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Functions and significance of nitric oxide in patho-physiological processes

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Nitric oxide (NO) is an important signaling molecule that regulates a diverse range of physiological processes in many tissues. NO is enzymatically synthesized from L-arginine by three isoforms of nitric oxide synthase (NOS). It plays an important role in a large number of normal physiological (regulation of blood pressure, neurotransmission, wound repair and host defense mechanisms) as well as in patho-physiological (inflammation, infection, apoptosis, neoplastic diseases, liver cirrhosis, diabetes) processes. With an unpaired electron, NO is a strong pro-oxidant produced in conditions like sepsis. It also exerts its effect through other free radicals such as superoxide and hydroxyl ions and causes oxidative stress within the cell. Reactive nitrogen intermediates play a central role in cell death (apoptosis), which is mediated by the induction of pro-inflammatory cytokines such as tumour necrosis factor. NO mediated apoptosis occurs in various cell types such as macrophages, lymphocytes, thymocytes, endothelial cells. At lower concentration (10 n*M*-1 μ*M*) NO has been shown to have antiapoptotic effect, which is cyclic GMP dependent. One of the most beneficial functions of NO is its implication in host defense against intracellular pathogens (*Salmonella* and *Leishmania*). Its derivatives such as per-oxynitrite are strong bactericidal in nature. Involvement of NO in inflammatory responses has been shown not only in experimental models but also in human inflammatory diseases. The antioxidant and antiapoptotic properties make this molecule of great therapeutic significance.

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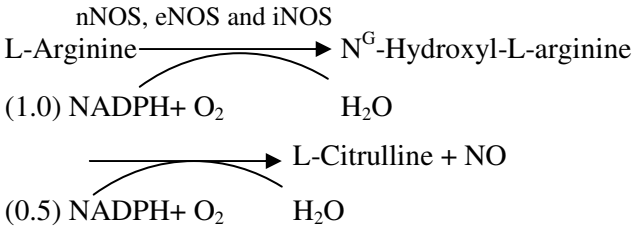
Introduction

Nitric oxide (NO) with a molecular weight of 30 is the smallest and short-lived molecular mediator in mammalian cells. It is synthesized in a highly regulated manner in a number of tissues and cell types and plays a major role in diverse physiological and pathological processes. The physiological processes include regulation of blood pressure, platelet adhesion, neutrophil aggregation, synaptic plasticity in brain, whereas NO and its secondary oxidants may act as cytoprotective, immunoregulatory, antimicrobial agents under pathological conditions[1].

In body, NO is produced during the metabolism of L-arginine. The terminal guanidino nitrogen atom of L-arginine is the donor for NO[2]. The possibilities of NO synthesis from compounds such as NO_2, NO_3, NH_3 and hydroxylamine were ruled out. It has been established that NO is the product of the five-electron oxidation of one guanidine nitrogen of L-arginine by the enzyme nitric oxide synthase (NOS), which leads to the production of citrulline and nitric oxide through an intermediate compound *N*-hydroxy-L-arginine (NHA).



The reaction catalyzed by NOS requires molecular oxygen (O_2) and NADPH. The availability of L-arginine determines cellular rate of NO synthesis. The uptake and synthesis of arginine can be enhanced when NOS expression is induced. Arginine transport increases in the macrophages stimulated to express inducible NOS (iNOS). This increase in transport may be due to induction of a new transporter, MCAT-2B, whereas normal arginine transport occurs via transporter MCAT-1. Arginine transport is stimulated by endotoxins in pulmonary artery endothelial cells and is increased in liver during sepsis. Further, it can be competitively inhibited by arginine analogues such as nitro-L-arginine methyl ester (L-NAME), and

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N ^{G}–monomethyl-L-arginine (L-NMMA) that are commonly used as inhibitors of NOS[3]. The major sites of arginine synthesis in vertebrates are liver and kidney. In liver, arginine is formed as well as rapidly metabolized by urea cycle while in kidney, citrulline (extracted from the plasma) is converted to arginine that is released into circulation[4]. Many other cell types also possess low levels of arginosuccinate synthetase (ASS) and arginosuccinate lyase (ASL) that together synthesize arginine from citrulline and can, therefore, play a role in NO synthesis[5].

Though, much information is available regarding NO and its physiological role, its involvement in the pathology and treatment of various diseases especially during infections, requires further reviewing. With this aim the present article reviews the available information on NO and its function in various patho-physiological processes such as infection, apoptosis and changed redox status.

Nitric Oxide Synthases (NOS) and NO

As discussed, NO is synthesized endogenously by NOS. Three major isoforms (nNOS, eNOS and iNOS) of this enzyme exist, which vary in calcium dependence, kinetics, and regulation. However, all NOS are flavoprotein in nature and require NADPH and tetrahydrobiopterin as cofactors. The neuronal NOS (nNOS, NOS1) and endothelial NOS (eNOS, NOS3), expressed constitutively in a variety of cells, are Ca^{2+}/calmodulin-dependent and together termed as constitutive NOS (cNOS). iNOS, NOS2, on the other hand, is Ca^{2+} independent and expressed in cells of the immune system and certain other cells in response to various stimuli[6]. The ability of some cells to regulate the expression of iNOS allows them to produce large amounts of NO on demand. The three isoforms (nNOS, eNOS and iNOS) are present in the cytoplasm. Another isoform of NOS, the mitochondrial NOS (mtNOS), is present exclusively in the mitochondrion. Co-stimulation of superoxide production and mtNOS can result in high levels of the highly reactive and damaging peroxynitrite[7]. All NOS enzymes are homodimers and activation of NOS requires binding of several co-factors[8].

The NOS genes from cow[9], rat[10], mouse[11], and human[12] have been cloned. The amino acid sequences deduced from the cDNAs of the endothelial and neuronal NOS and iNOS have been reported[13]. Various NOS isoforms have conserved domains in their structure. For example, cNOS and iNOS share sequence homology at the N-terminal region, which may be the arginine binding and catalytic sites[14]. Moreover, the C-terminal regions of endothelial NOS and iNOS show significant homology to FAD, FMN and NADPH binding regions of cytochrome P450 reductase from rat liver. Most of the P450s fall into a well described super gene family whose functions involve hydroxylation of endogenous and xenobiotic compounds. NOS is the first self-sufficient mammalian P450[15]. These C-terminal regions share multistranded β sheet surrounding α helices that are similar to the portions of nucleotide binding domains of the crystallized ferrodoxin NADPH reductase. However, the subcellular localization of various isoforms varies: for example, endothelial NOS is membrane bound whereas the neuronal NOS is present in cytosol of central and peripheral neurons. NO acts as a physiological regulator by relaxing vascular smooth muscles or by functioning as neurotransmitter. Constitutively expressed NOS produces low amounts (pico to nanomoles) of NO for short periods in a calcium/calmodulin-dependent manner in response to receptor stimulation (e.g., acetylcholine, bradykinin). In contrast, iNOS is expressed only upon exposure to diverse stimuli, such as the inflammatory cytokines (e.g., interleukin-1, tumour necrosis factor) and lipopolysaccharides (LPS). Once expressed, the inducible enzyme generates significantly larger amounts of NO than does the constitutive isoform. Expression of NOS in endothelium and brain is similar among different species but the expression of iNOS varies. Human monocytes express iNOS only after exposure to granulocyte stimulating factor (GSF) plus TNF or γ-interferon (INF-γ plus TNF or INF-γ plus LPS). In contrast, murine monocytes can express iNOS after exposure with LPS alone[16]. In addition, the quantity of NO produced by human monocytes/macrophages, lymphocytes, and neutrophils is considerably less than that by their murine counterparts. Hepatocytes and chondrocytes are two human cell types that can most readily be induced to express iNOS *in vitro.*

Oxidative Stress and NO

Oxidative stress is the metabolic status when the flux of partially reduced forms of reactive oxygen is greater than the ability of the biological system to cope with its production. Under stressed conditions biological molecules are exposed to pro-oxidants resulting in irreversible oxidation reactions. Such reactions result in chemical modification of biological molecules that can lead to cellular dysfunction.

Oxidative damage to DNA can result in gene mutation and is thought to be one of the causes of carcinogenesis. The redox status of a cell, which refers to the ratio of the reduced and oxidized forms of certain cellular components (GSH/GSSG, ubiquinol/ ubiquinone, ascorbate/dihydroascorbate, etc.), is an important signaling device in cellular homeostasis. An additional oxidative insult can change the redox-status of the cell that will consequently alter the expression and activity of cellular metabolic pathways[17].

Usually, cells have a battery of antioxidants and pro-oxidants. Antioxidants include glutathione, vitamins C and E, enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase and compounds of plant origin like retinol, flavonoids and, *N*-acetylcysteine, etc. The oxidants include various reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), peroxyl radical ($RO_2^{\bullet}$), hypochlorous acid (HOCl) and heme proteins[18]. Second type of pro-oxidants include reactive nitrogen species (RNS) such as NO, peroxynitrite ($OONO^-$), *S*-nitrosothiols (RSNO), nitrogen dioxide ($NO_2^{\bullet}$), dinitrogen trioxide (N_2O_3), and dinitrosyl-iron complexes (DNIC), etc.

NO inhibits enzymes such as cyclooxygenase, lipooxygenase and cytrochrome P450 by reduction of heme or non-heme iron in active site to its inactive ferrous form. These observations suggest that NO prevents many oxidative processes by reduction of ferryl heme[19]. NO effects are mediated through secondary oxidants, especially peroxynitrite in macrophages, neutrophils and Kupffer cells. Activated macrophages generate superoxide radicals through membrane bound NADPH oxidase along with NO leading to generation of peroxynitrite[20]. Inhibition of NO synthesis with N^G-methyl-L-arginine increases the amount of superoxide as two moles of oxygen are consumed for each mole of NO formed. Peroxynitrite is one electron reduced form of nitrosydioxyl radical ($ONOO^-$). This molecule is a powerful oxidant, which has been implicated in stroke, heart disease, inflammation and many other pathological conditions. During its decomposition at physiological *p*H, it can produce some of the strongest oxidants known in the biological system, resulting in the initiation of the reactions that are characteristics of hydroxyl radical ($OH^{\bullet}$), nitrosonium ion (NO^+) and $NO_2^{\bullet}$. Its unusual stability in the cis-conformation contributes to its toxicity by allowing peroxynitrite to diffuse far from its site of formation and to be selectively reactive with cellular targets[21]. Reaction of peroxynitrite with unsaturated fatty acid-containing liposomes results in initiation of lipid peroxidation. Reactive hydroxyl radicals also play an important role in lipid peroxidation,. Peroxynitrite rapidly oxidizes tocopherols. The α-tocopherol is oxidized to α-tocopherol quinone, a form that is not easily repaired by cellular reductants[22].

Proteins such as NF-κB are activated during oxidative stress, which produce NO through iNOS induction[23]. NF-κB; a transcription factor in higher eukaryotes, is a heterodimer composed of the DNA binding subunits p50 and p65 (also termed RelA)[24], and resides in cytoplasm in a latent form that is stabilized by an inhibitory protein termed IκB. As a consequence of cell stimulation, the IκB dissociates from the NF-κB, thereby allowing the liberated NF-κB to translocate to nucleus and bind to appropriate regulatory elements of the target gene. Rapid post translational activation of NF-κB can be initiated by a plethora of patho-physiological stimuli including inflammatory cytokines, bacterial and viral infections, radiation and certain oxidants[25].

NO and Antimicrobial Activity

NO has been implicated in host defense against intracellular pathogens such as *Leishmania*, *Mycobacteria* and *Salmonella*[26]. It plays a leading role in the pathogenesis of infections caused by viral pathogens including influenza virus and other RNA viruses[27]. Bacterial infection in human and experimental animals causes significant increase in systemic NO production as confirmed by the presence of nitric oxide oxidation products (nitrite and nitrate) in plasma and urine[28]. Microbial products (LPS, lipoteichoic acid and various cytokines such as IFN-α, TNF-α, IL-1 and IL-2) induce iNOS to produce large amounts of NO. Moreover, abrogation of iNOS activity produces dramatic increase in microbial burden[29].

Anti-leishmania activity is mediated by the release of free NO from *S*-nitrosothiol (RSNO) NO donors like *S*-nitroso-*N*-acetylpenicillamine (SNAP), *S*-nitrosoalbumin, *S*-nitrosoglutathione (GSNO), and *S*-nitroso-*N*-acetyl-l-cysteine (SNAC). Some of these like *S*-nitrosoalbumin, GSNO, and *S*-nitrosohemoglobin, have already been identified as endogenous NO carriers and donors in mammals. NO is covalently bound to a sulfur atom in a C–S–NO moiety and can be released through the homolytic or

heterolytic S–N bond cleavage. The homolytic cleavage is able to release free NO that can be transferred to specific receptors like iron-containing enzymes, to which it can coordinate as a ligand (nitrosylation reactions). The heterolytic reaction, on the other hand, allows the transfer of NO directly to thiol-containing proteins, to which it can bind as nitrosonium ion (NO^+) in transnitrosation reactions. In *Leishmania* NO donors have been shown to cause inhibition of mitochondrial respiration with decreased aconitase activity, probably triggered by iron loss[30]. It has also been demonstrated that NO-donors inhibit *Leishmania* cysteine proteinase (CP) activity[31].

NO causes toxicity to *Mycobacterium leprae*. Among others, NO mediates nitrosation of tyrosine residues. Both iNOS and nitrotyrosine are detected in lepromatous lesions[32]. NO from activated murine macrophages or exogenous sources exhibits antimycobacterial properties, and can irreversibly damage bacteria by inhibiting aerobic respiration. Nearly 50 genes are induced by NO in *M. tuberculosis*, a large subset of which has a suggested or documented role in promoting survival under nitrosative stress[33]. Remarkably, most of these genes are dysfunctional or absent in the obligate intracellular pathogen *M. leprae;* only superoxide dismutase reductase (*sodC*), alkylhydroperoxide reductase (*ahpC*), bifunctional thioredoxin reductase/thioredoxin (*trxB* and *trxC*), NADPH-ferredoxin reductase (*fprA* and *fprB*), truncated hemoglobin (trHb) and GlbO (*glbO*) orthologues are present in the *M. leprae* genome[34]. TrHbs; a family of widely distributed small oxygen-binding hemoproteins can be phylogenetically classified into three distinct groups (I–III). In *M. tuberculosis*, GlbN (group I trHb) has primarily been linked to NO detoxification, while GlbO (group II trHb) has been proposed to function in O_2 uptake/transport and/or redox sensing[35]. Having retained only one trHb, *M. leprae* GlbO has been proposed to represent merging of both O_2 uptake/transport and NO detoxification properties.

Non-thiol NO donors, such as L-arginine and L-citrulline have been shown to confer protection to mice against a challenge with lethal dose of *S. typhimurium*[36]. NO-related antimicrobial activity has been demonstrated against a remarkably broad range of pathogenic microorganisms including viruses, bacteria, fungi, and parasites[37]. Simultaneous production of reactive nitrogen and reactive oxygen intermediates may lead to the formation of a variety of antimicrobial molecules, each with distinct stability, subcelluler localisation and reactivity. These include NO, OONO, RSNO, nitrogen dioxide ($NO_2^{\bullet}$), dinitrogen trioxide (N_2O_3), dinitrosyl-iron complexes (DNIC), nitrous acid (HNO_2), etc.[38]. Interaction between reactive oxygen and reactive nitrogen intermediates provide a molecular basis for synergy between the respiratory burst and synthesis of NO. The combination of H_2O_2 and $NO^{\bullet}$ appears to possess particularly potent antimicrobial activity[39].

NO can easily cross membranes while superoxide cannot enter bacterial cells to any significant extent. NO-congener peroxynitrite can pass through membranes, probably as peroxynitrous acid (HOONO) and has greater reactivity for lipids and proteins as compared to NO. Gram negative bacteria such as *S. typhimurium* can actively take up S-nitroso-glutathione, which is a substrate for periplasmic enzyme γ-glutamyltranspeptidase and is converted to *S*-nitrosocysteinyl-glycine[40]. This is imported to bacterial cytoplasm across the inner membrane by a specific dipeptide permease (Dpp). The Dpp is an absolute requirement for *S*-nitrosoglutathione-mediated inhibition of *S. typhimurium* growth *in vitro*. NO and its congeners produce antimicrobial activity through multiple cellular targets that may include DNA, proteins, lipids and thiols. NO itself does not possess bactericidal activity against *S. typhimurium* or *E. coli,* however, *S*-nitrosothiol is bacteriostatic and peroxynitrite is bactericidal for these organisms. In contrast, *S*-nitrosothiol and NO are microbicidal for *S. aureus, L. major* and *G. lamblia,* under the conditions in which peroxynitrite does not exert an apparent antimicrobial effect[41].

DNA is an important target of reactive nitrogen species. NO can deaminate DNA *in vitro*[42]. NO_2 and peroxynitrite can also damage DNA oxidatively, resulting in abasic sites, strand breaks, and other DNA alterations[43]. However, no DNA damage has been shown in intact bacteria. Exposure of DNA repair-deficient *S. typhimurium* mutant TA1535 to NO donor compounds revealed mutagenicity consistent with a DNA deaminating mechanism[44]. *S. typhimurium* deficient in recombinational DNA repair has been found to be hyper susceptible to inhibition or killing by *S*-nitrosothiol and 3-morpholino-sydnonimine (a peroxynitrite generator) as well as attenuated for virulence in mice.

NO interaction with proteins can involve reactive thiols, heme groups, iron-sulfur cluster, phenolic or aromatic amino acid residues, tyrosyl radical, or amines. Peroxynitrite and NO can also non-specifically oxidize proteins at a variety of sites. NO-mediated cytotoxicity includes efflux of iron-nitrosyl complexes and inactivation of enzymes containing Fe-S clusters (e.g., aconitase, NADPH dehydrogenase, succinate dehydrogenase), suggesting that NO might directly release iron from metalloenzymes and promote iron depletion[45]. NO can interact with iron contained in heme proteins such as guanylate cyclase, which accounts for many of its roles in physiological signal transduction. Although guanylyl cyclase is activated by NO, NO-heme interaction can result in inactivation of other heme proteins, such as catalase and the cytochrome system. Ribonucleotide reductase, a non-heme metalloenzyme essential for DNA synthesis, has been implicated as major target for NO action in tumour cells[46].

Thiols are among the most important targets of NO, and *S*-nitrosylation (attachment or transfer of NO^+ to sulfhydryl groups) is favoured over *N*-nitrosylation under physiologic conditions. NO congeners including S-nitrosothiol, N_2O_3, and dinitrosyl-thiol-iron complexes are potent nitrosating species in this context. Nitration of tyrosine residues has received particular attention because this protein modification can be produced by peroxynitrite, although myeloperoxidase may also catalyze tyrosine nitration in the presence of NO and H_2O_2. Tyrosine nitration may disrupt the pathways involving tyrosine phosphorylation and modify protein functions or turnover of the proteins.

More recently the attenuated virulence of *S. typhimurium* lacking periplasmic SOD has been found to be restored by either NO synthase inhibition or absence of superoxide generating phagocyte oxidase (Phox)[47]. These observations suggest that peroxynitrite production from NO and superoxide (that is antagonized by the presence of bacterial SOD), might constitute an important component of the antimicrobial host defense in salmonellosis. NO can induce lipid peroxidation of membranes of mitochondria[48]. It may also mediate bacteriostasis through IFN-γ regulation especially by inhibiting and degrading indoleamine 2,3-dioxygenase[49].

Apoptosis and NO

NO plays a pivotal role in cell death and has the ability to both induce and protect against apoptosis as well as driving an apoptotic response into a necrotic one. NO can also combine with $O_2^{\cdot-}$ to form $ONOO^-$, which shares some of the properties of NO and can freely diffuse intra-and inter-cellularly and also act as powerful oxidant. It is well established that the generation or addition of ROS or reactive nitrogen intermediate (RNI) can cause cell death either by apoptosis or necrosis. Necrosis occurs usually in response to severe trauma/injury to the cell and is morphologically characterized by cytoplasmic and mitochondrial swelling, plasma membrane rupturing and release of the cellular contents into the extracellular space. Generation of an inflammatory response ensues, which can cause further injury or even death to neighbouring cells. Apoptosis, by contrast, is a tightly regulated pathway of cell death in which a cell effectively partakes in its own demise. The execution of the death programme is characterized by morphological and biochemical changes. These include mitochondrial depolarization and alterations in phospholipid asymmetry, chromatin condensation, nuclear fragmentation, membrane blebbing, cell shrinkage and the formation of membrane bound vesicles, the apoptotic bodies[50]. The mechanism of NO-induced apoptosis is presently under intensive investigation and several mechanisms have been elucidated. These include activation of Fas through up regulation of Fas-ligand expression[51], generation of peroxynitrite[52], inhibition of mitochondrial ATP synthesis[53] and inactivation of several antioxidant enzymes[54]. In addition, NO activation of guanylate cyclase and resultant increase in intracellular calcium through cGMP-gated channels has also been demonstrated as a mechanism of NO-induced apoptosis in retinal cells *in vivo*[55]. NO has also been reported to have protective effects against apoptosis in a variety of cell types including lymphocytes[56], hepatocytes[57], endothelial cells[58], neurons[59] and eosinophils[60]. Several mechanisms have been proposed to elucidate the ability of NO to confer protection against cell death. These can be divided into cGMP-dependent and cGMP-independent mechanisms.

Many of the morphological changes associated with apoptosis are orchestrated by activation of a cascade of proteases termed caspases[61]. The caspases are a family of cysteine proteases comprising of at least 14 members, all of which contain an active site thiol group necessary for their activity[62]. This thiol group renders them particularly susceptible to redox modification by *S*-nitrosylation or oxidation. Such

modifications result in the inhibition of their catalytic activities. Activation of caspase-3, one of the key executioners of apoptosis, results in cleavage of ICAD (inhibitor of caspase-activated DNase) and translocation of CAD (caspase-activated DNase) to the nucleus ultimately resulting in DNA fragmentation. Caspase-3 activation is often considered as the point of no return in apoptosis and, thus, inhibition of caspases might provide a mechanism to abort the apoptotic cascade. Caspases contain a highly conserved cysteine residue within their active site and therefore are a target for *S*-nitrosylation. Evidence in support of caspase inhibition via *S*-nitrosylation initially came from a study carried out on purified caspases[63] that demonstrated reversible inhibition of seven members of the caspase family. Dithiothreitol (DTT) removes the NO group bound to the thiol group on proteins and can reverse the inhibition of caspases by NO, thereby indicating direct *S*-nitrosylation of the caspase catalytic cysteine residue by NO[64]. NO inhibition of caspase activity has also been observed in a number of cells where NO donors were used. Caspase-3 was found to be nitrosylated intracellularly and denitrosylated on Fas/Fas-ligand cross-linking. Recently pro-caspase-3 was found to be *S*-nitrosylated on its catalytic-site cysteine *in vivo*. Finally, NO has been reported to protect against Fas-induced liver injury by inhibiting the caspase activity[65,66]. This caspase inhibition is DTT reversible, suggesting that cysteine *S*-nitrosylation is the underlying mechanism of caspase regulation by NO. Caspase-1 and caspase-8 have also been reported to be nitrosylated in cells. NO produced by SNAP or iNOS is sufficient to prevent TNF-induced apoptosis in hepatocytes. Both caspase-8 and caspase-3 were found to be reversibly inhibited and caspase-8 was found to be *S*-nitrosylated in this system. Caspase-1 is involved in both apoptosis and cytokine maturation. Caspase-1 inhibition by *S*-nitrosylation has shown to be reversed by DTT in macrophages[67]. It is evident that NO is a potent inhibitor of caspase activity both *in vitro* and *in vivo* and this inhibition is primarily due to *S*-nitrosylation of the cysteine present at the catalytic site that is conserved in all caspases. This discovery has significant implication for the regulation of apoptosis by NO. Under an oxidizing environment, the caspases get inactivated, and therefore the cell must maintain a reducing environment for the execution phase of apoptosis to occur.

ROS and RNI can be generated at many different organelles in response to various stimuli. Major sources of ROS production include the mitochondrion, endoplasmic reticulum, plasma membrane and cytosol, while RNIs are usually formed in the cytosol or at the mitochondrion. RNIs play a key role in both inducing and inhibiting TNF-mediated apoptosis. It is reported that NO generation is essential for TNF-mediated apoptosis. It was found that binding of TNF-α to TNFR1 induced the expression of iNOS. Inhibition of iNOS activity abolished the cytotoxicity of TNF-α in MCF7 and other TNF-susceptible cells[68]. In a separate study, HeLa cells transfected with constructs expressing eNOS were found to be resistant to TNF-mediated apoptosis. It was found that eNOS gets activated through ceramide formation. Generation of NO in this system was anti-apoptotic[69]. These conflicting results highlight the dual effects that RNI and ROS can have in different cells.

TNF-α is a pro-inflammatory cytokine that can bind to either TNF-α receptor 1 (TNFR1) or TNFR2. Binding to TNFR1 usually results in caspase activation and apoptosis. It is well established that both ROS and RNI are generated by TNF-α in many cells and tissue types. While ROS generation is not believed to be important in TNF-α-mediated apoptosis, generation of ROS by TNF-α is critical for the phagocytic immune response against invading pathogens[70]. TNF-α is involved in pathologies like septic shock, inflammatory bowel disease and rheumatoid arthritis. TNF-α and lipopolysaccharides can incite lethal shock, in which cardiovascular collapse is centrally orchestrated by the vasodilating action of NO free radical[71]. ROS/RNI generation plays an important role in functioning of immune system. Phagocytes, including macrophages and neutrophils, are capable of generating large quantities of RNI and ROS. These free radicals are important for phagocytic, antimicrobial and tumoricidal immune responses. It has been reported that NO generation is necessary for macrophage-induced apoptosis in some tumour cells[72]. Apoptosis in astrocytes mediated by IL-1β is associated with NO production. Inhibition of NOS can partially inhibit apoptosis in this system[73]. Pancreatic islet-β cells, however, require binding of both IL-1β and IFN-γ with their respective receptors to induce iNOS expression and NO generation. NOS also plays a role in TCR-mediated apoptotic death. In thymus, stimulation of TCR results in the depletion of

CD4, CD8 double-positive thymocytes. Expression of iNOS and subsequent NO generation is the primary pathway responsible for this process. NO either released by NO donors or endogenously produced by NOS induces apoptosis both *in vivo*[74] and *in vitro* in several cell types such as neuronal cells[75], macrophages[76] especially, group B streptococcus (GBS; *Streptococcus agalactiae*) induces apoptosis of macrophages[77], cardiac myocytes[78], endothelial cells[79], lymphocytes and thymocytes[80,81]. NO induces osteoblast apoptosis through *de novo* synthesis of Bax protein. The mechanism of NO-induced osteoblast apoptosis from the viewpoints of mitochondrial functions involves exposure of osteoblasts to sodium nitroprusside (SNP), an NO donor that significantly increases the amounts of lactate dehydrogenase in the culture medium and results in decreased cell viability in a concentration and time dependent manner[82].

Physiologically, high levels of NO are mutagenic and may contribute to carcinogenesis. The complexity of biological responses is a consequence of the multiple pathways through which NO causes damage to critical cellular macromolecules. Evidences are accumulating that NO modulates the biological functions of many other intracellular signaling proteins by *S*-nitrosylation. These include tissue transglutaminase (Ttg), the two cysteine-transcription factors NFκ B, and AP-1, both implicated in the regulation of apoptosis, several ion channels and the protease calpain[83]. In addition, NO has also been reported to impair p53 function possibly through aminoacid modifications such as *S*-nitrosylation[84].

Dual Nature of NO and its Therapeutic Role

As discussed, the concentration of NO beyond a threshold level has enormous potential to change cell redox status, which may result into inflammation and even cell death. NO at high concentrations readily combines with other oxidants to form reactive nitrogenous species, which can damage a variety of cellular targets such as DNA and proteins. This can ultimately lead to apoptosis, mutagenesis or carcinogenesis. Cells have mechanisms to resist nitro-oxidative stress that becomes either defective (as in certain diseases) or overwhelmed (as can occur in injury and inflammation)[85]. The participation of NO in inflammatory responses has been demonstrated in experimental models as well as in inflammatory diseases such as rheumatoid arthritis, where release of inflammatory mediators IL-β and PGE_2 occurs and results in cartilage damage[86]. During early phases of osteoarthritis, NO production induces chondrocyte apoptosis and can lead to cartilage degradation. L-NMMA inhibits apoptosis in explant cultures of synovium and cartilage. The inflammation of brain tissue in Alzheimer disease (AD) and other human dementia is mediated through the products of activated glial cells such as cytokines and NO[87]. The induction of iNOS by inflammatory reaction that exerts its effect through production of peroxinitrite, is thought to be an important contributor to neuronal damage in neurodegenerative disorders[88]. The nNOS, which is usually localized in interneurons in human cortex is aberrantly expressed in vulnerable pyramidal cells in AD. Moreover, eNOS expression is increased in astrogial cells and in nerve cells in AD. These would contribute to increased NO production, and possibly to neuronal dysfunction and/or cell death in AD.

Atherosclerosis is an inflammatory disease linked to abnormal oxidant-mediated signals in the vasculature[89]. In atherosclerosis, endothelial NO bioactivity is reduced and oxidative stress is increased resulting in endothelial dysfunction. Recent studies have suggested that changes in activity and regulation of endothelial NOS by tetrahydrobiopterin (BH4) is an important contributor to endothelial dysfunction[90]. The endothelium exerts a number of vasoprotective effects (vasodilation, suppression of smooth muscle cell growth, and inhibition of inflammatory responses) through NO. The effects of endothelium-derived vasoconstrictors are opposed by NO thereby inhibiting the oxidation of low-density lipoproteins (LDL). A defect in the production or activity of NO leads to endothelial dysfunction, signaled by impaired endothelium-dependent vasodilation. There are evidences to suggest that endothelial dysfunction is an early marker for atherosclerosis. Moreover, high levels of LDL may inhibit arterial function in terms of the release of NO from endothelium. This inhibition is enhanced by oxidation of these lipoproteins, which may occur during the development of atherosclerosis. Many of these effects are caused by lipid oxidation products. LDL may also enhance the activity of platelets, especially when they are mildly oxidized.

NO could also serve as protective species and the precursor for peroxynitrite formation with cytotoxic effects. Tissue protection by NO has been observed and cellular NO production as well as exogenous NO from NO donors protects epithelial cells from toxic effects of superoxide. Further, inhaled NO reduces rat

mortality during exposure to hyperoxia[91]. NO may up-regulate antioxidative thioredoxin system and antiapoptotic Bcl-2 protein through a cGMP-dependent mechanism. Antioxidative and antiapoptotic mechanisms of NO and *S*-nitrosothiols could mediate neuroprotection[92]. NO has a protective role against oxidative injury in acute inflammatory conditions through possible direct scavenging of oxygen radicals, modulation of glutathione levels or upregulation of heme oxygenase-1 (HO-1)[93].

The role of NO in tumour cytotoxicity is supported by a recent study indicating that induction of macrophage arginase promotes tumour cell growth by reduction of NO production[94]. Suppression of tumour formation and metastasis by IFN-γ has been linked to iNOS expression and NO production. Besides acting as an effecter of tumoricidal action of macrophages, NO could also produce tumour suppression by regulating the expression of genes related to tumour survival, invasion and angiogenesis[95]. Induction of iNOS is an essential component of TNFα-induced apoptosis in epithelial tumour cell lines. NO sensitizes human tumour cells to TNFα-induced cytotoxicity by interacting with superoxide thereby resulting in reduction of hydrogen peroxide generation. This mechanism interferes with NF-κB activation and results in the removal of antiapoptotic/resistance signals. In contrast, it has been reported that L-arginine inhibits apoptosis via NO-dependent mechanism, thus promoting the survival of growth-arrested lymphoblastoma cells[96]. Other observations define an important role of IFN-γ in controlling tumour development in a model of primary hepatocarcinoma associated with macrophage recruitment, iNOS induction and NO production. Further, *in vitro* experiments have shown that NO-donor compounds induce death of tumour hepatocytes[97]. NO exerts a protective role against apoptosis in transgenic hepatectomized mice. Local production of NO in liver cells, achieved by hydrodynamic-based transfection with an iNOS encoding plasmid, also resulted in delayed liver recovery after partial hepatectomy and conferred protection against Fas-mediated apoptosis[98].

In a physiological microenvironment in alveolar cells, NO (at picomolar concentrations) has a crucial regulatory role in airway physiology (bronchodilation, bronchoprotection and anti-inflammatory effects). As described earlier, pro-inflammatory cytokines activate transcription factors (nuclear factor-β, Janus activated kinase-signal transducer and activator of transcription and interferon regulatory factor-1) which leads to prolonged release of high amounts of NO (at nanomolar concentrations) through iNOS induction. This has both beneficial (host defense) and detrimental (inflammatory cell infiltration, vascular permeability and Th2 cell-driven inflammation) effects. In addition, iNOS-derived NO might react with radicals produced under conditions of oxidative stress, therefore, promoting the generation of reactive nitrogen species. In an allergic inflammatory microenvironment Th2 cytokines (IL-4 and IL-13) in conjunction with gene polymorphism might lead to over-expression of arginase I and II, leading to increased generation of proline and polyamines, which are able to provoke airway remodeling via mesenchymal cell proliferation and collagen production. Activation of the arginase pathway also results in amplification of inflammatory mechanisms through bio-limitation of arginine as a substrate for iNOS with subsequent release of both NO and superoxide anions (O_2^-). NO and O_2^- promote the formation of peroxynitrite. Finally, counter-regulatory mechanisms at pre- and post-transcriptional levels might regulate the functional NOS isoforms[99].

Conclusion

Production of NO in various organs such as brain, lungs and vasculature is constitutive and can be induced by microbial infection in different cells participating in inflammation. NO being a remarkably stable free radical, can involve in many patho-physiological reactions such as oxidative stress and apoptosis. Its role as pro-oxidant can be explained by its ability to react with the other secondary oxidants such as superoxide and transitional metals, which are co-factors of many enzymes. Most of these enzymes participate in maintaining the redox status of the cell. NOS catalyzed reaction that produces NO, utilizes NO-donor such as arginine and citrulline. Other cellular NO donors are *S*-nitroso-*N*-acetylpenicillamine, *S*-nitrosoalbumin and *S*-nitrosoglutathione. NO when combines with other free radical species brings about changes in redox status of cell, which triggers a cascade of events ultimately leading to cell death. Apoptosis may be initiated by death receptors such as Fas (CD 95 or Apo-1) and TNF-receptor-1.

NO produces deleterious effects inside phagocytic cells (macrophages, monocytes and polymor-

phonuclear cells and neutrophils) and can be instrumental in the death of invading pathogens (*Salmonella* and *Mycobacterium*) through highly toxic peroxinitrite, which is a strong oxidant. NO can play dual role, on one hand it is the suppressor of inflammation in various pathological conditions, and on the other hand it can change the redox status of the cell by generating free radicals. NO may have cytotoxic and cytostatic effects on tumour cells. This duality may provide a useful tool in therapeutics where controlled administration of NO-donors can help in maintaining the optimal NO levels. The diverse roles played by NO in normal and pathological physiology suggest that this molecule could have more significance and potential to understand the complexity of molecular mechanism of various diseases.

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