NO, hypoxia, and superoxide

# Nitric oxide, hypoxia, and superoxide: the good, the bad, and the ugly!

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## A possible role for NO in ARDS

Nitric oxide (NO) is endogenously synthesised by nitric oxide synthases (NOS) which convert L-arginine to L-citrulline and NO. Three NOS isoforms (types I, II and III) have been identified and all of them are expressed in the human lung.[1–8] NOS I (nNOS) and III (eNOS) are constitutively expressed in tissues and are dependent on increases in intracellular calcium for enzyme activation while NOS II (iNOS) is an inducible form that is calcium independent (table 1).[9] All NOS isoforms require oxygen, NADPH, FAD, FMN, tetrahydrobiopterin, and calmodulin for activity.[1 9] NO is recognised to have a key role in virtually all aspects of lung biology and has been implicated in the pathophysiology of lung diseases.[1 4 6 10–15] It is involved in pulmonary neurotransmission, host defence and bacteriostasis, airway and vascular smooth muscle relaxation, pulmonary capillary leak, inflammation, mucociliary clearance, airway mucus secretion, and cytotoxicity.[4 6 14]

Cellular sources of NO in the lung include epithelial cells, endothelial cells of pulmonary arteries and veins, inhibitory non-adrenergic non-cholinergic neurones, smooth muscle cells, mast cells, mesothelial cells, fibroblasts, neutrophils, lymphocytes, and macrophages.[4 6 14] Specifically, NOS I is located in inhibitory non-adrenergic non-cholinergic neurones in the lung while NOS III is found in endothelial cells and the brush border of ciliated epithelial cells.[1 5 7] NOS II is found in the epithelial cells of the airway. Although NOS II may be induced in several types of cells in response to cytokines, endotoxin, or reactive oxygen species, it is continuously expressed in normal human airway epithelium at basal airway conditions.[8 16]

Once produced, NO is freely diffusible and enters target cells activating soluble guanylate cyclase to produce guanosine 3′,5′-cyclic monophosphate (cGMP) which mediates most of the physiological effects of NO on smooth muscle including vasodilation and bronchodilation.[2 11] NO reaction products may also mediate other physiological and pathological functions in the lungs and many other organ systems. Due to the high reactivity, NO participates in a wide variety of reactions at different sites within the cell, lung tissue, extracellular fluids, and intravascular compartments. Primary reactions that may involve NO intracellularly and extracellularly include its reaction with oxygen, superoxide, haemoglobin, another molecule of NO, enzymes containing iron-sulfur centres, heme-containing proteins, and thiol proteins.[12] Notably, NO undergoes a direct bimolecular reaction with superoxide ($O_2^-$) yielding peroxynitrite ($ONOO^-$) at a rate that is even faster than the dismutation of $O_2^-$ by superoxide dismutases (SOD), which puts NO at the epicentre of oxidative metabolism and inflammation.

## REGULATION OF NO SYNTHESIS BY OXYGEN

All NOS isoforms require the presence of oxygen for activity.[9] Although it is recognised that oxygen is a substrate for NOS, its effects on the regulation of NOS activity are more complex than a simple enzyme-substrate interaction.[4 17] Interestingly, the effect of hypoxia on NO levels in the airway is primarily a result of airway and alveolar oxygen tension rather than vascular oxygen tension.[18 19] One proposed mechanism(s) for oxygen regulation of NOS activity is outlined in fig 1. NOS activity during the steady state includes an active cycle (A) that generates NO and an inactive cycle (B) that involves formation and decay of a heme-NO complex. In the active cycle, oxygen binding to ferrous heme ($Fe^{2+}$) is limiting for enzyme activity. In contrast, resolution of the inactive cycle and entry into the active cycle is oxygen-dependent due to effects on the stability of the heme-NO complex. This includes a reaction between the heme-NO complex and oxygen which results in loss of the heme-NO complex (fig 1).[17]

The oxygen concentration in intact tissues ranges from 1 to 150 μM,[4 17 20] with the highest levels found in the lung. Airway epithelial cells are unique in their exposure to oxygen since, above a thin layer of epithelial lining fluid, the airway cells are exposed directly to air containing 21% oxygen. Based on oxygen solubility and the low differential oxygen gradient between overlying fluid to intracellular endoplasmic reticulum (1–2 μM),[4] the levels of oxygen in airway epithelial cells may actually approach 260 μM. Thus, the Michaelis constant ($K_MO_2$) determined for NOS II (135 μM), but not NOS III (4 μM) or NOS I (400 μM), is well within the physiological range of oxygen concentrations in lung epithelial cells. Importantly, $K_MO_2$ for NO synthesis in the intact human lung (190 μM) is similar to NOS II $K_MO_2$ in vitro.[3 4 21]

## REGULATION OF NOS GENE EXPRESSION BY OXYGEN

The immediate effects of short term changes in oxygen concentration on the

**Abbreviations:** ARDS, acute respiratory distress syndrome; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; $K_MO_2$, Michaelis constant; NADPH, reduced nicotinamide-adenine dinucleotide phosphate; NO, nitric oxide; NOS, nitric oxide synthase; $O_2^-$, superoxide; $ONOO^-$, peroxynitrite; ROS, reactive oxygen species; SOD, superoxide dismutases

**Table 1** Nitric oxide synthase enzymes

| NOS isoforms | Numerical designation | Other designation | Expression | Regulation | NO output | Chromosome |
|---|---|---|---|---|---|---|
| Type I | 1 | nNOS | Constitutive | Calcium/CaM | Low (picomol) | 12 |
| Type II | 2 | iNOS | Inducible | Induced by cytokines, endotoxin, and oxidants | High (nanomol) | 17 |
| Type III | 3 | eNOS | Constitutive | Calcium/CaM | Low (picomol) | 7 |

NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neural nitric oxide synthase; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase; CaM, calmodulin.

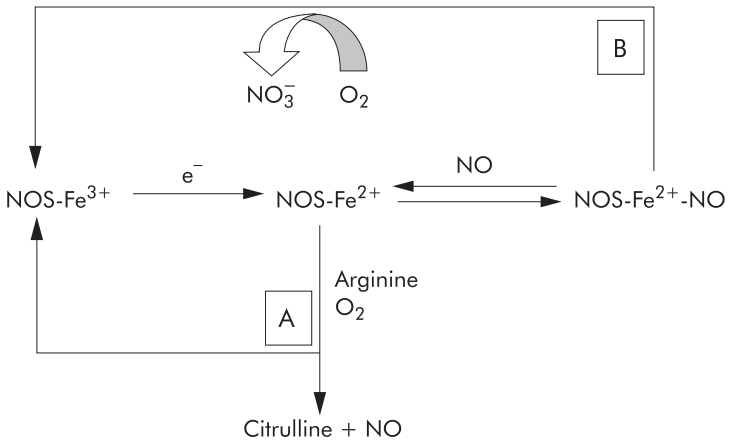


**Figure 1** Simplified proposed mechanism of oxygen regulation of NOS enzyme kinetics. NOS activity during the steady state includes an active cycle (A) that generates NO and an inactive cycle (B) that involves formation and decay of a heme-NO complex. In the active cycle, oxygen binding to ferrous heme ($Fe^{2+}$) is limiting for enzyme activity. In contrast, resolution of the inactive cycle and entry into the active cycle is oxygen-dependent due to effects on the heme-NO complex stability. This includes a reaction between the heme-NO complex and oxygen, which results in loss of the heme-NO complex.

activity of NOS enzymes are probably due to the effects of oxygen on NOS enzyme kinetics. However, prolonged hypoxia can have significant effects on the gene expression of the different NOS isoforms.[2 3 21–23] These transcriptional effects may vary among species or among organ systems in the same species.[2 3 21] For example, while hypoxia produces a progressive decline in constitutive NOS mRNA levels in bovine pulmonary artery endothelial cells,[22–24] chronic hypoxia upregulates constitutive NOS expression in rabbit heart[25] and rat lung pulmonary arteries.[26] Chronic hypoxia also increases NOS expression and NOS activity in rat carotid bodies.[27]

In this issue of *Thorax*, Muzaffar *et al*[28] describe the effect of hypoxia on the expression of endothelial nitric oxide synthase (NOS III) and $gp91^{phox}$ (the active catalytic subunit of NADPH oxidase), and the formation of superoxide in pig pulmonary artery segments, pulmonary artery smooth muscle cells, and pulmonary artery endothelial cells. They incubated pulmonary artery segments (with and without intact endothelium) and cells (endothelial and smooth muscle cells) in the absence of ambient oxygen for 2 hours and measured the formation of superoxide by ferricytochrome c reduction. They also measured the expression of proteins by Western blotting and immunocytochemistry. The absence of oxygen in the ambient air promoted the formation of superoxide in the studied tissues and cells. Various enzyme inhibitors were used to determine the source of superoxide production. They also pre-incubated the cells with several inflammatory mediators to determine if they could enhance the effects of hypoxia. A summary of the findings is that hypoxia upregulates NADPH oxidase and NOS III resulting in increased production of superoxide, NO, and peroxynitrite in their system.

A major component missing in the model studied by Muzaffar and colleagues is the role of NOS II. In humans NOS II is continuously expressed in the airway epithelium, is a major source of NO in the lung, and appears to be the most responsive to hypoxia in the physiological range.[2 3 21] Due to the free diffusion of NO and the close apposition of airways to pulmonary vessels, endogenous NO production in the airways can have significant effects on the pulmonary vessels. The authors comment on both eNOS (NOS III) and iNOS (NOS II) throughout the paper, but their system does not seem to be appropriate for the study of NOS II which is mainly expressed in the airway epithelium (which the authors did not study) and not in the endothelium or smooth muscle (reported here). The cells they studied do not express NOS II in detectable levels at baseline and that does not change with hypoxia. So, the additional use of NOS II inhibitors does not add much. While the authors emphasise the relevance of their findings to acute respiratory distress syndrome (ARDS), the link is rather speculative. They studied healthy piglets and evaluated their pulmonary artery rings or cells in isolation from the rest of the lung. Although they used pre-incubation with some inflammatory markers as a suggestion as to what happens in ARDS, it would have been more appropriate to study rings from piglets with and without ARDS. The weak link to ARDS, however, does not diminish the relevance of the findings.

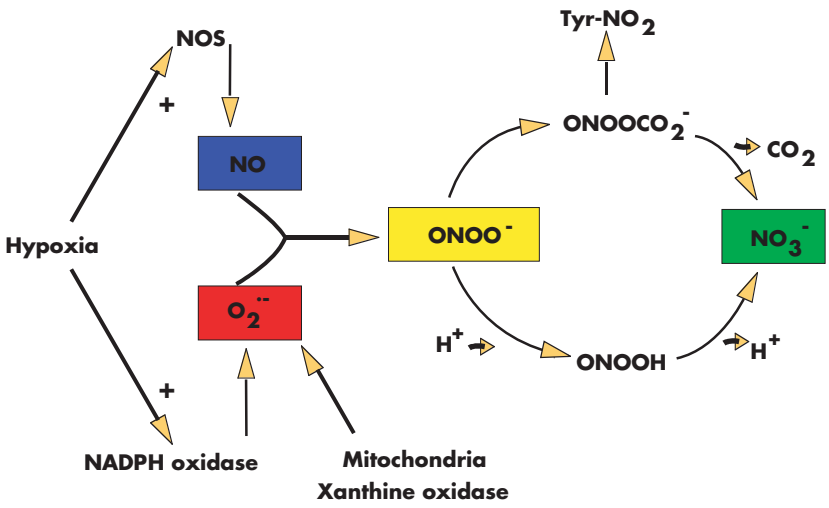


**Figure 2** Simplified proposed model of NO reaction with superoxide ($O^-$). NO undergoes a direct bimolecular reaction with $O_2^-$ yielding peroxynitrite ($ONOO^-$) at almost diffusion limited rates ($k = 6.7 - 19 \times 10^9$/M/s ). The rate constant is over 3.5 times faster than the dismutation of $O_2^-$ by superoxide dismutases. By rapidly consuming superoxide, NO produces $ONOO^-$/ONOOH, a far less reactive oxidant than superoxide that can be further metabolised to innocuous products like $NO_3^-$. Tyr-$NO_2$, nitrotyrosine.

## NO-SUPEROXIDE INTERACTION

Free radicals/reactive oxygen species (ROS) may be toxic in two ways. They can interact with metal or organic redox centres and promote irreversible oxidation reactions inactivating the target metabolic process, or they can initiate reactions which then become self-sustaining through the generation of propagating radicals. In either case, this can

result in deleterious effects on the cell. The most effective protection against oxidant mediated tissue damage is to scavenge the initiating radical.[15 29] Although NO is itself a radical, many of the same chemical and physical properties of NO that allow it to exert oxidant effects can also result in antioxidant actions. The role of NO as an oxidant or an antioxidant probably depends on the local tissue milieu. In an environment where the oxidant load is low, the highly reactive properties of NO give the molecule oxidant properties. However, in situations where the oxidant load is high (as in asthma and ARDS), NO plays an antioxidant role by scavenging superoxide and other ROS. NO undergoes a direct bimolecular reaction with $O_2^-$ yielding $ONOO^-$ at almost diffusion limited rates (rate constant ($k$) = $6.7 - 19 \times 10^9$/M/s).[12] The rate constant is over 3.5 times faster than the dismutation of $O_2^{\cdot -}$ by SOD. By rapidly consuming superoxide, NO produces $ONOO^-$/ONOOH, a far less reactive oxidant than superoxide that can be further metabolised to innocuous products like $NO_3^-$ (fig 2).[12 30]

Muzaffar and colleagues speculate that the upregulation of two enzymes with opposite effects may represent a protective mechanism to counteract the effect of hypoxia or a pathological mechanism leading to the progression of ARDS. There are no data in their study to favour one explanation over the other. However, based on models in other diseases and on our knowledge of the biology of NO and reactions in the lung, one would suspect that, if hypoxia upregulated NADPH oxidase without simultaneously upregulating NOS expression, the increased release of superoxide ($O_2^-$) would go unchecked resulting in serious tissue injury. By reacting with superoxide to form peroxynitrite ($ONOO^-$), NO produced by NOS serves as a scavenger of superoxide resulting in a net antioxidant effect (fig 2). The simultaneous upregulation of NOS is therefore probably a protective feature. Although the link to ARDS remains speculative, these findings have potential implications for a wide variety of lung diseases from asthma to pulmonary hypertension[12 13 15 31 32] in which oxidative stress and/or hypoxia have a role.

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