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Nitric Oxide : Biological Role and Clinical Uses

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Abstract : Nitric oxide is a product of the conversion of L-arginine by the enzyme nitric oxide synthase. Nitric oxide is involved in a variety of physiological situations and is produced by many different cell types. It is involved in neurotransmission, maintenance of vascular smooth muscle tone, and cytotoxicity. Nitric oxide has been suggested to play an anti-inflammatory role by inhibiting the expression of the genes for inflammatory cytokines. The pathophysiological role of nitric oxide is also evident in a variety of diseases, including septic shock, asthma, reperfusion injury, etc. Nitric oxide, by stimulating the production of cyclic GMP, relaxes smooth muscles of the cardiovascular, respiratory, gastrointestinal, and genito-urinary systems. Recent studies have provided important information on the use of inhaled nitric oxide for the management of several diseases characterized by the presence of abnormal pulmonary vascular tone, such as persistent pulmonary hypertension of the newborn. This review addresses the biology and clinical uses of inhaled nitric oxide. **(Indian J Pediatr 1998; 65 : 333-345)**

Key words *: L-arginine; Neurotransmission; Anti-inflammatory.*

The enzyme nitric oxide synthase (NOS) catalyzes the two-step oxidation of L-arginine to L-citrulline and free nitric oxide (NO)[1]. NO is known to be involved in a variety of physiological roles, including neurotransmission, maintenance of vascular smooth muscle tone, and cytotoxicity. Such diverse physiological functions dictate that the production of NO is regulated depending on the location of the enzymes responsible for its synthesis and the factors that induce their activity. In this review, we will discuss the biosynthesis of NO, the mechanisms by which NO causes smooth muscle relaxation, the experience to date with the use of inhaled NO in treating persistent pulmonary hypertension of the newborn, and some recommendations regarding its use.

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Regulation of NO Biosynthesis

Furchgott and Zawadzki[2] showed that vascular relaxation induced by acetylcholine is mediated by a factor released from the endothelium, which they termed endothelium-derived relaxing factor (EDRF) which was shown to be NO derived from vascular endothelium in response to a variety of stimuli, including shear stress[3]. NO is synthesized from L-arginine by NOS. During its synthesis, NOS is translated as a monomer, and in the presence of tetrahydrobiopterin it forms a dimer. In the pres-

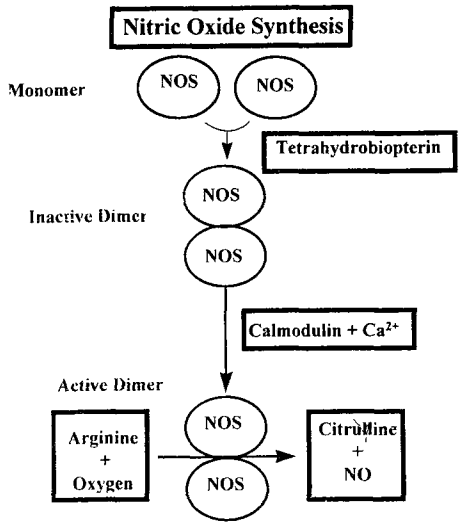

Fig. 1. Nitric oxide synthase (NOS) is synthesized as a monomer. In the presence of the co-factor tetrahydrobiopterin, the two NOS monomers form an inactive dimer. In the presence of calmodulin and calcium (required for the calcium-independent isoforms), the inactive dimer is converted to an active dimer. The active dimer enzyme converts L-arginine in the presence of molecular oxygen to NO and citrulline.

ence of calmodulin and molecular oxygen, NOS converts L-arginine to NO, with citrulline as a byproduct (Fig. 1). At least three isoforms of NOS have been described : neuronal NOS (n-NOS or NOS1), endothelial NOS (e-NOS or NOS3), and inducible NOS (i-NOS or NOS2)[4]. Several co-factors are involved in the synthesis of NO including calmodulin, FAD, FMN, NADPH, and tetrahydrobiopterin. NOS contains heme moiety in its structure.

Bredt *et al*[5] first isolated n-NOS from the brain. Although n-NOS has considerable homology in terms of amino acid sequence to the other NOS isoforms, the amino terminal region of this enzyme has a long sequence of amino acids whose precise function is as yet undefined. n-NOS is found in several parts of the brain, but appears to be more abundant in the cerebellum and as a result it has been speculated that NO may mediate motor function and coordination[6]. n-NOS is also found in several peripheral neurons. Activation of n-NOS in the peripheral neurons and the subsequent release of NO results in relaxation of smooth muscle[7]. For example, NO released from peripheral neurons regulates the function of smooth muscles of the gastrointestinal, respiratory, and vascular systems. NO release is also known to mediate penile erection.

n-NOS and e-NOS are constitutively expressed in neurons and endothelial cells respectively[8]. Under resting conditions, these enzymes do not synthesize appreciable quantities of NO. However, when intracellular calcium levels rise in response to excitation of the cells possessing these enzymes, the resultant calcium-calmodulin complex activates NOS, resulting in NO release. When the intracellular calcium levels fall, the activity of the enzyme decreases. Therefore, the constitutive NOS isoforms are described as calcium-dependent, since their activity is tightly regulated by the level of intracellular calcium. i-NOS, on the contrary, is not expressed to a significant extent by cells under resting conditions. Induction of i-NOS activity in inflammatory cells such as macrophages usually results in the production of large quantities of NO[9]. NO production by macrophages may be involved in host defense mechanisms. When i-NOS is induced, for example in response to inflammatory cytokines, immune complexes or LPS, it binds to calmodulin and produces NO even in the absence of

calcium[10]. Induction of i-NOS synthesis by LPS involves transcription and the NF-$_k$B family of transcription factors plays a key role in this process[11]. i-NOS has been described as calcium-independent, since its activity is not dependent on the level of intracellular calcium.

Mechanisms of NO Action in Smooth Muscle Cells

By interacting with the heme moiety of the enzyme soluble guanylyl cyclase, NO stimulates the production of cyclic GMP[12]. The resultant elevation of intracellular cGMP leads to activation of the cGMP-dependent protein kinase and phosphorylation of some key intracellular proteins, including ion channels. In vascular smooth muscle, recent studies have indicated that elevation of intracellular cGMP by NO and NO donors results in activation of caclium-activated K^+ channels, membrane hyperpolarization and relaxation of vascular tone[13]. The nitrovasodilators used in the treatment of hypertension and as coronary vasodilators act in a manner to decrease vascular resistance and thus effect greater blood flow to the affected organ. Previous studies from our laboratory have indicated that cGMP-dependent activation of the calcium-activated K^+ channels by NO released from neurons within the airways and s-nitroso-n-acetylpenillamine (SNAP), a NO donor, is also involved in the relaxation of airway smooth muscle[14].

Not all the effects of NO and NO donors in smooth muscle are mediated via elevation of intracellular cGMP. In a recent study, Bolotina *et al*[15] showed that NO directly activates calcium-dependent K^+ channels in vascular smooth muscle cells, causing relaxation. Exposure of aortic rings to NO caused an elevation of intracellular cGMP and relaxation. Pre-exposure to methylene blue, an inhibitor of the soluble guanylyl cyclase, did prevent the rise in the intracellular level of cGMP, without significantly affecting relaxation. However, pre-exposure to charybdotoxin, a toxin derived from the scorpion *Leiurus quinquestriatus herbraeus*, and a selective inhibitor of the calcium-dependent K^+ channels, reduced the relaxation response to NO. These results provided strong evidence for cGMP-independent pathways for vascular relaxation by NO involving direct activation of K^+ channels. In porcine airway smooth muscle, our earlier studies also support a cGMP-independent activation of the calcium-dependent K^+channels by NO donors and neurally released NO in the relaxation response. However, unlike vascular smooth muscle cells, activation of the calcium-dependent K^+ channels may not necessarily be associated with membrane hyperpolarization in airway smooth muscle cells. Therefore, other mechanisms may be involved in the relaxation response to NO donors in airway smooth muscle cells, such as changes in local intracellular calcium concentration, decreased calcium sensitivity of the contractile proteins, etc. Elucidation of these mechanisms may help us understand the basis for cellular specificity of NO action.

The effects of NO and NO donors are also characterized by inhibition of agonist stimulated elevation of intracellular calcium[16]. In smooth muscles of airways and vasculature, contractions to agonists involves elevation of intracellular calcium concentration above the basal level. Under normal, unstimulated conditions, smooth muscle cells maintain a relatively low

intracellular calcium level, usually in the range of 100-150 nM. In recent studies, we have shown that calcium release from intracellular structures known as the sarcoplasmic reticulum plays an important role in the maintenance of steady-state calcium levels during agonist stimulation[17]. This steady-state level of calcium is critical for the maintenance of force during agonist stimulation and intracellular calcium levels can reach 1000 mM or higher during the steady-state response to agonists in several smooth muscle cells. Inhibition of this steady-state level of calcium is expected to lead to smooth muscle relaxation and in the airways, such relaxation may result in decreased resistance to airflow. In airway smooth muscle, NO, NO donors, and β_2-adrenoceptor agonists that are used in the symptomatic treatment of asthma as bronchodilators, all cause relaxation by reducing intracellular calcium levels during agonist stimulation[16,18,19]. Furthermore, the effects of NO and NO donors are mimicked by exogenous membrane-permeant analogue of cGMP and that of β_2-adrenoceptor agonists by exogenous membrane-permeant analogue of cAMP. However, our recent studies have indicated that the mechanisms by which NO and NO donors lower intracellular calcium levels in airway smooth muscle cells are different from those induced by salbutamol, a β_2-adrenoceptor agonist. NO and NO donors, by increasing intracellular cGMP levels, decrease calcium release from the sarcoplasmic reticulum[16,18], while β_2-adrenoceptor agonist salbutamol increases intracellular cyclic AMP levels and thus decreases calcium influx through voltage-gated calcium channels and augments calcium efflux from the cell[19]. The net effect of inhibition of calcium release from the sarcoplasmic reticulum, inhibition of calcium influx and augmented calcium efflux is lower intracellular calcium level and smooth muscle relaxation.

The results of the studies outlined above clearly point to multiple mechanisms by which cyclic nucleotides, which mediate the effects of NO, NO donors, and β_2-adrenoceptor agonists, cause smooth muscle relaxation. Among the mechanisms associated with NO action are cGMP-dependent activation of K^+ channels, membrane hyperpolarization, reduction of intracellular calcium levels by inhibition of calcium release, calcium influx, and calcium efflux, and cGMP-independent mechanisms involving activation of K^+ channels. One or more of these mechanisms may be operative in a given cell. A thorough understanding of these various mechanisms is essential towards designing therapeutic strategies for the management of asthma, hypertension, etc.

Anti-inflammatory Role of NO

There is clear evidence of NO in inflammation. It is known to inhibit the production of several pro-inflammatory cytokines, including IL-6 and IL-8[20]. This action of NO appears to be independent of cGMP elevation. Evidence seems to indicate that NO inhibits transcription of these pro-inflammatory cytokine genes. The genes that encode the messenger RNAs for these pro-inflammatory cytokines contain DNA binding motifs in their promoter regions for the transcription factor NF-κB[20]. In a variety of cells, including macrophages and vascular endothelial cells, activation of NF-κB is an essential critical first step in the transcription of cytokine genes involved in inflammation. The binding of NF-KB to the pro-

moter regions of the genes encoding the messenger RNAs for these cytokines initiates transcription. It has been shown that NF-κB is located in the cytoplasm in an inactive form, bound to an inhibitory subunit known as IκB as well as an inhibitory prosequence[21]. Activation of NF-κB requires phosphorylation of these inhibitory subunits and the eventual translocation of the free NF-κB into the nucleus[22]. Although phosphorylation can be induced by several kinases within the cells, the role of tyrosine kinases in the phosphorylation of IκB is becoming increasing clear[22]. NO, by inhibiting NF-κB translocation and by increasing the expression of IκB[23], can prevent transcription of pro-inflammatory cytokine genes and thus can play a protective role in inflammation. Furthermore, agents that interfere with phosphorylation of these inhibitory subunits and thereby prevent NF-κB translocation, may also possess anti-inflammatory properties. In this regard, tyrosine kinase inhibitors are being investigated as potential anti-inflammatory agents. In experimental models of sepsis, Tyrphostin AG 556, a tyrosine kinase inhibitor, has been shown to decrease tumour necrosis factor (TNF) production, improve survival and reduce cardiopulmonary dysfunction[24,25]. NO donors such as S-nitrosoglutathione (GSNO) have been shown to inhibit not only NF-κB binding to promoter regions in the DNA, but to also induce IκB transcription and overxpression of the IκB protein[23]. The latter mechanism would lead to increased binding of NF-κB in the cytoplasm, maintaining it in an inactive form, thereby preventing transcription of the cytokine genes.

The anti-inflammatory role of NO is evident in some pathological situations such as atherosclerosis. Although atherogenesis has a complex pathophysiology, NO has been shown clearly to inhibit a variety of steps that contribute to vascular injury in atherogenesis, including vascular smooth muscle cell proliferation and platelet adhesion to endothelial cells. In addition, NO prevents the interaction of leukocytes with vascular endothelial cells by inhibiting chemotaxis and adhesion[20]. Recent studies have shown that NO decreases cytokine-induced induction of vascular endothelial cell adhesion molecule (VCAM-1), endothelial leukocyte adhesion molecule (ELAM-1), and intracellular adhesion molecule (ICAM-1)[20]. These surface receptors on inflammatory cells such as the eosinophils and endothelial cells are essential for the adhesion and diapedesis of cells to sites of inflammation. NO, by inhibiting the expression of these adhesion molecules, can also exert an anti-inflammatory effect in the host.

Pathophysiological Roles of NO

There is unequivocal evidence of NO as an effector of the inflammatory response. The mechanisms by which NO causes toxicity in the host are complex, but generation of reactive oxygen intermediates (ROIs) and its reaction with superoxide to generate peroxynitrite are potential mechanisms[26]. Peroxynitrite is more toxic than NO because of its potential for direct cytotoxicity through protein tyrosine nitration. Clearly, these actions of NO may be independent of cGMP elevation in the effector cells. The role of NO as a toxin has been delineated in several clinical entities. These include septic shock, various inflammatory diseases, atherosclerosis, reperfusion injury of the myocardium, acute renal failure, glomerulonephritis, and

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