Nitric oxide synthases: regulation in disease

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Nitric oxide in health and disease

The fundamental role of nitric oxide (NO) in the maintenance of normal human physiology and the attendant pathology resultant from its perturbation in disease states is becoming increasingly apparent. That NO mediates such diverse functions as maintenance of volume and pressure homeostasis, neurotransmission, and immunity is not surprising given its primordial origins. NO synthesis has been detected in species as diverse as slime mould, locusts, and man. This article will review the structure and regulation of the nitric oxide synthases and discuss selected aspects of the role of NO in human health and disease. The reader is referred to excellent recent reviews for more detailed discussions [1,2].

The L-arginine–nitric oxide pathway

The observation that the vascular endothelium produced a labile diffusible factor necessary for acetylcholine-dependent relaxation of vascular smooth muscle was first made by Furchgott and Zawadski in 1980 [3]. Further characterization of this endothelium-derived relaxing factor (EDRF) demonstrated its actions to be mediated through activation of soluble guanylate cyclase [4,5]. EDRF bioactivity can be inhibited by haem-containing proteins [6] and augmented by superoxide dismutase [7]. It is now generally accepted that EDRF and NO are, for the most part, the same entity.

The synthesis of NO from L-arginine is catalysed by the nitric oxide synthases. This reaction requires oxygen and reducing equivalents in the form of NADPH [8]. L-citrulline, which is produced in equimolar amounts with NO, may then pass through the urea cycle to regenerate L-arginine (Figure 1).

![Figure 1. The reaction catalysed by the nitric oxide synthases.](https://academic.oup.com/ndt/article-abstract/11/1/215/1867011)

The nitric oxide synthases

The nitric oxide synthases (NOS) are a family of apoenzymes, with at least three human isoforms, each of which catalyses the production of NO from L-arginine. Analysis of the genomic organization of each gene demonstrates a high degree of homology. This suggests that the NOS isoforms may reflect amplification events involving a primordial gene. Regulation of gene expression and tissue distribution vary for each of the isoforms demonstrating evolution and specialization of this fundamental signalling mechanism (Table 1).

Structurally the NOS enzymes consist of an oxidative domain at the amino-terminus, which is known to bind haem, and a reductase domain at the carboxy-terminus. Binding sites for NADPH, FAD and FMN exist in the reductase domain and act to shuttle electrons to the haem centre which catalyses the oxidation of L-arginine to form NO and L-citrulline [9–13]. Tetrahydrobiopterin and prosthetic haem (ferroprotoporphyrin IX) are necessary cofactors [14].

The two constitutively expressed members of the NOS family, neuronal NOS (nNOS, NOS1) and endothelial NOS (ecNOS, NOS3), have been localized to chromosomes 12 and 7 respectively [15]. Although expression of these genes is constitutive, activation is dependent on an elevation in the intracellular free calcium concentration. Increases in calcium stimulates transient binding of the calcium–calmodulin complex to NOS [16–18]. This binding results in a conformational change in the NOS protein allowing shuttling of electrons from NADPH in the reductase domain to the catalytic haem site within the oxidative domain.

As the constitutively expressed NOS isoforms are triggered by elevations in intracellular calcium concentration, their activity is transient. Furthermore, catalytic activity is low compared to inducible NOS [19]. Thus, ecNOS and nNOS may be characterized as a
NO in large amounts. This inducible NOS pathway is expression by lipopolysaccharide or cytokines takes place over hours and may be sustained for days [20,21]. It is now appreciated that iNOS induction produces transients for activation. Rather, iNOS mRNA synthesis is required for activity. Induction of iNOS does not depend on calcium.

The gene for neuronal NOS, the first NOS protein to be purified, was the last to be structurally characterized due to its immense size and complexity [22]. The nNOS gene consists of 29 exons and 28 introns spanning approximately 200 kb. The translation initiation and termination sites are located in exon 2 and exon 29 respectively. Regulation of nNOS gene expression is complex. Alternative promoters produce multiple first exons with each splicing onto a common exon 2 [23,24]. This allows for independent, perhaps tissue-specific, promoters to regulate transcription of a common nNOS protein. Further, cassette deletions of exons 9/10 and exon 10 have been described [22,25]. It remains to be determined whether these alternatively spliced mRNA transcripts are translated in vivo.

Expansion of triplet repeats has been demonstrated to be the cause of several neurodegenerative disorders. Three dinucleotide expansions of the (dC-dA)n variety have been identified within the nNOS gene. Of significance, one expansion is located in the 5'-flanking region of the gene and another in the 3'-untranslated region in exon 29. These dinucleotide repeats are located in regions important for regulation of gene transcription and mRNA stability and thus may have a role in inherited variability in nNOS expression.

The tissue distribution of nNOS is diverse. The mRNA transcript for nNOS has been detected in the central and peripheral nervous system, the non-adrenergic non-cholinergic system, macula densa of the kidney, adrenal medulla, skeletal muscle, male sex organ, and pancreatic B cells, among others [9,26–29].

**Endothelial NOS**

The ecNOS gene consists of 26 exons and 25 introns spanning approximately 21 kb of human genomic DNA [15]. Expression of ecNOS is largely restricted to vascular endothelial cells although there are recent reports of localization in the CA1 region of the hippocampus [30,31] and syncytiotrophoblast [32]. Sequence analysis of the 5'-flanking region of the promoter of the ecNOS gene reveals putative cis-regulatory DNA sequences: shear stress, steroid-regulatory and fos/jun responsive (AP-1) motifs, among others. Whether any of these cis-regulatory elements are involved in ecNOS gene expression remains to be determined.

The importance of post-transcriptional regulation of ecNOS is becoming more widely appreciated. In human vascular endothelial cells, NO production is significantly suppressed by hypoxia. Concomitant with this change is a 40–60% reduction in the steady-state mRNA levels of ecNOS. Nuclear run-off analyses and actinomycin D chase experiments have revealed that lower steady-state levels of ecNOS mRNA transcripts result from decreased transcription of the gene as well as reduced message stability [33]. In addition, TNFα treatment destabilizes ecNOS mRNA, shortening the half-life from 48 to 3 h in human umbilical vein endothelial cells. TNF-α-mediated decrease in ecNOS mRNA content in endothelial cells is dependent on new protein synthesis as it can be blocked by cycloheximide [34]. The observation that ecNOS mRNA transcripts are regulated post-transcriptionally at the level of RNA stability implies that RNA-binding proteins are involved in the processing of RNA transcripts in models of endothelial injury.

**Inducible NOS**

The human iNOS gene spans 37 kb and consists of 26 exons and 25 introns [35]. Multiple cell types have been demonstrated to express iNOS upon appropriate cytokine stimulation. These include, but are not limited to, vascular smooth muscle cells, macrophages, mesangial cells, renal tubular epithelium, cardiac myocytes, megakaryocytes and even endothelial cells, among others. However, the degree of activation of iNOS expression is less robust in cells of human origin.
The peripheral non-adrenergic non-cholinergic system mediates muscular relaxation in the gastrointestinal tract NO mediates neurotransmission [37]. In the presynaptic neuron, NO acts on the neurotransmitter release to modify neuronal interconnection and decrease protein stability [20].

Inducible NOS expression is largely transcriptionally regulated. Sequence analysis of the promoter region reveals consensus cis-regulatory DNA sequences implicated in cytokine-modulated gene expression, specifically interferon response factor-1 elements and NF-κB sites. In the unstimulated state, there is low basal transcription of the iNOS gene which can be markedly enhanced by treatment of cells with lipopolysaccharide or cytokines.

Post-transcriptional regulation of iNOS also appears to be important. Both lipopolysaccharide and γ-interferon have been shown to increase mRNA stability [20,36]. In contrast, transforming growth factor (TGF)-β has been demonstrated to destabilize iNOS mRNA as well as decrease iNOS protein translational efficiency and decrease protein stability [20].

**NO in human physiology**

*Regulation of blood flow and pressure*

Vasodilatory nitrates have been used in cardiovascular medicine for many years. It appears that these agents provide an exogenous source for NO. The discovery that EDRF, now generally accepted to be endogenous NO, is released by the vascular endothelium led to the realization that tonic vasodilatory forces were essential in the regulation of blood flow and pressure. This NO vasodilatory effect appears to be maintained through the physical stimuli of pulsatile flow and shear stress on vascular endothelial cells [2]. However, NO released from the non-adrenergic, non-cholinergic nervous system [37] and/or epithelial cells [38] may also contribute to regulation of blood flow in the lung (ventilation-perfusion matching) and kidney (tubuloglomerular feedback) [39].

*Neurotransmission*

The role of NO in neurotransmission in both the central and peripheral nervous systems has been clearly established. Stimulation of the excitatory N-methyl-D-aspartate (NMDA)-type glutamate receptor results in the formation of NO [40]. In the hippocampus, stimulation of NMDA-type glutamate receptors on postsynaptic neurons leads to formation of NO which then acts on the presynaptic neuron to modulate neurotransmitter release. This leads to a modification of synaptic transmission. Conceptually known as long-term potentiation, this modulation of neuronal interaction is the basis for the formation of memory [41,42]. The peripheral non-adrenergic non-cholinergic system also utilizes NO in neurotransmission [37]. In the gastrointestinal tract NO mediates muscular relaxation and is an important component of organized gut motility.

**Body fluid homeostasis**

NO plays a fundamental role in body fluid homeostasis. Neuronal NOS has been demonstrated to be localized to the macula densa of the glomerulus [39,43] where it may play a part in tubuloglomerular feedback. Measurements of proximal stop flow pressure during perfusion of the macula densa with L-NMMA (a competitive inhibitor of NOS enzymatic activity) reveal a reduction in the estimated glomerular capillary hydrostatic pressure [39]. Further, perfusion of the macula densa with a solution high in chloride resulted in afferent arteriolar constriction, which was augmented by addition of L-NAME (also a competitive inhibitor of NOS activity) to the tubular perfusate [44]. Together, this suggests that NO may function as an intercellular messenger counteracting the afferent arteriolar constriction seen in states of high tubular flow and solute delivery.

**Immunity and inflammation**

Production of NO by macrophages is a primary defense mechanism against pathogenic microbial organisms and tumour cells. Activation of macrophages by cytokines or lipopolysaccharide leads to induction of iNOS expression and generation of NO. Subsequent diffusion of NO into the pathogenic cells leads to inactivation of key iron-containing enzymes critical for the mitochondrial respiratory chain, oxidative metabolism and in DNA/RNA synthesis [45]. In many ways the induction of NO in inflammatory responses resembles a primitive defense mechanism. Unfortunately, damage to the host cells often accompanies the robust synthesis and action of NO.

**NO in disease states**

*Hypertension*

Blood pressure reflects a balance between tonic vasoconstrictor and vasodilatory forces. As nitric oxide produced by the vascular endothelium is a major vascular smooth muscle relaxant, it is not surprising that perturbations in NO have been noted in hypertension. Systemic infusions of competitive NOS antagonists result in sustained hypertension in animal models [46,47]. Reduction in forearm blood flow, in response to an infusion of L-NMMA, was diminished in hypertensives suggesting impaired basal NO generation [48]. Likewise, agonist-induced release of endothelium-derived NO is perturbed in varied animal models of hypertension and in patients with essential hypertension.

The nitric oxide synthases are logical 'candidate' genes in the pathogenesis of hypertension. In the stroke-prone hypertensive rat, a major blood pressure determinant was mapped to rat chromosome 10. This region of the rat genome is syntenic with the region of human chromosome 17 to which the iNOS gene has been localized [49]. However, initial assessment for

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linkage of ecNOS in hypertensive sib-pairs and for association in a hypertensive cohort and controls did not suggest a genetic contribution of endothelial NOS in human essential hypertension [50].

Atherosclerosis

Atherosclerotic vessels exhibit reduced NO mediated vasorelaxation [51,52]. Moreover, oxidized low-density lipoproteins have also been demonstrated to impair endothelium-dependent relaxation [53]. Oxidized LDL, which is highly toxic to endothelial cells, enters directly via unique cell surface receptors or may be modified from native LDL within endothelial cells. Oxidized LDL has recently been reported to decrease mRNA transcripts for ecNOS in cultured endothelium. Further, oxidized LDL can also bind and inactivate NO, thus preventing diffusion of NO to the underlying vascular smooth muscle where it is essential for maintenance of vasodilatory tone [54]. Finally, infusion of L-arginine resulted in improved endothelium-dependent vasodilatation in hypercholesterolaemic individuals but not in controls [55]. This novel finding implies that L-arginine may become substrate-limiting for NO synthesis. Because circulating levels of L-arginine average 80–100 μM in blood and the Kₘ for arginine in NOS catalysis is in the low μM range, this implies that transmembrane transport of L-arginine may be biologically relevant in disease. Complicating the pathophysiology further is the finding that production of oxidative end-products of NO, namely NO₂⁻ and NO₃⁻, are increased in atherosclerotic blood vessels and that nitration of tyrosine residues is enhanced in advanced atherosclerotic lesions. The molecular basis for these varied observations remains to be defined. However, the potential to pharmacologically augment or interrupt NO synthesis and action in diseased blood vessels suggests that novel therapeutic modalities may well develop.

Stroke

The cytotoxic effects of NO may be implicated in the neuronal destruction seen following occlusive cerebral ischaemia. This neurotoxicity is mediated in major part by glutamate, an activator of nNOS. It has been observed that NADPH diaphorase positive neurons are resistant to damage from cerebral ischaemia and some neurodegenerative disorders. NOS accounts for the long-described histochemical NADPH diaphorase response. Thus, NO produced by these neurons may mediate toxicity in neighbouring cells [56]. A recent study provides further insight into this phenomenon [57]. In response to cerebral ischaemia following middle cerebral artery occlusion, homozygous nNOS ‘knock out’ mice demonstrated significantly reduced infarct volumes compared to normal controls. This was accompanied by a corresponding reduction in neurological deficit. Further, infusion of a NOS antagonist to inhibit endothelial NOS activity led to an increase in infarct volume in the nNOS ‘knock out’ mice. These data suggest that neuronal NO production may mediate tissue damage following ischaemia but that endothelial-derived NO is necessary to maintain tissue perfusion in ‘watershed’ zones. A selective nNOS inhibitor may therefore prove to be beneficial in reducing ischaemic injury following stroke.

Sepsis

Profound hypotension with a marked reduction in peripheral vascular resistance is seen in bacterial sepsis and in patients receiving interleukin-2 therapy for cancer [58]. This state of marked vasodilatation reflects increased NO synthesis in response to cytokine stimulation. The observation that iNOS induction represents a major component of myocardial and vascular hypotension in states of sepsis has greatly advanced the understanding of the pathobiology of sepsis. Treatment with blockers of NOS (i.e. substituted L-arginine compounds) reverses the hypotension seen in septic shock but results in an overall increase in mortality [59,60]. This lack of overall benefit may be due to the beneficial role of NO in maintenance of tissue perfusion to vital organs. Indeed, in experimental models of sepsis, increases in blood pressure and evidence of reduced tissue perfusion correlated with increasing dose of NOS antagonist [61]. It appears that preservation of cardiac pump function and microvascular blood flow represent limiting features of acute systemic NO blockade. Selective iNOS antagonists may represent powerful therapeutic agents in the treatment of septic shock. Although aminoguanidine is a relatively selective iNOS inhibitor in rodents, this does not appear to be the case in man [62].

Conclusions

Nitric oxide is an integral messenger in many physiological systems. Its synthesis is catalysed from L-arginine by the nitric oxide synthases (NOS), a family of three distinct isoenzymes each of which has unique regulatory mechanisms and distribution in body tissues. Continuing insights into NOS regulation will enhance our understanding of important disease processes such as hypertension, atherosclerosis, infection and immunity. The development of selective NOS inhibitors holds promise for treatment of disease processes such as stroke and sepsis.

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