Abstract

Nitric oxide (NO) is synthesised from l-arginine by the enzyme NO synthase (NOS). The complex reaction involves the transfer of electrons from NADPH, via the flavins FAD and FMN in the carboxy-terminal reductase domain, to the haem in the amino-terminal oxygenase domain, where the substrate l-arginine is oxidised to l-citrulline and NO. The haem is essential for dimerisation as well as NO production. The pteridine tetrahydrobiopterin (BH4) is a key feature of NOS, affecting dimerisation and electron transfer, although its full role in catalysis remains to be determined. NOS can also catalyse superoxide anion production, depending on substrate and cofactor availability. There are three main isoforms of the enzyme, named neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS), which differ in their dependence on Ca2+, as well as in their expression and activities. These unique features give rise to the distinct subcellular localisations and mechanistic features which are responsible for the physiological and pathophysiological roles of each isoform. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Nitric oxide (NO), synthesised by the enzyme NO synthase (NOS), is a major factor in the cardiovascular system. Its multiple roles include regulation of vasomotor tone [1] and cell adhesion to the endothelium [2], and inhibition of platelet aggregation [3] and vascular smooth muscle cell proliferation [4]. At first glance, this list would appear to suggest that NO is a crucial factor in the prevention of cardiovascular damage such as that seen in atherosclerosis. Indeed, the loss of endothelial-derived NO arising from endothelial dysfunction is now thought to be a major cause of such pathological conditions. However, too much of a good thing should usually be avoided, and NO is no exception. Excess or inappropriate production of NO can be equally as deleterious as insufficient NO.

Hence, immense research efforts are currently being made to understand the regulation, production, and functions of NO. This review will concentrate on what is currently known about the enzyme which synthesises NO.

The complexity of NOS, with its distinct domains, its multitude of cofactors and prosthetic groups, and its unique reaction mechanism, has in recent years drawn the attention of a large number of biochemists and structural biologists. However, despite intense research efforts, several key features remain to be determined, most notably the structure of the intact enzyme. Other open questions include the complex role of the pterin cofactor tetrahydrobiopterin (BH4), the nature of the NOS products in vivo, and the mechanistic reasons for the subtle differences between the three isoforms. Recent progress that has been made towards these goals will be discussed.

2. Overview of the NO synthase family

NOS (EC 1.14.13.39) catalyses NO biosynthesis via a reaction involving the conversion of l-arginine to l-citrulline [5]. The enzyme functions as a dimer consisting of two identical monomers, which can be functionally (and structurally) divided into two major domains: a C-terminal reductase domain, and an N-terminal oxygenase domain.
(Fig. 1) [6]. The former contains binding sites for one molecule each of NADPH, FAD, and FMN, in close homology with cytochrome P-450 reductase, whereas the latter binds haem and BH4, as well as the substrate l-arginine. Between these two regions lies the calmodulin (CaM) binding domain, which plays a key role in both the structure and function of the enzyme.

There are three distinct isoforms of NOS which differ both in their structure and function [7]. Endothelial NOS (eNOS or NOS III, 2×134 kDa) and neuronal NOS (nNOS or NOS I, 2×160 kDa) are generally referred to as constitutively expressed, Ca2+-dependent enzymes, although eNOS can also be activated in a Ca2+-independent manner (discussed in Section 8.1) [8]. Inducible NOS (iNOS or NOS II, 2×130 kDa) is expressed at high levels only after induction by cytokines or other inflammatory agents, and its activity is independent of an increase in Ca2+. The three NOS isoforms are characterised by regions of high homology, namely the oxygenase and reductase domains, but at the same time each isoform exhibits distinctive features which reflect their specific in vivo functions. Although the molecular biology of these isoforms is discussed at length in another article in this issue, the main structural differences between the three enzymes will be referred to briefly in a later section of this article, since they have a major impact on the enzymatic function of each isoform.

3. The NOS-catalysed reaction

Biosynthesis of NO involves a two step oxidation of l-arginine to l-citrulline, with concomitant production of NO (Fig. 2). The reaction consumes 1.5 mol of NADPH, and 2 mol of oxygen per mol of l-citrulline formed. The proposed mechanisms are discussed at length by Griffith and Stuehr and others [9–11], and involve an initial hydroxylation of l-arginine, leading to the formation of \( \text{N}^\circ \)-hydroxy-l-arginine, which can also act as a substrate for NOS. This is followed by oxidation of the intermediate, using a single electron from NADPH [12], to form l-citrulline and NO. Although this scheme represents the reaction assumed to be catalysed by NOS, the enzyme is also capable of catalysing the production of additional products, notably superoxide anion (\( \text{O}_2^- \)), depending on the conditions [13–15]. The nature of the in vivo products of NOS is still under debate and is discussed in Section 7, as well as elsewhere in this issue.

4. The reductase and oxygenase domains

The isolated reductase domain is able to transfer electrons from NADPH via the flavins FAD and FMN to cytochrome c, while the oxygenase domain dimer can convert the reaction intermediate \( \text{N}^\circ \)-hydroxy-l-arginine to NO and l-citrulline [16–18]. Hence, the two domains perform catalytically distinct functions. While the reductase domain itself is highly homologous to enzymes such as the NADPH:cytochrome P450 reductase, its dependence on the CaM-binding domain for efficient electron transfer is unique [19]. The cofactor binding sites have been well-defined as a result of the close homology with related reductases as well as evidence obtained from mutagenesis studies [20–22]. In contrast, the binding sites for l-arginine, haem, and BH4 in the oxygenase domain are less well characterised, although several residues have been identified which are important for BH4 binding (C99 in eNOS [23], G450 and A453 in iNOS [24]). A polypeptide of this region (558–721 in rat nNOS) which shows similarity to the pterin-binding domain of dihydrofolate reductase and to a region in aromatic amino acid hydroxylases [24], was however unable to bind BH4, but could bind \( \text{N}^\circ \)-nitro-l-arginine [25]. Two residues important for l-arginine binding have been identified in this region: E371 and D376 in iNOS [26], and the analogous E361 in eNOS [27]. Several other acidic residues which affect l-arginine and BH4 binding were also identified in this region [26]. The loss of l-arginine binding by the E371 mutant was put to good use in a study of electron transfer in heterodimers consisting of a full length subunit and an oxygenase domain [28]. NO was synthesised when the oxygenase domain containing the mutation was in the same subunit as the reductase but not if it was in the opposite oxygenase domain, indicating that electrons are transferred from the reductase domain flavins on one subunit to the oxygenase domain haem on the second subunit.
The recently solved crystal structure of a dimeric iNOS oxygenase truncation mutant (residues 66–498) revealed a structure which is unusual for haem-binding proteins in that it contains a large amount of β-sheet [29]. The authors describe the structure in analogy with a baseball glove, with the haem cradled between the proximal ‘thumb’ and the distal ‘palm’. BH4 binds on the proximal side, while L-arginine is located on the distal side. Another notable feature of the oxygenase domain dimer, revealed in the recent crystal structure of the eNOS haem domain [30], is the presence of a zinc atom which is tetrahedrally coordinated to two pairs of Cys residues. The metal may be important in determining the stereospecificity of the BH4 binding site.

The reductase and oxygenase domains of NOS are therefore distinct catalytic units, which together provide the complete machinery required for NO production. This raises the question as to why the functional enzyme is a dimer rather than a monomer. The mutation experiments described above, showing that electrons are transferred from one subunit to another rather than within one subunit, provide a hint to this puzzle. The next section describes the factors important in the dimerisation process which leads to the fully functional enzyme.

5. The NO synthase dimer

An essential feature of NOS is that, despite the ability of the reductase and oxygenase domains to function independently under certain circumstances, NO synthase activity is carried out by the homodimer. Although the mechanistic reasons have not yet been resolved in detail, a significant amount is already known about the factors which govern dimerisation.

5.1. Role of haem

The haem plays an essential role in dimerisation (Fig. 3). In its absence, NOS exists as monomers which are essentially normal with respect to secondary structure. Furthermore, the ability to catalyse the NADPH-dependent reduction of cytochrome c is retained in nNOS monomers, indicating that the transfer of electrons within the reductase domain from NADPH via the two flavins is not dependent on the dimeric structure [31]. Monomers of all the isoforms are, however, unable to bind BH4 or a substrate analogue and do not catalyse L-citrulline/NO production [31–33]. Haem is the sole cofactor for which there is an absolute requirement for the formation of active nNOS dimers [31], and it is also the key factor in eNOS dimerisation [33]. Although the characteristics differ from those of the two constitutive isoforms, the haem plays a similarly essential role in iNOS dimerisation [32].

Resolution of the crystal structure confirmed that the haem is bound via a proximal cysteine thiolate ligand [29], the identity of which is known for all three isoforms [34–36]. The formation of this bond has been suggested to be a key step in the process of dimerisation [37]. Evidence obtained from studying the fluorescence dynamics of nNOS-bound flavins suggests that the haem is also essential in the interaction between the reductase and oxygenase domains [38], which form an interface in the quaternary structure.

The coordination state of the haem can be unequivocally identified through examination of the absorption spectrum. This depends upon the energetical ‘spin state’ of the unpaired electrons of the haem iron, which in turn is related to the geometry of the haem ligands. When these electrons are in the low-spin state, reflecting a six-coordinate haem, the maximum is observed at 394–397 nm. This form of NOS is inactive. Upon binding L-arginine and its analogues, as well as BH4 [39–47], the maximum shifts to 418 nm, indicative of a high-spin five-coordinate haem, which is necessary for NOS activity.

5.2. Role of BH4

The haem requirement for dimerisation is common to all three NOS isoforms. They do however differ with respect to the role of BH4 in dimerisation. Whereas nNOS and eNOS can form dimers in the absence of BH4 [48], iNOS dimerisation was reported to require the presence of the
pteridine [32], although dimers were formed in E. coli in the absence of BH$_4$ [49]. Furthermore, BH$_4$ stabilises the nNOS and eNOS dimers once formed, and also the iNOS dimer, although not to the same extent [33,48,50,51]. These data are supported by the reduced binding of BH$_4$ by an N-terminal deletion mutant of iNOS, demonstrating the importance of residues 66–114 in iNOS for binding of the cofactor and hence dimerisation [52]. The recent crystallographic data also show the location of BH$_4$ at the dimer interface [29]. Although these observations have had an impact on in vitro synthesis and reconstitution experiments, the functional implications remain uncertain. The close resemblance of 4-amino-BH$_4$, a novel pterin-based inhibitor of NOS [51,53], to BH$_4$ in terms of conformational changes induced (low-spin to high-spin conversion of the haem, dimer stabilisation, increased affinity for l-arginine) despite the inability to support NO production suggests a more complex role for BH$_4$ than merely inducing conformational changes [51,54]. The close proximity of BH$_4$ to the haem, as well as to the flavins at the domain–domain interface [38], hints at a possible role in electron transfer [55], although exogenously added BH$_4$ does not appear to provide electrons for the reaction [56]. In this respect, the role of BH$_4$ in NOS differs from that in aromatic amino acid hydroxylation [57]. A thorough analysis of the interaction of numerous pterins with iNOS revealed that the steps up to and including haem reduction are supported by dihydropterins as well as tetrahydropterins [58]. However, only the latter are able to support NO synthesis and NADPH oxidation. The role of BH$_4$ in electron transfer therefore remains to be settled, although it has been shown that the cofactor accelerates the decay of the ferrous–dioxy complex of nNOS, providing a novel hint to its role in NO synthesis [59]. Despite these clues, the full role of BH$_4$ in NOS catalysis remains to be elucidated.

5.3. Role of l-arginine

Binding of l-arginine to iNOS facilitates dimerisation [32]. Various arginine analogues as well as compounds containing the guanidinium moiety are also able to facilitate iNOS dimerisation [44]. Despite the inability to support NO synthesis, these compounds also alter the heme spin state, indicating a change in the haem geometry to the five-coordinate conformation, and increase the rate of NADPH oxidation, showing that occupation of the guanidinium-binding site in the enzyme alters electron flow. The extensive interactions of l-arginine, as revealed by the crystal structure [29], with hydrophilic side chains of the alpha helix involved in dimer formation, as well as with the haem propionate which hydrogen bonds to BH$_4$, provide the structural reasons for the stabilising effect on the dimer.

In summary, the key to dimerisation of NOS lies in the haem prosthetic group, although BH$_4$ and l-arginine are also important factors, with their relative contribution differing depending on the isoform in question. As a result of their stabilising interactions, these molecules endow the NOS dimer with an exceptionally stable quaternary structure.

6. Calcium dependence and the role of calmodulin

Dependence on Ca$^{2+}$ is a key distinguishing feature between the constitutive and inducible isoforms. eNOS and nNOS are both activated by an elevation in intracellular Ca$^{2+}$, followed by the subsequent binding of Ca$^{2+}$/CaM. In contrast, iNOS contains irreversibly bound CaM, and is hence largely independent of Ca$^{2+}$, although a 2-fold greater activity is observed in the presence of 2.5 nM Ca$^{2+}$ compared to that in 10 mM EGTA [60]. The molecular reasons for this fundamental difference have been investigated by swapping the respective CaM-binding regions [60]. The iNOS CaM-binding peptide enabled eNOS to bind CaM but did not confer Ca$^{2+}$-independence. Hence, only the Ca$^{2+}$-bound conformation of CaM can activate the enzyme. This dependence on Ca$^{2+}$ may be ultimately due to the presence of an autoinhibitory sequence which is present in the FMN-binding region of the two Ca$^{2+}$-dependent isoforms but is not found in iNOS [61]. The CaM-binding region in eNOS is directly involved in membrane association, specifically to anionic phospholipids such as phosphoserine, and this association prevents the binding of CaM to eNOS and hence catalytic activity [62]. CaM binds to both the isolated reductase domain of nNOS as well as to the full-length enzyme, and stimulates the rate of electron transfer within the reductase domain [19]. CaM is furthermore essential for the transdomain transfer of electrons to the haem [63]. Despite this knowledge, the exact mechanism by which CaM induces these changes is not understood.

7. Products of the reactions catalysed by NO synthase

nNOS is capable of producing not only NO, but also O$_2^-$, and peroxynitrite (Fig. 3). This unusual property is a consequence of the dimeric nature of the enzyme, in which the two subunits are able to function independently [64]. In fact, the purified nNOS dimer normally consists of one BH$_4$-containing subunit and one BH$_4$-free subunit, due to the large difference in binding affinity between the first and second BH$_4$-binding sites [47]. This negative cooperativity of BH$_4$ binding means that only one subunit will have BH$_4$ bound over a wide range of BH$_4$ concentrations (up to 1 μM). This has important implications for the outcome of the catalytic reaction, since only at very high BH$_4$ concentrations will NOS function purely as an
NO synthase (Fig. 3d). Although the following discussion concerning NO synthase products largely refers to nNOS, recent observations (see below) suggest that both eNOS and iNOS are also capable of catalysing the production of O$_2^-$, suggesting that this mechanism may be more widespread than originally thought.

7.1. The uncoupled reaction

In the presence of low concentrations or the absence of l-arginine, nNOS catalyses the uncoupled reduction of oxygen, leading to the production of O$_2^-$ and H$_2$O$_2$ (Fig. 3b) [13,14]. Although O$_2^-$ itself, as well as its metabolic products, can trigger a variety of signal transduction processes leading to pathogenic conditions [65], the detrimental effects of O$_2^-$ on endothelial function, vascular smooth muscle cell proliferation, and leukocyte adhesion, which are evident in vascular pathologies are likely to be mediated by the scavenging of NO. Evidence for the damaging role played by O$_2^-$ is provided by the protective effect of superoxide dismutase (SOD) delivery in a rat model of angiotensin II-induced hypertension [66] as well as in cholesterol-fed rabbits [67,68].

The only major difference between the NOS isoforms in terms of the reactions performed lies in the rate of this NADPH oxidation, termed the uncoupled reaction. Under these conditions, nNOS continues to transfer electrons to the haem and hence oxidise NADPH at a high rate, whereas in eNOS and iNOS, this reaction occurs at a much slower rate [33,40,63]. A mechanistic explanation for this difference is provided by a study examining the reduction potential of the haem [69]. The haem iron in nNOS has a significantly higher reduction potential than that in iNOS, which must first bind substrate and BH$_4$ in order to achieve a similar value. Hence, the haem iron of nNOS but not of iNOS is readily reduced in the absence of l-arginine and BH$_4$. The protective effect of manganese SOD in NO-mediated NMDA toxicity in cortical neurons [70] indicates that the uncoupled reaction catalysed by nNOS is pathologically relevant. The much lower rate at which this reaction is catalysed by iNOS, which is often present in situations where l-arginine is limited, for example in wounds [71], has been suggested to constitute a substrate-controlled safety mechanism to minimise O$_2^-$ production under such conditions [63]. However, recent data has shown that iNOS is in fact capable of catalysing substantial O$_2^-$ production [72]. This occurs via a different mechanism to that in nNOS, in that production is catalysed by the reductase domain and is only inhibitable by very high concentrations of l-arginine. A similar control mechanism for eNOS has been proposed, since the production of O$_2^-$ would be highly detrimental in the vasculature [40]. However, increased O$_2^-$ production by eNOS has been described in the context of the endothelial dysfunction seen in spontaneously hypertensive rats [73] and native LDL-treated endothelial cells [74]. The reason for this alteration in eNOS activity leading to an increase in O$_2^-$ production may be related to a decrease in BH$_4$ levels since two recent studies show that O$_2^-$ production by eNOS is inhibited by BH$_4$ but not l-arginine [75,76]. However, whereas l-arginine inhibits O$_2^-$ production by preventing uncoupled NADPH oxidation, BH$_4$ appears to act by directly scavenging superoxide [76]. The reasons for these isoform-specific differences in the regulation of O$_2^-$ production are not clear.

7.2. A peroxynitrite synthase?

Saturating l-arginine concentrations and the presence of sub-saturating levels of BH$_4$ lead to the simultaneous production of NO and O$_2^-$, by the BH$_4$-containing and the BH$_4$-free subunits respectively (Fig. 3c). Although these two products can react together extremely rapidly to form the potent oxidant peroxynitrite [77], the physiological outcome probably depends on the levels of GSH and SOD [78]. In the absence of these two molecules, peroxynitrite is formed. Free NO, which appears to feedback inhibit nNOS [79] by forming a ferrous–nitrosyl complex [80], is not detectable unless high concentrations of SOD are present [81]. However, very low levels of SOD are sufficient to allow enough NO to be formed to activate soluble guanylate cyclase, although the NO levels are below the limits of detection. In the presence of GSH and SOD, NO/O$_2^-$ reacts preferentially with the thiol to form the thionitrite GSNO, which is likely to act as a storage or transport form of NO [82,83]. Subsequent release of NO can be mediated by both enzymatic and non-enzymatic mechanisms, although the actual physiological route has not yet been clarified.

The formation of peroxynitrite from NO and O$_2^-$ has been implicated in the pathology of a large number of conditions involving oxidative stress such as atherosclerosis [84], using the presence of the marker nitrotyrosine as evidence. However, the true identity of the molecule responsible for the tyrosine nitration has recently been put into question, with the demonstration that peroxynitrite formed from the simultaneous production of NO and O$_2^-$ at physiological pH is unable to efficiently nitrate tyrosine [85]. NO$_2^-$ has been proposed as a possible alternative, with the demonstration that, in human polymorphonuclear neutrophils, NO$_2^-$ is converted by myeloperoxidase into the nitrating and chlorinating species NO$_2$Cl and NO$_3$ [86].

Therefore NOS can, in vitro, act as a peroxynitrite synthase. However, the name is unlikely to be applicable in vivo, given the ubiquitous presence of GSH and/or SOD, which will react with the NO synthase products, thereby preventing the formation of peroxynitrite. The ability to catalyse the production of O$_2^-$ is not, as was previously thought, limited to nNOS, and may in fact be pathologically highly relevant.
8. Unique features of the NO synthase isoforms

8.1. eNOS

The main source of endothelial NO, a crucial factor for the normal functioning of the cardiovascular system, is eNOS expressed by endothelial cells [87,88]. Other cellular sources relevant to the cardiovascular system include cardiac myocytes [89] and cardiac conduction tissue [90]. The particular properties of eNOS which enable it to perform its specialised functions include Ca\(^{2+}\) sensitivity, and the posttranslational modifications which mediate subcellular localisation. These enable the enzyme to respond not only to a variety of neurohormonal agents, but also to haemodynamic forces. In these respects, eNOS differs significantly from the other isoforms, and this section describes the molecular properties of the enzyme which account for these specialised features.

Although eNOS was often referred to earlier as constitutive NOS, a number of factors as diverse as hypoxia [91], estrogen [92] and exercise [93] are now known to alter its expression. Since endothelial control of vascular tone is a sensitive and highly tuned process, these changes are likely to be immensely important to cardiovascular function, particularly in pathophysiological situations. eNOS is active, albeit sub-maximally, at the concentration of Ca\(^{2+}\) found in resting endothelial cells (around 100 nM) [40], explaining the vasocostricting effect of a NOS inhibitor on basal blood flow [94]. The enzyme is generally fully activated by an increase in intracellular Ca\(^{2+}\), resulting either from an influx of extracellular Ca\(^{2+}\), or from release from intracellular stores. However, in a study using stretched segments of artery [8], shear stress-induced NO production, which was dependent on tyrosine phosphorylation, was independent of extracellular Ca\(^{2+}\). The mechanism behind this observation was suggested to be a change in the microenvironment of the protein, perhaps involving pH changes.

The activation of eNOS can be induced by hormones such as catecholamines and vasopressin, autacoids such as bradykinin and histamine, and platelet-derived mediators such as serotonin and ADP, via receptor-mediated activation of G proteins [95]. The activation of eNOS by mechanical forces including shear stress [96] and cyclic strain [97] is also mediated through G protein activation [98]. The subcellular targeting of eNOS plays a crucial role in this receptor-mediated mechanism of activation, by localising the enzyme in the proximity of the signaling molecules which mediate its activation [99]. This localisation to the plasmalemmal caveolae is regulated by the posttranslational modifications at the N-terminal myristoylation and palmitoylation sites which are unique to the endothelial isoform of NOS (see accompanying article by Papapetropoulos et al., pp. 509–520, this issue). The distinctive lipid content of the caveolae is important to their function, and, in light of the observation that NOS activity is negatively modified by anionic phospholipids [62], it is conceivable that in conditions such as hypercholesterolemia, the disruption in eNOS activity may be caused by alterations in the lipid surroundings. The association of eNOS with the resident coat protein of caveolae (caveolin-1 and caveolin-3 in endothelial cells and myocytes respectively) is mediated by the scaffolding domain in caveolin, and leads to inhibition of eNOS activity [100], apparently via functional interference with CaM binding and electron transfer [101]. A similar inhibitory association has recently been observed between eNOS and the bradykinin B2 receptor [102]. Interestingly, a putative consensus sequence for mediating the interaction with caveolin is present not only in eNOS (350–358 in bovine eNOS) but also in the other two NOS isoforms [103]. This sequence lies in the putative oxygenase/reductase interaction surface suggested by the crystal structure [29], supporting the proposed role of interference with electron transfer. It is the disruption of this acylation-independent eNOS-caveolin complex and not as earlier studies suggested depalmitoylation [104] which leads to the agonist-induced activation of eNOS [105].

8.2. nNOS

In addition to the well-studied role of NO in the process of penile erection [106], non-adrenergic non-cholinergic relaxation occurs in all vascular smooth cells, as a result of the widespread expression of nNOS in peripheral neurons [107]. The regulation of nNOS activity is unique, with the subcellular localisation of the enzyme being mediated by a completely different mechanism to the fatty acylation-mediated membrane association of eNOS. NOS is the largest of the three isoforms due to the addition of a 300 amino acid stretch at the N-terminus. This region contains a PDZ domain (named after three of the proteins in which it was first described [108], also called a discs-large homologous region (DHR) or GLGF amino acid repeat), which is an approximately 90-residue long protein-recognition module responsible for the association of nNOS with other proteins containing this motif, including dystrophin at the sarcolemmal membrane [109] and PSD-95, a channel-associated protein in the brain [110]. The Tyr77 residue in the NOS PDZ domain mediates selective binding to an Asp-X-Val consensus, which differs from the (Thr/Ser)-X-Val consensus favoured by PSD-95, and is present in glutamate and melatonin receptors [111]. Like eNOS, nNOS is also inhibited by the association with caveolin [100]. This inhibitory interaction with caveolin-3 in skeletal muscle can be mediated by two separate caveolin domains [112].

In terms of the enzymatic function of nNOS, it appears to differ from the other NOS isoforms by its readiness to catalyse the uncoupled oxidation of NADPH. Progress is being made in understanding the mechanism of this reaction (see Section 7). Although this reaction may help
to explain the damaging role of nNOS in ischaemia in the brain [113], the significance for nNOS-expressing cells outside the brain is not yet clear.

8.3. iNOS

The effects of iNOS in vivo are a result of its unique features of Ca$^{2+}$-independence and inducible, high level expression. Under normal physiological conditions, iNOS is unlikely to have much impact on the cardiovascular system because of its low or absent expression, a conclusion which is supported by the lack of phenotype of uninfected iNOS knockout mice [114]. However, iNOS expression can be induced by inflammatory mediators in most types of vascular cells, including endothelial cells [115], cardiac myocytes [116], and smooth muscle cells [117], as well as macrophages [118], which, as a result of the high NO output, can have potentially damaging consequences. The expression of iNOS by macrophages and smooth muscle cells in atherosclerotic lesions has been taken as evidence for its detrimental role in atherosclerosis [118]. Furthermore, iNOS expression is responsible for the impairment in eNOS-derived NO production in vessels treated with inflammatory mediators [119]. However, iNOS expression may in some cases be protective, as shown by the iNOS-mediated suppression of allograft arteriosclerosis, via the prevention of intimal hyperplasia [120]. In contrast to the two constitutive isoforms, iNOS contains neither of the specific membrane-targeting sequences. Despite this, it has been found to be membrane-associated in human neutrophils [121] and mouse macrophages [122,123]. However, the proportion of membrane-bound enzyme varies between cell type and species, with less than half of mouse macrophage iNOS being membrane-associated. The functional relevance of this association is not known.

The three distinct isoforms of NOS therefore show contrasting functions as a result of their sequence differences. Some of these lead to obvious structural changes, such as the fatty acylation of eNOS, whereas others are more subtle, for example the tendency to uncouple NADPH oxidation. Understanding these differences will enable us to exploit the unique features of each isoform, permitting selective stimulation or inhibition as required.

9. Control of NO synthase activity via substrate/cofactor regulation

Although an in depth discussion on the topic is outside the scope of this article, the regulation of cofactor availability in determining NOS activity deserves a mention here. Apparent paradoxes of decreased protein expression in the face of increased NO production, as seen in LDL-treated endothelial cells [124], illustrate the necessity of understanding the global regulation of NOS activity. The availability of substrate, particularly in the case of iNOS, can be regulated by changes in L-arginine uptake [125], or in the activities of argininosuccinate synthetase [126] or arginase [127]. BH$_4$ availability is controlled largely by GTP cyclohydrolase [128]. Regarding substrate levels, the fact that both the concentration of L-arginine in blood and the intracellular L-arginine concentration [129,130] are far greater than the $K_m$ of NO for L-arginine [131] would seem to suggest that substrate availability should never be a limiting factor under normal conditions. However, several studies have shown that supplementation with L-arginine can have beneficial effects, e.g. it reversed the increased adhesiveness of monocytes in hypercholesterolemic humans [132]. One possible mechanism by which L-arginine mediates these effects may be to out-compete the effects of the endogenous inhibitor, asymmetrical dimethylarginine, which is increased in hypercholesterolemia [133]. Since high doses of arginine can have additional effects which are unrelated to NO synthesis such as increasing the release of insulin [134,135], it is important to show that the effects of arginine are stereospecific in order to claim that increased NOS activity is mediating the observed changes.

Alterations in the pathways governing substrate and cofactor availability can have a significant impact on the outcome of NOS activity. Understanding these regulatory mechanisms will provide insights into both the physiological regulation of NOS activity as well as the reasons behind the many pathophysiological states in which alterations in NO production are postulated to play a role.

10. Conclusion

The complexity of NO biosynthesis is largely attributable to the multi-featured nature of the enzyme itself. Although much progress has recently been made into elucidating the biochemistry of this dimeric, multidomain molecule, it will be clear from this review that the mystery is far from solved. Outstanding questions which have been discussed here include the role of BH$_4$ and the nature of the NOS products in vivo. Despite the hints provided by the crystal structure and substances like 4-amino-BH$_4$, the precise function of the cofactor is still a matter of debate. The continuing development of various BH$_4$ analogues should in the near future enable us to at last understand the full role of this cofactor in NOS function. In contrast, with recent publications demonstrating the ability of eNOS and iNOS, as well as nNOS, to produce O$_2^-$/H$_2$O$_2$ [72,75,76], it appears that the struggle to clarify the nature of the actual products of NOS is far from over. Similarly, despite growing evidence concerning the structural differences which give the NOS isoforms their distinct functions, the aim of exploiting these differences in order to selectively modify NOS activity in an isoform-specific manner remains a target for the future.
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