REVIEW ARTICLE
Nitric oxide synthases in mammals

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INTRODUCTION

Nitric oxide is an inorganic free radical gas, of formula ·N=O (abbreviated as NO). The discovery in 1987/88 that vascular endothelial cells are able to synthesize NO from L-arginine as a transcellular signal [1–4] was initially received by most biologists with considerable scepticism. By now, however, the existence of the L-arginine:NO pathway has been thoroughly documented and its relevance in biology is slowly being unravelled. All of this has led to the appearance of a new and vigorous field of research, as evidenced by the increasing number of publications relating to NO and NO synthases (Figure 1).

This review will describe the known biochemical mechanisms involved in the synthesis of NO from L-arginine by the NO synthases and will also describe the nature of these enzymes, their inhibition and their molecular characteristics. For more extensive reviews about the biological roles of NO, see [5–7].

GENERAL PROPERTIES OF NO SYNTHASES

The enzymes responsible for the synthesis of NO from L-arginine in mammalian tissues are known as NO synthases (EC 1.14.13.39; they are not synthetases as the reaction does not utilize ATP). These enzymes are remarkable for three different reasons: the rapidity with which they have been characterized, purified and cloned (first described in 1989 [8–14], first purified in 1990 [15] and first cloned in 1991 [16]); for the complexity and number of reactions carried out (Table 1) by a medium-sized subunit of 125–155 kDa, albeit probably as a homodimer [25–30]; and for the number and range of physiological and pathological roles in which they are involved [5–7].

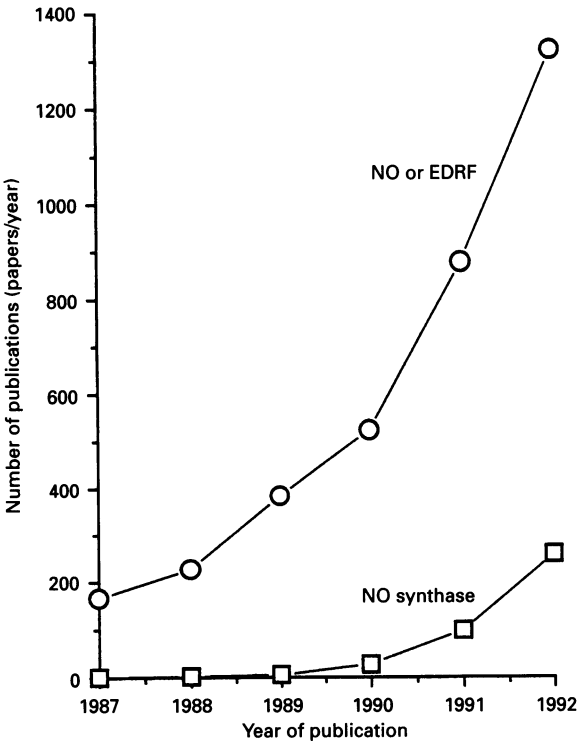

Figure 1 The annual rate of publications on nitric oxide or endothelium-derived relaxing factor (EDRF) and on NO synthases

Data derived from Medline.

Table 1 Reactions catalysed by NO synthase

Reactions 2–5 have so far only been demonstrated with brain NO synthase. Reaction 5 is controversial and may not occur from free dihydrobiopterin (BH_2), but only as part of the recycling of bound pterin to the tetrahydro form (BH_4). Cit, citrulline; cyt *c*, cytochrome *c*.

	Activity	Reaction	References
1a	Arginine N^{ω}-hydroxylase	$Arg + NADPH + H^+ + O_2 \rightarrow HydroxyArg + NADP^+ + H_2O$	8–14,17–20
1b	N^{ω}-Hydroxyarginine mono-oxygenase	$HydroxyArg + \frac{1}{2}(NADPH + H^+) + O_2 \rightarrow Cit + NO + H_2O$	8–14,17–20
2	NADPH diaphorase	$NitroBlue\ Tetrazolium\ (NBT) + NADPH + H^+ \rightarrow Reduced\ NBT + NADP^+$	21,22
3	Cytochrome *c* reductase	$Cyt\ c + NADPH + H^+ \rightarrow Reduced\ cyt\ c + NADP^+$	23
4	NADPH oxidase	$NADPH + H^+ + O_2 \rightarrow H_2O_2 + NADP^+$ $\frac{1}{2}NADPH + O_2 \rightarrow O_2^- + \frac{1}{2}(NADP^+ + H^+)$	20,23,24
5	Dihydropteridine reductase	$BH_2 + NADPH + H^+ \rightarrow BH_4 + NADP^+$	20

Abbreviations used: BH_4, tetrahydrobiopterin; L-NMMA, N^{ω}-monomethyl-L-arginine; L-ADMA, asymmetric $N^{\omega}N^{\omega}$-dimethyl-L-arginine; L-NNA, N^{ω}-nitro-L-arginine; L-NAME, N^{ω}-nitro-L-arginine methyl ester; L-NIO, N^{ω}-iminoethyl-L-ornithine; DAHP, 2,4-diamino-6-hydroxypyrimidine; nNOS, neuronal NO synthase; eNOS, vascular endothelial NO synthase; iNOS, inducible NO synthase; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; CaMK, calmodulin-dependent kinase.

Table 2 Participants in NO synthesis from L-arginine

Protein	Subunit molecular mass (kDa)	References
NO synthase	125–160	15,25–32
Calmodulin	17	15,31–34

Non-protein	Tightly bound*	Affinity (μM)	References
Protoporphyrin IX haem	Yes		35–37
FMN	Yes/no	0.10	20,26,28,31
FAD	Yes/no	0.04–0.10	20,26,28,31
BH_4	Yes/no	0.01–0.10	14,20,26–28,29,31,38–40
NADPH	No	0.1–1.0	8–15,25–32,41,42
L-Arginine	No	1–20	8–15,25–32,41,42

* 'Tightly bound' means that the cofactor remains at least partly bound to the enzyme even after purification to homogeneity; the degree of cofactor loss during purification varies among NO synthase isoenzymes.

The first descriptions of NO synthases demonstrated that the synthesis of NO requires L-arginine and NADPH, and results in the formation of citrulline [8–14]. NO synthesis requires not only these substrates but also four other coenzymes/cofactors, as well as the presence of calmodulin (Table 2).

Subsequent work demonstrated that molecular oxygen is also a substrate for this reaction, being incorporated into both NO and citrulline [17,19]; thus this reaction apparently constitutes an L-arginine $N^{\omega}C^{G}$ dioxygenation. However, as shown in Figure 2, it is now clear that NO synthesis from arginine is a reaction which involves two separate mono-oxygenation steps [17–19,41]. N^{ω}-Hydroxyarginine is an intermediate species formed by a reaction requiring one O_2 and one NADPH and the presence of tetrahydrobiopterin (BH_4) [17–20]. This reaction appears to be similar to those carried out by the aromatic amino acid hydroxylases, which also require BH_4. The second step in the NO synthase reaction results in the oxidation of N^{ω}-hydroxyarginine to form citrulline and NO (Figure 2). The detailed mechanism of this reaction remains to be determined [41]; however, the observation that the brain NO synthase can generate O_2^- and H_2O_2 in the absence of L-arginine, with continued oxidation of NADPH, suggests that the flavin coenzymes may be involved in the transfer of electrons to form a reduced oxygen species [20,23,24]. This hypothetical species could then react with N^{ω}-hydroxyarginine in the presence of arginine and BH_4, or in their absence form O_2^- or H_2O_2. This type of reaction would be similar to that of both the superoxide-generating NADPH oxidase and cytochrome *P*-450 reductase. In fact, the sequence similarity of NO synthase with cytochrome *P*-450 reductase [16,43–48], together with the presence of a haem centre with the spectral properties of a cytochrome *P*-450 [35–37], show that NO synthase is the first self-sufficient mammalian *P*-450 enzyme to be identified. In addition, there is evidence to suggest that N^{ω}-hydroxyarginine can be converted to NO and citrulline by liver microsomal cytochrome *P*-450/*P*-450 reductase [49].

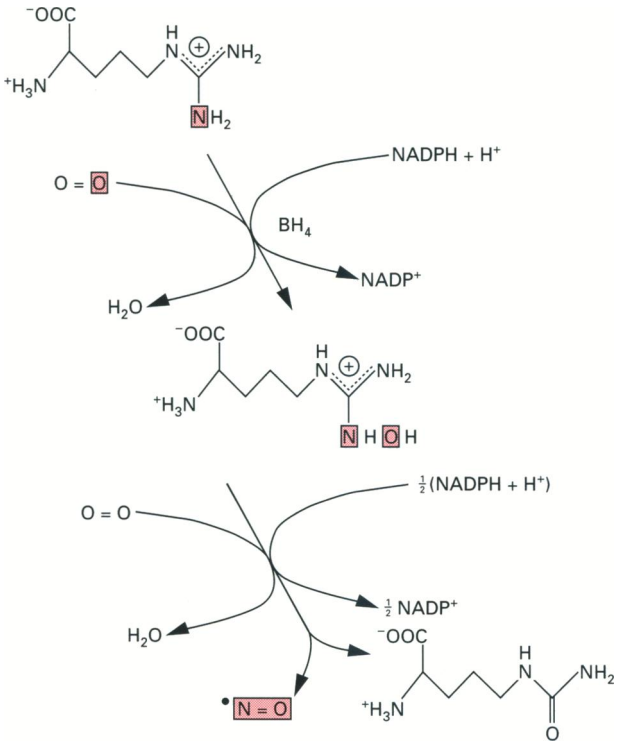

Figure 2 The NO synthase reaction

The boxed O and N atoms show the origin of the constituent atoms of NO (see the text and Table 1 for references).

ASSAYS FOR NO SYNTHASE ACTIVITY

NO synthase activity can be assayed by a range of methods, based on the formation of either citrulline or (indirectly) NO itself. Formation of ^3H- or ^{14}C-labelled citrulline from labelled L-arginine is perhaps the most widely used method, using a simple ion-exchange separation of substrate and product either in columns [15] or as batch addition [50]. This type of assay is sensitive (usually requiring the formation of < 100 nM product) and relatively robust, for it is unaffected by the presence of haemoglobin and does not require optically clear enzyme extracts. In order to obtain quantitative results, however, such assays of crude cell or tissue extract NO synthase carried out at a low L-arginine concentration (for maximum sensitivity) will require the L-arginine in the sample (e.g. ~ 100 μM in tissues; 500–1000 μM in cell culture media) to be either removed or accounted for. Moreover, some extracts (especially from liver) are able to metabolize further the labelled citrulline produced; this can be inhibited by the inclusion of 1 mM L-citrulline in the assay [50,51].

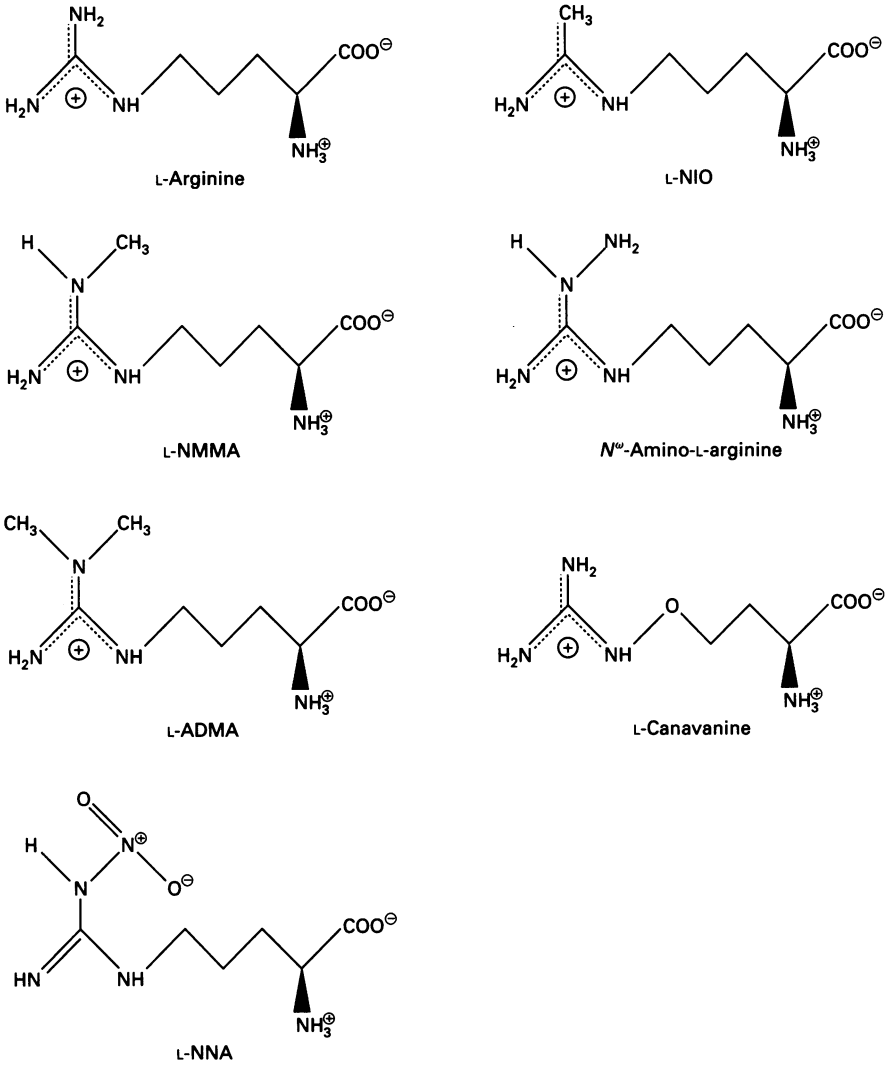

Figure 3 The structures of arginine and of the arginine analogues most frequently used as inhibitors of NO synthases

The predominant ionic species at neutral pH are shown.

There are two ways of measuring the generation of NO quantitatively: the oxidation of haemoglobin and the formation of $NO_2^- + NO_3^-$. NO rapidly reacts with oxyhaemoglobin to form NO_3^- and met-haemoglobin [52]; the resulting spectral changes have been used as the basis of a spectrophotometric assay of NO synthase which is highly sensitive [51]. With dual wavelength measurement at 401 and 421 nm (ϵ 77200 M^{-1}) this has a sensitivity of < 20 nM/min. However, this assay is somewhat less widely applicable than labelled citrulline formation because of interference by colour or opalescence in samples. In particular, contamination with blood that results in a haemoglobin concentration of $> 5\ \mu$M will reduce the sensitivity of the assay.

Formation of $NO_2^- + NO_3^-$, the oxygenation products of NO, can be measured by a variety of techniques. The simplest use reduction of NO_3^- to NO_2^- by nitrate reductase or metallic catalysts followed by the colorimetric Griess reaction [13,53,54] to measure NO_2^-. Other methods measure NO_2^- by chemiluminescence following reconversion to NO [1,8,12,55] or by h.p.l.c. techniques with u.v. absorption detection [56,57]. The sensitivity of these techniques is limited by the levels of NO_2^-/NO_3^- already present in buffers ($\sim 0.5\ \mu$M) and contributed by the enzyme samples (e.g. NO_3^- in tissues is present at $> 10\ \mu$M). One simplified variant of this type of assay measures only the NO_2^- formed. However, several reactions (especially that with oxyhaemoglobin) can result in NO and NO_2^- being converted to NO_3^- [52,58], and therefore this simplified assay must be used with great caution.

The stimulation of the soluble guanylate cyclase has been used as the basis of assays for NO synthase, both in a soluble form [8,10–12,42] and also within cells [33,59], such as the RFL-6 fibroblast cell line. Although such assays are sensitive and have yielded useful data, it is difficult to obtain quantitative NO synthase activity data. Moreover, the exquisite sensitivity of such assays to the presence of oxyhaemoglobin ($IC_{50} < 100$ nM), O_2^- or any other species that reacts with NO (thus neutralizing its effect on guanylate cyclase) limits their usefulness.

The components that are required for the full expression of NO synthase activity are shown in Table 2; which, if any, of these components need to be added to any particular NO synthase

Table 3 Types of NO synthase inhibitor

Inhibitor type	Type of inhibition	Comments
Arginine analogues (e.g. L-NMMA)	Competitive with arginine	Selective
Diphenyleneiodonium	Competitive with NADPH	Also inhibits some other NADPH-enzymes
Calmodulin antagonists (e.g. trifluoperazine, W7)	Competitive with calmodulin	Only inhibit some NO synthases, also inhibit other calmodulin-dependent enzymes
BH_4 synthesis inhibitors (e.g. DAHP, *N*-acetyl-5-hydroxytryptamine)	Indirect	
NO, CO	Not known	Probably inhibit by interacting with NO synthase haem

enzyme assay depends on the isoenzyme under study and whether endogenous cofactors are present or have been removed during any purification procedures. If the cell or tissue extracts contain arginase, then an arginase inhibitor such as L-valine may also need to be included to prevent depletion of L-arginine [51].

With all of the methods of assaying NO synthase, it is important to confirm that the product measured has been synthesized by this enzyme, by carrying out incubations in the presence of known inhibitors (see below). Moreover, it is also particularly important to check the time course of the reaction, because the time period for which linearity is maintained in the assay is dependent both on the particular NO synthase being studied and on the conditions of the assay (e.g. the L-arginine and BH_4 concentrations).

INHIBITORS OF NO SYNTHASE

The study of NO synthesis in mammalian systems has been greatly facilitated by the early identification of competitive inhibitors of NO synthase [60]. The most widely used of these inhibitors, N^{ω}-monomethyl-L-arginine (L-NMMA; Figure 3), was used in studies of the cytotoxicity of activated macrophages [61,62] before it was discovered that NO was involved in this process [63] and before the first descriptions of NO synthase [8–14]. L-NMMA has been shown to be a competitive inhibitor (competitive with L-arginine) of all the NO synthases so far examined, with a K_i of $\sim 1\ \mu$M [13–15,25,27,42,64–66]; this inhibition is enantiomer-specific. L-NMMA and asymmetric $N^{\omega}N^{\omega}$-dimethyl-L-arginine (L-ADMA; Figure 3), another NO synthase inhibitor, are both naturally occurring compounds [67] that can be derived from methylated protein arginine residues during protein turnover. Although the normal plasma concentrations of these compounds ($< 1\ \mu$M) are low, under some circumstances, e.g. during renal failure, L-NMMA and L-ADMA do accumulate in sufficient quantities to inhibit NO synthesis [67]. It is also possible that they could be present at higher concentrations in specific cells, tissues or individuals.

A range of structurally related compounds has been found to inhibit the NO synthases (Figure 3) [8,41,42,65–69]. Like L-NMMA, these compounds are competitive inhibitors. However, there are some differences between them. For example, N^{ω}-nitro-L-arginine (L-NNA) and its methyl ester (L-NAME), unlike L-NMMA, show progressive and irreversible or only slowly reversible inhibition of brain NO synthase following the initial (competitive) binding [70,71]. Similarly, the amidine N^{ω}-iminoethyl-L-ornithine (L-NIO) has been reported to inhibit progressively the inducible rodent macrophage and neutrophil NO synthases, again unlike L-NMMA [66]. These may be quantitative rather than qualitative differences, however, as L-NMMA has been reported to cause irreversible inhibition of macrophage and brain NO synthases during prolonged incubation [72,73]. The mechanism of this isoenzyme-specific irreversible inhibition is not known at present.

Whereas L-NMMA appears to be a non-selective inhibitor of the various NO synthase enzymes, some inhibitors do show some selectivity. L-Canavanine, N^{ω}-amino-L-arginine and L-NIO, for example, inhibit the inducible macrophage NO synthase more potently than the constitutive brain and endothelial NO synthases [8,41,65–69]. Another property which may only apply to L-NMMA and L-ADMA is that of being metabolized to L-citrulline and/or L-arginine in some cells and tissues [74–77]. This could result in reduced inhibition by those compounds, and could even conceivably lead to a paradoxical stimulation of NO synthesis under some conditions, if the concentration of L-arginine formed were high enough.

Another distinction within this related group of inhibitors is in the overall charge neutrality at physiological pH values of the guanido groups of L-NNA and L-NAME (because of their low $pK_a \approx 0$), which contrasts with the positively charged guanido and amidino groups of the others ($pK_a \approx 13$). One consequence of this is that L-NNA and L-NAME would not be expected to be transported on the y^+ amino acid transport system, which is responsible for a high proportion of the cellular uptake of positively charged amino acids such as L-arginine, L-ornithine and L-lysine [78,79]. This is consistent with data showing that, unlike L-NMMA, L-NNA does not compete with L-arginine for transport [80].

A recent report suggests that L-NAME and other alkyl ester analogues of arginine have an additional property which limits their usefulness as specific probes for NO synthesis *in vivo*. These compounds are muscarinic acetylcholine receptor antagonists, with a K_i value for L-NAME of approx. 100 μM [81]. Intracellular concentrations of L-NAME of this order are likely to be achieved following doses of 20–30 mg/kg.

A different type of inhibitor of NO synthase (Table 3) is diphenyleneiodonium [82]. This compound has no obvious structural similarity with arginine and is not competitive with this substrate; its inhibition of NO synthase is antagonized by high concentrations of NADPH, and it also inhibits other NAD(P)H-utilizing flavin enzymes, i.e. the superoxide-generating NADPH oxidase and mitochondrial NADH oxidase [83,84]. Diphenyleneiodonium acts as a progressive, irreversible inhibitor, such that the degree of inhibition depends not only on the concentration of the inhibitor but also on the time of exposure and the temperature [82].

Calmodulin antagonists such as trifluoperazine, chlorpromazine, calmidazolium, W7 and W13 have all been shown to inhibit some NO synthase isoenzymes, including the constitutive, Ca^{2+}-dependent neuronal and vascular endothelial NO synthases [15,33,85] and the induced Ca^{2+}-independent NO synthase in rat liver [31]. In contrast, the rodent macrophage and endothelial

Table 4 Functionally distinct NO synthases

Type/source	Ca^{2+}-dependent	CaM-dependent	Constitutive	Cytosolic/particulate
Constitutive vascular endothelial	Yes	Yes	Yes	Both
Constitutive neuronal	Yes	Yes	Yes	Cyto
Macrophage	No	No	No	Cyto
Liver	No	Yes/no	No	Cyto
Rabbit chondrocyte	Yes	No	No	Cyto
Human chondrocyte	No	No	No	Cyto

induced Ca^{2+}-independent enzymes [26,28,85] and the rabbit chondrocyte induced Ca^{2+}-dependent enzymes [86] are not inhibited by these compounds. This difference between isoforms of NO synthase is thus not simply related to their Ca^{2+}-dependence or -independence or to whether they are constitutive or inducible. The differential sensitivity to such inhibitors is probably based on the affinity of the different isoenzymes for calmodulin (see below).

Another distinct way of inhibiting NO synthase in biological systems is to limit the supply of one of its substrates or cofactors. This type of indirect inhibition has been demonstrated using inhibitors of BH_4 synthesis. Because of the turnover of BH_4 in intact cells and tissues, inhibition of its synthesis eventually results in depletion of BH_4 to concentrations that limit NO synthesis [85,87–90]. Examples of such inhibitors include 2,4-diamino-6-hydroxypyrimidine (DAHP), an inhibitor of the enzyme GTP cyclohydrolase-I which initiates *de novo* synthesis of BH_4, and methotrexate (an inhibitor of dihydropteridine reductase) or *N*-acetyl- or *N*-methoxyacetyl-5-hydroxytryptamine (inhibitors of sepiapterin reductase), both of which prevent salvage of sepiapterin to BH_4 [87,91–93]. The efficacy of such indirect inhibitors of NO synthesis will vary in different biological systems, depending on the saturation of NO synthase with BH_4, the rate of BH_4 turnover and the predominating pathway(s) of BH_4 synthesis.

NO synthase has, very recently, been demonstrated to be subject to feedback inhibition by NO: two distinct forms of NO synthase (brain constitutive and macrophage inducible) were shown to be inhibited by NO either produced by the enzyme itself or generated from chemical NO donors [94,95]. It is likely that this inhibition results from interaction with the haem of NO synthase. Other agents that are thought to interact with haem (carbon monoxide and Methylene Blue) have also been shown to inhibit NO synthase [35–37,96].

NO SYNTHASE ISOENZYMES

The mammalian systems in which NO synthesis was first demonstrated were the vascular endothelium, the brain and activated macrophages [1,2,63,97]. It was immediately apparent from the first studies of the NO synthases in these systems that the macrophage enzyme was different from the others, as it was Ca^{2+}-independent [8–14]. Differences in properties (Table 4) and differential inhibitor/substrate specificities suggest that there are more than just two forms of NO synthase, however, and this has recently been confirmed by the cloning and characterization of three distinct NO synthase cDNAs. This permits the definitive classification of the gene products so far characterized in this way as: nNOS (originally identified as constitutive in neuronal tissue, also known as type I NOS [98]); eNOS (originally identified as constitutive in vascular endothelial cells, also known as type III NOS); and iNOS (originally identified as being inducible by cytokines in macrophages and hepatocytes, also known as type II NOS). Examples of the physiological settings in which these three isoenzymes operate are shown in Figure 4; eNOS synthesizing NO in a vascular endothelial cell in response to acetylcholine, nNOS synthesizing NO in a neuron in response to glutamate; and iNOS synthesizing NO in a macrophage following its induction by a cytokine.

NEURONAL CONSTITUTIVE NO SYNTHASE: nNOS

The first isoenzyme to be purified [15] and cloned [16] was the rat nNOS. This enzyme is Ca^{2+}- and calmodulin-dependent [8,10,15] and is constitutively expressed at a high activity in the brain; the rat brain has the highest constitutive NO synthase activity of 20 rat tissues examined [50]. Both antibody staining and *in situ* mRNA hybridization techniques demonstrate that the expression of the gene encoding this isoenzyme is widespread in rat brain, with particularly large amounts of the protein and mRNA being found in the cerebellum [16,99,100]. The cDNA encoding the human nNOS has been cloned, and its mRNA shown to be expressed at high levels in human brain and also (more surprisingly) in human skeletal muscle [101]; in the rat, the nNOS mRNA is not readily detected in this tissue [16,101]. Histological studies suggest that this NO synthase isoenzyme is also widely distributed in peripheral neural systems (e.g. myenteric plexi in the gut) and in the spinal cord (e.g. in dorsal root ganglia) [99,102,103]. Partial purification and characterization of the NO synthase thought to be responsible for production of NO by non-adrenergic non-cholinergic nerves also suggests that the same isoenzyme is present in both the peripheral and central nervous systems [104]. Because of this wide expression in different tissues, and because of the high activity of nNOS in the brain and skeletal muscle, it is likely that this isoenzyme is responsible for the largest proportion of constitutive NO synthase activity in man.

The cloning, sequencing and characterization of the cDNA encoding rat and human nNOS reveal that they have mRNA species of > 10 kb which encode proteins of 1429 and 1433 amino acids respectively [16,101] (Table 5). The proteins from these two species show a high sequence identity (93%). They contain two consensus binding sequences for NADPH, two for FAD, one for FMN and one for calmodulin (Figure 5). They also have a consensus sequence (KRFGS) for phosphorylation by cyclic AMP-dependent protein kinase (PKA). Although it has been confirmed that not only PKA but also protein kinase C (PKC) and calmodulin-dependent kinase (CaMK) can indeed phosphorylate rat nNOS, the physiological significance of this phosphorylation remains uncertain as there is either no detectable effect of phosphorylation on enzyme activity (PKA) or else the effect on activity is controversial (PKC, CaMK) [105–107].

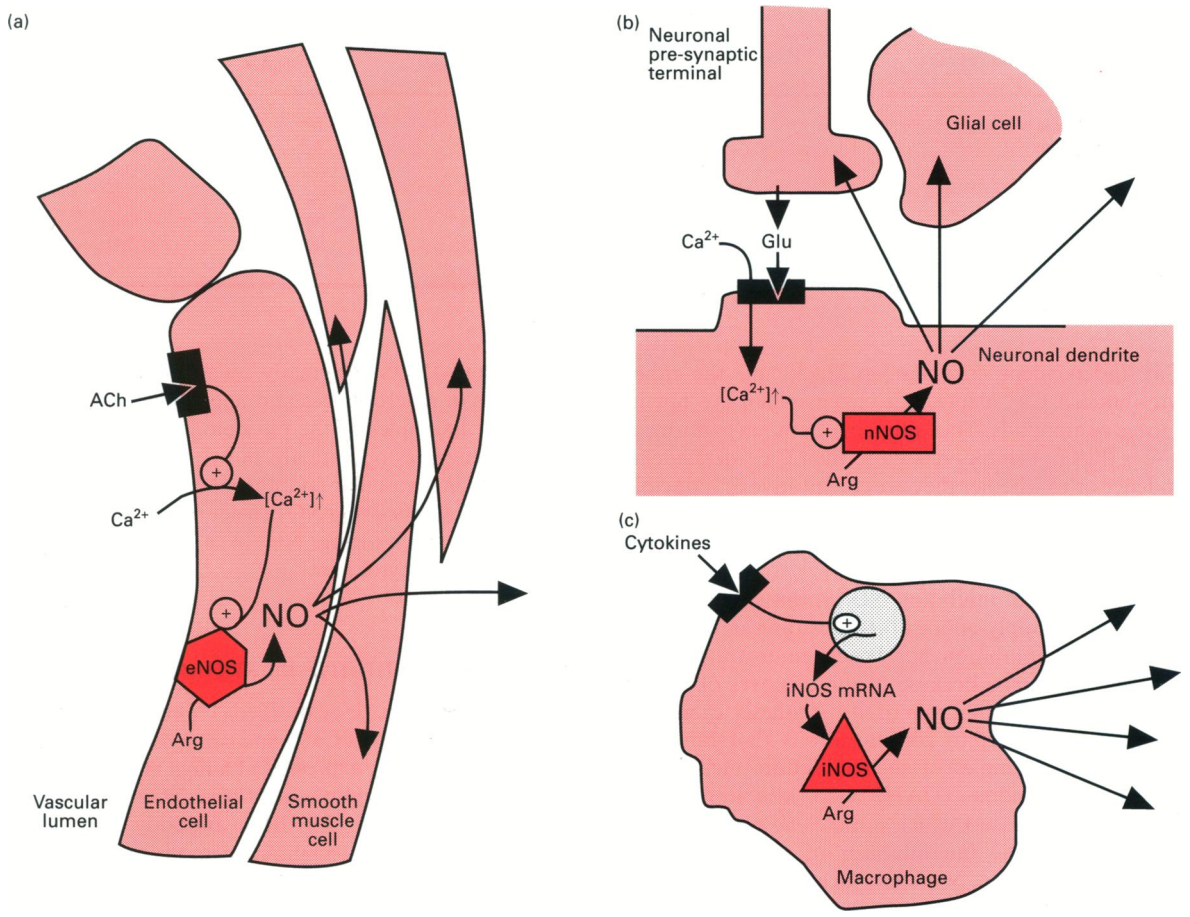

Figure 4 NO synthesis by the known NO synthase isoenzymes

Three examples of NO synthesis are shown: (**a**) synthesis of NO by eNOS in a vascular endothelial cell stimulated by acetylcholine (ACh); (**b**) NO synthesis by nNOS in a neuronal dendrite stimulated by glutamate (Glu); and (**c**) NO synthesis by iNOS in a macrophage following induction of iNOS mRNA and enzyme by cytokines.

ENDOTHELIAL CONSTITUTIVE NO SYNTHASE: eNOS

The constitutive NO synthase of vascular endothelial cells appears to be functionally similar to nNOS and distinct from iNOS, the induced isoenzyme found in activated macrophages. Thus both constitutive enzymes are clearly Ca^{2+}- and calmodulin-dependent, unlike the macrophage enzyme (Table 4). Therefore, until the endothelial enzyme had been cloned, it was not clear whether or not the neuronal and endothelial NO synthases were distinct isoenzymes, despite the lower apparent subunit molecular mass of the purified endothelial enzyme (135 kDa versus 155 kDa for the brain enzyme [15,27]).

Cloning of the bovine [43–45] and human [108,109] constitutive eNOS revealed that eNOS and nNOS are distinct gene products, with only 57% amino acid identity shared between the two human proteins. Northern analysis shows that the eNOS mRNA is 4.4 kb in length, less than half the size of the neuronal mRNA species (Table 5). eNOS, in common with nNOS, has consensus NADPH-, FMN-, FAD- and calmodulin-binding sites and a consensus PKA phosphorylation site (Figure 5). There are, however, significant differences in the N-terminal part of the enzymes: the eNOS N-terminus is shorter and has a consensus *N*-myristoylation site. This finding is in agreement with reports that eNOS is myristoylated [110], and this may explain why it is partially membrane-associated. This has been confirmed by site-directed mutagenesis at the putative myristoylation site, which results in the expression of a cytosolic NO synthase [111]. It seems likely that the 'soluble' and 'particulate' endothelial NO synthases (designated types Ib and III respectively by one group [98]) are the same gene product (eNOS) and that the enzyme is partly (and in some instances predominantly) membrane-associated because of binding of the N-terminal myristate to cell membranes.

This isoform of NO synthase is probably responsible for the NO synthase activity of vascular endothelium in blood vessels of all types: Northern blot analysis has revealed the presence of the mRNA for eNOS in human, bovine, rabbit and rat vascular endothelial cells derived from conduit arteries, microvessels or veins [43,45,108]. Moreover, immunohistochemistry with antibodies of different specificities for nNOS and eNOS has revealed the presence of eNOS immunoreactivity in human lung, liver and skin blood vessels [103].

INDUCIBLE NO SYNTHASE: iNOS

Following exposure of a wide range of cell types and tissues to cytokines or bacterial products, an NO synthase(s) is induced which is clearly different from nNOS and eNOS [5,41]. Rodent macrophages have been the source of the most extensively

Table 5 Cloned NO synthases

Type		Species	Size of mRNA (kb)	Calculated size of protein (kDa)	Amino acid identity (%) Between species	Between isoforms
Neuronal (constitutive)	nNOS	Rat	10.5	160.5	93	57
		Human	10.0	161.5		
Endothelial (constitutive)	eNOS	Bovine	4.4, 4.8	133.0	94	54
		Human	4.7	133.0		
Macrophage (inducible)	iNOS	Murine	4.0, 4.4, 5.0	130.6	81	51
Chondrocyte (inducible)		Human	4.4	131.2		

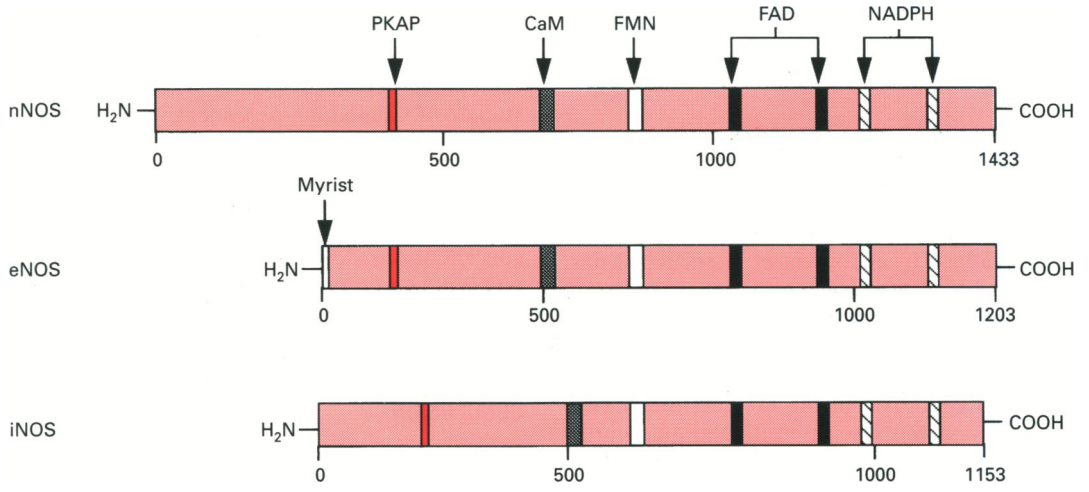

Figure 5 The primary structure of human NO synthases isoenzymes

This schematic representation shows the lengths and consensus binding sites for NADPH, FAD, FMN and calmodulin (CaM) as well as the consensus PKA phosphorylation site (PKAP) and the N-terminal myristoylation site (Myrist).

studied inducible Ca^{2+}-independent NO synthase. Macrophage NO synthase has been purified; it has a subunit molecular mass of approx. 130 kDa and is fully active in the absence of either Ca^{2+} or added calmodulin [26,28]. This enzyme has now been cloned by three independent groups [46–48]. Murine macrophage iNOS has an mRNA species of 4–5 kb which encodes a protein of 130600 Da (Table 5). The nucleotide sequence contains an alternative potential translation start site which could encode a smaller protein of 118 kDa, and in fact a slightly shorter RAW264.7 cell cDNA clone was identified by one group; however, no evidence was found for the expression of this form at the protein level [48]. The amino acid sequence predicted for the 130 kDa species shows that this inducible enzyme is quite distinct from the two known constitutive isoenzymes, sharing only 51 and 54% sequence identity with eNOS and nNOS respectively (Table 5). As for the other cloned isoenzymes, there are consensus sequences for binding of NADPH, FMN and FAD (Figure 5). Surprisingly, in view of the apparent calmodulin-independence of iNOS and its insensitivity to calmodulin inhibitors such as trifluoperazine, this isoenzyme also has a consensus sequence for calmodulin binding, although the sequence comparison of this 21-amino-acid region with that of nNOS shows only 43% identity. The solution of this paradox appears to be that iNOS binds calmodulin extremely tightly, in an apparently Ca^{2+}-independent manner [34]. It can be shown that calmodulin remains bound (non-covalently) to the NO synthase following boiling in SDS and PAGE. Thus calmodulin is a constitutive subunit of iNOS [34], as it is of phosphorylase kinase and one cyclic nucleotide phosphodiesterase.

Probes to the cDNA encoding mouse iNOS have recently been used to clone mRNA species from rat smooth muscle cells and hepatocytes [112,113]. These studies show that these two cell types contain an mRNA for an identical iNOS which is 94% identical to the murine macrophage enzyme. This suggests that the same NO synthase isoform can be induced in all three cell types.

Furthermore, an inducible NO synthase has been cloned, in these laboratories, from human chondrocytes [114]. Following exposure to interleukin-1, human chondrocytes express an NO synthase which is similar to the rodent iNOS in that it is Ca^{2+}-independent and not inhibited by the calmodulin antagonist W13 [115]. Analysis of the 4.4 kb mRNA for this enzyme shows that the 131200 Da protein is 81% identical (and 88% similar) to the rodent macrophage inducible enzyme (Table 5), suggesting

that it is likely to be the human homologue of the rodent macrophage/vascular smooth muscle/hepatocyte inducible NO synthase. This has been confirmed by a very recent report [116] on the cloning of human hepatocyte inducible NO synthase.

CHROMOSOMAL LOCALIZATION OF NO SYNTHASE GENES

The chromosomal location of the nNOS, eNOS and iNOS genes has been determined, using isoenzyme-specific cDNA probes, by Southern blotting analysis of a panel of human–rodent hybrid cell lines. The nNOS gene appears to be an intron-containing gene of at least 20 kb, and is unequivocally localized to a single position on human chromosome 12 [117,118]. The eNOS gene maps to chromosome 7 [119]. In contrast to nNOS and eNOS, iNOS cDNA probes identify several positive hybridization signals, located on either side of the centromere of chromosome 17 [119]. It is not clear whether these code for alternative forms of iNOS or whether some are intronless pseudogenes. The NO synthase genes therefore represent a dispersed gene family on three different chromosomes.

INDUCTION OF Ca^{2+}-DEPENDENT NO SYNTHASES

It is not clear that all of the induced NO synthase activities can be explained simply by the presence of the Ca^{2+}-independent, calmodulin-independent iNOS described above. Induction of a Ca^{2+}-dependent NO synthase by endotoxin or interleukin-1 has been demonstrated in rabbit articular chondrocytes *in vitro* [86]. This was demonstrated to be an induction process by its inhibition by cycloheximide, but it was not inhibited by glucocorticoids. Similarly, increased activity of Ca^{2+}-dependent enzyme has been observed in response to endotoxin in the rat intestine *in vivo* [50]. This enzyme induction was also insensitive to glucocorticoids. This response is different from that of the Ca^{2+}-independent iNOS, the induction of which is potently inhibited by glucocorticoids in macrophages, vascular smooth muscle, endothelial cells and EMT-6 adenocarcinoma cells *in vitro* [120–123] and in liver, lung, ileum and neutrophils *in vivo* [50,124,125]. Furthermore, as mentioned above, the Ca^{2+}-dependent enzyme induced in rabbit chondrocytes is not inhibited by the calmodulin inhibitors W13 or trifluoperazine, unlike the neuronal and endothelial constitutive Ca^{2+}-dependent NO synthases (Table 4). It therefore seems that this inducible Ca^{2+}-dependent NO synthase is biochemically distinct from the two other Ca^{2+}-dependent enzymes. A Ca^{2+}-dependent NO synthase activity has also been reported to be present in the microsomal fraction of activated rodent macrophages, in addition to the Ca^{2+}-independent cytosolic iNOS activity described above [126,127]. Because the sequence of the latter enzyme does not contain a consensus N-terminal myristoylation site [46–48], and because of the different Ca^{2+} sensitivities of these two activities, it again seems possible that this inducible, Ca^{2+}-dependent, particulate enzyme could be a distinct enzyme: either a novel gene product or a post-translationally modified enzyme.

Furthermore, it is now clear that the expression of the constitutive nNOS and eNOS is also subject to regulation, albeit by quite different stimuli from the cytokines and bacterial components that induce iNOS. We have found that the activity of Ca^{2+}-dependent NO synthase in the brain and in a variety of peripheral tissues is substantially increased during pregnancy, e.g. > 4-fold in the uterine artery and > 2-fold in the cerebellum, heart, kidney, skeletal muscle and oesophagus; these changes are accompanied by substantial increases in both eNOS and nNOS mRNAs [128]. Oestrogen also increases the Ca^{2+}-dependent NO synthase activity in a range of tissues, and eNOS and nNOS mRNAs in skeletal muscle. Thus the Ca^{2+}-dependent isoenzymes eNOS and nNOS are subject to induction by oestrogen [128]. In addition, the induction of both eNOS mRNA and protein has been demonstrated following exposure of cultured vascular endothelial cells to shear stress [44]. Significant increases in brain NO synthase activity and nNOS mRNA have also been demonstrated following treatment of rats with lithium and tacrine [129,130]. Further studies will be required to determine the physiological and pathophysiological significance of this regulation of the activity of Ca^{2+}-dependent NO synthases.

OTHER INDUCIBLE Ca^{2+}-INDEPENDENT NO SYNTHASES?

Endotoxin or certain micro-organisms cause a substantial induction of NO synthase in rat liver, and a large proportion of this is in the parenchymal liver cells (hepatocytes) [51,131]. Although this induced enzyme has a subunit molecular mass of approx. 130 kDa and is Ca^{2+}-independent like iNOS, data from two groups have nevertheless suggested that this may be a distinct form of NO synthase, because it is at least partly calmodulin-dependent [31,32] (Table 4). However, both of the studies of calmodulin-dependent, Ca^{2+}-independent, inducible NO synthase purified from rat liver used Ca^{2+} chelators in their extraction buffers, whereas studies in which the macrophage iNOS has not shown calmodulin dependence have not used Ca^{2+} chelators. It is therefore possible that at very low free Ca^{2+} concentrations, dissociation of calmodulin from the macrophage NO synthase occurs, giving rise to a calmodulin-stimulated NO synthase. Further work will be required to resolve whether or not the liver expresses a distinct inducible NO synthase isoenzyme.

CONCLUSION

Intensive study of the NO synthases during the last 5 years has revealed a family of enzymes, carrying out a complex reaction, which appear to be highly conserved between the mammalian species studied so far. Such conservation between species, together with the diversity of the isoenzymes and the large (and still growing) list of physiological roles that they serve, suggest that synthesis of NO from L-arginine is a regulatory and host defence mechanism of great importance. The combination of the types of role that NO plays, namely intracellular signal, transcellular signal and cytotoxic molecule, is unprecedented in biology.

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