with NO within the enzyme active site, as the addition of external NO scavengers has little effect on the inhibition. A unified model to explain these findings has recently been proposed by Stuehr's group [111]. In line with recent findings that a $Fe^{3+}$—NO complex is a final intermediate in the catalytic cycle [105], they propose that the dissociation of NO from this complex (normal catalysis) competes with its reduction to the $Fe^{2+}$—NO species (auto-inhibition).

In general, three strategies exist for preventing inhibition by NO in ferrous haem proteins [108]: kinetic prevention of the bond being formed, an increase in the dissociation rate of NO, or a modification of the chemical reactivity of the bond. In the case of NOS, as in some P450 enzymes, the latter course is followed, and the $Fe^{2+}$—NO bond reacts rather quickly with oxygen, generating nitrate and ferric iron [112]. This reaction is suggested to be fast in iNOS and slow in nNOS [111], with the consequence that in nNOS the rate of the oxygen reaction with the nitrosyl complex is one of the steps that limits turnover in the steady state. This may not be accidental; the presence of the conserved tryptophan residue ($Tyr^{409}$ in rat nNOS) that hydrogen bonds to the haem thiolate ligand appears to be designed to increase the stability of the nitrosyl complex and thus allow the enzyme to be controlled by its product [113]. By competing with oxygen, NO raises the $K_m$ for oxygen of nNOS, making NO synthesis oxygen-dependent throughout the physiological range [112].

## REGULATION OF NOSs

NO is now known to be synthesized in a large number of different tissues playing a wide variety of physiological roles. The regulation of NOS activity in order for NO to perform this variety of roles is complex. Cellular and tissue specific localization of the NOS isoforms can be regulated by transcriptional regulation, which has been reviewed elsewhere [114,115]. Table 3 summarizes the regulation of the activity, expression and localization of the NOS isoforms by protein–protein interactions, alternative mRNA splicing and covalent modifications

### Regulation of NOS activity

#### CaM

CaM was the first protein shown to interact with NOS [116] and is necessary for the enzymic activity of all three isoforms. The $Ca^{2+}$-dependence of NO synthesis distinguishes the NOS isoforms, with nNOS and eNOS having a much higher $Ca^{2+}$ requirement than iNOS. CaM binding increases the rate of electron transfer from NADPH to the reductase domain flavins [117,118] and artificial electron acceptors such as ferricyanide and cytochrome *c* [118]; it also triggers electron transfer from the reductase domain to the haem centre [119].

nNOS and eNOS differ in their primary structure from iNOS in the former having 40–50 amino acid inserts in the middle of the FMN-binding subdomain, which has been described as an auto-inhibitory loop [120]. Analysis of mutants of eNOS and nNOS with this loop deleted has shown that the insert acts by destabilizing CaM binding at low $Ca^{2+}$ and by inhibiting electron transfer from FMN to the haem in the absence of $Ca^{2+}$/CaM [121,122].

#### Phosphorylation

Phosphorylation of the nNOS and eNOS isoforms has an effect on NOS activity. Fluid shear stress elicits phosphorylation of eNOS and an increase in calcium-independent NOS activity [123,124]. Studies showed that $Ser^{1179}$ of eNOS is phosphorylated by protein kinase Akt [125,126], which results in an increase in electron flux through the reductase domain and an increase in NO production [127]. In contrast, the phosphorylation of nNOS at $Ser^{847}$ by CaM-dependent kinases leads to a decrease in NOS activity [128–130].

#### Protein inhibitor of NOS (PIN)

The N-terminal extension of nNOS also contains a binding site for the 89-amino-acid protein PIN [131]. NMR spectroscopy has been used to map the PIN-binding region of rat nNOS to $Met^{228}$–$His^{244}$ [132] and to determine that PIN binds nNOS with

**Table 3 Regulation mechanisms of NOSs**

For references see text. ENAP-1, eNOS-associated protein-1.

| Type of regulation | nNOS | eNOS | iNOS |
|---|---|---|---|
| Alternate mRNA splicing | Yes | Not reported | Yes |
| Protein–protein interactions | CaM/$Ca^{2+}$, PDZ domains, PIN, caveolin-1, Hsp90 | CaM/$Ca^{2+}$, caveolin-1 and -3, Hsp90, ENAP-1 | CaM/$Ca^{2+}$, kalirin |
| Covalent modifications | Phosphorylation | Myristoylation, palmitoylation, phosphorylation | Not yet reported |

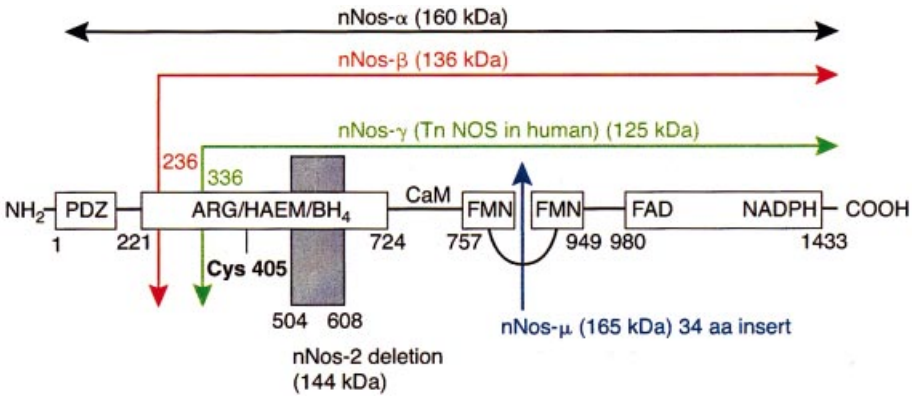



**Figure 3 Splice variants of rat nNOS**

The PDZ, oxygenase and reductase domains on rat nNOS are denoted by solid boxes and the protein products of the splice variants are shown by arrowed lines as follows: black, nNOS-$\alpha$ amino acids 1–1433; red, nNOS-$\beta$ amino acids 236–1433; green, nNOS-$\gamma$ amino acids 336–1433; blue, nNOS-$\mu$ amino acids 1–1433 with a 34-amino-acid insert in the FMN-binding domain. The deleted amino acid residues 504–608 in nNOS-2 (detected in mouse nNOS) amino acids 1–1433 are indicated by a grey box.

a 1:2 PIN:nNOS stoichiometry. However, the literature is contradictory on the ability of PIN to inhibit NOS activity. The initial report suggested that only nNOS associated with PIN and that PIN was inhibitory by destabilization of the nNOS [131]. However, recent reports claim that PIN neither inhibits nNOS nor promotes monomerization [133] or that PIN has no effect on nNOS dimerization but did inhibit both NOS and NADPH oxidase activity of nNOS, eNOS and iNOS in a time-dependent manner [134]. The identification of PIN as a light chain of myosin and dynein [135] has led to the suggestion of an alternative role for PIN as an axonal transport protein for nNOS rather than a regulator of nNOS [133,134].

### Heat-shock protein 90 (Hsp90)

The molecular chaperone Hsp90 has been identified as a regulator of eNOS activity, possibly as an allosteric modulator [136]. Activation by vascular endothelial growth factor, histamine or fluid shear stress in human endothelial cells increases the interaction between eNOS and Hsp90 and increases eNOS activity by approx. three-fold. The activity of purified eNOS was also increased by purified Hsp90, suggesting a direct interaction, the details of which are not yet clear. A previously identified protein that interacts with eNOS, eNOS-associated protein-1 ('ENAP-1') is a 90 kDa protein that is tyrosine phosphorylated in response to bradykinin stimulation of eNOS activity in bovine endothelial cells [137]. It remains to be demonstrated whether eNOS-associated protein-1 is Hsp90. Recently, nNOS–hsp90 heterocomplexes have been detected [138]. nNOS was not directly activated by hsp90 *in vitro*, but a role for Hsp90 in incorporation of the haem into nNOS in *in vivo* situations where haem is limited is suggested.

It has been thought that iNOS is primarily regulated at a transcriptional level but the identification of a protein interacting with iNOS in the central nervous system has recently been reported. Kalirin appears to inhibit iNOS by preventing the formation of iNOS dimers [139] and may play a neuroprotective role during inflammation.

## Regulation of NOS localization

### Myristoylation, palmitoylation

Of the three NOS isoforms, eNOS alone is acylated by both myristate and palmitate [140]. eNOS is co-translationally and irreversibly myristoylated at an N-terminal glycine residue while palmitoylation occurs post-translationally and reversibly at cysteine residues $Cys^{15}$ and $Cys^{26}$ [141,142]. Dual acylation of eNOS is required for efficient localization to the plasmalemmal caveolae of endothelial cells [143]. Palmitoylation is dynamically regulated by agonist (e.g. bradykinin)-induced changes in intracellular $Ca^{2+}$ [144].

### Caveolin

eNOS is localized to the caveolae, [143,145] which are microdomains of the plasmalemmal membrane that are implicated in a variety of cellular functions including signal transduction events. The caveolin proteins are the major coat proteins of

caveolae and in endothelial cells eNOS binds to caveolin-1, while in cardiac myocytes eNOS is associated with caveolin-3 [146]. Caveolin-1 and peptides from the caveolin-1 'scaffold' region directly inhibit eNOS activity and this interaction is regulated by $Ca^{2+}$/CaM [147,148]. A nine-amino-acid binding motif for caveolin has been identified in bovine eNOS, residues 350–358, and deletion mutation of these amino acids produced an active enzyme that was uninhibitable by caveolin-1 [149]. Additionally, an independent interaction between caveolin-1 and the reductase domain of eNOS has been described [150]. Caveolin-3 also binds to nNOS in skeletal muscle, inhibiting NO synthesis, and this inhibition is reversed by $Ca^{2+}$/CaM [151].

The N-terminal 220 amino-acids of nNOS are unique to the neuronal isoform (Figure 2) and contains a PDZ domain (where PDZ stands for **P**SD-95 **d**iscs large/**Z**O-1 homology domain, and where PSD-95 stands for post synaptic density protein 95) that targets nNOS to synaptic sites in brain and skeletal muscle. The membrane association of nNOS in neurons is mediated by the PDZ domain, since the mouse nNOS splice variants nNOS$\beta$ and nNOS$\gamma$, which lack this domain, occur in soluble fractions of the brain [152]. The PDZ domain of nNOS interacts with the second of several similar PDZ motifs in neuron-specific PSD-95 and PSD-93. Since PSD-95 also binds to the C-terminus of *N*-methyl-D-aspartate (NMDA) receptors through PDZ domains, PSD-95 may contribute to the co-localization and functional coupling of nNOS to NMDA receptors. Indeed PSD-95 assembles a ternary complex with the NMDA receptor and the nNOS PDZ domain [153] and peptides binding to either the nNOS PDZ domain or PSD-95 have been shown to uncouple NOS activity from NMDA receptor stimulation [154]. PDZ domain interactions also mediate the binding of nNOS to skeletal muscle $\alpha$1 syntrophin, which forms a complex with sarcolemmal dystrophin complex. The nNOS-PDZ consensus peptide-binding sequence has been identified as G(D,E)-X-V with $Tyr^{77}$ of nNOS mediating the preference for Asp at the $-2$ peptide position [155,156].

## Expression of NOS splice variants

nNOS is encoded by one of the most structurally diverse human genes, particularly in terms of promoter usage [157]. Post-transcriptional regulation of the nNOS gene occurs in the form of alternative mRNA splicing, and schematic representation of the predicted gene products of four nNOS splice variants detected so far (nNOS$\beta$, nNOS$\gamma$, nNOS$\mu$ and nNOS-2) and described below with the full length nNOS (designated nNOS$\alpha$) is shown in Figure 3. As yet, the determinants of alternative-splicing events or how these events are regulated and the biological significance of the nNOS splice variants is poorly understood.

### nNOS$\beta$ and nNOS$\gamma$

The detection of residual NOS activity in nNOS-knockout mice generated by deletion of exon 2 in embryonic stem cells [158] led to the identification of two splice variants of nNOS, nNOS$\beta$ and nNOS$\gamma$. Both lack the PDZ domain of nNOS which is encoded by exon 2, and do not bind PSD-95 from brain synaptic densities or associate with synaptic membranes [152]. Alternative splicing of exon 2 occurs in a region-specific manner in the mouse brain [159] and is developmentally regulated but the functions for nNOS$\beta$ and nNOS$\gamma$ are not yet clear. If translated *in vivo*, nNOS$\beta$ would produce a 136 kDa protein with six unique N-terminal amino acids and the deletion of the first 236 amino acids of nNOS$\alpha$, while nNOS$\gamma$ would produce a 125 kDa protein with the deletion of the first 335 amino acids of nNOS$\alpha$ (Figure 3). The catalytic activities of nNOS$\beta$ and nNOS$\gamma$ by heterologous expression by transfection into COS cells were reported to be 80% and 3% of nNOS$\alpha$ activity respectively [159].

An mRNA transcript encoding a human equivalent of the mouse nNOS$\gamma$, called TnNOS, has been detected at low levels specifically in testes [160]. Although the protein product is identical to nNOS$\gamma$, two unique exons are spliced to exon 4 of nNOS to produce a novel 5′-untranslated region which may contain a promoter for testis-specific regulation. Heterologous expression of TnNOS in Chinese hamster ovary cells gave a calcium-dependent NOS producing comparable amounts of NO as nNOS$\alpha$-transfected cell lines, which contrasts with the very low NOS activity described for mouse nNOS$\gamma$ [160].

### nNOS$\mu$

nNOS$\mu$ is selectively expressed in rat heart and is the predominant isoform in rat skeletal muscle [161], and in rat and human penis and urethra [162]. nNOS$\mu$ has an additional 34 amino acids inserted between the CaM- and flavin-binding domains (Figure 3) and is the most extensively characterized of the nNOS splice variants. Kinetic parameters, $V_{max}$ and $K_m$, and regulation by $Ca^{2+}$/CaM were found to be essentially identical for nNOS$\mu$ and nNOS$\alpha$ [161]. Rates of NADPH consumption and cytochrome *c* reduction by nNOS$\mu$ were approximately half those of nNOS$\alpha$, implying that uncoupled turnover and production of $H_2O_2$ may be somewhat decreased for nNOS$\mu$ [163]. The rates of degradation in rat brain and muscle homogenates show that nNOS$\mu$ is degraded rapidly, but four-fold more slowly than nNOS$\alpha$, with calpain, a $Ca^{2+}$-dependent protease, implicated as a major player in this degradation.

### nNOS-2

An mRNA variant of nNOS was detected in mouse brain (approx. 5% of transcripts contained this deletion) that, if translated, would result in a 105-amino-acid in-frame deletion of residues 504–608 [164]. This splice variant has been called nNOS-2 and has also been detected in human neuroblastoma cell lines [165]. The deletion in this highly conserved region of nNOS that is critical for L-arginine binding, has led to speculation that nNOS-2 may be catalytically inactive and may therefore function as a dominant negative regulator of nNOS activity [166]. The enzymic function of nNOS-2 has not yet been analysed. Antisense studies to selectively down-regulate NOS$\alpha$ and nNOS-2 to functionally differentiate between them have suggested that nNOS-2 plays a modulatory role in morphine analgesia but not morphine tolerance [167] and that the splice variants of nNOS play pharmacologically distinct roles.

### iNOS splice variants

Relatively little information exists on iNOS splice variants, compared with the data on the nNOS variants and as yet no splice variants of eNOS have been detected. Alternatively spliced mRNA transcripts of iNOS have been detected in human epithelial cells and alveolar macrophages which if translated would produce three proteins with deletions in the haem domain at positions 242–335 (denoted $iNOS_{8-9-}$) or 289–427 or in the FMN-binding region at positions 604–678 [168]. Heterologous expression of $iNOS_{8-9-}$ in human endothelial kidney (HEK) 293 cells yielded a monomeric protein that did not retain NOS activity but did retain NADPH diaphorase activty and CaM binding [169]. Heterodimers between full length iNOS and $iNOS_{8-9-}$ were not detected after co-expression, suggesting that

$iNOS_{8-9-}$ may not exert the suggested dominant negative effect on iNOS activity.

#### eNOS splice variants

As yet, no splice variants of eNOS have been reported.

## ARE THERE ANY OTHER FORMS OF NOS?

### Mitochondrial NOS?

There have been some suggestions in recent years that there might be a NOS in or associated with mitochondria. This was originally based on cytochemical and immuno-cytochemical evidence [170–172] showing association of either NADPH diaphorase- or NOS antibody-binding to mitochondrial membranes. More recently still there have been reports of NO synthesis within rat liver mitochondrial preparations, and of functional effects of this on respiration [173,174], as well as of characterization and purification of a NOS from such preparations [175,176]. It is still not absolutely certain, however, that this NOS protein is associated specifically with mitochondria or whether it is bound to or contained within other organelles present in substantial amounts in the mitochondrial fraction, e.g. lysosomes, as is normal for such preparations. While it remains to be formally proven, this NOS seems likely to consist of a membrane-associated iNOS, since it was recognized by antibodies to iNOS but not those to eNOS or nNOS [168]. This is then consistent with much earlier reports of non-cytosolic 'membrane-associated' iNOS in induced macrophages [177,178]. Although iNOS does not have fatty acylation sites like those of eNOS, membrane association via binding to membrane proteins such as the caveolins provide a plausible alternative mechanism for this.

### Osteoarthritic NOS?

Reports on the NOS within the chondrocytes and cartilage of osteoarthritic patients have provided some controversy. The original reports that interleukin-1 induced an NOS presumed that this was induction of iNOS, and indeed iNOS from interleukin-1-exposed human chondrocytes was one of the first to be cloned and sequenced [21]. In contrast, a study of chondrocytes isolated from patients with osteoarthritis [179] suggested that the osteoarthritic NOS ('OA-NOS') expressed in these cells resembled nNOS rather than iNOS, both from its immuno-reactivity and from its apparent molecular mass, and might represent a novel form. More recently, studies of human osteoarthritic cartilage both by immuno-histochemistry and NO synthesis, as assessed pharmacologically in primary culture with highly-selective iNOS inhibitors, has again, however, implicated iNOS [180,180a]. Thus the data on balance suggest that iNOS, rather than a novel osteoarthritic NOS or nNOS, is responsible for the increased NO synthesis within osteoarthritic tissue.

### Guinea-pig iNOS

The guinea pig homologue of human and rodent iNOS has recently been cloned and expressed, with some surprises resulting from the analysis of its expression *in vivo* and properties *in vitro* [181]. The guinea pig enzyme was closest to human and mouse iNOS in sequence (80 % and 79 % identity) and less similar to human nNOS (37 % identity) or eNOS (24 % identity), and it was potently inhibited by the highly-selective iNOS inhibitor *N*-[3-(aminomethyl)benzyl]acetamidine (1400W; see below). For rodent and human iNOS, calcium-independence of activity has been considered to be a defining characteristic which distinguishes this isoform from nNOS and eNOS. However, the distinction was much less clear for the guinea pig iNOS, since its calcium-dependence was intermediate between that of rat nNOS and human iNOS. Such behaviour has been reported for mutant versions of eNOS and nNOS lacking the auto-inhibitory loop described above [120–122]. The biochemical basis and physiological significance of this behaviour of guinea pig iNOS is at present unclear.

### Distinguishing the NOS isoforms

Unfortunately this has become in many ways less clear-cut since 1994. At that time it appeared that the three NOSs were distinguishable on the basis of several characteristics.

(1) Constitutive (eNOS, nNOS) versus inducible (iNOS) expression. It is now clear that all three can be induced, albeit by different stimuli, and all three can be constitutively expressed in some cells or tissues [114,115,182,183].

(2) Calcium dependence (eNOS, nNOS) versus independence (iNOS). This still holds for isolated human and rodent isoforms, although it is not so clear-cut in the guinea pig (see above) and is not known for many other species.

(3) Cytosolic (iNOS, nNOS) versus particulate or mixed (eNOS) cellular location. It is now clear that all three can have either cytosolic or particulate locations, with protein–protein interactions playing an important role (see above).

(4) Subunit molecular mass of $\approx 160$ kDa (nNOS) versus $\approx 130$ kDa (eNOS, iNOS). Again, as described above, this is only true for the major splice variant nNOS$\alpha$ ($\approx 160$ kDa) but not for nNOS$\beta$ and nNOS$\gamma$ ($\approx 130$ kDa).

## NOS INHIBITORS

There are a bewildering array of NOS inhibitors described in the literature and in use as pharmacological tools. Of these the most widely used have been L-NMMA, L-NNA and its methyl ester prodrug ($N^G$-nitro-L-arginine methyl ester, 'L-NAME') and aminoguanidine. Table 4 shows the efficacy of some of these in inhibiting the three human NOS isoforms. In this section we will describe some of the pitfalls in the published research on selective NOS inhibitors, the types of interaction of different inhibitors, as well as describing some of the recently discovered selective inhibitors of the iNOS and nNOS isoforms.

### Selective NOS inhibitor pitfalls

#### Definitions of selectivity

Unfortunately, the literature on NOS inhibitors has many misleading statements and claims about the selectivity or otherwise of many of these inhibitors. This has been a consequence of differing criteria for what constitutes selectivity (does 'selective' imply 2-fold, 10-fold or 100-fold?), and how selectivity is defined (ratio of $IC_{50}$ values at constant substrate concentration or ratio of $K_i$ values or ratio of pharmacologically-effective doses) or determined (effects on isolated enzymes or in cells/tissues or *in vivo* pharmacological properties). For the purposes of this review we define of levels of selectivity on pragmatic grounds, and primarily on the basis of relating their potency under identical conditions in the physiological range (L-arginine concentration etc). Inhibitors with less than 10-fold selectivity are unlikely to be useful as selective agents because of the difficulties inherent in attempting to use them in such a way that only one isoform is affected, and should be regarded as non-selective. Agents which have 10-50-fold selectivity can be useful as 'partially selective' inhibitors, as long as great care is taken over the necessary

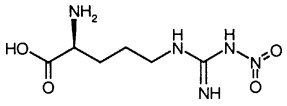
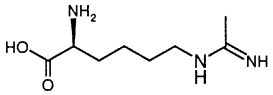
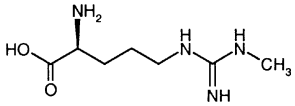
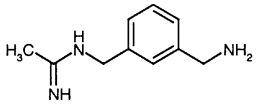
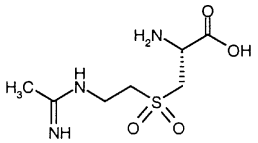
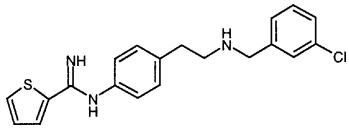
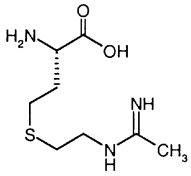
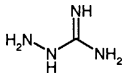
**Table 4 Selectivity (a) and structures (b) of inhibitors of NOSs**

The data shown are for inhibition of the human NOS isoforms in the presence of 30 $\mu$M L-arginine at 37 °C over 15 min after a 15 min pre-incubation with inhibitor under turnover [228]. This permits the contribution of progressive inhibitory mechanisms to be at least partially reflected by the assay. Data are from Young et al. [229] except for * (J. Dawson and R. G. Knowles, unpublished work). The human NOS isoforms were expressed in the baculovirus expression system, and cell lysates (after treating with Dowex ion-exchange resin to remove endogenous arginine) were used as the enzyme source. †, iNOS potency and selectivity known to be underestimates for these compounds because of the progressive inhibition of iNOS but not eNOS or nNOS; e.g. for 1400W the steady-state values of iNOS $IC_{50}$ and selectivity have been estimated to be $< 0.1$ $\mu$M, $>$ 250-fold (versus nNOS) and $>$ 5000-fold (versus eNOS) [203]. ‡, Partially-selective, 10–50-fold; §, highly-selective, $>$ 50-fold.

(a)

| | $IC_{50}$ ($\mu$M) | | | Selectivity (fold) | | |
|---|---|---|---|---|---|---|
| Inhibitor | iNOS | nNOS | eNOS | iNOS versus nNOS | iNOS versus eNOS | nNOS versus eNOS |
| L-NNA* | 3.1 | 0.29 | 0.35 | 0.09 | 0.11 | 1.2 |
| L-NMMA | 6.6 | 4.9 | 3.5 | 0.7 | 0.5 | 0.7 |
| 7-NI* | 9.7 | 8.3 | 11.8 | 0.9 | 1.2 | 1.4 |
| ARL 17477* | 0.33 | 0.07 | 1.6 | 0.2 | 5 | 23‡ |
| Aminoguanidine* | 31 | 170 | 330 | 5.5 | 11‡ | 1.9 |
| L-NIL | 1.6 | 37 | 49 | 23‡ | 49‡ | 1.3 |
| 1400W | 0.23† | 7.3 | 1000 | 32†‡ | $>$ 4000†§ | $>$ 130§ |
| GW273629 | 8.0† | 630 | 1000 | 78†§ | $>$ 125†§ | $>$ 1.6 |
| GW274150 | 1.4† | 145 | 466 | 104†§ | 333†§ | 3.2 |

(b)

| Inhibitor | Structure | Inhibitor | Structure |
|---|---|---|---|
| L-NNA |  | L-NIL |  |
| L-NMMA |  | 1400W |  |
| 7-NI |  | GW273629 |  |
| ARL 17477 |  | GW274150 |  |
| Aminoguanidine |  | | |

controls and concentrations/doses used. With compounds of over 50- or 100-fold selectivity, inhibitors become much simpler to use to inhibit the activity of a single isoform without affecting others, and also start to have potential as selective therapeutic agents without the potential for side effects that might arise from inhibiting the other isoforms. Since the literature has used 'selective' rather indiscriminately, we perhaps need to refer to such inhibitors as 'highly selective' in order to differentiate them.

### Apparent *in vivo* selectivity

Several *in vivo* pharmacological effects of NOS inhibition have been associated with the functioning of one or other of the three isoforms, and effects or lack of them on these is sometimes used to infer isoform selectivity of action. The classic example is that when the activity of eNOS expressed in vascular endothelial cells is inhibited, resulting in decreased release of NO as a mediator of the relaxation of vascular smooth muscle, leading to constriction, and increased vascular resistance, and thence to increases in blood pressure. However, it is prudent to only refer to inhibitors as being selective for a particular NOS isoform if selectivity of effect has been shown at the isolated enzyme level, as well as (ideally) on pharmacological effects in cells or tissues and *in vivo*. Thus the compound 7-nitroindazole (7-NI) should not be described as a selective nNOS inhibitor, since at the isolated enzyme level it demonstrates no selectivity at all (Table 4). The

confusion has arisen because of the interesting pharmacology of this compound, inhibiting some processes inferred to be nNOS-dependent without causing increases in blood pressure, thought to be eNOS-dependent. Since 7-NI is not actually nNOS-selective this is presumably because of some other explanation, e.g. cell-type specificity of effect (neuronal verus endothelial), perhaps depending on intracellular $BH_4$ concentration, or depending on specific cellular transport or metabolism (for recent discussion of this see [184]). Since all three NOS isoforms can be expressed in neurons and both eNOS and iNOS in endothelial cells, cell-type specificity is clearly a very distinct phenomenon from isoform selectivity.

A related pitfall is that of inferring selectivity against eNOS from a lack of significant effect on blood pressure at a particular dose. This is inappropriate because increasing blood pressure with NOS inhibitors appears to require significantly higher doses than for some other effects. This is not surprising given the tight homeostatic control on blood pressure. For example, in humans, the non-selective NOS inhibitor L-NMMA causes a five-fold increase in vascular resistance with only a 10% change in blood pressure, because of reflex decreases in cardiac output [185–187]. The dose of L-NMMA required for 50% of the maximum effect on blood pressure in rodents is 17–30 mg/kg [188,189], whereas it has powerful effects (attributable to constitutive NOS as well as iNOS) on vascular leakage in response to endotoxin which are maximal at 5 mg/kg [190]. Thus it is not valid to carry out 'apples versus pears' comparisons of blood pressure effects with e.g. suppression of endotoxin-induced plasma nitrate (thought to be mediated predominantly by iNOS) and to infer selectivity from them. Over-reliance on the absence of effects on blood pressure has led to inhibitors being described as being selective which have no useful degree of selectivity, e.g. *S*-ethylisothiourea [44].

### Selectivity against only one other target

Another source of confusion is defining an inhibitor as selective for, e.g. iNOS versus eNOS, and then ignoring its non-selectivity for nNOS or completely distinct enzyme targets. Examples include ARL 17477 (*N*-[4-(2-{[(3-chlorophenyl)methyl]amino}-ethyl)phenyl]-2-thiophenecarboximide dihydrochloride) (see below) and aminoguanidine. Whereas aminoguanidine has partial selectivity for iNOS versus eNOS ($\approx$ 10-fold, Table 4), the selectivity over nNOS is minimal. It has a wide range of other effects, inhibiting advanced glycosylation end-product formation, diamine oxidase and polyamine metabolism [191–193] and catalase [194] and having anti-oxidant effects [195,196]. Depending on your temperament it is either distressing or funny that different papers describe aminoguanidine as a 'selective iNOS inhibitor', a 'specific inhibitor of diamine oxidase' or 'an inhibitor of advanced glycosylation end-product formation', frequently without mentioning any of its other targets! Clearly aminoguanidine should not be being described as a selective inhibitor. In the more subtle instances of agents which have selectivity versus only one NOS isoform, this needs to be made explicit when describing and using them.

### Time-dependence of inhibition

Having established such pragmatic definitions of selectivity, one final difficulty that arises in assessing efficacy and selectivity of NOS inhibitors, and of comparing such data from different groups in the literature, is the frequent finding of time-dependent inhibition which may vary significantly between isoforms (for examples see below and review by Bryk and Wolff [197]). This means that the different potencies and selectivities will be observed depending on the protocol of the assay, especially the time of exposure to the inhibitor. Screens for NOS inhibitors have often used assays over short periods (0–10 min or 0–15 min) with no pre-incubation, and this can lead to a very significant under-estimation of the potency of slow-onset inhibitors. Since exposure times *in vivo* in experimental or clinical use would be likely to be $> 30$ min it is crucial that such time-dependence is assessed in order to predict the potency and selectivity of inhibitors in this context. Pre-incubation of inhibitor with enzyme and substrates (e.g. for 15 min as for the data in Table 4) gives a much better prediction of the efficacy and selectivity in intact tissues and *in vivo* (although it may still underestimate the steady-state potency of slow-onset inhibitors), whilst full analysis of the time course of inhibition permits estimation of the true steady-state potency and selectivity.

## NOS inhibitor interactions with the NOS enzymes

Inhibitors of NOS have been described which interact with the NOS enzymes in a variety of ways: different sites, as well as differing time- and substrate-dependence, and mechanism of inhibition.

### L-Arginine site

Most inhibitors identified so far are competitive with the substrate L-arginine and have therefore been inferred to be binding at the arginine-binding site. In the instances in which data on NOS haem-domain crystal structures have been published with such inhibitors (aminoguanidine, *S*-ethylisothiourea, thiocitrulline), they do indeed bind in the active site, interacting with the conserved glutamate ($Glu^{363}$ of bovine eNOS, $Glu^{371}$ of murine iNOS) which also interacts with the guanidino group of L-arginine [31,32,35].

However, with many arginine-site NOS inhibitors there are mechanisms involved in their effects on NOS beyond simple binding in competition with L-arginine. A substantial body of work has been published on the mechanisms of iNOS inhibition by aminoguanidine [197–199] and by the acetamidine inhibitors, $N^5$-iminoethyl-L-ornithine (L-NIO) and $N^6$-iminoethyl-L-lysine (L-NIL) [200–203], GW273629 (*S*-[2-[(1-iminoethyl)-amino]ethyl]-4,4-dioxo-L-cysteine) and GW274150 (*S*-[2-[(1-iminoethyl)amino]ethyl]-L-homocysteine) [204,205] (see Table 4). These are all mechanism-based inhibitors of iNOS, requiring active enzyme and NADPH substrate to permit inhibition to proceed from the initial relatively weak binding to the enzyme:

$$E + I \leftrightarrow EI$$

to potent inhibition/inactivation:

$$EI \rightarrow EI^*$$

where E is iNOS, I is the inhibitor, EI is the initial non-covalent complex and EI* is a modified complex, either with a conformational change to tight binding or with covalent changes to the enzyme, inhibitor or both. In the case of aminoguanidine this appears to occur through multiple pathways of covalent modification of the enzyme [206], consistent with the promiscuity of this compound in interacting with many targets (see above). This is not the case with the acetamidines, which proceed from initial binding ($K_i$ 2–20 $\mu$M) to potent inhibition without incorporation of radiolabelled inhibitor into protein. Work with both L-NIO and L-NIL suggest that this may involve reaction with and loss of haem, perhaps by forming an unstable haem adduct which breaks down to biliverdin, inactive iNOS and (via the unstable carboxy-acetamidine) the original acetamidine [206]:

$$E + I \leftrightarrow EI \rightarrow EI^* \rightarrow E^* + I$$

where EI is the initial non-covalent complex, EI* is the unstable haem adduct and E* is inactive enzyme without intact haem cofactor.

### Biopterin site

There is also significant interest in inhibitors of NOS which act at its $BH_4$-binding site; this is located adjacent to the arginine-binding region and the haem cofactor (see Figure 2). A range of compounds have been identified which appear to interact with the pterin site as assessed by a range of criteria, e.g. mutual antagonism with $BH_4$ on activity, competition for binding of radiolabelled $BH_4$ to full-length or haem domains of NOS. Some are obvious pterin analogues, e.g. 4-amino-$BH_4$, $BH_2$ and a range of synthetic analogues thereof, whereas others are not, e.g. 7-NI, 2,4-diamino-5-(3′,4′dichlorophenyl)pyrimidine (11U50) [85,207,208]. The interactions of some of these pterin-site inhibitors with NOS reveals unexpected complexity. For example, some exhibit partial inhibition whilst others fully inhibit [207,208]. As another example, 7-NI competes with the binding of both [$^3$H]nitro-L-arginine and $B^3H_4$ to nNOS haem domains, whereas the arginine-site inhibitor *S*-ethylisothiourea inhibits the binding of [$^3$H]nitro-L-arginine but promotes the binding of $B^3H_4$ [85], making it clear that there are allosteric interactions between the two binding sites.

### Haem-binding inhibitors

A further group of compounds interact directly with the haem. In monomeric murine-iNOS haem domain crystallized in the presence of the inhibitor imidazole, two imidazole molecules have been shown to be bound to each monomer, one to the haem iron and one to the arginine-binding region ($Glu^{371}$) [31]. Various anti-fungal imidazoles have been shown to inhibit NOS activity, not only by interacting with the haem at the active site but also by acting in competition with CaM [209]; these compounds have also been shown to affect the assembly of iNOS monomers into active dimer, either promoting or inhibiting dimerization [50]. Most recently, a class of substituted pyrimidine imidazoles have been identified which do not directly inhibit NOS activity, but very potently inhibit dimerization of iNOS during its synthesis and assembly [52]. An example was shown from the crystal structure of its complex with monomeric iNOS haem domain to be ligated to the haem iron of iNOS.

### Flavoprotein and CaM inhibitors

A range of inhibitors which have effects on a variety of either flavoproteins (e.g. diphenyleneiodonium) or CaM (e.g. trifluoperazine) have been shown to inhibit NOS (see [2] for references). At present these would not appear to be a promising avenue for the discovery of selective inhibitors of NOSs.

## Partially and highly isoform-selective NOS inhibitors

Identification of selective inhibitors of iNOS and nNOS has been a goal of both academic and pharmaceutical scientists for some years now. The observation of very high structural homology between the haem domains of iNOS and eNOS [33] has led to 'retrospective pessimism' about whether selective inhibition of iNOS is feasible [43]. Given that several inhibitors of $>$ 100-fold selectivity for iNOS versus eNOS have already been identified (see below), this pessimism would seem to be somewhat inappropriate. A large number of patents claiming a range of structural classes as selective NOS inhibitors have been published [210,211]. Table 4 shows inhibition data for a range of compounds for the three human isoforms under identical conditions.

### Partially-selective nNOS inhibitors

In the search for selective nNOS inhibitors [212], some amino acids have been described which are partially selective for nNOS versus eNOS and iNOS. For example, *S*-ethyl- and *S*-methyl-L-thiocitrulline [213,214] and vinyl-L-NIO [215] all show time-dependent inhibition of nNOS with significant selectivities versus isolated eNOS and iNOS enzymes. However, *S*-ethyl- and *S*-methyl-L-thiocitrulline appeared less selective in intact rat tissues and *in vivo*, raising questions over their usefulness as pharmacological tools. The non-amino acid ARL 17477 has been reported to be both a selective nNOS inhibitor *in vitro* and effective *in vivo* in animal models of brain damage in stroke [216,217]; however, these papers do not report whether this compound inhibits iNOS. The data in Table 4 suggest that this compound is partially selective for nNOS versus eNOS (23-fold) but only 5-fold versus iNOS, so that 'selective' in this context is primarily relative to eNOS only. Given the potential role of iNOS in stroke [218,219] this may be significant.

### Partially-selective iNOS inhibitors

The acetamidine-containing analogues of arginine, L-NIO and L-NIL (Table 4), discussed above in terms of their mechanism, have been known for some time to be partially selective inhibitors of iNOS versus eNOS and nNOS [200,220]. They are both 30–50-fold selective for iNOS versus eNOS, and $\approx$ 20-fold versus nNOS, with no other known pharmacological actions apart from competition with L-arginine for cellular uptake. They have been widely used to probe the effects of iNOS inhibition, but caution is necessary in attributing the effects observed purely to iNOS inhibition. The relatively stringent selectivity test of comparing the effects of L-NIO or aminoguanidine on early exacerbation of endotoxin-provoked vascular leakage (an effect of eNOS/nNOS inhibition) and on the later suppression (an effect of iNOS inhibition) show that neither of these agents is acting selectively at the doses used (15–50 mg/kg) [221].

More recently, compounds have been described that have high selectivity for iNOS versus eNOS, while not being very selective against nNOS. For example, some 2-iminohomopiperidines and 2-iminopyrrolidines with high (100–900-fold) selectivity for iNOS versus eNOS, but similar potency on iNOS and nNOS (1–13-fold selectivity), have been reported [222,223], such that these arguably class as dual action iNOS/nNOS inhibitors. It will be very interesting to contrast the pharmacology of such dual action inhibitors with highly selective iNOS or nNOS inhibitors.

### Highly-selective iNOS inhibitors

The first 'highly selective' iNOS inhibitors versus eNOS were the bis-isothioureas reported by Garvey et al. [44]. Of these, *S*,*S*′-[1,3-phenylenebis-(1,2-ethanediyl)]bisisothiourea ('PBITU') is an L-arginine-competitive, rapidly reversible inhibitor of human iNOS with a $K_i$ of 47 nM, and a selectivity (in $K_i$ terms) of 190-fold versus eNOS, although it is only $\approx$ 5-fold selective versus nNOS ($K_i$ 250 nM). This compound is a clear demonstration that the substrate-binding sites of full-length human iNOS and eNOS in solution are in fact significantly different from each other, and poses a challenge to the structural biologists to explain the basis of this. It may be revealing to study NOS crystal structures with this compound bound. Unfortunately, the utility of this series of compounds as pharmacological agents is

limited by the poor cellular and tissue penetration of the more selective compounds, as well as by significant acute toxicity.

1400W (Table 4), also identified by Garvey et al. [203], has proved to be a further step forward, since it is not only highly selective as an iNOS inhibitor versus both eNOS and nNOS, but also penetrates cells and tissues. Inhibition of human iNOS by 1400W was competitive with L-arginine, NADPH-dependent and developed relatively slowly, with a maximal rate constant of $0.028\ s^{-1}$, and no significant reversal of this inhibition was observed after 2 h. Thus it is either an irreversible, or only very slowly reversible, mechanism-based inhibitor with a $K_d$ value $\leqslant 7$ nM and steady-state selectivity against eNOS and nNOS of $> 5000$-fold and $> 250$-fold respectively. Efficacy and selectivity were maintained *in vivo* in the stringent test of its differential effects on vascular leakage; unlike L-NIO and aminoguanidine it suppressed the late, iNOS-driven phase of endotoxin-provoked leakage ($ED_{50} < 1$ mg/kg) with no exacerbation of the early phase as is seen when eNOS and nNOS are inhibited [203,221]. 1400W does exhibit an acute toxicity at high doses, which is likely to prevent its safe therapeutic use in humans, but there is a significant therapeutic window such that it can be used as a pharmacological tool in a variety of animal models [203,221,224–228].

GW273629 and GW274150 are two novel NOS inhibitors that have been identified from a series of acetamidine amino acids which, like 1400W, have a very high degree of selectivity for iNOS versus both eNOS and nNOS (Table 4) [229]. Both are sulphur-substituted acetamidine amino acids (Table 4) acting in competition with L-arginine. Like with 1400W the inhibition of human iNOS is NADPH-dependent and develops relatively slowly, whereas the inhibition of human eNOS and nNOS is rapidly reversible. Again like 1400W this efficacy and selectivity is maintained in intact cells and tissues, but there was no indication of the acute toxicity seen with 1400W (and several other non-amino acid NOS inhibitors). GW273629 and GW274150 have been used to probe the roles of iNOS in several animal models of diseases in which iNOS has been implicated [204,205].

The haem-binding substituted pyrimidine imidazoles discussed in the previous section are not direct inhibitors, but instead inhibit assembly of active dimeric iNOS during its synthesis. Because of this, it is not straightforward to determine their isoform selectivity, but studies with transient transfection of the three isoforms suggested that high selectivity was achievable (one compound being 1000-fold less potent on eNOS than on iNOS) [52]. It will be interesting to see what the pharmacology and utility of such compounds will be, and whether other compound series are discovered with this unusual mechanism of action.

## PERSPECTIVES FOR THE FUTURE

A decade of study of the NO synthases has brought us a long way in understanding the structure, function and inhibition of this family of isoenzymes, but we still have some important outstanding questions:

- What is the three-dimensional structure of the reductase domain and of the full-length enzyme, and thus how do the haem and reductase domains interact?
- How does the three-dimensional structure of the haem domain of nNOS compare with those of iNOS and eNOS?
- Does the distribution between monomer and dimer vary in cells or *in vivo*, and if so what is its significance in the regulation of the three NOS isoforms?
- What are the RNS directly produced from L-arginine by NOS (NO, nitroxyl, peroxynitrite, or all three) and the stoichiometry of the reaction?
- What is the mechanism of NO formation from NHA (the monooxygenase II reaction)?
- What are the key roles played by the pterin cofactor, and is it redox cycling between $BH_4$ and $BH_3$ during the NOS reaction?
- Which post-translational modifications are significant in the regulation of the three isoforms?
- What is the significance and basis of the sub-cellular localization of the NOSs?
- Which cells and tissues express splice variants of the NOS isoforms and what role(s) do these play?
- What is the molecular and structural basis of the high isoform selectivity of some NOS inhibitors?
- Will selective iNOS, nNOS or dual iNOS + nNOS inhibitors prove to be of value in the treatment of human diseases, and if so, which diseases, and what side-effects might result?

If research on NOSs continues for another decade at anything like the current level, then it will surely have answers for all of these questions, and may well have resulted in the development of new medicines to fight disease.

We would like to acknowledge GlaxoSmithKline scientists Norman Gray for help with preparation of Figure 2 and John Dawson for the unpublished data in Table 4. C. E. C. would also like to thank Diana Nicol, Gary Silkstone and Mike Wilson (University of Essex) for helpful comments.

## REFERENCES

1 Anon (1999) Citation Superstars of the UK. Science Watch **10**, 1–2
2 Knowles, R. G. and Moncada, S. (1994) Nitric oxide synthases in mammals. Biochem. J. **298**, 249–258
3 Marletta, M. A. (1994) Nitric oxide synthase: aspects concerning structure and catalysis. Cell (Cambridge, Mass.) **78**, 927–930
4 Nathan, C. and Xie, Q. W. (1994) Nitric oxide synthases: roles, tolls, and controls. Cell (Cambridge, Mass.) **78**, 915–918
5 Feelisch, M. and Stamler, J. S. (eds) (1996) Methods in Nitric Oxide Research, John Wiley, Chichester
6 Packer, L. (ed.) (1996) Nitric Oxide Part B Physiological and Pathological Processes. Methods Enzymol. **269**
7 Packer, L. (ed.) (1996) Nitric Oxide Part A Sources and detection of NO; NO synthase. Methods Enzymol. **268**
8 Titheradge, M. A. (ed.) (1998) Nitric Oxide Protocols. Methods Mol. Biol. **100**
9 Moncada, S. and Higgs, A. (1993) The L-arginine-nitric oxide pathway. N. Engl. J. Med. **329**, 2002–2012
10 Kroncke, K. D., Fehsel, K. and Kolb-Bachofen, V. (1998) Inducible nitric oxide synthase in human diseases. Clin. Exp. Immunol. **113**, 147–156
11 Hobbs, A. J., Higgs, A. and Moncada, S. (1999) Inhibition of nitric oxide synthase as a potential therapeutic target. Annu. Rev. Pharmacol. Toxicol. **39**, 191–220
12 Muscara, M. N. and Wallace, J. L. (1999) Nitric oxide. V. Therapeutic potential of nitric oxide donors and inhibitors. Am. J. Physiol. **276**, G1313–G1316
13 Feelisch, M. (1998) The use of nitric oxide donors in pharmacological studies. Naunyn-Schmiedebergs Arch. Pharmacol. **358**, 113–122
14 Cutruzzola, F. (1999) Bacterial nitric oxide synthesis. Biochim. Biophys. Acta **1411**, 231–249
15 Watmough, N. J., Butland, G., Cheesman, M. R., Moir, J. W., Richardson, D. J. and Spiro, S. (1999) Nitric oxide in bacteria: synthesis and consumption. Biochim. Biophys. Acta **1411**, 456–474
16 Millar, T. M., Stevens, C. R., Benjamin, N., Eisenthal, R., Harrison, R. and Blake, D. R. (1998) Xanthine oxidoreductase catalyses the reduction of nitrates and nitrite to nitric oxide under hypoxic conditions. FEBS Lett. **427**, 225–228
17 Nakane, M., Schmidt, H. H., Pollock, J. S., Forstermann, U. and Murad, F. (1993) Cloned human brain nitric oxide synthase is highly expressed in skeletal muscle. FEBS Lett. **316**, 175–180
18 Hall, A. V., Antoniou, H., Wang, Y., Cheung, A. H., Arbus, A. M., Olson, S. L., Lu, W. C., Kau, C. L. and Marsden, P. A. (1994) Structural organization of the human neuronal nitric oxide synthase gene (NOS1). J. Biol. Chem. **269**, 33082–33090
19 Geller, D. A., Lowenstein, C. J., Shapiro, R. A., Nussler, A. K., Di Silvio, M., Wang, S. C., Nakayama, D. K., Simmons, R. L., Snyder, S. H. and Billiar, T. R. (1993) Molecular cloning and expression of inducible nitric oxide synthase from human hepatocytes. Proc. Natl. Acad. Sci. U.S.A. **90**, 3491–3495

20 Sherman, P. A., Laubach, V. E., Reep, B. R. and Wood, E. R. (1993) Purification and cDNA sequence of an inducible nitric oxide synthase from a human tumor cell line. Biochemistry **32**, 11600–11605

21 Charles, I. G., Palmer, R. M., Hickery, M. S., Bayliss, M. T., Chubb, A. P., Hall, V. S., Moss, D. W. and Moncada, S. (1993) Cloning, characterization and expression of a cDNA encoding an inducible nitric oxide synthase from the human chondrocyte. Proc. Natl. Acad. Sci. U.S.A. **90**, 11419–11423

22 Janssens, S. P., Shimouchi, A., Quertermous, T., Bloch, D. B. and Bloch, K. D. (1992) Cloning and expression of a cDNA encoding human endothelium-derived relaxing factor/nitric oxide synthase. J. Biol. Chem. **267**, 14519–14522

23 Marsden, P. A., Schappert, K. T., Chen, H. S., Flowers, M., Sundell, C. L., Wilcox, J. N., Lamas, S. and Michel, T. (1992) Molecular cloning and characterization of human endothelial nitric oxide synthase. FEBS Lett. **307**, 287–293

24 McMillan, K. and Masters, B. S. (1995) Prokaryotic expression of the heme- and flavin-binding domains of rat neuronal nitric oxide synthase as distinct polypeptides: identification of the heme-binding proximal thiolate ligand as cysteine-415. Biochemistry **34**, 3686–3693

25 Richards, M. K. and Marletta, M. A. (1994) Characterization of neuronal nitric oxide synthase and a C415H mutant, purified from a baculovirus overexpression system. Biochemistry **33**, 14723–14732

26 Ghosh, D. K. and Stuehr, D. J. (1995) Macrophage NO synthase: characterization of isolated oxygenase and reductase domains reveals a head-to-head subunit interaction. Biochemistry **34**, 801–807

27 Sheta, E. A., McMillan, K. and Masters, B. S. (1994) Evidence for a bidomain structure of constitutive cerebellar nitric oxide synthase. J. Biol. Chem. **269**, 15147–15153

28 Lowe, P. N., Smith, D., Stammers, D. K., Riveros-Moreno, V., Moncada, S., Charles, I. and Boyhan, A. (1996) Identification of the domains of neuronal nitric oxide synthase by limited proteolysis. Biochem. J. **314**, 55–62

29 Chen, P. F., Tsai, A. L., Berka, V. and Wu, K. K. (1996) Endothelial nitric-oxide synthase. Evidence for bidomain structure and successful reconstitution of catalytic activity from two separate domains generated by a baculovirus expression system. J. Biol. Chem. **271**, 14631–14635

30 Ghosh, D. K., Abu-Soud, H. M. and Stuehr, D. J. (1995) Reconstitution of the second step in NO synthesis using the isolated oxygenase and reductase domains of macrophage NO synthase. Biochemistry **34**, 11316–11320

31 Crane, B. R., Arvai, A. S., Gachhui, R., Wu, C., Ghosh, D. K., Getzoff, E. D., Stuehr, D. J. and Tainer, J. A. (1997) The structure of nitric oxide synthase oxygenase domain and inhibitor complexes. Science **278**, 425–431

32 Crane, B. R., Arvai, A. S., Ghosh, D. K., Wu, C., Getzoff, E. D., Stuehr, D. J. and Tainer, J. A. (1998) Structure of nitric oxide synthase oxygenase dimer with pterin and substrate. Science **279**, 2121–2126

33 Fischmann, T. O., Hruza, A., Niu, X. D., Fossetta, J. D., Lunn, C. A., Dolphin, E., Prongay, A. J., Reichert, P., Lundell, D. J., Narula, S. K. and Weber, P. C. (1999) Structural characterization of nitric oxide synthase isoforms reveals striking active-site conservation. Nat. Struct. Biol. **6**, 233–242

34 Li, H., Raman, C. S., Glaser, C. B., Blasko, E., Young, T. A., Parkinson, J. F., Whitlow, M. and Poulos, T. L. (1999) Crystal structures of zinc-free and -bound heme domain of human inducible nitric-oxide synthase. Implications for dimer stability and comparison with endothelial nitric-oxide synthase. J. Biol. Chem. **274**, 21276–21284

35 Raman, C. S., Li, H., Martasek, P., Kral, V., Masters, B. S. and Poulos, T. L. (1998) Crystal structure of constitutive endothelial nitric oxide synthase: a paradigm for pterin function involving a novel metal center. Cell (Cambridge, Mass.) **95**, 939–950

36 Cubberley, R. R., Alderton, W. K., Boyhan, A., Charles, I. G., Lowe, P. N. and Old, R. W. (1997) Cysteine-200 of human inducible nitric oxide synthase is essential for dimerization of haem domains and for binding of haem, nitroarginine and tetrahydrobiopterin. Biochem. J. **323**, 141–146

37 Chen, P. F., Tsai, A. L., Berka, V. and Wu, K. K. (1997) Mutation of Glu-361 in human endothelial nitric-oxide synthase selectively abolishes L-arginine binding without perturbing the behavior of heme and other redox centers. J. Biol. Chem. **272**, 6114–6118

38 Gachhui, R., Ghosh, D. K., Wu, C., Parkinson, J., Crane, B. R. and Stuehr, D. J. (1997) Mutagenesis of acidic residues in the oxygenase domain of inducible nitric-oxide synthase identifies a glutamate involved in arginine binding. Biochemistry **36**, 5097–5103

39 Crane, B. R., Arvai, A. S., Ghosh, S., Getzoff, E. D., Stuehr, D. J. and Tainer, J. A. (2000) Structures of the *N*(omega)-hydroxy-L-arginine complex of inducible nitric oxide synthase oxygenase dimer with active and inactive pterins. Biochemistry **39**, 4608–4621

40 Ghosh, S., Wolan, D., Adak, S., Crane, B. R., Kwon, N. S., Tainer, J. A., Getzoff, E. D. and Stuehr, D. J. (1999) Mutational analysis of the tetrahydrobiopterin-binding site in inducible nitric-oxide synthase. J. Biol. Chem. **274**, 24100–24112

41 Chen, P. F., Tsai, A. L. and Wu, K. K. (1995) Cysteine 99 of endothelial nitric oxide synthase (NOS-III) is critical for tetrahydrobiopterin-dependent NOS-III stability and activity. Biochem. Biophys. Res. Commun. **215**, 1119–1129

42 Hemmens, B., Goessler, W., Schmidt, K. and Mayer, B. (2000) Role of bound zinc in dimer stabilization but not enzyme activity of neuronal nitric-oxide synthase. J. Biol. Chem. **275**, 35786–35791

43 Anon (1999) No news is not necessarily good. Nat. Struct. Biol. (Editorial) **6**, 201–202

44 Garvey, E. P., Oplinger, J. A., Tanoury, G. J., Sherman, P. A., Fowler, M., Marshall, S., Harmon, M. F., Paith, J. E. and Furfine, E. S. (1994) Potent and selective inhibition of human nitric oxide synthases. Inhibition by non-amino acid isothioureas. J. Biol. Chem. **269**, 26669–26676

45 Crane, B. R., Rosenfeld, R. J., Arvai, A. S., Ghosh, D. K., Ghosh, S., Tainer, J. A., Stuehr, D. J. and Getzoff, E. D. (1999) N-terminal domain swapping and metal ion binding in nitric oxide synthase dimerization. EMBO J. **18**, 6271–6281

46 Venema, R. C., Ju, H., Zou, R., Ryan, J. W. and Venema, V. J. (1997) Subunit interactions of endothelial nitric-oxide synthase. Comparisons to the neuronal and inducible nitric-oxide synthase isoforms. J. Biol. Chem. **272**, 1276–1282

47 Masters, B. S., Martasek, P. and Roman, L. J. (1999) Structural variations on a theme of nitric oxide production by three isoforms of nitric oxide synthase. In Pathophysiology and Clinical Applications of Nitric Oxide, Part A (Rubyani, G. M., ed.), pp. 17–37, Harwood Academic Publishers, (Amsterdam)

48 Stuehr, D. J. (1999) Mammalian nitric oxide synthases. Biochim. Biophys. Acta **1411**, 217–230

49 Rusche, K. M., Spiering, M. M. and Marletta, M. A. (1998) Reactions catalyzed by tetrahydrobiopterin-free nitric oxide synthase. Biochemistry **37**, 15503–15512

50 Sennequier, N., Wolan, D. and Stuehr, D. J. (1999) Antifungal imidazoles block assembly of inducible NO synthase into an active dimer. J. Biol. Chem. **274**, 930–938

51 Klatt, P., Schmidt, K., Lehner, D., Glatter, O., Bachinger, H. P. and Mayer, B. (1995) Structural analysis of porcine brain nitric oxide synthase reveals a role for tetrahydrobiopterin and L-arginine in the formation of an SDS-resistant dimer. EMBO J. **14**, 3687–3695

52 McMillan, K., Adler, M., Auld, D. S., Baldwin, J. J., Blasko, E., Browne, L. J., Chelsky, D., Davey, D., Dolle, R. E., Eagen, K. A. et al. (2000) Allosteric inhibitors of inducible nitric oxide synthase dimerization discovered via combinatorial chemistry. Proc. Natl. Acad. Sci. U.S.A. **97**, 1506–1511

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

54 Schmidt, H., Hofmann, H., Schindler, U., Shutenko, Z. S., Cunningham, D. D. and Feelisch, M. (1996) No NO from NO synthase. Proc. Natl. Acad. Sci. U.S.A. **93**, 14492–14497

55 Hobbs, A. J., Fukuto, J. M. and Ignarro, L. J. (1994) Formation of free nitric oxide from L-arginine by nitric oxide synthase: direct enhancement of generation by superoxide dismutase. Proc. Natl. Acad. Sci. U.S.A. **91**, 10992–10996

56 Murphy, M. E. and Sies, H. (1991) Reversible conversion of nitroxyl anion to nitric oxide by superoxide dismutase. Proc. Natl. Acad. Sci. U.S.A. **88**, 10860–10864

57 Koppenol, W. H., Moreno, J. J., Pryor, W. A., Ischiropoulos, H. and Beckman, J. S. (1992) Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. Chem. Res. Toxicol. **5**, 834–842

58 Miller, R. T., Martasek, P., Roman, L. J., Nishimura, J. S. and Masters, B. S. (1997) Involvement of the reductase domain of neuronal nitric oxide synthase in superoxide anion production. Biochemistry **36**, 15277–15284

59 Xia, Y. and Zweier, J. L. (1997) Direct measurement of nitric oxide generation from nitric oxide synthase. Proc. Natl. Acad. Sci. U.S.A. **94**, 12705–12710

60 Vasquez-Vivar, J., Hogg, N., Martasek, P., Karoui, H., Pritchard, K. A. and Kalyanaraman, B. (1999) Tetrahydrobiopterin-dependent inhibition of superoxide generation from neuronal nitric oxide synthase. J. Biol. Chem. **274**, 26736–26742

61 Vasquez-Vivar, J., Kalyanaraman, B., Martasek, P., Hogg, N., Masters, B. S., Karoui, H., Tordo, P. and Pritchard, K. A. (1998) Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. Proc. Natl. Acad. Sci. U.S.A. **95**, 9220–9225

62 Komarov, A. M., Wink, D. A., Feelisch, M. and Schmidt, H. H. (2000) Electron-paramagnetic resonance spectroscopy using N-methyl-D-glucamine dithiocarbamate iron cannot discriminate between nitric oxide and nitroxyl: implications for the detection of reaction products for nitric oxide synthase. Free Radical Biol. Med. **28**, 739–742

63 Pufahl, R. A., Wishnok, J. S. and Marletta, M. A. (1995) Hydrogen peroxide-supported oxidation of NG-hydroxy-L-arginine by nitric oxide synthase. Biochemistry **34**, 1930–1941

64 List, B. M., Klosch, B., Volker, C., Gorren, A. C., Sessa, W. C., Werner, E. R., Kukovetz, W. R., Schmidt, K. and Mayer, B. (1997) Characterization of bovine endothelial nitric oxide synthase as a homodimer with down-regulated uncoupled NADPH oxidase activity: tetrahydrobiopterin binding kinetics and role of haem in dimerization. Biochem. J. **323**, 159–165

65 Stroes, E., Hijmering, M., van Zandvoort, M., Wever, R., Rabelink, T. J. and van Faassen, E. E. (1998) Origin of superoxide production by endothelial nitric oxide synthase. FEBS Lett. **438**, 161–164

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

67 Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A. and Freeman, B. A. (1990) Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc. Natl. Acad. Sci. U.S.A. **87**, 1620–1624

68 Sharpe, M. A. and Cooper, C. E. (1998) Reactions of nitric oxide with mitochondrial cytochrome *c*: a novel mechanism for the formation of nitroxyl anion and peroxynitrite. Biochem. J. **332**, 9–19

69 Polte, T., Abate, A., Dennery, P. A. and Schroder, H. (2000) Heme oxygenase-1 is a cGMP-inducible endothelial protein and mediates the cytoprotective action of nitric oxide. Arterioscler. Thromb., Vasc. Biol. **20**, 1209–1215

70 Wink, D. A., Feelisch, M., Fukuto, J., Chistodoulou, D., Jourd'heuil, D., Grisham, M. B., Vodovotz, Y., Cook, J. A., Krishna, M., DeGraff, W. G. et al. (1998) The cytotoxicity of nitroxyl: possible implications for the pathophysiological role of NO. Arch. Biochem. Biophys. **351**, 66–74

71 Ma, X. L., Gao, F., Liu, G. L., Lopez, B. L., Christopher, T. A., Fukuto, J. M., Wink, D. A. and Feelisch, M. (1999) Opposite effects of nitric oxide and nitroxyl on postischemic myocardial injury. Proc. Natl. Acad. Sci. U.S.A. **96**, 14617–14622

72 Chabrier, P. E., Demerle-Pallardy, C. and Auguet, M. (1999) Nitric oxide synthases: targets for therapeutic strategies in neurological diseases. Cell. Mol. Life Sci. **55**, 1029–1035

73 Heinzel, B., John, M., Klatt, P., Bohme, E. and Mayer, B. (1992) $Ca^{2+}$/calmodulin-dependent formation of hydrogen peroxide by brain nitric oxide synthase. Biochem. J. **281**, 627–630

74 Adak, S., Ghosh, S., Abu-Soud, H. M. and Stuehr, D. J. (1999) Role of reductase domain cluster 1 acidic residues in neuronal nitric-oxide synthase. Characterization of the FMN-free enzyme. J. Biol. Chem. **274**, 22313–22320

75 Matsuda, H. and Iyanagi, T. (1999) Calmodulin activates intramolecular electron transfer between the two flavins of neuronal nitric oxide synthase flavin domain. Biochim. Biophys. Acta **1473**, 345–355

76 Noble, M. A., Munro, A. W., Rivers, S. L., Robledo, L., Daff, S. N., Yellowlees, L. J., Shimizu, T., Sagami, I., Guillemette, J. G. and Chapman, S. K. (1999) Potentiometric analysis of the flavin cofactors of neuronal nitric oxide synthase. Biochemistry **38**, 16413–16418

77 Siddhanta, U., Wu, C., Abu-Soud, H. M., Zhang, J., Ghosh, D. K. and Stuehr, D. J. (1996) Heme iron reduction and catalysis by a nitric oxide synthase heterodimer containing one reductase and two oxygenase domains. J. Biol. Chem. **271**, 7309–7312

78 Pou, S., Pou, W. S., Bredt, D. S., Snyder, S. H. and Rosen, G. M. (1992) Generation of superoxide by purified brain nitric oxide synthase. J. Biol. Chem. **267**, 24173–24176

79 Stuehr, D. J., Cho, H. J., Kwon, N. S., Weise, M. F. and Nathan, C. F. (1991) Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. Proc. Natl. Acad. Sci. U.S.A. **88**, 7773–7777

80 Schmidt, H. H. and Murad, F. (1991) Purification and characterization of a human NO synthase. Biochem. Biophys. Res. Commun. **181**, 1372–1377

81 Baek, K. J., Thiel, B. A., Lucas, S. and Stuehr, D. J. (1993) Macrophage nitric oxide synthase subunits. Purification, characterization, and role of prosthetic groups and substrate in regulating their association into a dimeric enzyme. J. Biol. Chem. **268**, 21120–21129

82 Lee, C. M., Robinson, L. J. and Michel, T. (1995) Oligomerization of endothelial nitric oxide synthase. Evidence for a dominant negative effect of truncation mutants. J. Biol. Chem. **270**, 27403–27406

83 Giovanelli, J., Campos, K. L. and Kaufman, S. (1991) Tetrahydrobiopterin, a cofactor for rat cerebellar nitric oxide synthase, does not function as a reactant in the oxygenation of arginine. Proc. Natl. Acad. Sci. U.S.A. **88**, 7091–7095

84 Klatt, P., Schmid, M., Leopold, E., Schmidt, K., Werner, E. R. and Mayer, B. (1994) The pteridine binding site of brain nitric oxide synthase. Tetrahydrobiopterin binding kinetics, specificity, and allosteric interaction with the substrate domain. J. Biol. Chem. **269**, 13861–13866

85 Alderton, W. K., Boyhan, A. and Lowe, P. N. (1998) Nitroarginine and tetrahydrobiopterin binding to the haem domain of neuronal nitric oxide synthase using a scintillation proximity assay. Biochem. J. **332**, 195–201

86 McMillan, K. and Masters, B. S. S. (1993) Optical difference spectrophotometry as a probe of rat brain nitric oxide synthase heme-substrate interaction. Biochemistry **32**, 9875–9880

87 Wang, J., Stuehr, D. J., Ikeda-Saito, M. and Rousseau, D. L. (1993) Heme coordination and structure of the catalytic site in nitric oxide synthase. J. Biol. Chem. **268**, 22255–22258

88 Wang, J., Stuehr, D. J. and Rousseau, D. L. (1995) Tetrahydrobiopterin-deficient nitric oxide synthase has a modified heme environment and forms a cytochrome P-420 analogue. Biochemistry **34**, 7080–7087

89 Reif, A., Frohlich, L. G., Kotsonis, P., Frey, A., Bommel, H. M., Wink, D. A., Pfleiderer, W. and Schmidt, H. H. (1999) Tetrahydrobiopterin inhibits monomerization and is consumed during catalysis in neuronal NO synthase. J. Biol. Chem. **274**, 24921–24929

90 Bec, N., Gorren, A. C., Voelker, C., Mayer, B. and Lange, R. (1998) Reaction of neuronal nitric-oxide synthase with oxygen at low temperature. Evidence for reductive activation of the oxy-ferrous complex by tetrahydrobiopterin. J. Biol. Chem. **273**, 13502–13508

91 Hurshman, A. R., Krebs, C., Edmondson, D. E., Huynh, B. H. and Marletta, M. A. (1999) Formation of a pterin radical in the reaction of the heme domain of inducible nitric oxide synthase with oxygen. Biochemistry **38**, 15689–15696

92 Witteveen, C. F., Giovanelli, J. and Kaufman, S. (1999) Reactivity of tetrahydrobiopterin bound to nitric-oxide synthase. J. Biol. Chem. **274**, 29755–29762

93 Presta, A., Siddhanta, U., Wu, C. Q., Sennequier, N., Huang, L. X., AbuSoud, H. M., Erzurum, S. and Stuehr, D. J. (1998) Comparative functioning of dihydro- and tetrahydropterins in supporting electron transfer, catalysis and subunit dimerization in inducible nitric oxide synthase. Biochemistry **37**, 298–310

94 Kaufman, S. (1993) The phenylalanine hydroxylating system. Adv. Enzymol. Related Areas Mol. Biol. **67**, 77–264

95 Mayer, B., Wu, C., Gorren, A. C., Pfeiffer, S., Schmidt, K., Clark, P., Stuehr, D. J. and Werner, E. R. (1997) Tetrahydrobiopterin binding to macrophage inducible nitric oxide synthase: heme spin shift and dimer stabilization by the potent pterin antagonist 4-amino-tetrahydrobiopterin. Biochemistry **36**, 8422–8427

96 Pfeiffer, S., Gorren, A. C., Pitters, E., Schmidt, K., Werner, E. R. and Mayer, B. (1997) Allosteric modulation of rat brain nitric oxide synthase by the pterin-site enzyme inhibitor 4-aminotetrahydrobiopterin. Biochem. J. **328**, 349–352

97 Presta, A., Siddhanta, U., Wu, C., Sennequier, N., Huang, L., Abu-Soud, H. M., Erzurum, S. and Stuehr, D. J. (1998) Comparative functioning of dihydro- and tetrahydropterins in supporting electron transfer, catalysis and subunit dimerization in inducible nitric oxide synthase. Biochemistry **37**, 298–310

98 Sono, M., Roach, M. P., Coulter, E. D. and Dawson, J. H. (1996) Heme-containing oxygenases. Chem. Rev. **96**, 2841–2887

99 Poulos, T. L., Li, H. and Raman, C. S. (1999) Heme-mediated oxygen activation in biology: cytochrome *c* oxidase and nitric oxide synthase. Curr. Opin. Chem. Biol. **3**, 131–137

100 Adams, D. R., Brochwicz-Lewinski, M. and Butler, A. R. (1999) Nitric oxide: physiological roles, biosynthesis and medical uses. Fortschr. Chem. Org. Naturst. **76**, 1–211

101 Griffith, O. W. and Stuehr, D. J. (1995) Nitric oxide synthases: properties and catalytic mechanism. Annu. Rev. Physiol. **57**, 707–736

102 Kerwin, J. F., Lancaster, J. R. and Feldman, P. L. (1995) Nitric oxide: a new paradigm for second messengers. J. Med. Chem. **38**, 4343–4362

103 Presta, A., Weber-Main, A. M., Stankovich, M. T. and Stuehr, D. J. (1998) Comparative effects of substrates and pterin cofactor on the heme midpoint potential in inducible and neuronal nitric oxide synthases. J. Am. Chem. Soc. **120**, 9460–9465

104 Abu-Soud, H. M., Gachhui, R., Raushel, F. M. and Stuehr, D. J. (1997) The ferrous-dioxy complex of neuronal nitric oxide synthase. Divergent effects of L-arginine and tetrahydrobiopterin on its stability. J. Biol. Chem. **272**, 17349–17353

105 Boggs, S., Huang, L. and Stuehr, D. J. (2000) Formation and reactions of the heme-dioxygen intermediate in the first and second steps of nitric oxide synthesis as studied by stopped-flow spectroscopy under single-turnover conditions. Biochemistry **39**, 2332–2339

106 Korth, H. G., Sustmann, R., Thater, C., Butler, A. R. and Ingold, K. U. (1994) On the mechanism of the nitric oxide synthase-catalyzed conversion of *N*omega-hydroxyl-L-arginine to citrulline and nitric oxide. J. Biol. Chem. **269**, 17776–17779

107 Clague, M. J., Wishnok, J. S. and Marletta, M. A. (1997) Formation of N delta-cyanoornithine from NG-hydroxy-L-arginine and hydrogen peroxide by neuronal nitric oxide synthase: implications for mechanism. Biochemistry **36**, 14465–14473

108 Cooper, C. E. (1999) Nitric oxide and iron proteins. Biochim. Biophys. Acta **1411**, 290–309

109 Hurshman, A. R. and Marletta, M. A. (1995) Nitric oxide complexes of inducible nitric oxide synthase: spectral characterization and effect on catalytic activity. Biochemistry **34**, 5627–5634

110 Abu-Soud, H. M., Wang, J., Rousseau, D. L., Fukuto, J. M., Ignarro, L. J. and Stuehr, D. J. (1995) Neuronal nitric oxide synthase self-inactivates by forming a ferrous-nitrosyl complex during aerobic catalysis. J. Biol. Chem. **270**, 22997–23006

111 Santolini, J., Adak, S., Curran, C. M. and Stuehr, D. J. (2001) A kinetic simulation model that describes catalysis and regulation in nitric-oxide synthase. J. Biol. Chem. **276**, 1233–1243

112 Abu-Soud, H. M., Rousseau, D. L. and Stuehr, D. J. (1996) Nitric oxide binding to the heme of neuronal nitric-oxide synthase links its activity to changes in oxygen tension. J. Biol. Chem. **271**, 32515–32518

113 Adak, S., Crooks, C., Wang, Q., Crane, B. R., Tainer, J. A., Getzoff, E. D. and Stuehr, D. J. (1999) Tryptophan 409 controls the activity of neuronal nitric-oxide synthase by regulating nitric oxide feedback inhibition. J. Biol. Chem. **274**, 26907–26911

114 Geller, D. A. and Billiar, T. R. (1998) Molecular biology of nitric oxide synthases. Cancer Metastasis Rev. **17**, 7–23

115 Forstermann, U., Boissel, J. P. and Kleinert, H. (1998) Expressional control of the 'constitutive' isoforms of nitric oxide synthase (NOS I and NOS III). FASEB J. **12**, 773–790

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

117 Gachhui, R., Presta, A., Bentley, D. F., Abu-Soud, H. M., McArthur, R., Brudvig, G., Ghosh, D. K. and Stuehr, D. J. (1996) Characterization of the reductase domain of rat neuronal nitric oxide synthase generated in the methylotrophic yeast *Pichia pastoris*. Calmodulin response is complete within the reductase domain itself. J. Biol. Chem. **271**, 20594–20602

118 Gachhui, R., Abu-Soud, H. M., Ghosha, D. K., Presta, A., Blazing, M. A., Mayer, B., George, S. E. and Stuehr, D. J. (1998) Neuronal nitric-oxide synthase interaction with calmodulin-troponin C chimeras. J. Biol. Chem. **273**, 5451–5454

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

120 Salerno, J. C., Harris, D. E., Irizarry, K., Patel, B., Morales, A. J., Smith, S. M., Martasek, P., Roman, L. J., Masters, B. S., Jones, C. L. et al. (1997) An autoinhibitory control element defines calcium-regulated isoforms of nitric oxide synthase. J. Biol. Chem. **272**, 29769–29777

121 Nishida, C. R. and Ortiz de Montellano, P. R. (1999) Autoinhibition of endothelial nitric-oxide synthase. Identification of an electron transfer control element. J. Biol. Chem. **274**, 14692–14698

122 Daff, S., Sagami, I. and Shimizu, T. (1999) The 42-amino acid insert in the FMN domain of neuronal nitric-oxide synthase exerts control over Ca(2+)/calmodulin-dependent electron transfer. J. Biol. Chem. **274**, 30589–30595

123 Corson, M. A., James, N. L., Latta, S. E., Nerem, R. M., Berk, B. C. and Harrison, D. G. (1996) Phosphorylation of endothelial nitric oxide synthase in response to fluid shear stress. Circ. Res. **79**, 984–991

124 Fisslthaler, B., Dimmeler, S., Hermann, C., Busse, R. and Fleming, I. (2000) Phosphorylation and activation of the endothelial nitric oxide synthase by fluid shear stress. Acta Physiol. Scand. **168**, 81–88

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

126 Fulton, D., Gratton, J. P., McCabe, T. J., Fontana, J., Fujio, Y., Walsh, K., Franke, T. F., Papapetropoulos, A. and Sessa, W. C. (1999) Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. Nature (London) **399**, 597–601

127 McCabe, T. J., Fulton, D., Roman, L. J. and Sessa, W. C. (2000) Enhanced electron flux and reduced calmodulin dissociation may explain "calcium-independent" eNOS activation by phosphorylation. J. Biol. Chem. **275**, 6123–6128

128 Komeima, K., Hayashi, Y., Naito, Y. and Watanabe, Y. (2000) Inhibition of neuronal nitric-oxide synthase by calcium/calmodulin-dependent protein kinase IIalpha through Ser847 phosphorylation in NG108–NG115 neuronal cells. J. Biol. Chem. **275**, 28139–28143

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

130 Nakane, M., Mitchell, J., Forstermann, U. and Murad, F. (1991) Phosphorylation by calcium calmodulin-dependent protein kinase II and protein kinase C modulates the activity of nitric oxide synthase. Biochem. Biophys. Res. Commun. **180**, 1396–1402

131 Jaffrey, S. R. and Snyder, S. H. (1996) PIN: an associated protein inhibitor of neuronal nitric oxide synthase. Science (Washington, D.C.) **274**, 774–777

132 Fan, J. S., Zhang, Q., Li, M., Tochio, H., Yamazaki, T., Shimizu, M. and Zhang, M. (1998) Protein inhibitor of neuronal nitric-oxide synthase, PIN, binds to a 17-amino acid residue fragment of the enzyme. J. Biol. Chem. **273**, 33472–33481

133 Rodriguez-Crespo, I., Straub, W., Gavilanes, F. and Ortiz de Montellano, P. R. (1998) Binding of dynein light chain (PIN) to neuronal nitric oxide synthase in the absence of inhibition. Arch. Biochem. Biophys. **359**, 297–304

134 Hemmens, B., Woschitz, S., Pitters, E., Klosch, B., Volker, C., Schmidt, K. and Mayer, B. (1998) The protein inhibitor of neuronal nitric oxide synthase (PIN): characterization of its action on pure nitric oxide synthases. FEBS Lett. **430**, 397–400

135 King, S. M., Barbarese, E., Dillman, J. F., Patel-King, R. S., Carson, J. H. and Pfister, K. K. (1996) Brain cytoplasmic and flagellar outer arm dyneins share a highly conserved Mr 8,000 light chain. J. Biol. Chem. **271**, 19358–19366

136 Garcia-Cardena, G., Fan, R., Shah, V., Sorrentino, R., Cirino, G., Papapetropoulos, A. and Sessa, W. C. (1998) Dynamic activation of endothelial nitric oxide synthase by Hsp90. Nature (London) **392**, 821–824

137 Venema, V. J., Marrero, M. B. and Venema, R. C. (1996) Bradykinin-stimulated protein tyrosine phosphorylation promotes endothelial nitric oxide synthase translocation to the cytoskeleton. Biochem. Biophys. Res. Commun. **226**, 703–710

138 Bender, A. T., Silverstein, A. M., Demady, D. R., Kanelakis, K. C., Noguchi, S., Pratt, W. B. and Osawa, Y. (1999) Neuronal nitric-oxide synthase is regulated by the Hsp90-based chaperone system *in vivo*. J. Biol. Chem. **274**, 1472–1478

139 Ratovitski, E. A., Alam, M. R., Quick, R. A., McMillan, A., Bao, C., Kozlovsky, C., Hand, T. A., Johnson, R. C., Mains, R. E., Eipper, B. A. and Lowenstein, C. J. (1999) Kalirin inhibition of inducible nitric-oxide synthase. J. Biol. Chem. **274**, 993–999

140 Michel, T. (1999) Targeting and translocation of endothelial nitric oxide synthase. Braz. J. Med. Biol. Res. **32**, 1361–1366

141 Liu, J., Garcia-Cardena, G. and Sessa, W. C. (1995) Biosynthesis and palmitoylation of endothelial nitric oxide synthase: mutagenesis of palmitoylation sites, cysteines-15 and/or -26, argues against depalmitoylation-induced translocation of the enzyme. Biochemistry **34**, 12333–12340

142 Liu, J., Hughes, T. E. and Sessa, W. C. (1997) The first 35 amino acids and fatty acylation sites determine the molecular targeting of endothelial nitric oxide synthase into the Golgi region of cells: a green fluorescent protein study. J. Cell Biol. **137**, 1525–1535

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

144 Prabhakar, P., Thatte, H. S., Goetz, R. M., Cho, M. R., Golan, D. E. and Michel, T. (1998) Receptor-regulated translocation of endothelial nitric-oxide synthase. J. Biol. Chem. **273**, 27383–27388

145 Garcia-Cardena, G., Oh, P., Liu, J., Schnitzer, J. E. and Sessa, W. C. (1996) Targeting of nitric oxide synthase to endothelial cell caveolae via palmitoylation: implications for nitric oxide signaling. Proc. Natl. Acad. Sci. U.S.A. **93**, 6448–6453

146 Feron, O., Belhassen, L., Kobzik, L., Smith, T. W., Kelly, R. A. and Michel, T. (1996) Endothelial nitric oxide synthase targeting to caveolae. Specific interactions with caveolin isoforms in cardiac myocytes and endothelial cells. J. Biol. Chem. **271**, 22810–22814

147 Ju, H., Zou, R., Venema, V. J. and Venema, R. C. (1997) Direct interaction of endothelial nitric-oxide synthase and caveolin-1 inhibits synthase activity. J. Biol. Chem. **272**, 18522–18525

148 Michel, J. B., Feron, O., Sacks, D. and Michel, T. (1997) Reciprocal regulation of endothelial nitric-oxide synthase by $Ca^{2+}$-calmodulin and caveolin. J. Biol. Chem. **272**, 15583–15586

149 Garcia-Cardena, G., Martasek, P., Masters, B. S., Skidd, P. M., Couet, J., Li, S., Lisanti, M. P. and Sessa, W. C. (1997) Dissecting the interaction between nitric oxide synthase (NOS) and caveolin. Functional significance of the NOS caveolin binding domain *in vivo*. J. Biol. Chem. **272**, 25437–25440

150 Ghosh, S., Gachhui, R., Crooks, C., Wu, C., Lisanti, M. P. and Stuehr, D. J. (1998) Interaction between caveolin-1 and the reductase domain of endothelial nitric-oxide synthase. Consequences for catalysis. J. Biol. Chem. **273**, 22267–22271

151 Venema, V. J., Ju, H., Zou, R. and Venema, R. C. (1997) Interaction of neuronal nitric-oxide synthase with caveolin-3 in skeletal muscle. Identification of a novel caveolin scaffolding/inhibitory domain. J. Biol. Chem. **272**, 28187–28190

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

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

154 Bredt, D. S., Christopherson, K. S. and Brenman, J. E. (1997) Jpn. J. Pharmacol. **75** (suppl 1), 7P (abstract 20)

155 Schepens, J., Cuppen, E., Wieringa, B. and Hendriks, W. (1997) The neuronal nitric oxide synthase PDZ motif binds to -G(D,E)XV* carboxyterminal sequences. FEBS Lett. **409**, 53–56

156 Stricker, N. L., Christopherson, K. S., Yi, B. A., Schatz, P. J., Raab, R. W., Dawes, G., Bassett, D. E., Bredt, D. S. and Li, M. (1997) PDZ domain of neuronal nitric oxide synthase recognizes novel C-terminal peptide sequences. Nat. Biotechnol. **15**, 336–342

157 Wang, Y., Newton, D. C., Robb, G. B., Kau, C. L., Miller, T. L., Cheung, A. H., Hall, A. V., VanDamme, S., Wilcox, J. N. and Marsden, P. A. (1999) RNA diversity has profound effects on the translation of neuronal nitric oxide synthase. Proc. Natl. Acad. Sci. U.S.A. **96**, 12150–12155

158 Huang, P. L., Dawson, T. M., Bredt, D. S., Snyder, S. H. and Fishman, M. C. (1993) Targeted disruption of the neuronal nitric oxide synthase gene. Cell (Cambridge, Mass.) **75**, 1273–1286
159 Eliasson, M. J., Blackshaw, S., Schell, M. J. and Snyder, S. H. (1997) Neuronal nitric oxide synthase alternatively spliced forms: prominent functional localizations in the brain. Proc. Natl. Acad. Sci. U.S.A. **94**, 3396–3401
160 Wang, Y., Goligorsky, M. S., Lin, M., Wilcox, J. N. and Marsden, P. A. (1997) A novel, testis-specific mRNA transcript encoding an NH2-terminal truncated nitric-oxide synthase. J. Biol. Chem. **272**, 11392–11401
161 Silvagno, F., Xia, H. and Bredt, D. S. (1996) Neuronal nitric-oxide synthase-mu, an alternatively spliced isoform expressed in differentiated skeletal muscle. J. Biol. Chem. **271**, 11204–11208
162 Magee, T., Fuentes, A. M., Garban, H., Rajavashisth, T., Marquez, D., Rodriguez, J. A., Rajfer, J. and Gonzalez-Cadavid, N. F. (1996) Cloning of a novel neuronal nitric oxide synthase expressed in penis and lower urinary tract. Biochem. Biophys. Res. Commun. **226**, 145–151
163 Laine, R. and de Montellano, P. R. (1998) Neuronal nitric oxide synthase isoforms alpha and mu are closely related calpain-sensitive proteins. Mol. Pharmacol. **54**, 305–312
164 Ogura, T., Yokoyama, T., Fujisawa, H., Kurashima, Y. and Esumi, H. (1993) Structural diversity of neuronal nitric oxide synthase mRNA in the nervous system. Biochem. Biophys. Res. Commun. **193**, 1014–1022
165 Fujisawa, H., Ogura, T., Kurashima, Y., Yokoyama, T., Yamashita, J. and Esumi, H. (1994) Expression of two types of nitric oxide synthase mRNA in human neuroblastoma cell lines. J. Neurochem. **63**, 140–145
166 Brenman, J. E., Xia, H., Chao, D. S., Black, S. M. and Bredt, D. S. (1997) Regulation of neuronal nitric oxide synthase through alternative transcripts. Dev. Neurosci. **19**, 224–231
167 Kolesnikov, Y. A., Pan, Y. X., Babey, A. M., Jain, S., Wilson, R. and Pasternak, G. W. (1997) Functionally differentiating two neuronal nitric oxide synthase isoforms through antisense mapping: evidence for opposing NO actions on morphine analgesia and tolerance. Proc. Natl. Acad. Sci. U.S.A. **94**, 8220–8225
168 Eissa, N. T., Strauss, A. J., Haggerty, C. M., Choo, E. K., Chu, S. C. and Moss, J. (1996) Alternative splicing of human inducible nitric-oxide synthase mRNA. Tissue-specific regulation and induction by cytokines. J. Biol. Chem. **271**, 27184–27187
169 Eissa, N. T., Yuan, J. W., Haggerty, C. M., Choo, E. K., Palmer, C. D. and Moss, J. (1998) Cloning and characterization of human inducible nitric oxide synthase splice variants: a domain, encoded by exons 8 and 9, is critical for dimerization. Proc. Natl. Acad. Sci. U.S.A. **95**, 7625–7630
170 Bates, T. E., Loesch, A., Burnstock, G. and Clark, J. B. (1995) Immunocytochemical evidence for a mitochondrially located nitric oxide synthase in brain and liver. Biochem. Biophys. Res. Commun. **213**, 896–900
171 Bates, T. E., Loesch, A., Burnstock, G. and Clark, J. B. (1996) Mitochondrial nitric oxide synthase: a ubiquitous regulator of oxidative phosphorylation? Biochem. Biophys. Res. Commun. **218**, 40–44
172 Kobzik, L., Stringer, B., Balligand, J.-L., Reid, M. B. and Stamler, J. S. (1995) Endothelial type nitric oxide synthase in skeletal muscle fibers: mitochondrial relationships. Biochem. Biophys. Res. Commun. **211**, 375–381
173 Giulivi, C. (1998) Functional implications of nitric oxide produced by mitochondria in mitochondrial metabolism. Biochem. J. **332**, 673–679
174 Giulivi, C., Poderoso, J. J. and Boveris, A. (1998) Production of nitric oxide by mitochondria. J. Biol. Chem. **273**, 11038–11043
175 Ghafourifar, P. and Richter, C. (1997) Nitric oxide synthase activity in mitochondria. FEBS Lett. **418**, 291–296
176 Tatoyan, A. and Giulivi, C. (1998) Purification and characterization of a nitric oxide synthase from rat liver mitochondria. J. Biol. Chem. **273**, 11044–11048
177 Hiki, K., Yui, Y., Hattori, R., Eizawa, H., Kosuga, K. and Kawai, C. (1991) Cytosolic and membrane-bound nitric oxide synthase. Jpn. J. Pharmacol. **56**, 217–220
178 Hecker, M., Walsh, D. T. and Vane, J. R. (1992) Characterization of a microsomal calcium-dependent nitric oxide synthase in activated J774.2 monocyte/macrophages. J. Cardiovasc. Pharmacol. **20**, S139–S141
179 Amin, A. R., Di Cesare, P. E., Vyas, P., Attur, M., Tzeng, E., Billiar, T. R., Stuchin, S. A. and Abramson, S. B. (1995) The expression and regulation of nitric oxide synthase in human osteoarthritis-affected chondrocytes: evidence for up-regulated neuronal nitric oxide synthase. J. Exp. Med. **182**, 2097–2102
180 Melchiorri, C., Meliconi, R., Frizziero, L., Silvestri, T., Pulsatelli, L., Mazzetti, I., Borzi, R. M., Uguccioni, M. and Facchini, A. (1998) Enhanced and coordinated *in vivo* expression of inflammatory cytokines and nitric oxide synthase by chondrocytes from patients with osteoarthritis. Arthritis Rheum. **41**, 2165–2174
180a Vuolteenaho, K., Moilanen, T., Al-Saffar, N., Knowles, R. G. and Moilanen, E. (2001) Regulation of the nitric oxide production resulting from the glucocorticoid-insensitive expression of iNOS in human osteoarthritic cartilage. Osteoarthr. Cartilage, in the press
181 Shirato, M., Sakamoto, T., Uchida, Y., Nomura, A., Ishii, Y., Iijima, H., Goto, Y. and Hasegawa, S. (1998) Molecular cloning and characterization of $Ca^{2+}$-dependent inducible nitric oxide synthase from guinea-pig lung. Biochem. J. **333**, 795–799
182 Weiner, C. P., Lizasoain, I., Baylis, S. A., Knowles, R. G., Charles, I. G. and Moncada, S. (1994) Induction of calcium-dependent nitric oxide synthases by sex hormones. Proc. Natl. Acad. Sci. U.S.A. **91**, 5212–5216
183 Lundberg, J. O., Farkas-Szallasi, T., Weitzberg, E., Rinder, J., Lidholm, J., Anggaard, A., Hokfelt, T., Lundberg, J. M. and Alving, K. (1995) High nitric oxide production in human paranasal sinuses. Nat. Med. (N.Y.) **1**, 370–373
184 Handy, R. L. and Moore, P. K. (1998) A comparison of the effects of L-NAME, 7-NI and L-NIL on carrageenan-induced hindpaw oedema and NOS activity. Br. J. Pharmacol. **123**, 1119–1126
185 Haynes, W. G., Noon, J. P., Walker, B. R. and Webb, D. J. (1993) Inhibition of nitric oxide synthesis increases blood pressure in healthy humans. J. Hypertens. **11**, 1375–1380
186 Hansen, J., Jacobsen, T. N. and Victor, R. G. (1994) Is nitric oxide involved in the tonic inhibition of central sympathetic outflow in humans? Hypertension **24**, 439–444
187 Stamler, J. S., Loh, E., Roddy, M. A., Currie, K. E. and Creager, M. A. (1994) Nitric oxide regulates basal systemic and pulmonary vascular resistance in healthy humans. Circulation **89**, 2035–2040
188 Rees, D. D., Palmer, R. M., Schulz, R., Hodson, H. F. and Moncada, S. (1990) Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. Br. J. Pharmacol. **101**, 746–752
189 Rees, D. D., Monkhouse, J. E., Cambridge, D. and Moncada, S. (1998) Nitric oxide and the haemodynamic profile of endotoxin shock in the conscious mouse. Br. J. Pharmacol. **124**, 540–546
190 Laszlo, F., Whittle, B. J. and Moncada, S. (1994) Time-dependent enhancement or inhibition of endotoxin-induced vascular injury in rat intestine by nitric oxide synthase inhibitors. Br. J. Pharmacol. **111**, 1309–1315
191 Seiler, N., Bolkenius, F. N. and Knodgen, B. (1985) The influence of catabolic reactions on polyamine excretion. Biochem. J. **225**, 219–226
192 Bieganski, T., Kusche, J., Lorenz, W., Hesterberg, R., Stahlknecht, C. D. and Feussner, K. D. (1983) Distribution and properties of human intestinal diamine oxidase and its relevance for the histamine catabolism. Biochim. Biophys. Acta **756**, 196–203
193 Nilsson, B. O., Kockum, I. and Rosengren, E. (1996) Inhibition of diamine oxidase promotes uptake of putrescine from rat small intestine. Inflamm. Res. **45**, 513–518
194 Ou, P. and Wolff, S. P. (1993) Aminoguanidine: a drug proposed for prophylaxis in diabetes inhibits catalase and generates hydrogen peroxide *in vitro*. Biochem. Pharmacol. **46**, 1139–1144
195 Giardino, I., Fard, A. K., Hatchell, D. L. and Brownlee, M. (1998) Aminoguanidine inhibits reactive oxygen species formation, lipid peroxidation, and oxidant-induced apoptosis. Diabetes **47**, 1114–1120
196 Yildiz, G., Demiryurek, A. T., Sahin-Erdemli, I. and Kanzik, I. (1998) Comparison of antioxidant activities of aminoguanidine, methylguanidine and guanidine by luminol-enhanced chemiluminescence. Br. J. Pharmacol. **124**, 905–910
197 Bryk, R. and Wolff, D. J. (1999) Pharmacological modulation of nitric oxide synthesis by mechanism-based inactivators and related inhibitors. Pharmacol. Ther. **84**, 157–178
198 Misko, T. P., Moore, W. M., Kasten, T. P., Nickols, G. A., Corbett, J. A., Tilton, R. G., McDaniel, M. L., Williamson, J. R. and Currie, M. G. (1993) Selective inhibition of the inducible nitric oxide synthase by aminoguanidine. Eur. J. Pharmacol. **233**, 119–125
199 Wolff, D. J. and Lubeskie, A. (1995) Aminoguanidine is an isoform-selective, mechanism-based inactivator of nitric oxide synthase. Arch. Biochem. Biophys. **316**, 290–301
200 McCall, T. B., Feelisch, M., Palmer, R. M. and Moncada, S. (1991) Identification of *N*-iminoethyl-L-ornithine as an irreversible inhibitor of nitric oxide synthase in phagocytic cells. Br. J. Pharmacol. **102**, 234–238
201 Grant, S. K., Green, B. G., Stiffey-Wilusz, J., Durette, P. L., Shah, S. K. and Kozarich, J. W. (1998) Structural requirements for human inducible nitric oxide synthase substrates and substrate analogue inhibitors. Biochemistry **37**, 4174–4180
202 Wolff, D. J., Lubeskie, A., Gauld, D. S. and Neulander, M. J. (1998) Inactivation of nitric oxide synthases and cellular nitric oxide formation by $N^6$-iminoethyl-L-lysine and $N^5$-iminoethyl-L-ornithine. Eur. J. Pharmacol. **350**, 325–334
203 Garvey, E. P., Oplinger, J. A., Furfine, E. S., Kiff, R. J., Laszlo, F., Whittle, B. J. R. and Knowles, R. G. (1997) 1400W is a slow, tight binding and highly selective inhibitor of inducible nitric-oxide synthase *in vitro* and *in vivo*. J. Biol. Chem. **272**, 4959–4963
204 Alderton, W., Angell, A., Clayton, N., Craig, C., Dawson, J., Frend, A., McGill, J., Mangel, A., Moncada, S., Rees, D. et al. (2000) GW274150 is a potent, long-acting, highly-selective inhibitor of inducible nitric oxide synthase (NOS-2) with therapeutic potential in post-operative ileus. In Biology of Nitric Oxide, vol. 7 (Moncada, S., Gustaffson, L. E., Wiklund, N. P. and Higgs, E. A., eds.), pp. 22, Portland Press, London

205 Knowles, R., Dawson, J., Waslidge, N., Russell, R., Angell, A., Craig, C., Schwartz, S., Evans, S., Whittle, B., Garvey, E. et al. (2000) GW273629 is a highly-selective, short-acting iNOS (NOS-2) inhibitor both *in vitro* and *in vivo*: beneficial effects in endotoxin shock. In Biology of Nitric Oxide, vol. 7 (Moncada, S., Gustaffson, L. E., Wiklund, N. P. and Higgs, E. A., eds.), pp. 21, Portland Press, London

206 Bryk, R. and Wolff, D. J. (1998) Mechanism of inducible nitric oxide synthase inactivation by aminoguanidine and L-$N^6$-(1-iminoethyl)lysine. Biochemistry **37**, 4844–4852

207 Werner, E. R., Pitters, E., Schmidt, K., Wachter, H., Werner-Felmayer, G. and Mayer, B. (1996) Identification of the 4-amino analogue of tetrahydrobiopterin as a dihydropteridine reductase inhibitor and a potent pteridine antagonist of rat neuronal nitric oxide synthase. Biochem. J. **320**, 193–196

208 Bommel, H. M., Reif, A., Frohlich, L. G., Frey, A., Hofmann, H., Marecak, D. M., Groehn, V., Kotsonis, P., La, M., Koster, S. et al. (1998) Anti-pterins as tools to characterize the function of tetrahydrobiopterin in NO synthase. J. Biol. Chem. **273**, 33142–33149

209 Wolff, D. J., Datto, G. A., Samatovicz, R. A. and Tempsick, R. A. (1993) Calmodulin-dependent nitric-oxide synthase. Mechanism of inhibition by imidazole and phenylimidazoles. J. Biol. Chem. **268**, 9425–9429

210 Anon (1999) Nitric oxide synthase inhibitors with cardiovascular therapeutic potential. Expert Opin. Ther. Pat. **95**, 537–547

211 Anon (1999) The inhibition of isoforms of nitric oxide synthase: non-cardiovascular aspects. Expert Opin. Ther. Pat. **95**, 549–556

212 Babu, B. R. and Griffith, O. W. (1998) Design of isoform-selective inhibitors of nitric oxide synthase. Curr. Opin. Chem. Biol. **2**, 491–500

213 Furfine, E. S., Harmon, M. F., Paith, J. E., Knowles, R. G., Salter, M., Kiff, R. J., Duffy, C., Hazelwood, R., Oplinger, J. A. and Garvey, E. P. (1994) Potent and selective-inhibition of human nitric-oxide synthases – selective-inhibition of neuronal nitric-oxide synthase by S-methyl-L-thiocitrulline and S-ethyl-L-thiocitrulline. J. Biol. Chem. **269**, 26677–26683

214 Narayanan, K., Spack, L., McMillan, K., Kilbourn, R. G., Hayward, M. A., Masters, B. S. and Griffith, O. W. (1995) S-alkyl-L-thiocitrullines. Potent stereoselective inhibitors of nitric oxide synthase with strong pressor activity *in vivo*. J. Biol. Chem. **270**, 11103–11110

215 Babu, B. R. and Griffith, O. W. (1998) $N_5$-(1-imino-3-butenyl)-L-ornithine. A neuronal isoform selective mechanism-based inactivator of nitric oxide synthase. J. Biol. Chem. **273**, 8882–8889

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

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

218 Iadecola, C., Zhang, F., Casey, R., Nagayama, M. and Ross, M. E. (1997) Delayed reduction of ischemic brain injury and neurological deficits in mice lacking the inducible nitric oxide synthase gene. J. Neurosci. **17**, 9157–9164

219 Nagayama, M., Zhang, F. and Iadecola, C. (1998) Delayed treatment with aminoguanidine decreases focal cerebral ischemic damage and enhances neurologic recovery in rats. J. Cereb. Blood Flow Metab. **18**, 1107–1113

220 Moore, W. M., Webber, R. K., Jerome, G. M., Tjoeng, F. S., Misko, T. P. and Currie, M. G. (1994) L-$N_6$-(1-iminoethyl)lysine: a selective inhibitor of inducible nitric oxide synthase. J. Med. Chem. **37**, 3886–3888

221 Laszlo, F. and Whittle, B. J. (1997) Actions of isoform-selective and non-selective nitric oxide synthase inhibitors on endotoxin-induced vascular leakage in rat colon. Eur. J. Pharmacol. **334**, 99–102

222 Hagen, T. J., Bergmanis, A. A., Kramer, S. W., Fok, K. F., Schmelzer, A. E., Pitzele, B. S., Swenton, L., Jerome, G. M., Kornmeier, C. M., Moore, W. M. et al. (1998) 2-Iminopyrrolidines as potent and selective inhibitors of human inducible nitric oxide synthase. J. Med. Chem. **41**, 3675–3683

223 Hansen, D. W., Peterson, K. B., Trivedi, M., Kramer, S. W., Webber, R. K., Tjoeng, F. S., Moore, W. M., Jerome, G. M., Kornmeier, C. M., Manning, P. T. et al. (1998) 2-Iminohomopiperidinium salts as selective inhibitors of inducible nitric oxide synthase (iNOS). J. Med. Chem. **41**, 1361–1366

224 Thomsen, L. L., Scott, J. M., Topley, P., Knowles, R. G., Keerie, A. J. and Frend, A. J. (1997) Selective inhibition of inducible nitric oxide synthase inhibits tumor growth *in vivo*: studies with 1400W, a novel inhibitor. Cancer Res. **57**, 3300–3304

225 Wray, G. M., Millar, C. G., Hinds, C. J. and Thiemermann, C. (1998) Selective inhibition of the activity of inducible nitric oxide synthase prevents the circulatory failure, but not the organ injury/dysfunction, caused by endotoxin. Shock **9**, 329–335

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

227 Koarai, A., Ichinose, M., Sugiura, H., Yamagata, S., Hattori, T. and Shirato, K. (2000) Allergic airway hyperresponsiveness and eosinophil infiltration is reduced by a selective iNOS inhibitor, 1400W, in mice. Pulmon. Pharmacol. Ther. **13**, 267–275

228 Kankuri, E., Vaali, K., Knowles, R., Lähde, M., Korpela, R., Vapaatalo, H. and Moilanen, E. (2001) Suppression of acute experimental colitis by an iNOS-selective inhibitor *N*-3-(aminomethyl)benzyl]acetamidine (1400W). J. Pharmacol. Exp. Ther., in the press

229 Young, R. J., Beams, R. M., Carter, K., Clark, H. A., Coe, D. M., Chambers, C. L., Davies, P. I., Dawson, J., Drysdale, M. J., Franzman, K. W. et al. (2000) Inhibition of inducible nitric oxide synthase by acetamidine derivatives of hetero-substituted lysine and homolysine. Bioorg. Med. Chem. Lett. **10**, 597–600

230 Dawson, J. and Knowles, R. G. (1998) A microtiter-plate assay of human NOS isoforms. Methods Mol. Biol. **100**, 237–242