

## THE ROLE OF NITRIC OXIDE IN HYPEROXIA-INDUCED INJURY TO THE DEVELOPING LUNG

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### 1. ABSTRACT

Exogenous or inhaled NO (iNO) has been successfully used, as a selective pulmonary vasodilator, in a wide variety of clinical situations especially in the management of persistent pulmonary hypertension in the newborn. A better understanding of the role of endogenous and exogenous NO in the lungs of surfactant-deficient animals exposed to hyperoxia could result in novel strategies for the better management of RDS in premature babies with the ultimate aim to decrease chronic lung disease in these infants. This review will focus on the effects of NO, when used in combination with hyperoxia, on lung injury; information on the effects on cell culture systems and animal models will be used to highlight the unique responses of the developing lung. Most of the data from cell culture systems and adult animal models of hyperoxia-induced lung injury suggests that endogenous NO has a protective role. In the newborn animal, endogenous NO appeared to be harmful, had no effect or was protective in hyperoxia-induced lung injury. The data are conflicting on the issue of whether exogenous NO is protective or damaging in the presence of hyperoxia on lung cells and animal models. Despite the variability in the studies, it would appear that low dose exogenous NO for short duration is beneficial in hyperoxic lung injury in adult and newborn animals. In the human newborn, use of iNO in infants < 34 weeks of gestation should be considered experimental, pending results of ongoing trials.

### 2. INTRODUCTION

Nitric oxide (NO), an important inter- and intracellular messenger in the body, has been identified in a wide variety of physiologic and pathophysiologic processes (1). Exogenous or inhaled NO (iNO) has been successfully used, as a selective pulmonary vasodilator, in a wide variety of clinical situations especially in the management of persistent pulmonary hypertension in the newborn (2- 4)

and in congenital heart disease (5,6). In an animal model of surfactant deficiency, Kinsella *et al* (7) found that iNO improved oxygenation and improved pulmonary blood flow. Use of a 15-minute administration of iNO in premature infants with surfactant deficiency after exogenous surfactant replacement showed that iNO caused significant improvement in oxygenation with no obvious side effects (8). Two recent studies, however, did not show any significant benefit after using iNO in the management of respiratory distress syndrome (RDS) (9,10). Use of iNO has been associated with EEG abnormalities (11). There is also concern regarding the outcome of premature neonates treated with iNO (12).

Except for its use in the management of pulmonary hypertension, the use of NO remains investigational as it has not been FDA approved for any of the other disease processes. It is imperative that more data be gathered regarding the use of iNO in surfactant-deficient neonates before it's widespread use (13 -15). In addition to the vascular smooth muscle, other pulmonary structures are exposed to iNO, resulting in suppression of NO synthesis in a variety of pulmonary cells and this is potentially toxic (16). There is a need for understanding the deleterious and salutary properties of NO (17). This is especially important in the context of long-term use of NO in the presence of hyperoxia, which is akin to the clinical situation of premature infants with RDS being treated with high concentrations of oxygen. A better understanding of the role of endogenous and exogenous NO in the lungs of surfactant-deficient animals exposed to hyperoxia could result in novel strategies for the better management of RDS in premature babies with the ultimate aim to decrease chronic lung disease in these infants.

This review will focus on the effects of NO, when used in combination with hyperoxia, on lung injury;

information on the effects on cell culture systems and animal models will be used to highlight the unique responses of the developing lung. The effects of NO as a pulmonary vasodilator is beyond the scope of this review.

### 3. NITRIC OXIDE AND HYPEROXIA IN CELL CULTURE

#### 3.1. Effect of cytokines on iNOS induction and nitric oxide production

Lung epithelial cells (murine lung epithelial cell line LA-4) have been shown to release NO in response to cytokine stimulation (18). An increased number of cells stained for inducible nitric oxide synthase (iNOS) and an increase in iNOS mRNA was also observed (18). Cytokine stimulation of A549 cells also produced a similar response (19). Expression of the iNOS gene has been shown to be under cytokine control and is transcriptionally regulated (20). Synergistic effect of the cytokines has been proposed to explain NO production from A549 cells (21). Release of NO and iNOS expression can be attenuated by dexamethasone (18,19,22) as well as interleukin-4 (IL-4) and IL-13 but not IL-10 (22). Endogenous NO has also been shown to be protective against cytokine induced damage to LA-4 cells (23).

It has been reported that a combination of cytokines induces production of a high concentration of the NO oxidation products (nitrite and nitrate) by cultured fetal rat TIIP cells (24). On the other hand, it has been shown that exogenous NO was not appreciably cytotoxic to fetal TIIP (17). Possible mechanisms for this protective effect includes its action as an iron chelator, activation of guanylate cyclase with subsequent induction of cGMP-dependent effects, potent inhibition of oxidant-induced membrane and lipoprotein oxidation by annihilation of lipid radical species or diverting superoxide-mediated toxic reactions to other oxidative and less damaging pathways (17).

The explanation for these different results is probably that NO is a free radical with both antioxidant and pro-oxidant properties - a molecular chameleon (16).

#### 3.2 Effect of hyperoxia on iNOS induction and nitric oxide production

Hyperoxia may cause lung inflammation, superoxide generation and increased alveolar to capillary permeability (22,23,25). Exposure of adult rat TIIP to hyperoxia increased iNOS mRNA production >5 fold (26). Hyperoxia was shown to increase NO production and iNOS mRNA and protein expression in adult rat alveolar macrophages after stimulation with lipopolysaccharide (LPS) and/or interferon-gamma (IFN- $\gamma$ ) (27). In a study on human airway epithelial cells and alveolar macrophages obtained from healthy volunteers exposed to 100% oxygen for 12 hours, iNOS mRNA was increased 2.5 fold in the airway epithelial cells (28).

#### 3.3. Interaction between nitric oxide and oxygen (and reactive oxygen species)

NO is a free radical that together with the superoxide radical can form peroxynitrite which may be responsible for damaging proteins, lipids and DNA (16).

By increasing the formation of peroxynitrite, superoxide potentiates the toxicity of NO (29,30). By releasing nonheme iron from ferritin (31), NO has the potential to enhance the formation of hydroxyl radicals, known to damage the surfactant system (16). Besides hemoglobin, the effects of NO on other heme proteins are likely to modify the oxidant stress. Peroxynitrite additionally decreases the oxygen uptake and sodium transport in TIIP (32). So, while NO may be a critical intermediary in the production of oxidant tissue damage (33), NO-dependent protective effects have been observed in processes having increased rates of superoxide generation and for which oxidant injury has been proposed to play an etiological role (34-36).

It is likely that the production and metabolism of both reactive oxygen and nitrogen species in the intracellular compartments of each cell type that populates the lung could influence the progression of lung diseases influenced by inflammatory-immune activation (37). The dose and duration of exposure to endo- or exogenous NO, in the presence of hyperoxia, may determine its beneficial or detrimental role. The developmental age of the lung would also have a modifying effect.

#### 3.4. Effect of nitric oxide and hyperoxia on surfactant

It has been shown that exposure of isolated type II pneumocytes (TIIP) from adult rats to NO for 2 hours (using NO donor compounds) results in inhibition of alveolar TIIP ATP and surfactant synthesis (38). On addition of exogenous NO (as S-nitroso-N-acetylpencillamine or SNAP 1 mM), to isolated alveolar TIIP from adult rats, there was a 60-70% reduction of the synthesis of disaturated phosphatidylcholine (DSPC) and cell ATP levels (39). On the other hand, exposure of TIIP to lung surfactant or DPPC vesicles lead to increased production of NO and increased the rate of ATP synthesis and cell ATP levels (40). Most studies on TIIP using NO-donor compounds (mainly using S-Nitroso-N-acetylpencillamine or SNAP and 3-Morpholinosydnonimine or SIN-1) have been of short duration of 1-4 hours (17,32,38,39,40).

We found that exposure of adult rat TIIP to exogenous NO for 24 hours resulted in no untoward effect on surfactant synthesis (41). We have recently reported on the effects of hyperoxia and NO exposure on rat fetal TIIP (42). Exposure to 95% O<sub>2</sub> and increasing doses of NO (akin to doses used in clinical practice of approximately 5-20 ppm) resulted in no change in surfactant synthesis; however, there was decreased surfactant proteins A, B and C as well as iNOS mRNA expression (42).

#### 3.5. Effect of nitric oxide and hyperoxia on inflammation/mediators

A recent study has reported on the response of fetal TIIP to hyperoxia and NO in terms of cytokine release (43). The release of IL-1 $\beta$ , IL-6, and IL-10 was not significantly different in room air versus hyperoxia alone or in combination with NO at 24 hours (43). However, it was noted that IL-10 release was significantly increased in the

presence of dexamethasone and hyperoxia compared to hyperoxia alone (43).

### 3.6. Effect of nitric oxide and hyperoxia on cell viability

NO has been shown to enhance cell death by apoptosis in pulmonary neutrophils (44,45) and vascular smooth muscle cells (46). In contrast, depletion of endogenous NO has been shown to induce apoptosis in rat lung epithelial and mesothelial cells (47). We found that exposure of adult rat TIIP to exogenous NO for 24 hours resulted in increased cell death and a trend towards decreased catalase activity (41).

One study by Narula *et al* (48) has looked at the synergistic cytotoxicity of NO and hyperoxia over 6 days. Their results suggest that combined NO and hyperoxia exposure result in cell death after day 2 and rapidly thereafter and this cell death did not occur by apoptosis (48). However, they used a cultured cell line (A549 cells) and used a much higher dose (2 mM) of SNAP than other investigators (50–100  $\mu$ M). They conceded the fact that the levels of NO exposure in their experiments are high relative to those experienced by cells in the intact lung (48). The precise relationship between cell culture experiments and the experimental use of NO is not yet clear, and the full range of NO doses and oxygen levels is yet unexplored (48).

Exposure of HeLa-80 cells to NO (using the chemical donor, 0.5–7.5 mM DETA NONOate) and hyperoxia (80% O<sub>2</sub>) for up to 6 days suggested that NO and hyperoxia-induced apoptotic cell death was by suppression of NF-kappaB activity (49).

There was a tendency towards increased apoptotic cell death of rat fetal TIIP upon exposure to hyperoxia (95% O<sub>2</sub>) and NO (using the chemical donor, Glyco-SNAP 2) (42).

## 4. NITRIC OXIDE AND HYPEROXIA IN ANIMAL MODELS

The cellular distribution of all 3 isoforms of NOS was similar in fetal, newborn and adult lamb lungs (50). Thus, all may be important sources of endogenous NO (50). Use of L-NAME (N<sup>G</sup>-nitro-L-arginine methyl ester; a non-specific blocker of NOS) in rats exposed to hyperoxia resulted in their earlier death (51). In adult mice exposed to hyperoxia, there was a decrease in constitutive endothelial cell NOS (ecNOS); L-NAME treatment worsened the lung injury, as measured by lung compliance and survival (52). This suggests that endogenous NO has a protective effect in hyperoxia-induced lung injury (52). Alveolar macrophages and TIIP release both NO and superoxide which react to form peroxynitrite which can damage the alveolar epithelium and inactivate surfactant; peroxynitrite nitrates tyrosine to form nitrotyrosine. Lungs of adult rats exposed to hyperoxia (for 60 hours) exhibited a 2-fold increase in nitrotyrosine staining compared to controls (53). This nitrotyrosine formation could be replicated *in vitro* by incubation of the rat lungs with peroxynitrite, but not NO or reactive oxygen species (53).

In isolated perfused adult rabbit lungs ventilated with 100% O<sub>2</sub>, L-arginine (which is the substrate for formation NO via NOS) caused significant edema (54). These effects could be attenuated with L-NAME treatment (54). The authors speculated that the mechanism of lung injury by L-arginine in the presence of hyperoxia probably involves peroxynitrite formation (54).

Hyperoxia induced iNOS expression in adult rat lungs; however, there was no increase in NO concentration in the exhaled air, suggesting that NO is not synthesized in rats exposed to hyperoxia (55). However, another study found no increase in iNOS in adult rats exposed to hyperoxia; both isoforms of arginase (L-arginine can be metabolized by NOS to produce NO or by arginase to produce urea and L-ornithine) were, however, upregulated and had increased activity (56). Hyperoxia induced lung injury in both adult wild-type and iNOS deficient mice (57). It was greater in the iNOS-deficient mice and associated with increased polymorphonuclear leukocytes in the bronchoalveolar lavage fluid (BAL) (55). It appeared that iNOS induction served as a protective mechanism to minimize the effects of acute exposure to hyperoxia (55). On the other hand, a recent study reported no difference in the amount of surfactant or surfactant components obtained via BAL when comparing iNOS deficient with wild type mice, after exposure to hyperoxia for 48 hours (58).

Adult rats were exposed to hyperoxia for 40 hours and had evidence of lung injury; inhalation of 10 and 100 ppm of NO abrogated the effects to some extent (59). Inhalation of NO, however, did not improve the survival of the rats exposed to hyperoxia (59). Treatment with L-NAME or aminoguanidine (a specific blocker of iNOS) reduced survival (59). The authors concluded that endogenous NO was protective in the presence of hyperoxia; furthermore, depending on its concentration, iNO can either reduce or increase hyperoxic lung injury (59).

NO has been implicated in the pathogenesis of hyperoxia-induced lung damage in adult rabbits (60) as well as improved the survival of adult rats in hyperoxia when used in the dose of 100 ppm (61). In a dose of 20 ppm, iNO was found to protect the lung endothelium and alveolar epithelium in adult rats exposed to hyperoxia (62). Adult rats exposed to hyperoxia developed severe lung injury as evident by pronounced vascular leak and alveolar cell apoptosis; the addition of NO (20 ppm) significantly attenuated the lung injury (63). In adult Wistar rats, 6 hours of exposure to hyperoxia and NO (40 ppm) significantly decreased free-radical mediated effects in the lung (64).

In the presence of carbon dioxide, the major product of the reaction of peroxynitrite with proteins leads to the formation of 3-nitrotyrosine; another important reaction is the S-nitrosylation of cysteine residues (65). Both of above products have been implicated in the regulation of cellular function and injury (65). In a murine model, 3-nitrotyrosine production was enhanced in airway epithelium and alveolar interstitium when exposed to

hyperoxia; administration of iNO lead to increased production of S-nitrosocysteine with no apparent change in 3-nitrotyrosine formation (65). The authors speculated that this might account for the protective effect of NO in the presence of hyperoxia (65).

In a recent study on 2-week old rat pups, it was reported that exposure to hyperoxia appears to impair the ability of endogenous NO to modulate constriction of central (but not distal) airways (66). Hyperoxic exposure to rat pups results in upregulation of NOS in the lungs (67, 68). In newborn (3 days old) rats, exposure to hyperoxia for 7 and 14 days resulted in significant lung injury; L-NAME treatment of the hyperoxic animals reduced lung edema and epithelial proliferation (67). It was, therefore, concluded that increased generation of (endogenous) NO contributes to the pathogenesis of hyperoxia-induced lung damage in newborn rats (67). Interestingly, in rat pups (21 days old) exposed to hyperoxia, NO blockade [using aminoguanidine or N<sup>ω</sup>-nitro-L-arginine i.e.L-NNA] did not abrogate the pathologic consequences of hyperoxic exposure (68).

In premature rats exposed to hyperoxia or room air, with or without L-NAME treatment for 3 and 7 days, L-NAME treatment worsened hyperoxic lung injury and also had a deleterious effect in the room air exposed rats (69). This suggests that endogenous NO has a protective effect not only during exposure to hyperoxia but also under physiological conditions. On the other hand, hyperoxia upregulated iNOS and eNOS and this may have contributed to the lung damage (69).

In a premature lamb model, exposure to NO (20 ppm) alone for 5 hours did not reveal any impairment of gas exchange or pulmonary mechanics; however, there was some increase in lung vascular protein leak (70). Compared to hyperoxia exposure alone, the combination of NO/hyperoxia did not exacerbate nor attenuate lung injury in newborn guinea pigs (71). A low dosage of iNO (14 ppm) decreased or prevented hyperoxia induced detrimental effects on alveolar surfactant and alleviated the oxidant stress in preterm rabbits (72). In contrast, in newborn (8-15 days old) piglets, the combination of NO (50 ppm) and hyperoxia increased alveolar permeability (73), apoptosis (74) and collagen content in the lung (75). In another study of newborn piglets (1-2 days old) exposed to hyperoxia, the iNO (at 20 ppm) group had higher extravascular albumin space and dry weight of the lung; however, there were no alterations in the extravascular lung water content or respiratory mechanical variables (76). The results do not show any benefit of iNO in the presence of hyperoxia. Another cautionary note was reported in newborn piglets exposed to iNO (40 or 80 ppm) and acetaminophen, phytomenadione or EMLA cream (all commonly used in human newborn infants); use of either of the drugs in combination with iNO lead to a significant increase in methemoglobinemia (77).

The role of monocyte chemoattractant protein-1 (MCP-1) and its receptor, C-C chemokine receptor 2 (CCR-2) has been described in young piglet lungs exposed

to hyperoxia and NO (78). Hyperoxia alone had a depressant effect on CCR-2 abundance. This may have potential implications for lung repair after injury, because MCP-1 acting through CCR-2 participates in angiogenesis and in wound repair (78). They also showed that combined exposure to hyperoxia and NO did not exacerbate the depressant effect of hyperoxia on CCR-2 but may actually be protective (78). This suggests a potential beneficial role for NO in minimizing the injurious effect of hyperoxia on angiogenesis and repair.

It is possible that the protective effect of NO could be due to transcriptional inhibition of proinflammatory mediators with the net result being enhanced survival in a model of acute lung injury as observed from *in vivo* studies (17). Peroxynitrite has been shown to have direct stimulatory effects on Mn superoxide dismutase (SOD) transcript expression (79). On the other hand, iNO may inhibit catalase activity or decrease the activity of iNOS (80). Recombinant SOD has been shown to decrease lung injury caused by iNO and hyperoxia in newborn piglets (81). NO has been shown to decrease IL-1 $\beta$  and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) production by lung macrophages (82). NO inhalation has been shown to transiently elevate pulmonary levels of cGMP, iNOS RNA and TNF- $\alpha$  (83). Long term exposure of adult rodents to a low dose of NO decreases lung interstitial cells, connective tissue, and alveolar septae (84). Pretreatment with NO potentiated acute lung injury in an isolated rabbit lung model (85). Animals exposed to iNO for 24 hours showed evidence of surfactant dysfunction (86). In contrast, exposure of isolated surfactant complex to NO during surface cycling strikingly decreases the inactivation of surfactant (86). Hence, NO may either activate or inhibit the pulmonary surfactant system. Under conditions favoring generation of peroxynitrite, surfactant is degraded (84). Thiols and nitrosothiols in the epithelial lining fluid are likely to control NO homeostasis. By becoming nitrosylated, the thiols neutralize the toxic effects of peroxynitrite (84). The interaction of NO with reduced thiol groups of proteins is critical in maintaining the balance between protein tyrosine nitration and protein cysteine nitrosylation and possibly preventing cellular and tissue injury (65). This may explain some of the beneficial effects of iNO and the lack of toxicity when administered in the presence of hyperoxia (65).

## 5. NITRIC OXIDE AND HYPEROXIA IN THE HUMAN PREMATURE NEWBORN

Plasma 3-nitrotyrosine levels were found to be elevated in premature infants who developed bronchopulmonary dysplasia (BPD), suggesting that peroxynitrite-mediated oxidant stress may contribute to the development of BPD (87). In contrast, 20 ppm iNO had a favorable response in 11 out of 16 infants with BPD (88). In a recent study, use of iNO (20 ppm) in premature infants with developing chronic lung disease, improved oxygenation without any changes in intraventricular hemorrhage, oxidative injury or markers of inflammation (89).

For preterm infants, iNO is not routinely recommended (90), although clinical trials are underway. An earlier report on the use of iNO in premature infants had a disconcerting high incidence of intracranial hemorrhage (91). In 2 recent studies (9,10), low-dose (5 and 10 ppm respectively) iNO was used. Both these randomized multicenter studies showed an improvement in oxygenation in the iNO treated infants, but with no decrease in mortality (9,10). In both studies, there was tendency to decrease chronic lung disease (9,10). In a meta-analysis (n=210), the OR (95% CI) for mortality was 0.97 (0.54-1.75) and for death or chronic lung disease was 0.77 (0.41-1.45) when comparing NO-treated with non-treated infants (92). There was also no significant difference in the rates of intracranial hemorrhage between the 2 groups (92).

## 6. SUMMARY AND CONCLUSIONS

Hyperoxia appears to upregulate iNOS in the lungs. Research is needed to clarify whether this induction of iNOS is dependent on or independent of cytokine release. Most of the data from cell culture systems and adult animal models of hyperoxia-induced lung injury suggests that endogenous NO has a protective role. In the newborn animal, endogenous NO appeared to be harmful (67), had no effect on hyperoxia-induced lung injury (68) or was protective (69).

The data is conflicting on the issue of whether exogenous NO is protective or damaging in the presence of hyperoxia on lung cells and animal models. The effects of exogenous NO (dose, duration, developmental stage of the lung) on endogenous NO release has important implications and needs study. The variability in the reports probably reflect the fact that the dose, duration of exogenous NO (as well as hyperoxia) and the developmental age of the lung, all have important influences. It would appear that low dose exogenous NO for short duration appeared to be beneficial in hyperoxic lung injury in adult and newborn animals.

As for the human newborn, use of iNO in infants < 34 weeks of gestation should be considered experimental, pending results of ongoing trials (90, 93).

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