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Minireview

iNOS-mediated nitric oxide production and its regulation

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Abstract

This review focuses on the production of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) and its regulation under physiological and pathophysiological conditions. NO is an imporant biological mediator in the living organism that is synthesized from L-arginine using NADPH and molecular oxygen. However, the overproduction of NO which is catalyzed by iNOS, a soluble enzyme and active in its dimeric form, is cytotoxic. Immunostimulating cytokines or bacterial pathogens activate iNOS and generate high concentrations of NO through the activation of inducible nuclear factors, including NFkB. iNOS activation is regulated mainly at the transcriptional level, but also at posttranscriptional, translational and postranslational levels through effects on protein stability, dimerization, phosphorylation, cofactor binding and availability of oxygen and L-arginine as subsrates. The prevention of the overproducion of NO in the living organism through control of regulatory pathways may assist in the treatment of high NO-mediated disorders without changing physiological levels of NO.

Keywords: Nitric oxide; iNOS; Inflammation; NFkB; LPS

Introduction

Nitric oxide (NO) is an important intracellular and intercellular signalling molecule involved in the regulation of diverse physiological and pathophysiological mechanisms in cardiovascular, nervous and immunological systems. NO has contrasting roles in living organisms. It acts as a biological mediator similar to neurotransmitters in the neuronal system, can regulate blood vessel tone in vascular systems, and is an important host defence effector in the immune system. On the other hand, it is a free oxygen

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radical (NO·) and can act as a cytotoxic agent in pathological processes, particularly in inflammatory disorders (Alderton et al., 2001; Bogdan, 2001; Dawn and Bolli, 2002; Moncada and Higgs, 1991). Inhibition of iNOS may be beneficial for the treatment of inflammatory disease (Aktan et al., 2003; Bogdan, 2001; Kroncke et al., 1998). In this review, the overproduction of NO by iNOS and its regulation are examined.

NO is a short-lived free radical and a very small compound that diffuses freely within cells from its site of formation to its site of action. The biosynthesis of NO in the organism is carried out from L-arginine and molecular oxygen utilizing NADPH as an electron donor and using heme, FMN, FAD and tetrahydrobiopterin (H_4B) as cofactors through a reaction that consumes five electrons. The overall reaction consists of a two step oxidative conversion of L-arginine to NO and L-citrulline via N^w-hydroxy-L-arginine (NOHarginine) as an intermediate, with monooxygenase I and monooxygenase II, each step representing a mixed-function oxidation (Fig. 1) (Alderton et al., 2001; Mayer and Hemmens, 1997; Stuehr et al., 1991).

The production of NO in the body is catalysed by a family of enzymes called nitric oxide synthases (NOSs) (130–160 kDa). NOS enzymes consist of different subtypes depending on the tissue type, although all share between 50–60% sequence homology (Alderton et al., 2001). Currently, at least three distinct isoforms of NOS have been isolated and cloned: eNOS (endothelial NOS, NOS I), iNOS (inducible NOS, NOS II) and nNOS (neuronal NOS, NOS III). nNOS and iNOS are soluble whereas eNOS is membrane bound, with its N-terminal myristoylated (Liu et al., 1995). Two enzymes, eNOS and nNOS isoforms, designated as constitutive NOS, are constantly present in resting cells, and are activated by calcium and calmodulin (CaM) (Alderton et al., 2001). Upon demand by a signal molecule, NO is synthesized in low concentrations by constitutive NOS, binds to heme iron of soluble guanylate cyclase to yield the second messenger cGMP, which in turn modulates an array of mediators, including various ion channels, phoshodiesterases and protein kinases, decreasing intracellular calcium levels, and allows smooth muscle to relax (Ignarro, 1992). Stimulation of soluble guanylate cyclase by NO also inhibits platelet aggregation and vessel wall adhesion of platelets (Chiang et al., 2002). The third isoform, inducible NOS (iNOS), is not present in resting cells but can be induced by immunostimulatory

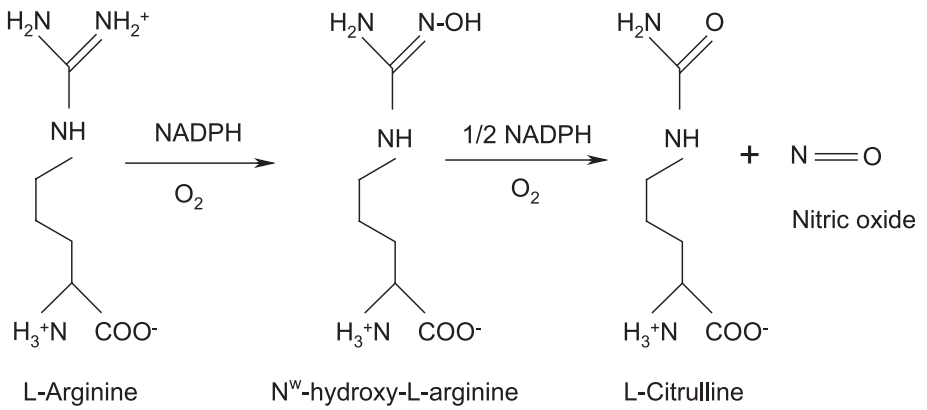

Fig. 1. The reaction of NO synthesis from L-arginine. NO is synthesized endogenously by the conversion of L-Arginine to L-citrulline. During this reaction, NADPH (1.5 molecules) is used as an electron donor and NOHarginine is generated as an intermediate.

cytokines, bacterial products or infection in a number of cells, including endothelium, hepatocytes, monocytes, mast cells, macrophages and smooth muscle cells. It generates NO independently of intracellular calcium concentrations (Alderton et al., 2001; Mayer and Hemmens, 1997). There is some evidence of a different NOS enzyme, mitochondrial NOS (mtNOS). Recently, the existence of mtNOS, its Ca^{2+} dependence, and its relevance for mitochondrial bioenergetics was reported in rat and mouse tissues, such as liver, thymus, skeletal muscle, heart, and brain. (Elfering et al., 2002; Ghafourifar et al., 1999; Giulivi et al., 1998). Upon uptake of Ca^{2+} mtNOS is stimulated, peroxynitrite ($ONOO^{-}$) is formed, and Ca^{2+} is subsequently released from intact mitochondria, suggesting a feedback loop which prevents overloading of mitochondria with Ca^{2+} (Bringold et al., 2000). Enhanced NO production and $ONOO^{-}$ formation by mtNOS also lead to protein nitration in the mitochondrial matrix during mitochondrial dysfunction and contractile failure (Boveris et al., 2002). However, its relation to other NOS isoforms and how it is regulated is still unclear (Brown, 2001).

NO is not only synthesized enzymatically, but can also be produced nonenzymatically from nitrite at low pH under reducing conditions. Nonenzymatic NO production may play a role in similar biological events as when NO is generated from L-arginine by NOS enzymes, such as in the stomach, on the surface of skin, in the ischemic heart, and in infected nitrite-containing urine, but its importance in regulation of iNOS activity and NO production is unclear (Weitzberg and Lundberg, 1998).

The overproduction of NO independently of intracellular calcium concentrations is due to iNOS that can result in either protective or damaging effects, although activation of NO production depends on the cell type. Many cell types can express iNOS for their function in host defense against microbial and viral pathogens (Bogdan, 2001), leading to the formation of NO radicals or S-nitrosothiols or ONOO- in the host cell or in the microbe itself. In addition to the protective effects of iNOS, iNOS expression in macrophages is activated by particular inducers, participating in the pathology of inflammatory diseases including atherosclerosis, rheumatoid arthritis, diabetes, septic shock, transplant rejection, and multiple sclerosis, leading to cell death (Buttery et al., 1994; Hubbard and Giardina, 2000; Luoma et al., 1998).

iNOS was first purified and cloned from mouse macrophage. Although mouse and rat macrophages are different from human macrophage in their NO production, iNOS consists of a bi-domain protein in which the C-terminal half of NOS acts as a reductase domain that contains the binding sites for CaM, NADPH, FAD and FMN; and the N-terminal half of the enzyme acts as an oxygenase domain that contains the binding sites for heme, H_4B, and L-arginine (Sennequier and Stuehr, 1996). L-arginine or substrate analogs bind near the heme in the oxygenase domain to influence its reduction or its interaction with small ligands such as NO or carbon monoxide (CO) (Prabhakar, 1998). L-arginine binding alters electron flux through NOS by a change in oxygen consumption or NADPH oxidation (Alderton et al., 2001; Sennequier and Stuehr, 1996). During NO synthesis, arginine and NOHarginine roughly lie parallel to the heme plane, with the guanidino group interacting with heme binding O_2 ($Fe^{II}O_2$ intermediate) to cause a shift in the heme iron (Lefevre-Groboillot et al., 2003). In the structure of the murine iNOS oxygenase domain, the guanidinium group contributes two hydrogen bonds to carboxy oxygens of Glu 371 (Glu 377 in human iNOS) and one oxygen bond to the carbonyl of Trp 366, localizing the substrate over the heme (Lefevre-Groboillot et al., 2003). H_4B transfers an electron to $Fe^{II}O_2$ intermediate for rapid hydroxylation of arginine, since the reduction of $Fe^{II}O_2$ by H_4B is faster than heme reduction by the NOS reductase domain (Hurshman and Marletta, 2002; Wang et al., 2002). H_4B is oxidized at the N-5 position to yield $H_3B^{\cdot}$ (Fig. 2) (Gorren et al., 2001).

The C-terminal reductase domain of NOS displays close homology with cytochrome P450 reductase and transfers NADPH-derived electrons that are required for reductive activation of molecular oxygen to

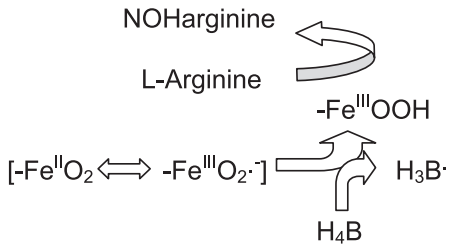

Fig. 2. Conversion of L-arginine to NOHarginine by H_4B in NOS oxygenase domain. L-arginine is positioned above the heme with heme binding O_2 ($Fe^{II}O_2$). H_4B transfers an electron to $Fe^{II}O_2$, yielding H_3B for hydroxylation of arginine. This oxidizing product oxidizes L-arginine to NOHarginine.

the N-terminal oxygenase domain, resulting in oxygen insertion into L-arginine and NOHarginine (Alderton et al., 2001). The CaM-binding site is situated approximately in the center of NOS and separates the two domains. CaM effects as a molecular switch, inducing a conformational change in the structure for electron flow (Ruan et al., 1996). It controls communication between the two domains in that its binding triggers flavin-to-heme electron transfer.

The iNOS enzyme appears to be active only in its dimeric form (Alderton et al., 2001; Panda et al., 2002). Dimerization created between two oxygenase domains of NOS is one of several posttranslational steps required for conversion into an active enyme (Siddhanta et al., 1996). iNOS dimerization is initiated by insertion of heme, resulting in rapid conformational changes. The heme is buried in the protein interior and makes extensive van der Waals interaction with hydrophobic and aliphatic side changes. Biochemical and crystallographic studies of the iNOS oxygenase domain suggest that its N-terminal β-hooks can swap across the dimer to interact with their partner subunits and, thus, stabilize the dimer (Panda et al., 2002). The incorporation of heme into the oxygenase domain creates the binding sites for arginine and H_4B (Chen et al., 2002; Ghosh et al., 1996; Siddhanta et al., 1996). The binding of arginine and H_4B cause a transition from loose to tight NOS dimer (Fig. 3). L-arginine does not directly participate in the dimer interface, whereas the H_4B cofactor binding bridges across the dimer interface, π-stacking, and hydrogen-bonding with residues in both subunits of the dimer (Panda et al., 2002). H_4B

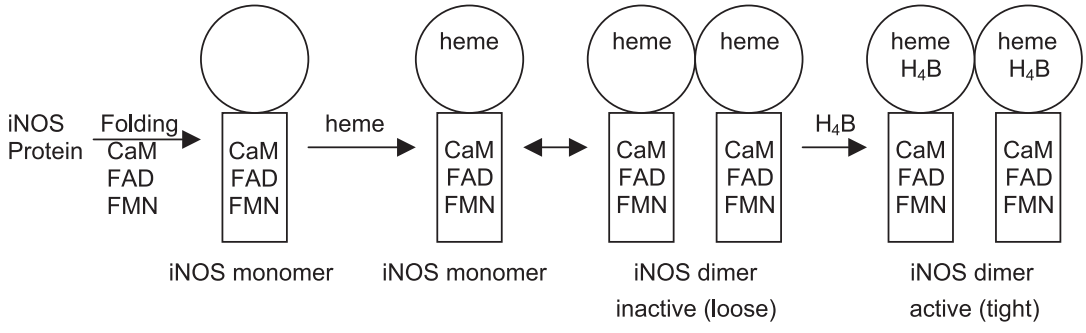

Fig. 3. Model for the generation of active iNOS dimer. After the synthesis of iNOS protein, the heme free monomer that contains a functional reductase domain is generated in the presence of CaM, FAD and FMN. The heme incorporation into the oxygenase domain initiates dimerization. H_4B and Arginine generate active tight dimer iNOS form.

interacts with one of the heme propionate side-chains causing a complete spin-state conversion of the heme and stabilizing the NOS dimer (Hurshman and Marletta, 2002). Zn^{2+} incorporation at the dimer interface can provide additional stabilization (Ruan et al., 1996). Human iNOS appears to act by a similar mechanism, in that H_4B is required for the active dimer (Stuehr, 1999).

Higher concentrations of NO synthesized by iNOS expressed during a variety of inflammatory diseases will interact with numerous substances. NO generated by iNOS reacts with oxygen by a third order reaction up to 300-fold greater in the membrane in hydrophobic conditions, compared to reactivity in the cytosol or in an aqueous medium, due to concentrated oxygen in the membrane. This interaction is important in membranes in order that $NO^{\cdot}$ and oxygen can concentrate 2- to 8- fold. The interaction between NO and oxygen generates reactive nitrogen oxide intermediates (RNOIs) such as $NO_2^{\cdot}$ and N_2O_3, N_2O_4 (Eqs. (1) and (2)). Under normal conditions, NO is converted to nitrite (NO_2^-) and nitrate (NO_3^-) (Darrah et al., 2000).

$$2NO^{\cdot} + O_2 \rightarrow 2NO_2^{\cdot}, \text{ where } k = 2 \times 10^6 (mol/L)^{-2} \cdot s^{-1}. \quad (1)$$

$$NO^{\cdot} + NO^{\cdot} \rightarrow N_2O_3 \quad (2)$$

$$NO_2^{\cdot} + NO_2^{\cdot} \rightarrow N_2O_4$$

$$R - SH + NOx \rightarrow R - SNO. \quad (3)$$

$NO_2^{\cdot}$ and RNOI can react with other biomolecules such as thiols and amines which may result in conformational changes in structure due to formation of S-nitrosothiols (R-S-NO) in proteins (Eq. (3), reactive nitrogen oxide intermediates donated as NOx) such as occuring in nitrosoglutathione production (Kroncke et al., 1998; Reichenbach et al., 2001). RNOI or S-nitrosothiols, the latter via a trans-nitrosylation mechanism, can S-nitrosylate cystein thiols involved in Zn^{2+} complexation in zinc finger structures, leading to conformational changes and modifications of functional activities (Coffey et al., 2002; Kroncke, 2001). Zinc finger domains are important for protein-DNA or protein-RNA interactions. Some transcription factors have zinc finger domains (Berendji et al., 1999). Complexation of S-nitrosylate cystein with Zn^{2+} causes a conformational change, leading to inhibition of transcription factor-DNA binding activities (Kroncke, 2001). In addition, in inflammatory-mediated reactions, $NO_2^{\cdot}$ reacts with hypochlorous acid (HOCl) to form a nitryl chloride ($Cl\text{-}NO_2$). Also, myeloperoxidase in neutrophils and monocytes generates additional $NO^{\cdot}$ and HOCl-derived NO metabolites (Whiteman et al., 2003). Recently, an additional RNOI was postulated to contribute to 3-nitrotyrosine formation in vivo by a nitryl chloride being formed via the reaction of nitrite and neutrophil myeloperoxidase-derived HOCl. Neutrophil infiltration (indicated by myeloperoxidase activity in the colon) was associated with formation of nitrotyrosine and marked apoptosis (Whiteman et al., 2003). The main reactions of NO are summarized in Table 1.

$NO^{\cdot}$ can react with iron-protein structures such as in heme containing enzymes. The binding of $NO^{\cdot}$ to constitutive NOS inhibits its activity by interacting directly with enzyme rather than with any of the soluble cofactors or substrates, suggesting that $NO^{\cdot}$ may act as a negative feedback modulator through its interaction with enzyme-bound heme (De Alba et al., 1999). Within the vascular system, $NO^{\cdot}$ may be removed by reacting with oxyhemoglobin, thus preventing the generation of oxidant $NO^{\cdot}$ metabolites and protecting against oxidative damage. $NO^{\cdot}$ binds to iNOS-heme iron and inhibits its activation at the

Table 1
Main reactions of NO

Reaction with	Product
Fe^{2+}	$Fe^{2+}-NO$
O_2	N_2O_3, N_2O_4, NO_2^-, NO_3^-, $ONOO^-$
R-SH	R-S-NO
HOCl	$Cl\text{-}NO_2$

NO reacts with Fe2+ within heme containing protein, with oxygen to produce reactive nitrogen oxide intermediates and peroxynitrite, and with thiols to generate nitrosothiols.

posttranslational level in the absence of inducers such as cytokines and/or endoxin (Rao, 2000). In cell respiration, the interaction of $NO^{\cdot}$ and iron-containing enzyme is most important (Brown, 2001). High levels of NO and $ONOO^-$ inhibit the activity of complexes I and II in the respiratory complex, ATP synthase, aconitase, creatine kinase, superoxide dismutase, and damage mitochondrial DNA and mitochondrial membranes, which are an important in oxidative phosphorylation (Borutaite et al., 2000; Brown, 2001).

NO at physiological concentrations inhibits proinflammatory platelet aggregation, integrin-mediated adhesion, and proinflammatory-induced gene expression, factors that control vascular inflammation and oxidative injury. However, $NO^{\cdot}$ and NO_2^- at high concentrations can display pathogenic properties due to the production of $ONOO^-$, $NO^{\cdot}$, and other reactive oxidizing compounds in the presence of superoxide radicals or peroxidases, causing the reversal of NO effects from protective to deleterious (Grisham et al., 1999). $NO^{\cdot}$ reacts with superoxide to form $ONOO^-$ that can interact with proteins, lipids, carbohydrates, and DNA, and with carbon dioxide to form highly reactive nitrosoperoxocarbonate, which through oxidation and nitration mechanisms change the structure and function of these compounds to lead to oxidative damage of tissues (Bloodsworth et al., 2000). Peroxynitrite can irreversibly inhibit mitochondrial respiration and damage a variety of mitochondrial components via oxidizing reactions (Brown, 2001). The oxidant metabolites of $NO^{\cdot}$ can also inhibit the activity of antioxidant enzymes such as glutathione peroxidase and reduce the levels of some cellular antioxidants such as ascorbic acid, uric acid and plasma thiols (Han et al., 2001). Reactive nitrogen compounds and $ONOO^-$ can also modulate some enzymes in the inflammatory process and in vascular functions, including prostaglandin endoperoxide synthase, cytochrome P450 and 5-lipooxygenase metabolism (Bloodsworth et al., 2000; Coffey et al., 2002).

Regulation of NO synthesis by iNOS

iNOS activity in macrophage is first regulated and modulated by cellular receptor molecules such as Toll-like receptors and CD14. CD14 is the receptor for lipopolysaccharide (LPS) and plays an essential role in pro-inflammatory responses in monocytes and macrophages via activation of the NFkB pathway (Schroder et al., 2000). CD14 has two distinct forms; mCD14 (GPI-anchored form) and sCD14 (soluble CD14). mCD14 is believed to directly effect LPS simulation via interaction with Toll-like receptor-4 (TLR-4). (Du and Low, 2001). IFN-γ induces iNOS via the Jak-STAT signalling pathway. Activated Jak-STAT signalling pathway increases iNOS induction and NO production (Fig. 4) (Rao, 2000).

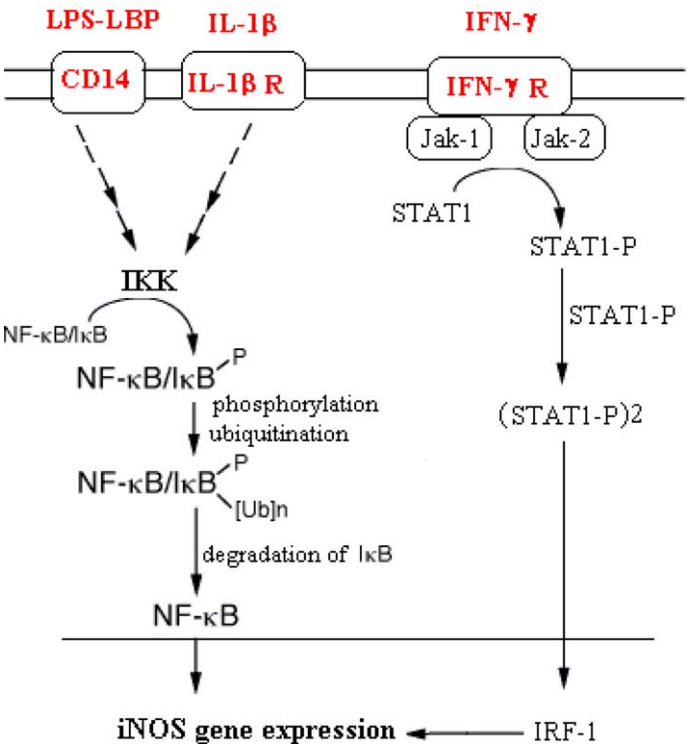

Fig. 4. Activation of NFkB and STAT signalling pathway. LPS binding protein (LBP) transfers LPS to CD14 receptor on the cell membrane. Binding LPS activates NFkB or AP-1. IkB-NFkB complex is inactive in the cytosol. After activation of IkB-NFkB complex, free NFkB transfers into the nucleus and induces the expression of iNOS. IFN-γ binds to IFN-γ R and phosphorylates STAT1. Activated STAT1 translocates into the nucleus and increases iNOS induction and NO production.

The regulation of NO synthesis by iNOS differs according to the strain and species of animals and depends on the inducers. Bovine and murine macrophages generate considerable amounts of iNOS in response to cytokine stimulation, but human and pig macrophages are resistant (Jungi et al., 1996). Recent developments are reviewed and show that NO biosynthesis is regulated by a variety of mechanisms at the transcriptional and posttranslational levels in activated macrophages and other cells.

Transcriptional and posttranscriptional regulation

The level of NO produced by iNOS is mainly regulated at the transcriptional level. The molecular mechanisms for transcription of the iNOS gene have been studied in different cells. Depending on the stimulator or cell type, different signalling pathways activate transcriptional factors, activators such as protein kinase C, tyrosine kinase, janus kinases, raf-1 protein kinase, mitogen activated protein kinases (MAP kinase), and inhibitors such as protein tyrosine phosphatases, phosphoinositide-3-kinase (Lahti et al., 2002; Marks-Konczalik et al., 1998; Momose et al., 2000; Muller et al., 2001; Wang et al., 1999). The promoter region of the mouse iNOS gene contains several binding sites for transcription factors like NFkB as well as Jun/Fos heterodimers, some C/EBT, CREB and the STAT family of transcription

factors, within its proximal and distal regions (Hecker et al., 1999; Kleinert et al., 1996; Kleinert et al., 1998; Marks-Konczalik et al., 1998). The human iNOS gene promoter contains sequences homologous to mouse proximal and distal regions (Rao, 2000).

Transcription factors like NF-kB (nuclear factor kappaB) and AP-1 (activating protein-1) mediate the expression of iNOS and other inducible genes such as COX-2 (cyclooxygenase-2), VCAM-1 (Vascular Cell Adhesion Molecule-1), ICAM-1 (Intercellular Cell Adhesion Molecule-1) in immune and inflammatory responses (Xia et al., 2001). NFkB is present in the cytosol as an inactive complex IkB-NFkB (Fig. 4). The IkB-NFkB complex is phosphorylated by IkB kinase (IKK) through activation by stimulator(s) such as potent exogenous inducer (LPS), endogenous inducers (cytokines, interferon-γ (IFNγ), and tumor necrosis factor-α (TNFα)), which facilitate the translocation of free NFkB from cytosol to the nucleus and the induction of iNOS gene expression (Huang et al., 1998; Marks-Konczalik et al., 1998; Obermeier et al., 1999). LPS down-regulates the DNA binding activity of the inducible transcription factors NFkB, CREB and AP-1, whereas LPS up-regulates the DNA binding activity of constitutive transcription factors Sp1 (promoter selective transcription factor) and AP-2 (activating protein-2) in a time-dependent manner (Ye and Liu, 2001). The regulation of iNOS via the NFkB pathway is an important mechanism in inflammatory processes and a potential site for intervention in inflammatory disease. We studied the reversal effect of gypenosides, a herbal medicine, on LPS-induced iNOS expression through NFkB activity, suggesting its use in treatment of inflammatory diseases (Aktan et al., 2003). In addition, some compounds suppress iNOS induction via degradation of IkB. H_2O_2 increases degradation of IkB and activation of the NFkB pathway, whereas lactacystin prevents iNOS induction by the blocking degradation of IkB (Bowie and O'Neill, 2000; Musial and Eissa, 2001). Therefore, the degradation of IkB, phosphorylation/dephosphorylation of the NFkB-IkB complex and the translocation of free NFkB to the nucleus are important in regulating NO production by iNOS. Activation of the C/EBP family transcription factors by protein kinases increases intracellular cAMP concentrations, inducing iNOS in rat vascular smooth muscle cells (Hecker et al., 1999). Also, NFkB and C/EBP can also cooperate in a synergistic manner to induce the iNOS gene (Hecker et al., 1997).

Combinations of cytokines or LPS with IFNγ induce iNOS expression synergistically. Suppressor of cytokine signalling-1 (SOCS-1) is a negative regulator of the Jak-STAT signalling pathway activated by cytokines and interferons. Exposure of cells to IFNγ activates janus kinases, Jak-1 and Jak-2, which phosphorylate and activate STAT1. The activated STAT1 translocates into the nucleus, increases IRF-1 levels and induces iNOS gene in murine macrophages, mouse and human islets, hepatocytes, and renal mesangial cells (Fig. 4) (Chong et al., 2002; Rao, 2000). The overproduction of SOCS-1 inhibits cytokine signals that activate this pathway (Rao, 2000).

NO can regulate its own production. It has biphasic effects on NO synthesis by modulating iNOS mRNA expression (Connelly et al., 2001). The positive feedback regulation of iNOS expression in renal mesangial cells and vascular smooth muscle cells is mediated indirectly through increased intracellular cAMP levels via inhibition of PDE III (phosphodiesterase III) (Perez-Sala et al., 2001). The negative feedback regulation of iNOS expression by NO in macrophages and hepatocytes is mediated by the inhibition of NFkB activation. In contrast to rat renal mesangial cells, in rat aortic vascular smooth muscle cells, NO overproduction is up-regulated by negative feedback regulation as well (Hecker et al., 1999). NO-releasing agents and cGMP analogs can modulate the transcription factors AP-1 and NF-kB in a positive or negative manner, depending on the tissue type (Lee et al., 2002). On the other hand, H_2O_2 and ROS activate NFkB with enhanced iNOS expression and this

is suppressed by antioxidant compounds and enzymes. Thus, H_2O_2 production by LPS in macrophages participates in the upregulation of iNOS (Bowie and O'Neill, 2000; Han et al., 2001).

Some neuropeptides such as vasoactive intestinal peptide (VIP) and the pituitary adenylate cyclase activating polypeptide (PACAP) can function as macrophage deactivating factors. VIP and PACAP use the cAMP-dependent pathway to stimulate CREB phosphorylation, causing a change in the CRE binding complex, a binding site for IL-10 as a macrophage deactivating factor, and inhibiting IFNγ-induced interferon regulatory factor-1 (IRF-1) expression. A cAMP-independent pathway is also involved in inhibition of IKK activity and NFkB binding, thus reducing iNOS expression (Leceta et al., 2000). cAMP induction of iNOS has been documented for macrophages, vascular smooth muscle cells, and renal mesangial cells (Hecker et al., 1999).

iNOS expression is related to peroxisome proliferator-activated receptors (PPARs), which may antagonize the activities of transcriptional factors including AP-1, STAT and NFkB. TNFα and IFNγ may modify PPAR activity, leading to a modified activity on p65/RelA subunit of the NFkB signalling pathway (Chinetti et al., 1998).

Also important may be the post-transcriptional regulation of iNOS expression. Steady-state iNOS mRNA levels depend on its synthesis, stability, and degradation. The 3'-untranslated region (3′-UTR) of iNOS mRNA contains AU-rich sequences, known to destabilize iNOS mRNA (Rodriguez-Pascual et al., 2000; Taylor et al., 1998). The exposure of cells to transforming growth factor β (TGFβ) inhibits iNOS induction because it causes iNOS mRNA destabilization (Perrella et al., 1994). The posttranscriptional effects of TGF-β might be mediated via a modification of IRF-1 activation through the activation of nucleases acting on the AU rich sequence (Vodovotz, 1997). In addition, protein kinase α (PKCα) has been shown to play an essential role in iNOS mRNA stabilization in pancreatic β-cells (Carpenter et al., 2001). Conversely, iNOS mRNA stability is enhanced by elevation of intracellular cAMP levels, but reduced by increases in intracellular Ca2+ (Oddis et al., 1995). Additionally, NO and/or cGMP can modulate iNOS gene expression by posttranscriptional mechanisms (Perez-Sala et al., 2001). iNOS mRNA destabilization by cGMP limitas iNOS induction during late treatment times of cells supplemented with NO or cGMP donors, and NO and/or cGMP generation and cGMP appears to control iNOS expression by positive and negative feedback mechanisms (Perez-Sala et al., 2001).

Translational and posttranslational regulation

The biosynthesis of NO is also controlled at the translational and posttranslational levels. Recently, some compounds such as dexamethasone and sodium salicylate were found to be effective in modulating iNOS mRNA translation (Bogdan, 2001; Rao, 2000). However, mechanisms of translational regulation of NO biosynthesis are unclear. iNOS can trigger phosphorylation of eIF-2α which is related to protein synthesis at the translational level (Petrov et al., 2001). The reduction of protein synthesis results in reduction of iNOS activity. This regulation is another important step in the downregulation of NO production by iNOS.

Posttranslational regulation of NO synthesis is important in controlling of iNOS protein availability through effects on protein stability, dimerization, phosphorylation, cofactor binding, and availability of O_2 and L- arginine as substrates.

Enhancement or blockade of degradation of iNOS protein is another posttranslational regulatory mechanism. TGF-β increases the degradation of iNOS protein as well as decreasing stability and

translation of iNOS mRNA (Matsuno et al., 2001; Perrella et al., 1994). The effect of TGF-β on the protein may be mediated indirectly by proteases. TGF-β increases the deposition of extracellular matrix in order to repair damage at inflammatory sites of myocardial tissue injury (Lijnen et al., 2000). This occurs by the stimulation of the production of new matrix proteins through the inhibition of synthesis of proteases and stimulation of production of protease inhibitors. iNOS protein is degraded under the control of the proteasome pathway directly (Musial and Eissa, 2001). Lactacystin, a proteasome inhibitor, prevents iNOS induction by blocking the degradation of IkB at the transcriptional level and by blocking the degradation of iNOS protein, form enzymic degradation by proteases at the posttranslational level. The cysteine protease calpain plays an important role in iNOS protein degradation (Walker et al., 2001).

The regulation of iNOS activity through effects on its structural stability has been considered recently. Kalirin has a neuroprotective role in inflammation and appears to inhibit iNOS by preventing formation of the iNOS dimer (Ratovitski et al., 1999a). Also, several proteins inhibit the dimerization, including the macrophage product, the inducible nitric-oxide synthase (NOS)-associated protein (NAP 110) (Ratovitski et al., 1999b). Antifungal imidazoles also prevent iNOS dimerization, but smaller compounds such as imidazole itself and 1-phenylimidazole inhibit the activity of dimeric iNOS (Blasko et al., 2002).

NO synthesis can be regulated through effects on the substrate L-arginine, cofactors such as heme, H_4B, and the electron donor NADPH. Overproduction of NO by NOS depends on extracellular L-arginine concentrations (Mori and Gotoh, 2000). In macrophages, CAT1 and CAT2A, cationic amino-acid transporter proteins upregulated by LPS stimulation, increase L-arginine uptake. Also, in macrophages, LPS stimulation can generate arginine from citrulline because of induction of argininosuccinate synthase, the rate-limiting enzyme of the citrulline-NO cycle (Nagasaki et al., 1996). Moreover, LPS activates arginase that degrades arginine to urea and ornithine, potentially limiting NO production, although the Km value of arginine for arginase is much higher than its Km value for iNOS (Nicholson et al., 2001).

NO synthesis can also be regulated via the subunit heme. CO inhibits NO synthesis by coordinating with the heme iron. This reaction is important in regulating NO production by heme oxygenase (HO), the enzyme that catalyzes the conversion of heme to biliverdin, CO, and free iron (Watts et al., 2003). NOS can also bind NO as a sixth ligand in ferrous or ferric states of its heme iron providing a negative feedback regulation (Connelly et al., 2001). The dissociation of the Fe^{3+}-NO complex competes with its reduction to the Fe^{2+}-NO structure to inhibit iNOS activation. In addition, cell-produced NO can block heme incorporation into iNOS monomer although this inhibition by NO is reduced in the presence of NO scavengers, O_2 or superoxide (Bowie and O'Neill, 2000).

NO synthesis can be regulated via H_4B, essential for NOS catalysis, as another posttranslational NOS regulation. Guanosine triphosphate cyclohydrolase, the key enzyme for H_4B synthesis, is induced or suppressed by particular cytokines. Another enzyme related to iNOS that uses H_4B is phenylalanine hydroxylase, which catalyses the conversion of phenylalanine to tyrosine, but has little impact on iNOS activity. Suppression of H_4B levels results in increased H_4B synthesis by phenylalanine, but not by arginine (Taylor et al., 1998).

NO synthesis can also be regulated by modification of phosphorylation, although, the role of phosphorylation under physiological conditions for iNOS is unclear. Posttranslational modification of macrophage iNOS by tyrosine kinases has been demonstrated. Protein kinase phosphorylation may be important for cellular localization and/or function of iNOS (Pan et al., 1996).

Conclusion

The overproduction of NO by iNOS is important in inflammation and its related processes High levels of NO are markers for the treatment of inflammatory disorders, and its prevention is a target for the design of new drugs acting on iNOS. In the design of NOS inhibitors, inhibition of NFkB activation was initially considered important, because it is the main regulatory step for iNOS expression. Recently, in addition to this regulation at the transcriptional level, many translational and posttranslational modifications of iNOS have been demonstrated. These regulatory steps may be important point for inhibition of the overproducion of NO, depending on the cell type and other related pathways, in the treatment of disorders related to high level of NO, and for the design of new drugs against these disorders. Thus, research has focused on the control of physiological levels of NO and for the design of new drugs that inhibit iNOS to prevent overproduction of NO for the treatment of NO-mediated diseases. This review on iNOS and its regulation may assist the development of strategies to treat such diseases.

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