NITRIC OXIDE MODULATION OF GENE EXPRESSION

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1. INTRODUCTION

The free radical nitric oxide ($\cdot\text{NO}$) is the major form of the endothelial-derived relaxing factor that is enzymatically synthesized from arginine oxidation by an NADPH-dependent nitric oxide synthase. $\cdot\text{NO}$ causes smooth muscle relaxation by activating soluble guanylate cyclase, and inhibits platelet aggregation and adhesion to endothelium by increasing cGMP⁴¹. Because of the vasorelaxant properties of $\cdot\text{NO}$ and its rapid inactivation in the blood by its reaction with hemoglobin, $\cdot\text{NO}$ inhalation has been advocated as a means of selectively reducing pulmonary hypertension and improving ventilation/perfusion mismatching in a variety of clinical situations^{9,40}.

In spite of its therapeutic value, there is reason to believe that in pathological conditions where there is increased production of oxygen free radicals by lung cells, inhalation or overproduction of $\cdot\text{NO}$ may damage alveolar and airway cells. Since $\cdot\text{NO}$ is a free radical, it can undergo a radical-radical reaction with superoxide (O_2^-) at near diffusion-limited rates, to yield peroxynitrite (ONOO^-)⁴. Peroxynitrite is a potent oxidizing agent known to initiate lipid peroxidation in biological membranes, at rates that are a thousand fold higher than for hydrogen peroxide (H_2O_2), hydroxylation, and nitration of aromatic amino acid residues and sulfhydryl oxidation of proteins^{50,51}. Furthermore, ONOO^- may decompose homolytically to yield nitrogen dioxide ($\cdot\text{NO}_2$), and species with hydroxyl radical-like reactivity ($\cdot\text{OH}$) without the need for metal catalysis⁴. Following stimulation by cytokines and lipopolysaccharide, alveolar macrophages and epithelial cells produce large amounts of $\cdot\text{NO}$ and O_2^- for prolonged periods of time^{26,45,49} and can act as foci for the intense and localized production of ONOO^- and its toxic intermediates in close proximity to lung cells. Herein, we will: (1) summarize the cellular and molecular mechanisms responsible for the regulation of $\cdot\text{NO}$ synthesis; and (2) review evidence for the beneficial and detri-

mental functions of $\cdot\text{NO}$ during oxidant stress in the lung. We will point out existing controversies and whenever possible, attempt to reconcile existing differences.

2. $\cdot\text{NO}$: A FREE RADICAL SIGNAL TRANSDUCING AGENT

$\cdot\text{NO}$ is synthesized from the five electron oxidation of either of the two equivalent guanidine nitrogens of L-arginine. The reaction is catalyzed by one of three isozymes of nitric oxide synthase (NOS), using reduced NADPH as the source of electrons and cofactors, including tetrahydrobiopterin (H_4B) and flavin nucleotides (FMN; FAD). Molecular oxygen is a co-substrate and N^G -hydroxy-L-arginine is formed as a short-lived intermediate with L-citrulline as a byproduct. All isoforms of NOS bind calmodulin and contain heme. Electrons are supplied by NADPH, transferred along the flavins and calmodulin and presented to the catalytic heme. Details of the biochemical pathway and electron flow schema appear in several recent reviews.¹⁸

Our understanding of how the simple, small, diffusible, lipophilic, short-lived molecule, $\cdot\text{NO}$ performs an extraordinarily diverse array of physiologic and pathophysiologic functions remains quite rudimentary. Nonetheless, it appears that regulation of $\cdot\text{NO}$ biosynthesis subserves function, and thus significant advances have been made by studying unique characteristics of NOS isoforms. Collectively, NOS are homodimeric cytochrome P450-like heme proteins that have oxygenase and reductase domains in the NH_2 and COOH termini, respectively, and are 50% homologous by amino acid sequence. The active dimeric form is hypothesized to be dependent upon H_4B ⁶⁰. The domains are separated by a Ca^{2+} /calmodulin binding region. The reductase domain is homologous to NADPH-cytochrome P450 including binding sites for FMN, FAD and NADPH. The oxygenase domain contains similar binding sites amongst the isozymes for heme, L-arginine and H_4B . The constitutive forms have similar phosphorylation sites, while the endothelial cell isozyme has a unique NH_2 terminal myristoylation site.

Differences between the isozymes, however, truly underscore the function of $\cdot\text{NO}$ in various biological systems. The nomenclature evolved from descriptions of cellular sources of the enzyme (e.g. neuronal, macrophage or endothelial) or its expression (constitutive vs. inducible) and by many conventions is now relegated to the chronology in which the enzyme was purified and cloned. NOS I and III are constitutively expressed and their activity is regulated by intracellular calcium; prototypic sources are neuronal and endothelial cell, respectively. NOS II is induced by cytokines, its activity is largely independent of calcium and it is regulated at the transcriptional level. The prototypic source is macrophage. Although the human genes are present on discrete chromosomes, a considerable degree of homology exists between the three isoforms suggesting common ancestral origin with subsequent gene duplication and transposition. Comparison of their calcium dependence and gene regulation are manifested in functional differences. NOS-I and III derived $\cdot\text{NO}$ are produced in small quantities for brief periods of time and underscore intra- and intercellular signaling events such as neurotransmission or vascular homeostasis. In contrast, NOS-II produces large amounts of $\cdot\text{NO}$ for prolonged periods of time (assuming availability of substrate and cofactors) and contributes to more diffuse physiological roles associated with inflammation or infection.

A recent study has shown that normal human and rat airway epithelium in large, cartilaginous airways, unstimulated by cytokines, immunostained in a specific manner with an antibody to inducible NOS, suggesting a constitutive role for a NOS isozyme that shares epitopes with NOS-II³⁴. Thus a new methodology for identifying the NOS isoforms has been developed. The constitutive and inducible forms of NOS from a number of species, including smooth muscle and endothelial cells, neurons and macrophages, have been cloned and sequenced.

3. PHYSIOLOGICAL EFFECTS OF ·NO

3.1. Biological Targets

The biologic actions of ·NO are dictated by the reactions it undergoes with different target molecules in cells, membranes, and the extracellular milieu. For each target, depending on the amount and duration of induction, ·NO can exert beneficial or detrimental effects. Known targets for ·NO include:

3.1.1. Guanylate Cyclase. ·NO binds the heme group of soluble guanylate cyclase leading to an increase in cyclic guanosine-3'-5'-monophosphate (cGMP) levels. Many effects of cGMP are mediated via a group of enzymes, cGMP-associated protein kinases PKGs. PKGs act to reduce intracellular calcium causing smooth muscle relaxation. ·NO-mediated increased cGMP levels also prevent platelet aggregation and decreases adhesion of neutrophils³⁶. However, excessive ·NO-mediated cGMP production has been implicated in sepsis-induced refractory hypotension and shock⁶³.

3.1.2. Hemoglobin. The major route for the destruction of ·NO *in vivo* is the fast and irreversible reaction with oxy-hemoglobin (Hb-Fe²⁺+O₂) or oxy-myoglobin to produce nitrate and methemoglobin according to the following reaction:

Because of its vasorelaxant properties and rapid inactivation in the blood by its reaction with hemoglobin, ·NO inhalation has been advocated as a means of selectively reducing pulmonary hypertension and improving systemic oxygenation in a variety of clinical situations including bronchopulmonary dysplasia and the Acute Respiratory Distress Syndrome (ARDS)⁵⁴. However, patients with diminished methemoglobin reductase activity, such as neonates, are unable to efficiently convert methemoglobin to ferrous hemoglobin, and could therefore be at greater risk for developing methemoglobinemia and decreased oxygen transport⁴³.

3.1.3. Iron/Sulfur (4Fe/4S) Centers of Enzymes. Production of ·NO by activated macrophages defends the host against infectious agents including bacteria²⁸, parasites¹⁷, and viruses²⁹ and destroys tumor cells⁵⁸. The proposed mechanisms responsible for these effects involve the reaction of ·NO with the nonheme iron of iron-sulfur complexes, resulting in the inactivation of iron-sulfur containing enzymes including mitochondrial aconitase¹³, cytochrome c oxidase¹⁰, and the DNA synthesis rate limiting enzyme, ribonucleotide reductase³⁷. Inhibition of these critical enzymes leads to suppression of mitochondrial respiration, energy metabolism, and cell replication.

3.1.4. Other Free Radicals. NO has an unpaired electron, and thus can readily react with other free radicals, as specified below:

a. Reaction with Oxygen. If a tank of ·NO is opened and allowed to leak into the air, a cloud of lethal and highly reactive orange-brown nitrogen dioxide (·NO₂) is formed according to the following reaction:

The production of $\cdot\text{NO}_2$ is a third order reaction and the rate of conversion of $\cdot\text{NO}$ in the gas phase can be calculated by: $d[\cdot\text{NO}]/dt = 4 k_3 [\cdot\text{NO}]^2 [\text{O}_2]$ where k_3 is $2 \times 10^6 \text{ M}^{-2} \text{ s}^{-1}$.¹⁵ Thus, the rate of $\cdot\text{NO}_2$ formation, at a given concentration of oxygen, will be determined by the square of the $\cdot\text{NO}$ concentration. At low $\cdot\text{NO}$ concentrations ($<5 \mu\text{M}$), observed *in vivo* at physiologic and most pathologic conditions, the low probability of any two $\cdot\text{NO}$ molecules encountering each other makes the formation of $\cdot\text{NO}_2$ extremely slow and can, therefore, be discarded. Instead, $\cdot\text{NO}$ may react with a single molecule of oxygen in a second order reaction to form a nitrosyldioxyradical ($\text{ONOO}\cdot$).

b. Reaction with O_2^- . $\cdot\text{NO}$ reacts with O_2^- at a near diffusion-limited rate constant of about $7 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ to form ONOO^- . Initially, this reaction was mistakenly thought to be protective, since it decreased the amount of O_2^- detected. ONOO^- has a pK_a of 6.8 at 37°C and thus may remain stable for months in alkaline solutions. The protonated form of ONOO^- , peroxyxynitrous acid (ONOOH), decomposes to form $\cdot\text{NO}_2$ and an intermediate with a reactivity equivalent to the hydroxyl radical ($\cdot\text{OH}$), derived from the trans isomerization of ONOOH , as shown in Equation 3:⁴

The $\cdot\text{OH}$ -like reactivity of ONOOH is mediated by a vibrationally excited intermediate that does not physically separate into a free $\cdot\text{OH}$ and $\cdot\text{NO}_2$. This intermediate has the reactivity of $\cdot\text{OH}$ and in some cases the toxicity of ONOO^- has been mitigated by $\cdot\text{OH}$ scavengers^{21,50}.

Under physiological conditions, a minimum of 25% of ONOO^- decomposes to form " $\text{OH}\cdots\cdot\text{NO}_2$ ", with the remainder recombining to form nitrate⁴. While being highly reactive, its modest rate of decomposition under physiological conditions allows ONOO^- to diffuse for up to several cell diameters to critical cellular targets before becoming protonated and decomposing. ONOO^- initiates iron-independent lipid peroxidation and oxidizes thiols at rates at least a thousand fold greater than that of hydrogen peroxide (H_2O_2) at pH 7,⁵¹ damages the mitochondrial electron transport chain⁵², is a key mediator of macrophage-mediated cytotoxicity towards bacteria and *Trypanosoma cruzi*⁵⁵, and causes lipid peroxidation of human low density lipoproteins¹⁶. In addition, metal ions, such as Fe^{3+} EDTA and copper in the active site of superoxide dismutase (SOD), catalyze the heterolytic cleavage of ONOO^- to form a nitronium ion-like species (N^+Q) which nitrates phenolics including tyrosine in proteins⁵. A recent report indicates that carbonated species accelerate the oxidation and nitration reactions of ONOO^- , via the formation of the nitrosoperoxy carbonate anion ($\text{O}=\text{N}-\text{OOCO}_2^-$)⁶¹.

Under normal conditions, intracellular O_2^- concentrations are kept at remarkably low levels (10 pM) because eukaryotic cells contain large amounts of SOD (4–10 μM). Under these conditions, ONOO^- formation is prevented. However, pathological conditions can greatly increase the synthesis of both $\cdot\text{NO}$ and O_2^- . When the concentration of $\cdot\text{NO}$ increases to the micromolar range, it can effectively compete with SOD for O_2^- to form ONOO^- (rate constant of the reaction of O_2^- with $\cdot\text{NO}$ is three times faster than that with SOD). Indeed, activated rat alveolar macrophages²⁶, human neutrophils⁸, and agonist-stimulated bovine endothelial cells³⁵ have been shown to produce ONOO^- .

3.2. Effect of $\cdot\text{NO}$ on Gene Expression

In addition to the above considerations regarding regulation of gene expression in L-arginine-dependent $\cdot\text{NO}$ biosynthesis, $\cdot\text{NO}$ itself is now considered to be a modulator of

gene expression and also affects the activity of various transactivating factors and DNA binding proteins. $\cdot\text{NO}$ decreases cytokine-induced endothelial cell activation by inhibiting NF- κB expression and, subsequently, either vascular cell adhesion molecule (VCAM)¹² or NOS-II expression¹¹. $\cdot\text{NO}$ has been reported to affect not only NF- κB function (in part by altering I $\kappa\text{B}\alpha$ expression)⁴⁶, but other redox sensitive transcription factors, including AP-1 components⁴⁷. In addition, $\cdot\text{NO}$ has been reported to modulate inflammation by altering the production of soluble cytokines such as TNF- α ,¹⁴ IL-8,³ IL-1, IL-6,⁵³ IFN- γ ,⁶² and chemo-attractant factors (MCP-1) and by inhibiting adhesion molecule expression in various cells.

A potential coupling between $\cdot\text{NO}$ -iron interactions, signal transduction and gene expression has been shown by Weiss et al.⁶⁴ and Drapier et al.¹³. It is well known that iron is an important target and signaling molecule for $\cdot\text{NO}$. The iron-sulfur clusters of many proteins are affected by $\cdot\text{NO}$ and in particular, *cis* aconitase serves as a bifunctional protein depending upon its iron-sulfur status. In its 4Fe-4S conformation, *cis* aconitase functions as an enzyme facilitating the interconversion of isocitrate and citrate. While in its 3Fe-4S complex, it functions as the iron-regulatory protein (IRP). In this latter apo-IRP form (after $\cdot\text{NO}$ attack), it can bind to iron responsive elements (IRE) and lead to: (a) inhibition of translation of ferritin and aminolevulinate synthase mRNA by forming an IRE/IRP complex in their 5' untranslated regions (UTRs); and (b) stabilizing transcription of transferrin receptor mRNA against targeted endonucleolytic degradation by interacting with five IREs in the 3' UTR⁴⁴. In murine macrophages, endogenous $\cdot\text{NO}$ (after cytokine stimulation), exogenous $\cdot\text{NO}$ (via $\cdot\text{NO}$ donors), or $\cdot\text{NO}$ produced by fibroblasts transfected with an expression vector containing NOS-II activates IREs resulting in repression of ferritin mRNA and enhanced transferrin receptor mRNA. Accordingly, iron acts as both a sensory and regulatory molecule in $\cdot\text{NO}$ signaling and IRP is the switch in affecting gene expression. Although less is known regarding similar mechanisms in the lung, a recent report indicates that exposure of pulmonary artery endothelial cells in culture to S-nitrosoacetylpenicillamine (SNAP) resulted in induction of heme oxygenase, a transient increase in iron, and a repression in the binding activity of IRP as manifested by an enhanced ferritin and EPR-detectable dinitrosyl-iron-sulfur complex.³²

3.3. $\cdot\text{NO}$ Modulation of Adenovirus-Mediated Gene Transfer

A recent study by Knowles et al.³³ showed that adenovirus-mediated transfer of the cystic fibrosis gene did not result in correction of the functional defect in nasal epithelium and that local inflammatory responses limited the dose of adenovirus that could be administered to overcome inefficient transduction by the adenovirus vector. Endogenous $\cdot\text{NO}$ has been found in exhaled air of both animals and humans with the largest contribution from the upper airway and nasal epithelium in adult humans³⁷, especially during upper respiratory tract infections³¹ and lung inflammation². In addition to modification of the inflammatory process, $\cdot\text{NO}$ may also directly alter the efficiency of adenovirus-mediated gene transfer by several mechanisms. Firstly, $\cdot\text{NO}$ may alter binding and internalization of the adenovirus complex by S-nitrosylation of adenoviral proteins or adenoviral-specific cell receptors. S-nitrosylation of critical thiol groups has been reported to modify protein and receptor functions³⁹. Secondly, $\cdot\text{NO}$ may modify gene transfer by regulating the expression of the transgene. We recently reported that $\cdot\text{NO}$ produced by murine fibroblasts, stably transfected with the human NOS-II, decreased the efficiency of reporter gene expression following infection with recombinant replication-deficient adenovirus (E1-deleted) expressing either the luciferase or Lac Z reporter genes (AdCMV-Luc and AdCMV-Lac Z) to approximately 60% of their corresponding control values (Figures 1

Figure 1. Luciferase expression 24 hours following infection of cultured NIH3T3 fibroblasts, stably transfected with the gene for human NOSII with a recombinant replication-deficient adenovirus expressing the luciferase reporter gene (AdCMV-Luc; 10 pfu/cell). The addition of sepiapterin (SEP; 50 μ M) increased nitrite/nitrate in the medium (solid line) and decreased reporter protein expression. L-NMMA is NG-monomethyl-L-arginine at 1 mM. Luciferase activity is expressed in relative light units (RLU) per mg cell proteins (hatched bars). Nitrite/nitrate levels were expressed in μ M/mg protein. Values are means + SEM for $n = 3$ different experiments performed in triplicate. * = $p < 0.05$ compared to control cells. Figure reproduced from reference 20 with permission.

and 2).²⁰ Pretreatment of cells with N^G-monomethyl-L-arginine (L-NMMA; 1 mM), an inhibitor of NOS, prevented the sepiapterin-mediated induction of $\cdot$ NO and restored gene transfer to baseline values. Furthermore, parenteral steroid administration to mice enhanced the efficiency of adenoviral gene transfer upon re-administration of the virus, partly by inhibition of NOS-II induction.⁵⁹

3.4. Cytotoxic Potential of $\cdot$ NO/ONOO

The neutrality of $\cdot$ NO and its low Stoke's radius, permits facile transmembrane diffusion and its relatively long biological half-life allows it to react with targets distant from its point of origin. Thus, smooth muscle cell and endothelial cell derived $\cdot$ NO may

Figure 2. β -galactosidase expression 24 hours following infection of cultured NIH3T3 fibroblasts, stably transfected with the gene for human NOSII, with a recombinant replication-deficient adenovirus expressing the Lac Z reporter gene (AdCMV-LacZ; 1 pfu/cell). The addition of sepiapterin SEP; 50 : M) increased nitrite/nitrate in the medium (solid line) and decreased reporter protein expression identified by the 4 characteristic histochemical response to the substrate X-gal. Number of β -galactosidase positive cells is shown as percent infected cells (hatched bars). L-NMMA is NG-monomethyl-L-arginine at 1 mM. Nitrite/nitrate levels were expressed in μ M/mg protein. Values are means + SEM for $n = 3$ different experiments in triplicate. * = $p < 0.05$ compared to control cells. Figure reproduced from reference 20 with permission.

combine with epithelial cell-derived O_2^- to form $ONOO^-$ in the lung epithelial lining fluid. The following key observations establish the potential involvement of $\cdot NO$, $ONOO^-$ and various reactive nitrogen species in the generation and propagation of pulmonary epithelial injury in a variety of pathological situations including: 1) induction of immune complex alveolitis in rat lungs⁴²; 2) paraquat induced injury⁷; 3) ischemia-reperfusion injury; 4) *Bordetella pertussis* infection of airway cells *in vitro*²⁵; 5) pathogenesis of influenza virus-induced pneumonia¹; and 6) injury to pulmonary surfactant and surfactant apoproteins.^{19,22-24,38}

4. INHIBITION OF OXIDANT-MEDIATED INJURY

$\cdot NO$ reaction with O_2^- may, in some cases, also serve to protect O_2^- -sensitive target molecules. This reaction of $\cdot NO$, which kinetically can out compete SOD, forces O_2^- through $ONOO^-$ oxidation and decomposition pathways. At the same time this limits the accumulation of H_2O_2 and decreases the formation of secondary reactive species derived from H_2O_2 reaction with transition metals. For example, it has recently been reported that $\cdot NO$ donors inhibit reactive oxygen species-mediated cellular damage and cytotoxicity.⁶⁵ $\cdot NO$ can also react with other free radicals, such as lipid alkoxyl ($LO\cdot$) and peroxy ($LOO\cdot$) radicals, decreasing their steady state concentration and terminating free radical lipoprotein oxidation propagation chain reactions. Rubbo et al.⁵⁶ showed that when the molar concentration of $\cdot NO$ exceeds that of O_2^- , $\cdot NO$ protects against O_2^- - and $ONOO^-$ -dependent lipid peroxidation. These reactions are especially important to consider inasmuch as $\cdot NO$ significantly concentrates in lipophilic cell compartments, having a lipid-water partition coefficient of ~ 6.5 . $\cdot NO$ may also ameliorate tissue injury by activating guanylate cyclase with subsequent induction of cGMP-dependent effects, such as reducing platelet and neutrophil adhesion to endothelium³⁶ and reacting with iron to form iron-nitrosyl compounds. By binding free coordination sites of iron, $\cdot NO$ can limit Fenton chemistry and iron-dependent electron transfer reactions²⁷.

In buffer-perfused isolated rabbit lungs, inhaled $\cdot NO$ (24 ppm) ameliorated the increase in pulmonary vascular permeability produced by the intravascular generation of H_2O_2 ⁴⁸. Kavanagh et al.³⁰ investigated the effect of inhaled $\cdot NO$ on the increase in capillary filtration coefficient ($K_{f,c}$) arising from the perfusion of isolated rabbit lungs with purine and xanthine oxidase. Inhaled $\cdot NO$ (90–120 ppm) attenuated, and inhibition of endogenous $\cdot NO$ exacerbated, the oxidant-mediated increase in $K_{f,c}$. In patients with acute lung injury, inhaled $\cdot NO$ has been shown to decrease permeability pulmonary edema⁶ and to suppress pro-inflammatory cytokine production in the lung. We investigated the effects of aminoguanidine (100 mg/kg per dose injected intraperitoneally every 12 h), a nucleophilic compound and presumed selective inhibitor of NOS-II, on indices of hyperoxic lung injury assessed at 48 h. Aminoguanidine significantly increased the total protein concentrations in the BAL. In further experiments, all rats that received aminoguanidine during hyperoxia (n=3) died between 48 and 60 h. In contrast, only one rat that received saline during hyperoxia (n=7) died during that same time period. This data suggests that, in this model of hyperoxic lung injury, upregulation of $\cdot NO$ production may serve a protective role. However, this conclusion is complicated by the fact that inhibitors of NOS-II activity also affect NOS-III, to varying degrees. Furthermore, aminoguanidine, is known to interfere with glucose metabolism and thus may have diverse biologic functions in addition to blocking NOS-II⁶⁶.

5. SUMMARY AND CONCLUSIONS

Studies aimed at identifying regulation of the NOS genes will help us understand the wide range of functional manifestations of NOS-derived $\cdot\text{NO}$ from modulating perfusion to mediating cytotoxicity. The reaction of $\cdot\text{NO}$ with O_2^- , on the other hand, is critical to our understanding of the role of $\cdot\text{NO}$ in oxidant stress and inflammation.

$\cdot\text{NO}$ plays a dual role in the modification of oxidant-mediated injury. $\cdot\text{NO}$ acts as a chain reaction-breaking antioxidant to inhibit O_2^- -mediated lipid peroxidation by scavenging alkoxyl and peroxyl radicals. However, under similar experimental conditions, $\cdot\text{NO}$ may, at the same time, promote oxidant injury to proteins by rapidly reacting with O_2^- to form a potent oxidizing and nitrating agent, ONOO^- . These results clarify, in part, the often confusing literature describing $\cdot\text{NO}$ reactivity during oxidant stress and provide evidence that the target molecule is an important determinant of the final outcome. The efficacy of inhaled $\cdot\text{NO}$ should not be judged only by the physiological parameters noted at the bedside, but also by the variety of other non-cGMP-dependent biochemical reactions including modulation of gene expression.

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