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COMMENTARY

ISOFORMS OF NITRIC OXIDE SYNTHASE

PROPERTIES, CELLULAR DISTRIBUTION AND EXPRESSIONAL CONTROL

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NO†, the smallest known bioactive product of mammalian cells, can be produced by most cell types. Despite its almost ubiquitous occurrence, this simple molecule can act in a fairly specific manner controlling vital functions such as neurotransmission or vascular tone (via activation of soluble guanylyl cyclase) [1, 2], gene transcription [3] and mRNA translation (via iron-responsive elements) [4]. NO can produce post-translational modifications of proteins (via ADP-ribosylation) [5] and is capable of destroying parasites and tumor cells by inhibiting iron-containing enzymes [6] or directly interacting with the DNA of these cells. [7, 8]. In view of this multitude of molecular targets, effectors and functions of NO, it is clearly important to understand the mechanisms by which cells accomplish and regulate their NO production. In 1991, the first commentary on "Isoforms of Nitric Oxide Synthase" was published [9]. At that time only protein chemistry data were available to classify the NOS isozymes. While the basic characterization withstood the test of time, some aspects (such as the subclassification of NOS I and a postulated inducible NOS IV [9]) had to be abandoned as knowledge progressed. During the last 4 years, information on NOS has increased to such an extent that this new commentary had to be restricted to some important biochemical aspects of the NOS enzymes, namely their protein and cDNA structure, their cellular distribution, and the mechanism controlling their expression. Functional aspects of the enzymes have been reviewed elsewhere [2].

Cellular expression of NOS protein and regulation of enzymatic activity

Three isozymes of NOS (L-arginine, NADPH: oxygen oxidoreductases, nitric oxide forming; EC 1.14.13.39) have been identified. Different, sometimes confusing and overlapping nomenclatures for the NOS isozymes are currently being used by the scientific community. We use the numerical nomenclature suggested in 1991 [9], which is based on the historical order of purification (and cDNA isolation) of the isoforms (Table 1). Yet many authors prefer descriptive terms based on the cell or tissue from which the enzyme derives, or its constitutive or inducible expression. Table 1 tries to list the different nomenclatures including a brief definition.

NOS I was first purified from rat and porcine cerebellum [10–12]. Immunohistochemistry using specific antibodies to NOS I suggests that the isoform is also expressed in certain areas of the spinal cord [13], in sympathetic ganglia and adrenal glands [14, 15], in peripheral nitrergic nerves [16–18], in epithelial cells of lung, uterus and stomach [19] including human lung epithelial cells [20], in kidney macula densa cells [19], in pancreatic islet cells [21], and in human and rat skeletal muscle [22, 23]. In many neurons, NOS I seems to be co-localized with other neurotransmitters (or neurotransmitter-synthesizing enzymes). For example, in dentate hilar neurons of the rat hippocampus, NOS I is co-localized with somatostatin [24], and in the peripheral autonomic nervous system, NOS I has been found in pre- and postganglionic sympathetic neurons together with choline acetyltransferase and dopamine-β-hydroxylase, respectively [14, 15].

NOS I from brain is mainly a soluble enzyme that migrates with a molecular mass of 150–160 kDa in SDS–PAGE [10–12]. Some particulate activity can be detected in the presence of FAD [25]. Because the particulate enzyme shows the same immunological characteristics as the soluble form, it is likely to represent the same protein [26]. In skeletal muscle, most of the NOS I protein seems to be membrane associated [22, 23]. The post-translational modification that conveys the membrane association is unknown. NOS I is a Ca^{2+}- and calmodulin-dependent enzyme [11, 27], whose activity is regulated by physiological changes in the intracellular Ca^{2+} concentration. The enzyme can

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† Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; LPS, lipopolysaccharide; IL, interleukin; NF-κB, nuclear factor-κB; IFN-γ, interferon-γ; FMN, flavin mononucleotide; BH_4, 6(*R*)-5,6,7,8-tetrahydrobiopterin; TNF-α, tumor necrosis factor-α; and TGF-β, transforming growth factor-β.

Table 1. NOS nomenclatures

Numerical	Descriptive*	Definition of isoform
NOS I (or NOS 1)	b-NOS (for brain NOS), c-NOS (for constitutive or Ca^{2+}-regulated NOS), bc-NOS (for brain constitutive NOS), n-NOS (for neuronal NOS), nc-NOS (for neuronal constitutive NOS)	A low output NOS that is constitutively expressed and whose activity is regulated by Ca^{2+}, the prototypical enzyme being present in neurons.
NOS II (or NOS 2)	i-NOS (for inducible NOS), mac-NOS (for macrophage NOS), hep-NOS (for hepatocyte NOS)	A high output NOS whose expression is induced by cytokines, whose activity is largely or completely Ca^{2+} independent, the prototypical enzyme being expressed by murine macrophages.
NOS III (or NOS 3)	e-NOS (for endothelial NOS), c-NOS (for constitutive or Ca^{2+}-regulated NOS; overlap with nomenclature for NOS I) ec-NOS or EC-NOS (for endothelial constitutive NOS)	A low output NOS that is constitutively expressed and whose activity is regulated by Ca^{2+}, the prototypical enzyme being found in endothelial cells.

* List may not be complete.

be phosphorylated at serine and threonine residues by Ca^{2+}/calmodulin-dependent protein kinase II, and protein kinases A, C and G [28, 29]. *In vitro*, phosphorylation by Ca^{2+}/calmodulin kinase II and protein kinases A and G reduces catalytic activity of the enzyme [28, 29].

NOS II can be induced in many cell types by cytokines, bacterial LPS and a variety of other agents (see below). It was first isolated from murine macrophages [30, 31]. Immunohistochemical localization of NOS II in rats treated with *Propionibacterium acnes* and LPS demonstrated the enzyme in macrophages, occasional lymphocytes, neutrophils and eosinophils in red pulp of spleen, Kupffer cells, endothelial cells and hepatocytes in liver, alveolar macrophages in lung, macrophages and endothelial cells in adrenal glands, and histiocytes, eosinophils, mast cells and endothelial cells in colon [32]. NOS II immunoreactivity has also been reported in pancreatic islets of diabetic BB rats, but not Wistar rats; the immunoreactivity was restricted to areas of islet infiltration by macrophages [33]. Kobzik *et al.* [34] found strong NOS II labeling of macrophages from LPS-treated, but not untreated rats. *Human* alveolar macrophages were occasionally NOS II immunoreactive, especially in areas of inflammation. NOS II immunoreactivity was also present in murine and human lung epithelial cells after cytokine stimulation [20, 35]. Experiments with our NOS II-specific antibody demonstrated strong immunoreactivity in alveolar macrophages from a patient with acute bronchopneumonia, whereas no immunoreactivity was detected in grossly normal human lung tissue [36]. Thus, despite the frustrating attempts of many laboratories to induce *human* monocytes or macrophages *in vitro*, nature seems to be able to do so.

NOS II has a denatured molecular mass of 125–135 kDa and, like NOS I, it is a predominantly soluble enzyme [30, 31]. Some particulate activity is found in induced murine macrophages [37, 38]. Based on immunological data this represents the same protein [36, 39], and *not* a separate NOS as previously assumed [9]. Also for this enzyme, the post-translational modification that conveys the membrane association of part of the protein is not clear. Once expressed, no regulatory mechanisms are known for the *activity* of NOS II. Interestingly, the amino acid sequence of the murine enzyme demonstrates a binding site for calmodulin [40, 41] despite the Ca^{2+} independence of its activity. The *murine* enzyme seems to bind calmodulin tightly, even in the absence of Ca^{2+} [42]. The induced NOS from *human* hepatocytes and DLD-1 adenocarcinoma cells loses some activity following Ca^{2+} chelation and/or exposure to a calmodulin antagonist [43, 44], suggesting that the human NOS II sequence does not convey the same tight binding of calmodulin as the mouse enzyme (cf. Table 2).

NOS III was first identified in endothelial cells. Our immunohistochemical studies using a specific antibody to NOS III located the enzyme to various types of arterial and venous endothelial cells in many tissues, including human tissues [45]. NOS III immunoreactivity has also been detected in syncytiotrophoblasts of human placenta [46], LLC-PK_1 kidney tubular epithelial cells [47], and interstitial cells of the canine colon [48]. Surprisingly, NOS III immunoreactivity has also been described in neurons of the rat hippocampus and other brain regions [49].

Unlike NOS I and II, the endothelial NOS III is more than 90% particulate. The solubilized enzyme shows a denatured molecular mass of 135 kDa [50, 51]. Myristylation at the N-terminal glycine (glycine-2 before removal of the initiation methionine by a specific aminopeptidase) as well as palmitylation contribute to the membrane association of most of the enzyme [52, 53, 53a]. Like NOS I, NOS III is regulated by physiological concentrations of Ca^{2+} (via calmodulin) [50, 51]. NOS III can undergo

Table 2. Kinetic properties of the three purified isoforms of NOS

Isoform	Protein source	Calcium dependence of activity (EC_{50}, μM)	Calmodulin dependence of activity (EC_{50}, nM)	K_m L-arginine (μM)	V_{max} (μmol citrulline or NO/mg protein/min)	References
NOS I (nc-NOS)	Rat cerebellum	0.2	10	1.5	0.96	12
	Rat cerebellum	0.35	3.5	2.2	0.107	11
	Porcine cerebellum	0.4	35–70		0.73	10
	Rat NOS I expressed in pCIS kidney cells	Decrease (>80%) in the absence of Ca^{2+}	Decrease (>80%) by trifluoperazine (100 μM)			65
	Human NOS I expressed in COS-1 cells	Activity abolished by EGTA	Activity abolished by trifluoperazine (100 μM)			22
NOS II (i-NOS)	Mouse RAW 264.7 macrophages	Increase (<20%) at 2 mM Ca^{2+} vs Ca^{2+}-free	None	2.8	1.3*	31
	Mouse RAW 264.7 macrophages			16		30
	Rat peritoneal macrophages	None	None	32.3	1.052	66
	Human DLD-1 adenocarcinoma cells	Decrease (60%) by EGTA	No effect of trifluoperazine (50 μM)			44
	Human NOS II expressed in 293 embryonic kidney cells	Decrease (50–70%) by EGTA or EDTA	Decrease (50%) by trifluoperazine (50 μM)			43
NOS III (ec-NOS)	Bovine aortic endothelial cells	0.3	3.5	2.9	0.015	51
	Bovine NOS III expressed in COS-1 cells	Total dependence	Total dependence	2.8		53,67

* Measured as NO_2^- plus NO_3^-.

serine phosphorylation in response to bradykinin, and a translocation from the particulate to the cytosolic fraction [54]. Phosphorylation of endothelial NOS has also been described in response to shear stress [55]; the physiological relevance of NOS III phosphorylation remains to be determined.

NOS catalysis

The mechanism of NOS catalysis is still incompletely understood; the chemical steps are likely to be the same for all three isoforms because of identical cofactor requirements. Current biochemical evidence from several laboratories favors the model of a 5-electron oxidation of a guanidino-nitrogen of L-arginine resulting in NO and its coproduct L-citrulline. The first step in this reaction seems to be the hydroxylation of L-arginine [56]. Molecular oxygen and reduced NADPH participate in NOS catalysis as cosubstrates. All three isoforms of NOS contain FAD, FMN [30, 31, 57, 58], and heme iron [59–61] (Pollock JS, Nakane M and Förstermann U, unpublished observation, 1993, for NOS III) as prosthetic groups, and also require the cofactor BH_4 [57, 58, 62]. Recently, Sheta *et al.* [63] demonstrated a bidomain structure for NOS I, with the heme-binding consensus sequence in the N-terminal half of NOS, and the binding sequences for the flavins in the C-terminal half. The calmodulin-binding sequence lies between the heme- and flavin-binding domains, suggesting a role for calmodulin in modulating a spatial orientation of these domains that is required for catalytic activity. Biopterin is bound to all NOS isozymes in its totally reduced form, BH_4 [57, 58, 62]. Neither dihydrofolate reductase nor dihydropteridine reductase increases NOS activity [62], suggesting that biopterin is either recycled by NOS itself or is not a stoichiometric reactant in NOS catalysis, but merely an allosteric regulator or stabilizer of the enzyme [62, 64]. Reported K_m values for L-arginine and V_{max} values for NO- or L-citrulline formation are shown in Table 2. Half-saturating L-arginine concentrations seem to be similar for the two constitutive NOS I and III, and higher for the inducible NOS II.

cDNAs encoding the three different NOS

The cDNAs encoding NOS I have been isolated from rat and human brain (Table 3). The deduced amino acid sequences predict proteins of 160 and 161 kDa, respectively, which is in good agreement

Table 3. cDNAs encoding the three isoforms of NOS

Isoform	cDNA source		Predicted protein		Size of mRNA	References
	Cell, tissue	Species	Amino acids	Mol. mass (kDa)		
NOS I	Brain	Rat	1429	160	10.5 kb	64
(nc-NOS)	Brain	Human	1433	161	10 kb	22
NOS II	Macrophage	Mouse	1144	131	4.4 kb	41
(i-NOS)	Macrophage	Mouse	1144	131	5 kb	40
	Macrophage	Mouse	1144	130	4 kb	75
	Smooth muscle cell	Rat	1147	131	4 kb	69
	Hepatocyte	Rat	1147	131	4.5 kb	68
	Liver	Rat	1147	131	4.2 kb	76
	Hepatocyte	Human	1153	131	4.5 kb	43
	DLD-1 adenocarcinoma cell	Human	1153	131		44
	Articular chondrocyte	Human	1153	131		70
	Articular chondrocyte	Human	1153	131	4.5 kb	71
	Glioblastoma cell line A-172	Human	1153	131		72
NOS III	Endothelium	Bovine	1205	133	4.4 kb	67
(ec-NOS)	Endothelium	Bovine	1205	133	4.8 kb	74
	Endothelium	Bovine	1205	133	4.8 kb	77
	Endothelium	Human	1203	133	4.3 kb	78
	Endothelium	Human	1203	133	4.052 kb	73,79

with protein purification results [10–12]. The enzyme is highly conserved between species; 93% identity of the amino acid sequences was found between rat and human.

The cDNAs encoding NOS II have been cloned by several groups from mouse, rat and human cells (Table 3). The deduced amino acid sequences predict proteins of 130–131 kDa molecular mass, confirming data obtained for the purified protein from murine macrophages [30, 31]. Sequence analyses of the NOS II cDNAs from *different* cells of the *same* species are available for rat (smooth muscle cells and hepatocytes) and human (four cell types). The two cDNA sequences from rat [68, 69], which were from different rat strains and both obtained with polymerase chain reaction (which can introduce mutations), were 98.8% identical. The five human NOS II cDNA sequences published at the time of submission of this manuscript [43, 44, 70–72] show over 99.7% identity, including a 206 nucleotide 5′-untranslated sequence that is identical in human hepatocytes and chondrocytes [43, 70]. Finally, Southern blots of human genomic DNA digested with four different restriction enzymes give single bands when hybridized with a 240 bp cDNA fragment of hepatocyte NOS [43]. These data strongly indicate that NOS II expressed in different cells of the same species represents the product of a single gene. The few nucleotide mismatches between the different cDNAs may represent experimental errors or polymorphisms (for example, the two published chondrocyte sequences are also not more than 99.7% identical [70, 71]).

The cDNAs encoding NOS III have been isolated from bovine and human endothelial cells (Table 3). The deduced amino acid sequences predict proteins of 133 kDa molecular mass for both species, again in good agreement with the molecular mass determined by protein purification [51]. The amino acid sequences of bovine and human NOS III are 94% identical, and both show a consensus motif for N-terminal myristylation (MGNLKSV[G or A]Q) [73, 74]. Single amino acid substitution of the myristic acid acceptor site, glycine-2, with alanine by site-directed mutagenesis converts NOS III into a 92% cytosolic enzyme [53]. Kinetic analysis of the wild-type and mutated enzymes revealed similar K_m values for L-arginine (2–4 μM), demonstrating that the mutation did not alter the function of this NOS.

Structures of the NOS genes and their chromosomal location

Three human genes have been identified for the three isoforms of NOS, and their structures have been mapped. The *human* gene for NOS I is by far the biggest of the three NOS genes, spanning over 150 kb of DNA [80]. The mRNA is encoded by 29 exons, with translation initiation and termination sites in exons 2 and 29, respectively. Sequence analyses of cDNAs derived from several human tissues demonstrated 5′-structural diversity and alternate promoter usage. Eight unique exons 1 resulting in eight different messenger RNAs have been isolated. *In situ* hybridization with antisense cRNAs localized the exon 1 variants to different cell populations, indicating that cell type-specific transcription/splicing factors may control NOS I expression [80]. Analysis of a human–rodent genomic DNA somatic cell hybrid panel and fluorescent *in situ* hybridization indicated that the NOS I gene is located on human chromosome 12 (Table 4).

The *human* NOS II gene has been isolated from

Table 4. Structure and chromosomal location of the three human NOS genes

Isoform	Gene: Size (kb)	Gene: Number of exons encoding the open reading frame	Chromosomal location: Number	Chromosomal location: Region	References
NOS I (nc-NOS)	≥150	28	12	12q24.2	79,80
			12	12q24.2–24.31	85
NOS II (i-NOS)	37	26	17	17cen–q11.2	81
			17	17cen–17q11	86
			17	17q11.2–q12	87
NOS III (ec-NOS)	21	26	7	7q35–7q36	79
	22	26	7	Between the genetic markers AFM199zd4 and AFM074xg5*	82
	>20		7	7q36	83
			7		86

* Genetic localization corresponding to the locus symbols named D7S505 and D7S483 (Soubrier F, personal communication, 1994; cited with permission).

a human genomic cosmid library [81]. The isolated cosmids were part of a single genomic locus, and no other genomic loci were identified. The human NOS II gene is about 37 kb in length and consists of 26 exons. Primer extension analyses with the RNA of cytokine-induced human hepatocytes mapped the transcriptional initiation site 30 bp downstream of a TATA sequence. The human NOS II gene has been localized to chromosome 17 (Table 4).

Genomic clones encoding *human* and *bovine* NOS III have also been isolated, and their structural organization determined [79, 82–84]. The human NOS III mRNA is encoded by 26 exons spanning 21–22 kb of genomic DNA [79, 82]. The gene is present as a single copy in the haploid human genome. A highly polymorphic dinucleotide repeat $(CA)_n$ (n = 14–42) was found in intron 13 of the human NOS III gene [82]. The human NOS III gene has been assigned to chromosome 7 (Table 4). The bovine NOS III gene spans >20 kb and also contains 26 exons. Two transcription start sites have been determined, which are located 170 and 240 bp upstream, respectively, from the methionine translational initiation codon [84].

Regulation of NOS I expression

Little information is available on the possible regulation of NOS I expression. One report suggests that estrogens can up-regulate NOS I mRNA and activity in various tissues [88], but the estrogen concentrations used were above physiological levels. In rat brain, there was a long-lasting up-regulation of NOS I expression (up to 159 days) following axotomy. In most regions investigated, this enhanced expression was associated with an increased expression of the transcription factor c-jun [89]. Occlusion of the middle cerebral artery of the rat also led to an up-regulation of NOS I mRNA and immunoreactive protein [90]. Chronic salt loading of rats up-regulated NOS I mRNA in the supraoptic and paraventricular nuclei of the hypothalamus. At the same time, NOS activity increased in the posterior pituitary [91]. Thus, NOS I expression can be regulated under some circumstances, but the molecular mechanisms of this regulation remain to be elucidated.

Regulators of NOS II expression

Unlike NOS I and NOS III, NOS II is regulated mainly at the expressional level. In uninduced cells, expression of NOS II is usually very low (mRNA merely detectable by northern blot analysis, but detectable by reverse transcription-polymerase chain reaction) or the enzyme is not expressed at all. The first agents that were found to induce expression of this enzyme in macrophages and other cells were LPS and cytokines such as IL-1, IFN-γ and TNF-α. The cytokines (or cytokine combinations) that produce good NOS II expression vary between species and between cell types within the same species. In addition, in some cell types, agents other than cytokines are efficacious inducers of NOS II. For example, in murine 3T3 fibroblasts and vascular smooth muscle cells, NOS II is expressed in response to cyclic AMP (cAMP)-elevating agents such as forskolin or dibutyryl cAMP [92, 93], protein kinase C stimulating agents such as tetradecanoyl phorbol-13-acetate (TPA) [92, 94], and growth factors such as platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) [92].

On the other hand, compounds that prevent the cytokine induction of NOS II are legion. First, there is the group of inhibitory cytokines and growth factors. IL-4 [95], IL-8 [96], IL-10 [97], monocyte chemotactic protein-1 (MCP-1) [98], and macrophage deactivating factor (MDF) [99] are inhibitors of NOS II induction in macrophages and neutrophils (IL-8). Three isoforms of TGF-β (TGF-β 1, 2, and 3, in macrophages and smooth muscle cells) [37, 39, 99, 100], PDGF-AB and -BB and insulin-like growth

factor I (in rat vascular smooth muscle cells) [100, 101], and basic and acidic FGF (in bovine retinal pigmented epithelial cells) [102] also prevent NOS II induction. Drugs such as the tyrosine kinase inhibitors genistein, herbimycin A and tyrphostin [103], and the inhibitors of NF-κB pyrrolidine dithiocarbamate (PDTC) and diethyl-dithiocarbamate (DETC) [104–106] prevent NOS II induction in macrophages, indicating that tyrosine kinases and NF-κB are involved in induction. Glucocorticoids are effective inhibitors of NOS II induction in endothelial cells, macrophages, fibroblasts, and smooth muscle cells [92, 100, 107–109], and nifedipine has been shown to reduce NOS II induction in macrophages [110].

Confusingly, some agents may stimulate NOS II induction in one cell type and inhibit it in another. TGF-β and PDGF-AB and -BB, for example, are inhibitors of NOS II induction in mouse macrophages and rat vascular smooth muscle cells [37, 99, 100, 111], but stimulate induction in 3T3 fibroblasts [92, 111]. TGF-β also stimulates induction in bovine retinal pigmented epithelial cells [102]. Cyclic AMP elevating agents prevent induction in primary rat astrocytes [112], but produce induction in 3T3 cells and vascular smooth muscle cells [92, 93]. Thus, signal transduction pathways leading to NOS II induction seem to differ markedly between cells.

Molecular mechanisms regulating NOS II gene expression

We are only beginning to understand the molecular actions of compounds that stimulate or inhibit induction. In murine macrophages, LPS, IFN-γ and other agents increase transcription of the NOS II gene. Parts of the 5′-flanking region of the *murine* NOS II gene have been cloned [113, 114]. The promoter of the gene contains a "TATA box" and numerous consensus sequences for the binding of transcription factors (some in multiple copies) such as IFN-γ response element (IFN-γ-RE), γ-site, NF-κB-binding motifs, nuclear factor IL-6 (NF-IL6) binding sites, IFN-α-stimulated RE, activating protein 1 (AP1) site, and TNF-RE. Many of these sequences are associated with stimuli that induce NOS II expression [113]. To localize functionally important sequences of the regulatory region, mutants of the NOS II 5′-flanking region were constructed and placed upstream of a reporter gene. The degree of expression of the reporter gene was dependent on two regulatory regions upstream of the putative "TATA box." The first region (position −48 to −209) contains LPS-related response elements such as the putative binding sites for NF-IL6 and NF-κB. This region was responsive to LPS, suggesting that it regulates LPS-induced expression of the gene [114]. The NF-κB binding site on the promoter sequence begins 55 bp upstream of the "TATA box" [106]. Oligonucleotide probes containing the NF-κB site plus the 9 or 47 nucleotides downstream bound proteins that rapidly appeared in the nuclei of LPS-treated macrophages. The NF-κB inhibitor PDTC blocked both the activation of proteins binding to the NF-κB binding site and the production of NO in LPS-treated macrophages, indicating that NF-κB activation is essential for transcription of the NOS II gene in murine macrophages [106]. The second region (position −913 to −1029) mediated the potentiation of the LPS induction by IFN-γ, and thus probably is responsible for IFN-γ-mediated regulation of NOS II induction [114]. In positions −951 to −911, Martin *et al.* [115] identified a cluster of four enhancer elements known to bind IFN-γ-responsive transcription factors, including an interferon regulatory factor binding site (IRF-E) at nucleotides −913 to −923. Site-specific mutagenesis of two nucleotides within the IRF-E abolished the enhancement of transcription by IFN-γ. Gel shift analyses demonstrated IRF-E-binding protein(s) in the nuclei of IFN-γ-treated macrophages, one of which was immunochemically identified as IRF-1. In another study, macrophages from mice with a targeted disruption of the IRF-1 gene produced little or no NO and synthesized barely detectable NOS II mRNA in response to stimulation, also indicating that IRF-1 is important for NOS II activation in murine macrophages [116]. Fragments (up to 1090 bp) of the 5′-flanking region of the *human* NOS II gene have been cloned [81, 117]. They show some structural similarity with the murine NOS II promoter sequence, including a "TATA box" 30 bp upstream of the transcription initiation site and several consensus sequences for the binding of transcription factors involved in the cytokine-mediated induction of other genes (IFN-γ-RE, NF-IL-6- and NF-κB-binding motifs). Interestingly, the human NOS II gene contains a shear-stress responsive element (GAGACC), which is also present in the human NOS III gene but not in the murine NOS II gene. The structural similarity of the known *human* and *murine* promoter fragments contrasts with the different cytokine patterns required to induce NOS II in *human* or *murine* NOS II.

Different cells may use different signal transduction pathways for the induction of NOS II. In several cell types, NOS II *mRNA production* is prevented by the protein synthesis inhibitor cycloheximide, suggesting the need for *de novo* synthesis of transcription factors [109, 118–121]. In 3T3 fibroblasts, however, NOS II induction by a variety of agents is resistant to cycloheximide. Thus in these cells, NOS II seems to be a primary response gene [92]. Also, in rat glomerular mesangial cells, cycloheximide had little effect on induction of NOS II mRNA by LPS [122], and in rat aortic smooth muscle cells, cycloheximide even potentiated NOS II mRNA induction by LPS and IFN-γ [123].

In addition to transcriptional events, post-transcriptional phenomena can regulate the expression of NOS II. Weisz *et al.* [124] reported that LPS, in addition to promoting NOS II transcription, also prolonged the half-life of NOS II mRNA about 4-fold, thereby contributing to enhanced NOS II protein formation. In mouse peritoneal macrophages, three mechanisms have been described for the inhibition of NOS II induction by TGF-β. The growth factor reduced NOS II mRNA by decreasing its stability without affecting transcription. It also reduced NOS II mRNA translation and increased the degradation of NOS II protein [39]. Other inhibitory mechanisms have been

reported for IL-4. This cytokine did not affect NOS II mRNA early after exposure to IFN-γ (4–6 hr), but strongly reduced NOS II mRNA at later times (24–72 hr) [95]. Dexamethasone reduced NOS II mRNA in stimulated 3T3 fibroblasts, hepatocytes and smooth muscle cells [92, 109, 118], presumably by inhibiting transcription. The published DNA sequences of the *murine* and *human* NOS II promoters contain no consensus sequence for the binding of glucocorticoid receptors. However, binding sites of glucocorticoid receptors on promoters can differ from the established consensus sequence, and DNAse I footprint and gel-shift analyses are required to identify a binding site for a glucocorticoid receptor on a specific promoter [125]. In insulin-producing RINm5F cells, post-transcriptional effects have been reported for dexamethasone [126].

Regulation of NOS III expression

NOS III is found constitutively expressed in endothelial cells and some other cell types (see below). Some mechanisms have been described that also regulate the expression of this gene. Shear stress produced by the flowing blood not only increases endothelial NO release acutely [127], but it also up-regulates NOS III expression [77]. A putative shear stress-responsive element has been described in the promoter sequence of NOS III [79]. In cultured bovine aortic endothelial cells, we and others have observed that TNF-α down-regulates NOS mRNA, protein and activity [74, 77, 128]. In our bovine aortic endothelial cell line, no parallel induction of NOS II was observed, indicating that the down-regulation of NOS III is independent from NOS II induction [128]. The mechanism of action of TNF-α has been ascribed recently to a destabilization of NOS III mRNA with no effect on transcription [129]. In guinea pigs, near-term pregnancy and treatment with estradiol (but not progesterone) increased calcium-dependent NOS activity in various tissues [88]. The mRNA for NOS III was increased in skeletal muscle as shown by northern blots (along with NOS I; cf. above). Also, in coronary artery rings from oophorectomized rabbits, 17 β-estradiol produced endothelium-dependent, NO-mediated vasorelaxation [130]. In postmenopausal women, physiological levels of 17 β-estradiol selectively potentiated endothelium-dependent vasodilatation in both large coronary conductance arteries and coronary microvascular resistance arteries [131]. These data point to a stimulatory effect of estrogens on NOS III activity. The molecular mechanisms of this regulation are still unclear. Furthermore, one report on uterine NO production in the rat suggests that progesterone increases NO formation during pregnancy, and a rise in estrogen at term inhibits NOS to initiate labor [132]. Finally, endothelial NOS in cerebral blood vessels was up-regulated markedly during cerebral ischemia [133].

Genomic clones encoding the *human* NOS III have been isolated [79, 82, 83]. Characterization of the 5′-flanking genomic region indicates that the endothelial NO synthase promoter is "TATA-less" and exhibits proximal promoter elements consistent with a constitutively expressed gene found in endothelial cells, such as Sp1 and GATA motifs. The promoter region contains putative AP-1, AP-2, nuclear factor-1, heavy metal, acute-phase response, shear stress, and sterol-regulatory *cis* elements [79, 82]. The *bovine* NOS III gene plus 2.9 kb of the 5′-flanking sequence has also been isolated and characterized [84]. The first 1.6 kb of the 5′-flanking sequence show 75% nucleotide identity with the corresponding human sequence. Like the human promoter, it lacks a typical "TATA box," but contains numerous putative transcription factor binding sites.

Summary and concluding remarks

Three isozymes of NOS have been identified, and their protein structure and their cDNA and genomic DNA structures have been elucidated. In humans, NOS I, II and III are encoded by three different genes located on chromosomes 12, 17 and 7, respectively. Based on Southern blot results in human cells, it seems unlikely that any additional bona fide NOS genes will be discovered. The cDNA-deduced amino acid sequences of the three *human* isozymes show less than 59% identity. Across species, amino acid sequences are more than 90% identical for NOS I and III, and greater than 80% identical for NOS II, demonstrating high phylogenetic conservation. All NOS oxidize a guanidino nitrogen of L-arginine to NO, utilizing molecular oxygen and NADPH as cosubstrates. All isoforms contain FAD, FMN and heme iron as prosthetic groups and require the cofactor BH_4. NOS I (originally discovered in neurons) and NOS III (originally discovered in endothelial cells) are low-output, Ca^{2+}-activated enzymes whose physiological function is signal transduction. They are constitutively expressed in various cells, but recent evidence suggests that their level of expression can be subject to up- or down-regulation. NOS II (originally discovered in macrophages) produces high, toxic amounts of NO that represent an important component of the antimicrobial and antineoplastic activity of these cells. Depending on the species, NOS II activity is largely or completely Ca^{2+} independent. Expression of NOS II can be induced with suitable agents (LPS, cytokines, and others) in almost any cell type. Whether some cells can express NOS II constitutively is not clear at this time. The widespread cellular distribution of the three NOS isozymes, which reaches far beyond the cell types in which they were originally discovered, renders cell-based nomenclatures increasingly ambiguous and confusing. It makes little sense to term an enzyme in epithelial cells n(euronal)NOS, or to describe the e(ndothelial)NOS in central neurons. Also, cell-based abbreviations of inducible enzymes, such as mac-NOS or hep-NOS, suggest structural differences that are unlikely to exist. The numerical nomenclature used here (NOS I, II, III or NOS 1, 2, 3, as preferred by some authors [81, 83, 85–87, 134]) avoids ambiguity and facilitates communication in this rapidly expanding field.

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