Nitric Oxide Synthase Isozymes
Characterization, Purification, Molecular Cloning, and Functions

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Abstract Three isozymes of nitric oxide (NO) synthase (EC 1.14.13.39) have been identified and the cDNAs for these enzymes isolated. In humans, isozymes I (in neuronal and epithelial cells), II (in cytokine-induced cells), and III (in endothelial cells) are encoded for by three different genes located on chromosomes 12, 17, and 7, respectively. The deduced amino acid sequences of the human isozymes show less than 59% identity. Across species, amino acid sequences for each isoform are well conserved (>90% for isoforms I and III, >80% for isoform II). All isoforms use L-arginine and molecular oxygen as substrates and require the cofactors NADPH, 6(R)-5,6,7,8-tetrahydrobiopterin, flavin adenine dinucleotide, and flavin mononucleotide. They all bind calmodulin and contain heme. Isoform I is constitutively present in central and peripheral neuronal cells and certain epithelial cells. Its activity is regulated by Ca2+ and calmodulin. Its functions include long-term regulation of synaptic transmission in the central nervous system, central regulation of blood pressure, smooth muscle relaxation, and vasodilatation via peripheral nitricergic nerves. It has also been implicated in neuronal death in cerebrovascular stroke. Expression of isoform II of NO synthase can be induced with lipopolysaccharide and cytokines in a multitude of different cells. Based on sequencing data there is no evidence for more than one inducible isoform at this time. NO synthase II is not regulated by Ca2+; it produces large amounts of NO that has cytostatic effects on parasitic target cells by inhibiting iron-containing enzymes and causing DNA fragmentation. Induced NO synthase II is involved in the pathophysiology of autoimmune diseases and septic shock. Isoform III of NO synthase has been found mostly in endothelial cells. It is constitutively expressed, but expression can be enhanced, eg, by shear stress. Its activity is regulated by Ca2+ and calmodulin. NO from endothelial cells keeps blood vessels dilated, prevents the adhesion of platelets and white cells, and probably inhibits vascular smooth muscle proliferation. (Hypertension. 1994;33(part 2):1121-1131.)

Key Words • L-arginine • brain • nitricergic nerves • endothelial cells • induced macrophages • blood pressure • septic shock • endothelium-derived relaxing factor

The initial evidence for the production of nitrogen oxides in mammals came from experiments demonstrating nitrate production in germ-free rats. This triggered the search for mammalian cells capable of synthesizing nitrogen oxides and in 1985 led to the discovery that macrophages could be induced with lipopolysaccharide to produce significant amounts of both nitrite and nitrate. Further work demonstrated that L-arginine was the substrate for this pathway and that L-citrulline was formed as a coproduct. One year later nitric oxide (NO) was identified as the initial product that is subsequently oxidized to nitrite and nitrate. In parallel, Furchgott and coworkers had discovered endothelium-derived relaxing factor (EDRF). It had been established that, similar to nitrovasodilators, the EDRF-mediated vasodilatation was associated with increased levels of cyclic GMP and activation of cyclic GMP kinase activity in smooth muscle cells and that the EDRF could directly stimulate soluble guanylyl cyclase. In 1987 it was concluded that NO can account for the biologic activity of EDRF and, analogous to the macrophage pathway, L-arginine was established as a substrate for EDRF/NO synthesis in endothelial cells. In the late 1980s physiological research demonstrated that stimulation of neuronal cells and brain slices with agonists leads to the release of a labile mediator that stimulates guanylyl cyclase and has the properties of NO. During the past 4 years, significant progress has been made elucidating the mechanism of NO synthesis, the NO synthases involved, and the functions of NO in different biologic systems. The present review attempts to summarize this progress with some emphasis on the cardiovascular system.

Isozymes of NO Synthase

Many cells are capable of synthesizing NO. Three isozymes of NO synthase (EC 1.14.13.39) have been identified as being responsible for this synthesis. We termed them isoforms I (in neuronal and epithelial cells), II (in cytokine-induced cells), and III (in endothelial cells) according to the order of their first purification and the first isolation of their cDNAs. All isoforms use L-arginine as the substrate, with molecular oxygen being the cosubstrate. NO synthesis represents a five-electron oxidation of a guanidino-nitrogen of L-arginine, resulting in NO and the coproduct L-citrulline. Half-saturating L-arginine concentrations were reported as 1.4 to 2.2 μmol/L for isoform I, 2.8, 16, and 32.3 μmol/L for isoform II, and 2.9 μmol/L for isoform III of NO synthase. Cofactors required include NADPH, bioperin, flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN). Flavins and bioperin are found bound to all three isozymes; biop-
terin is always found as the totally reduced biopterin derivative 6(R)-5,6,7,8-tetrahydrobiopterin. All three isoforms of NO synthase contain heme, whose function in the catalytic process is not completely understood (J.S.P. and M.N., unpublished observation for isoform III, 1993).

**Isoform I**

Isoform I is constitutively expressed in brain and was first purified from rat and porcine cerebellum. In the literature, this enzyme is also being referred to as b-NOS (for brain NO synthase), c-NOS (for constitutive or Ca^2+/-regulated NO synthase), bc-NOS (a combination of both), or n-NOS (for neuronal NO synthase). We do not favor this nomenclature because isoform I is also expressed by tissues and cells other than brain and neurons (see below) and the endothelial enzyme (isoform III) is also constitutively expressed and Ca^2+ regulated. Isoform I in brain is mostly a soluble enzyme that migrates with a molecular mass of 150 to 160 kD on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Some particulate activity can be detected in the presence of added FAD that shows the same immunochemical characteristics as the soluble form. Therefore, it probably represents the same protein. cDNAs encoding isoform I have been cloned from rat  and human brain. The deduced amino acid sequences predict proteins of 160 and 161 kD molecular mass, which is in good agreement with data obtained for the purified protein from murine macrophages. At the time this article was submitted, sequence analyses of cDNAs from different cells of the same species were available for rat (smooth muscle versus hepatocyte) and human (chondrocyte versus adenocarcinoma cells). The two cDNA sequences from rat, which were from different rat strains and both obtained with polymerase chain reaction (which can introduce mutations), were 98.8% identical. The three human cDNA sequences showed 99.9% identity (chondrocyte versus adenocarcinoma cells) and 99.7% identity (hepatocyte versus adenocarcinoma cells).

**Isoform II**

Isoform II of NO synthase is usually not constitutively expressed but can be induced in macrophages and many other cells with bacterial lipopolysaccharide and/or cytokines. The enzyme is also referred to as i-NOS or mac-NOS. Like isoform I, isoform II is predominantly a soluble enzyme. On SDS-PAGE the enzyme purified from induced mouse macrophages migrates with a molecular mass of 125 to 135 kD. Some particulate activity is found in induced murine macrophages and probably represents the same protein. Several groups have cloned cDNAs encoding isoform II from mouse macrophages, rat smooth muscle, rat liver, and human hepatocytes, and a human colorectal adenocarcinoma cell line (DLD-1). The deduced amino acid sequences predict proteins of 130 to 131 kD molecular mass, which is in good agreement with data obtained for the purified protein from murine macrophages. At the time this article was submitted, sequence analyses of cDNAs from different cells of the same species were available for rat (smooth muscle versus hepatocyte) and human (chondrocyte versus hepatocyte versus adenocarcinoma cells). The two cDNA sequences from rat, which were from different rat strains and both obtained with polymerase chain reaction (which can introduce mutations), were 98.8% identical. The three human cDNA sequences showed 99.9% identity (chondrocyte versus adenocarcinoma cells) and 99.7% identity (hepatocyte versus adenocarcinoma cells).
Gene contains 26 exons spanning almost 21 kb of genomic DNA and encodes a messenger RNA of 4052 nucleotides. Characterization of the 5'-flanking region indicates that the endothelial NO synthase promoter is "TATA-less" and exhibits regulatory elements consistent with the constitutive expression in endothelial cells. The human endothelial NO synthase gene is present as a single copy in the haploid human genome and localized to the 7q35->7q36 region of chromosome 737,48,61 (see Table 1). When this article was submitted, the human species was the only species for which CDNA sequences were available for all three isozymes. Within the human species, deduced amino acid sequences for all three isoforms are 52% to 58% identical, confirming that they are three distinct proteins. The proteins predicted from the amino acid sequences of all three NO synthase isoforms in all species investigated contain consensuses sequences for the binding of NADPH, flavins, and calmodulin (cf. regulation of NO synthase activity).

Mapping of NO Synthase Isozymes in Tissues

Isoform I of NO synthase is not only found in brain. Immunohistochemical staining using specific antibodies to this isozyme suggest that it is also present in certain areas of the spinal cord; in sympathetic ganglia and adrenal glands; in peripheral nitrergic nerves; in epithelial cells of lung, uterus, and stomach; in kidney macula densa cells; and in pancreatic islet cells. In addition, Northern and Western blot analyses localized the enzyme to human skeletal muscle. Many laboratories are using NADPH-diaphorase staining to localize NO synthase (especially isoform I) histochemically.

The technique is based on the ability of enzymes including NO synthase to reduce soluble tetrazolium salts to an insoluble, visible formazan. The C-terminal portion of all three NO synthase isoforms conveys NADPH-diaphorase activity, but several other enzymes show this activity as well. In fixed tissues, NADPH-diaphorase staining often correlates with NO synthase I immunoreactivity. But it is important to note that NADPH-diaphorase staining is not specific for NO synthases and does not prove that such enzymes are present in a given preparation.

Many cell types can be induced to express NO synthase isoform II. In each case its expression seems to depend on the exposure of cells and tissues to lipopolysaccharide and cytokines. Only a limited number of immunohistochemical studies have been reported so far. NO synthase II immunoreactivity has been reported in pancreatic islets of diabetic BB rats but not Wistar rats. The immunoreactivity was restricted to areas of islet infiltration by macrophages. Immunohistochemical localization of NO synthase II in rats treated with Propionibacterium acnes and lipopolysaccharide 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. Another immunohistochemical study done on lipopolysaccharide-treated rats found induced NO synthase II immunoreactivity predominantly in macrophages distributed in liver, kidney, and lung. In addition, mesangial cells and a small number of Kupffer cells and hepatocytes were stained, as were nerves supplying mesenteric blood vessels. Kobzik et al found strong labeling of rat macrophages after...
lipopolysaccharide treatment of the animals. Human alveolar macrophages were occasionally NO synthase II immunoreactive, especially in areas of inflammation. They also found NO synthase II immunoreactivity in the epithelium of large airways in rat and humans. 66

Immunohistochemical studies using a specific antibody to isoform III77 indicated that this enzyme was relatively specific for endothelial cells. It is found in various types of endothelial cells (arterial and venous) in many tissues, including human tissues. Recently, it has also been detected in syncytiotrophoblasts of human placenta78 and LLC-PK1 kidney tubular epithelial cells.79

**Regulation of the Activity of NO Synthase Isozymes**

Isoform I is constitutively expressed in several cell types. It is a Ca2+- and calmodulin-dependent enzyme that is inactive at 100 nmol/L Ca2+ and fully active at 500 nmol/L.22 This represents typical changes in intracellular Ca2+ concentrations upon receptor stimulation of excitatory cells such as neurons. The enzyme is phosphorylated by Ca2+-calmodulin–dependent protein kinase II, protein kinase C, and cyclic AMP–dependent protein kinase.80-81 Phosphorylation of NO synthase I by protein kinase C significantly reduced the activity of that enzyme.82

NO synthase II is transcriptionally regulated by various cytokines. Once expressed, no regulatory mechanism is known for the activity of this enzyme. Interestingly, the amino acid sequence of the enzyme demonstrates a binding site for calmodulin82,83 despite the Ca2+ independence of its activity. In mouse macrophages, calmodulin has been found stochiometrically associated with the enzyme. Apparently, its binding is so tight (even in the absence of Ca2+) that it can be considered a subunit of that enzyme.83 On the other hand, the induced NO synthase from human hepatocytes showed a loss in activity after Ca2+ chelation and exposure to a calmodulin antagonist, indicating a looser binding of calmodulin to this enzyme.84 Also, the human NO synthase II from DLD-1 adenocarcinoma cells lost activity in the presence of a Ca2+ chelator.85

NO synthase III is found constitutively expressed in endothelial cells. Like isoform I, it is regulated by Ca2+ (between 100 and 500 nmol/L) and calmodulin.86,87 Recently, some mechanisms have been described that regulate the expression of the gene. The shear stress of the flowing blood not only increases endothelial NO release acutely,88,89 it also upregulates NO synthase III expression.90 A putative shear stress–responsive element has been described in the promoter sequence of NO synthase III.91 NO synthase III has been shown to undergo serine phosphorylation in response to bradykinin as well as translocation from the particulate to the cytosolic fraction of endothelial cells with agonist activation.92 Also, endothelial cells exposed to shear stress increase their incorporation of radiolabeled phosphate.93 The physiological importance of phosphorylation for the regulation of NO synthase III activity remains to be determined. Recently, we have observed in cultured bovine aortic endothelial cells that tumor necrosis factor-α (TNF-α) alone or in combination with interleukin-1β (IL-1β) or IFN-γ, downregulates NO synthase activity, protein, and mRNA.94 Unlike native rat aortic endothelial cells, in which isoform II–like activity is induced with TNF-α, IL-1β, or IFN-γ,95 no such induction was found in our bovine aortic endothelial cell line. Other researchers have also reported a decrease in NO synthase III mRNA in response to TNF-α, but this was associated with an increase in NO production.96-98 Club muscle and meninges induction of isoform II–like activity. The mechanism of action of TNF-α has recently been ascribed to a destabilization of the mRNA of NO synthase III.99

**Physiological Functions of NO Synthase Isoforms**

**Isoform I**

Synaptic plasticity in the central nervous system has been associated with the NO produced by isoform I of NO synthase. With the use of inhibitors of NO synthesis, long-term potentiation of synaptic transmission by NO has been demonstrated in rat and guinea pig hippocampus90-92; long-term depression has been shown in rat cerebellar slices.93 NO synthase inhibitors have been reported to block synaptic responses to low-frequency stimulation, indicating that NO is not involved in normal neurotransmission. Retrograde communication across synaptic junctions is presumed to be involved in memory formation, and there is now evidence that inhibitors of NO synthase impair learning and can produce amnesia in chicks and rats.949797a

Evidence is also accumulating that NO formed in the central nervous system (most probably by isoform I of NO synthase) is involved in the regulation of blood pressure. Intracerebroventricular administration of low doses of the NO synthase inhibitor Nω-nitro-L-arginine or Nω-methyl-L-arginine increased blood pressure, sympathetic renal nerve activity, and heart rate in rats.98-100 Spinal cord transection at C4 to C5 prevented this effect.9899 and it could not be mimicked by intravenous administration of the same low doses of NO synthase inhibitors.100 Thus, central NO seems to play an important role in the regulation of blood pressure by reducing vascular sympathetic tone. NO produced by neuronal cells in the central nervous system may also regulate local cerebral blood flow by a direct paracrine action on adjacent vascular smooth muscle cells.

In the periphery, most smooth muscle tissues are innervated by nerves whose electrical stimulation produces relaxations that are not mediated by adrenergic or cholinergic transmitters. Many of these nerves are nitricergic; i.e., they generate and release NO.101-103 Purification and characterization of NO synthase from a prototypical tissue rich in nonadrenergic, noncholinergic nerves, the bovine retractor penis muscle, has indicated that these nitricergic nerves contain isoform I of NO synthase.104 Immunohistochemistry using an isoform I–specific antibody105 has demonstrated that this isoform is also responsible for NO production in nitricergic nerves in other smooth muscle tissues.22,58,106 Hence, NO produced by isoform I in nitricergic nerves can be viewed as an unorthodox neurotransmitter that decreases the tone of various types of smooth muscle.

Blood vessels are also innervated by nitricergic nerves that release NO as their neurotransmitter.107-109 These vascular nitricergic nerves are functionally antagonistic to
sympathetic nerves in that their stimulation produces vascular relaxation.\textsuperscript{104-106} Histochemical detection of NO synthase I immunoreactive fibers in the wall of canine arteries provides further evidence that blood vessels are innervated by nitrergic nerves.\textsuperscript{110} Also, NADPH-diaphorase-positive nerve fibers were found in cerebral vessels and ganglia innervating cerebral vessels.\textsuperscript{111}

**Isoform II**

When induced in macrophages, isoform II of NO synthase produces large amounts of NO that represent a major cytotoxic principle of those cells.\textsuperscript{112} Because of its affinity to protein-bound iron, NO can inhibit a number of key enzymes that contain iron in their catalytic centers. These include ribonucleotide reductase (rate-limiting in DNA replication), iron-sulfur cluster-dependent enzymes (complex I and II) involved in mitochondrial electron transport, and citrullase in the citric acid cycle.\textsuperscript{112} In addition, higher NO concentrations as produced by induced macrophages can directly interfere with the DNA of target cells and cause strand breaks and fragmentation.\textsuperscript{113,114} A combination of these effects probably forms the basis of the cytostatic and cytotoxic effects of NO on parasitic microorganisms and tumor cells. Interestingly, NO also seems to determine the life span of the induced macrophages themselves. Lipopolysaccharide- and cytokine-induced macrophages exhibited the typical ladder pattern of DNA fragmentation of apoptotic cells. DNA fragmentation was inhibited by N\textsuperscript{2}-methyl-L-arginine and mimicked by authentic NO gas.\textsuperscript{115,116} However, in order to be cytotoxic to other cells, macrophages have to be more resistant to NO than their target cells. The biochemical basis of this “NO resistance” is not known yet.

**Isoform III**

NO is produced by vascular endothelium under basal conditions, and its production can be further stimulated by a variety of receptor agonists as well as the shear stress produced by the flowing blood. NO dilates all types of blood vessels studied by stimulating soluble guanylyl cyclase and increasing cyclic GMP in smooth muscle cells.\textsuperscript{5,11,12,117} NO released by endothelial cells and nitrergic nerves is a major endogenous vasodilator system counterbalancing the vasoconstriction produced by the sympathetic nervous system and renin-angiotensin system. A study in rabbits indicates that the counterregulation against peripheral vasodilatation by NO accounts for 69\% of the basal norepinephrine release,\textsuperscript{118} thus demonstrating the powerful nature of the endothelial NO system. Blockade of NO synthesis with inhibitory l-arginine analogues leads to significant resistance in vivo is usually abrogated by inhibitory l-arginine analogues,\textsuperscript{124,125} although in one vascular bed no such blockade was seen.\textsuperscript{126} NO released toward the vascular lumen is a potent inhibitor of platelet aggregation and adhesion to the vascular wall.\textsuperscript{117,118} NO can also inhibit leukocyte adhesion to vascular endothelium either by interfering with the ability of the leukocyte adhesion molecule CD11/CD18 to form an adhesive bond with the endothelial cell surface or by suppressing CD11/CD18 expression on leukocytes.\textsuperscript{131,132} White cell adherence is an early event in the development of atherosclerosis, and therefore, NO may protect against the onset of atherogenesis. Furthermore, NO has been shown to inhibit DNA synthesis, mitogenesis, and proliferation of vascular smooth muscle cells.\textsuperscript{133-136} These antiproliferative effects are probably mediated by cyclic GMP, although one report failed to confirm this.\textsuperscript{137} Also, the prevention of platelet adhesion protects the smooth muscle from exposure to platelet-derived growth factors, thereby inhibiting the proliferation of vascular smooth muscle and their production of matrix molecules. Therefore, NO could protect against a later step in atherogenesis—fibrous plaque formation. Based on the combination of these effects, NO produced in endothelial cells can be considered an “antiatheroergic principle.”

**NO Synthase Isoforms in Pathophysiology**

**Isoform I**

In the central nervous system, NO is released on activation of the N-methyl-D-aspartate (NMDA) subtype of the glutamate receptor.\textsuperscript{138} During cerebral ischemia and cerebrovascular stroke large amounts of glutamate are released and act on neuronal cells already deprived of oxygen. Some of these cells contain NO synthase, and NO has been implicated in NMDA receptor-mediated neuronal death. Evidence for this assumption comes from experiments demonstrating that NO synthase inhibitors and the NO scavenger hemoglobin prevent neuronal death.\textsuperscript{139} Nowicki et al\textsuperscript{140} injected small doses of an NO synthase inhibitor into mice immediately after initiating cerebral ischemia and found that the NO synthase inhibitor reduced neuronal damage to a larger degree than a potent NMDA receptor antagonist. The usefulness of nonspecific NO synthase inhibitors in cerebrovascular stroke may be limited, however, because decreased release of (endothelium-derived) NO could favor neutrophil adherence to the endothelium, leading to oxygen radical–induced cerebral injury.\textsuperscript{141}

Some disturbances of smooth muscle tone within the gastrointestinal tract (e.g., gastroparesis and reflux disease) may be related to an overproduction of NO by isoform I in peripheral nitrergic nerves.\textsuperscript{142}

**Isoform II**

Most cell types seem to be inducible to express NO synthases whose activity is not regulated by Ca\textsuperscript{2+}. Once induced, these enzymes tend to produce large amounts of NO for prolonged periods of time. This marked NO production is likely to be involved in various pathophysiological disorders. The high levels of NO produced by activated macrophages (and probably neutrophils and other cells) not only may be toxic to undesired microbes, parasites, or tumor cells (see above) but may also harm healthy cells. Most inflammatory and autoimmune lesions are characterized by an abundance of activated macrophages and neutrophils. Thus, high levels of NO will be secreted, leading to damage of the surrounding tissue. The lysis of pancreatic islet cells by activated macrophages is a model of inflammatory death of healthy tissue. Inhibitory l-arginine analogues prevent
Hypercholesterolemia and atherosclerosis are associated with impaired endothelial-mediated vasodilatation in vitro and in vivo in laboratory animals and humans. Arteries, especially human coronary arteries, may thereby be predisposed to vasoconstriction and vasospasm. Indeed, the paradoxical vasoconstriction to the endothelium-dependent vasodilator acetylcholine can be used as a diagnostic indicator of beginning coronary atherosclerosis during coronary catheterization. The reduced ability of the endothelium to produce active NO would also predispose the vascular wall to platelet adhesion and the constrictor effects of substances released as a result of platelet disruption. Interestingly, coronary arteries showing marked intimal thickening and plaque formation usually maintain a morphologically intact endothelial cell layer. Studies on arterial segments from hypercholesterolemic animals have indicated that endothelial synthesis of NO is not impaired under these conditions (or may even be increased) but that there is increased oxidative breakdown of NO due to enhanced formation of superoxide radicals in the vascular wall. Thus, vascular superoxide dismutase activity, but not catalase or glutathione, seems to be required for the release of biologically active NO. An imbalance between intrinsic superoxide dismutase activity and the generation of superoxide radicals in atherosclerotic arteries seems to result in a more rapid oxidation of NO. Treatment with polyethylene-glycolated superoxide dismutase can partially restore the impaired NO-mediated vasodilatation of atherosclerotic arteries. Recent immunohistochemical results obtained with our monoclonal antibody to isoform III seem to confirm this concept. Staining of endothelial NO synthase in response to the cytokines may occur in the vascular system of these patients. The underlying process or outcome. It is clear, however, that the presence of immune complexes in a variety of organs is associated with inflammation, macrophage and neutrophil invasion, and tissue damage. Autoimmune diseases involving immune complex deposition include rheumatoid arthritis, systemic lupus erythematosus, certain forms of glomerulonephritis, and various forms of vasculitides (Henoch-Schönlein purpura, periarteritis nodosa, allergic granulomatosis, tumor-associated vasculitides).

Adherent, activated macrophages are also known to play an important role in in vivo allograft rejection. In a model comparing the infiltration of allogeneic versus syngeneic grafts in rats, allogeneic graft fluid was found to contain higher levels of nitrite and nitrate (the oxidation products of NO). Evaluation of the cultured graft-invading cells revealed that allogeneic graft-infiltrating cells (mainly macrophages and granulocytes) spontaneously produced higher amounts of NO than syngeneic graft-infiltrating cells and showed higher cytotoxicity toward the allogeneic donor splenocytes. Thus, NO seems to be an important mediator of non-specific allograft rejection.

Finally, induced isoform II of NO synthase probably plays an important role in septic shock, which is characterized by massive arteriolar vasodilatation, hypotension, and microvascular damage. Bacterial endotoxins are likely to be responsible for the symptoms. The mechanism by which endotoxemia causes the above vascular effects is not completely understood. A number of mediators are elevated in septic shock and have been implicated in its pathophysiology. These include platelet-activating factor, thromboxane A2, prostanoids, and cytokines such as IL-1, TNF-α, and INF-γ. More recently, a new concept has emerged indicating that endotoxin alone or in combination with cytokines induces the expression of large amounts of Ca2+-independent NO synthase in the vascular wall (presumably in smooth muscle cells). This is backed by in vitro experiments that demonstrate induction of smooth muscle NO synthase by IL-1 and TNF-α as well as in vivo studies showing the reversal of endotoxin-induced hypotension by inhibitory L-arginine analogues. A study in terminally ill patients showed that Nω-methyl-L-arginine or Nω-nitro-L-arginine methyl ester can decrease blood pressure in septic shock in humans. It has also been shown that the treatment of patients suffering from malignant melanoma and renal carcinoma with IL-2 leads not only to an increase in the plasma levels of TNF-α but also to a marked increase of nitrate in plasma and urine. Metabolic tracer studies with 15N2O. L-arginine demonstrated that the nitrate was derived from the terminal guanidino nitrogen atom of L-arginine. Among other problems, IL-2 therapy is complicated by hypotension, suggesting that an induction of NO synthase in response to the cytokines may occur in the vascular system of these patients.

Isoform III

Hypercholesterolemia and atherosclerosis are associated with impaired endothelial-mediated vasodilatation in vitro and in vivo in laboratory animals and humans. Arteries, especially human coronary arteries, may thereby be predisposed to vasoconstriction and vasospasm. Indeed, the paradoxical vasoconstriction to the endothelium-dependent vasodilator acetylcholine can be used as a diagnostic indicator of beginning coronary atherosclerosis during coronary catheterization. The reduced ability of the endothelium to produce active NO would also predispose the vascular wall to platelet adhesion and the constrictor effects of substances released as a result of platelet disruption. Interestingly, coronary arteries showing marked intimal thickening and plaque formation usually maintain a morphologically intact endothelial cell layer. Studies on arterial segments from hypercholesterolemic animals have indicated that endothelial synthesis of NO is not impaired under these conditions (or may even be increased) but that there is increased oxidative breakdown of NO due to enhanced formation of superoxide radicals in the vascular wall. Thus, vascular superoxide dismutase activity, but not catalase or glutathione, seems to be required for the release of biologically active NO. An imbalance between intrinsic superoxide dismutase activity and the generation of superoxide radicals in atherosclerotic arteries seems to result in a more rapid oxidation of NO. Treatment with polyethylene-glycolated superoxide dismutase can partially restore the impaired NO-mediated vasodilatation of atherosclerotic arteries. Recent immunohistochemical results obtained with our monoclonal antibody to isoform III seem to confirm this concept. Staining of endothelial NO synthase was not diminished in arteries from hypercholesterolemic animals, but there was a marked invasion of inflammatory cells in the media and thickened intima. Reduced endothelium-mediated vasodilatation has also been found in arteries from hypertensive and diabetic animals, and impaired responses to endothelium-dependent vasodilators have been demonstrated in the forearm of hypertensive patients. The underlying

References:
2. AfG-methyl-L-arginine protected against lung and skin vascular injury, whereas L-arginine increased the damage. It is unclear at this time whether the ultimate tissue-damaging role is related to the NO radical (• NO) itself or an interaction of • NO with • O2 leading to the formation of peroxynitrite (ONOO⁻) and eventually • HO, but NO formation seems to be crucial for the tissue damage observed. In humans, the key properties of antigen-antibody complexes with respect to immune complex disease are activation of the complement system, interactions with cell receptors, and deposition in tissues. The characteristics of immune complexes in human disease have not been examined in sufficient detail to relate their features to the disease processes or outcome. It is clear, however, that the presence of immune complexes in a variety of organs is associated with inflammation, macrophage and neutrophil invasion, and tissue damage. Autoimmune diseases involving immune complex deposition include rheumatoid arthritis, systemic lupus erythematosus, certain forms of glomerulonephritis, and various forms of vasculitides (Henoch-Schönlein purpura, periarteritis nodosa, allergic granulomatosis, tumor-associated vasculitides).

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mechanism is unclear but is probably a consequence rather than a cause of the diseases.\textsuperscript{158}

The Arginine Paradox

The substrate for the NO synthases, L-arginine, is supplied to cells by carriers that mediate facilitated diffusion and are specific for cationic amino acids. This transport system, referred to as system \textit{y} \textsuperscript{+},\textsuperscript{159} has recently been shown to consist of at least two membrane proteins, CAT-1 and CAT-2B (cationic amino acid transporter), that are differentially expressed in mouse tissues.\textsuperscript{160,161} In lipopolysaccharide-activated macrophages, NO production depends on extracellular arginine.\textsuperscript{162-164} An increased transport of L-arginine has been reported in these cells,\textsuperscript{165,166} as well as in lipopolysaccharide-activated hepatocytes,\textsuperscript{167} suggesting that the L-arginine supply could be rate limiting for NO production by NO synthase II. In contrast, NO production in endothelial cells in vitro seems to depend less on extracellular L-arginine.\textsuperscript{17} In cultured endothelial cells, intracellular L-arginine concentrations have been reported in the range of 100 to 800 \textmu mol/L.\textsuperscript{168-170} Even after 24 hours in L-arginine-free medium, endothelial L-arginine concentrations were still 200 \textmu mol/L,\textsuperscript{148} and the NO production of such cells was only marginally reduced.\textsuperscript{17} This is to be expected because the half-saturating L-arginine concentration \( (K_m) \) for NO synthase III was determined as 2.9 \textmu mol/L.\textsuperscript{26} In freshly isolated endothelial cells, up to 2 mmol/L L-arginine has been measured.\textsuperscript{170} Consequently, NO synthase III should be saturated in these cells, and additional L-arginine should not increase the activity any further. Surprisingly, in vivo data suggest that increasing serum L-arginine (which is normally around 100 \textmu mol/L) can augment endothelial NO production. Endothelium-dependent vasodilatation is impaired in hypercholesterolemia (see above), and a correction of this endothelial dysfunction with L-arginine has been demonstrated in hypercholesterolemic animals\textsuperscript{171,172} and humans.\textsuperscript{173,174} Similarly, in patients with essential and secondary hypertension, L-arginine lowered blood pressure and increased indicators of NO production such as plasma citrulline and urinary excretion of nitrite and nitrate.\textsuperscript{175} Even in a study in young healthy volunteers, L-arginine but not \( \textit{p} \)-arginine was found to increase basal forearm blood flow. L-Arginine also stereospecifically augmented the effect of the endothelium-dependent vaso- dilator acetylcholine but not of the endothelium-independent vasoconstrictor sodium nitroprusside.\textsuperscript{176} These findings are hard to explain based on the above biochemical data. The reason for these discrepancies is not readily apparent; the intracellular \( K_m \) value for L-arginine, the intracellular L-arginine concentrations, and transmembrane transport of L-arginine will have to be determined simultaneously under controlled conditions to solve this paradox.

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