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**INHALED NITRIC OXIDE PROTECTS AGAINST HYPEROXIA-INDUCED  
APOPTOSIS IN THE RAT LUNG**

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This thesis is submitted in partial fulfillment of the requirements for the degree of  
Master of Science (M.Sc.) in the Physiology graduate program

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## **Abstract**

Inhaled nitric oxide (NO) is frequently administered in combination with hyperoxic gas mixtures to effect pulmonary vasodilation in patients with pulmonary hypertension. NO was recently shown to have a protective effect against the injurious consequences of prolonged hyperoxia. The present study investigated the possibility that this protective effect may be attributable to the ability of NO to block apoptosis. Rats exposed to 100 % O<sub>2</sub> for 60 h developed severe lung injury consisting of pronounced vascular leak and alveolar apoptosis, as inferred from the presence of DNA fragmentation (positive ISEL staining and DNA ladders in agarose gels), and a decrease in constitutive pro-caspase-3 levels. However, the inclusion of NO (20 ppm) in the hyperoxic gas mixture significantly attenuated both the vascular leak and apoptosis. NO reversed the hyperoxia-associated changes in the activity of the redox-sensitive transcription factors NF- $\kappa$ B, AP-1 and Sp1 after 24 h. This protective effect was accompanied by a decrease in the level of the pro-inflammatory protein ICAM-1 and an increase in the cellular antioxidant glutathione. This study shows for the first time that NO can protect against both hyperoxia-induced apoptosis and vascular leak. The data suggest that the protection may occur at the transcriptional and caspase activation level.

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## **List of Abbreviations**

<b>ANOVA</b>	<b>analysis of variance</b>
<b>ARDS</b>	<b>acute respiratory distress syndrome</b>
<b>AP-1</b>	<b>activator protein 1</b>
<b>ATP</b>	<b>adenosine triphosphate</b>
<b>cGMP</b>	<b>cyclic guanosine monophosphate</b>
<b>DNA</b>	<b>deoxyribonucleic acid</b>
<b>DTNB</b>	<b>dithio-nitrobenzoic acid</b>
<b>EDRF</b>	<b>endothelium derived relaxing factor</b>
<b>EMSA</b>	<b>electrophoretic mobility shift assay</b>
<b>FITC</b>	<b>fluorescein isothiocyanate</b>
<b>DTT</b>	<b>dithiothreitol</b>
<b>GSH</b>	<b>glutathione</b>
<b>GSSG</b>	<b>glutathione disulfide</b>
<b>HPS</b>	<b>hematoxylin-phloxine-saffron</b>
<b>I<math>\kappa</math>B</b>	<b>inhibitory kappa B</b>
<b>ICAM-1</b>	<b>intercellular adhesion molecule</b>
<b>ISEL</b>	<b>in situ end label</b>
<b>IL-1<math>\beta</math></b>	<b>interleukin-1beta</b>
<b>mRNA</b>	<b>messenger ribonucleic acid</b>
<b>NADPH</b>	<b>nicotinamide-adenine dinucleotide phosphate</b>

NF- $\kappa$ B	nuclear factor kappa B
NO	nitric oxide
NOS	nitric oxide synthase
PBS	phosphate buffered saline
PMSF	phenyl methyl sulfonyl fluoride
PVDF	polyvinylidene difluoride
ROI	reactive oxygen intermediates
Sp1	specificity protein 1
TBST	tris buffered saline with tween
TCA	trichloroacetic acid
TE	tris EDTA
TNF- $\alpha$	tumor necrosis factor alpha
TSH	total sulfhydryl

# **INHALED NITRIC OXIDE PROTECTS AGAINST HYPEROXIA-INDUCED APOPTOSIS IN THE RAT LUNG**

## **Introduction**

### **1. Hyperoxic Lung Injury**

#### **1.1 Hyperoxia**

In critical care medicine, hyperoxic gas mixtures are routinely administered to mechanically ventilated, critically ill patients with pulmonary hypertension and acute respiratory distress syndrome (ARDS) in order to increase the oxygen tension in arterial blood and tissues. The loss of vital capacity, lung compliance, and diffusing capacity associated with lung injury significantly impairs the exchange of gas between the alveolar air and the capillary blood. These patients require supplemental O<sub>2</sub> to meet the metabolic demands of their tissues and vital organs. However, prolonged exposure to hyperoxia has well described cytotoxic effects. Medical advances have drastically improved the treatment and mortality rate associated with ARDS making the use of hyperoxic therapy much more prevalent in the clinical setting. Consequently, pulmonary oxygen toxicity has increasingly become an important clinical problem.

## 1.2 Oxidative Injury

The deleterious effects of oxygen are thought to be mediated by the reactive intermediates formed as it is reduced within the cell. Molecular oxygen ( $O_2$ ) readily accepts electrons from other molecules giving rise to highly toxic reactive oxygen intermediates (ROI) such as superoxide anions ( $O_2^-$ ), hydroxyl radicals ( $OH\cdot$ ) and hydrogen peroxide ( $H_2O_2$ ) (1-4). These oxygen metabolites are routinely generated during normal cellular respiration which reduces oxygen to water through the sequential addition of four electrons (5,6). Each step in this reaction gives rise to a new potential reactive oxygen intermediate. However, under physiological conditions the intracellular antioxidant defenses are sufficient to maintain a reduced environment.

A full complement of enzymatic and nonenzymatic antioxidants are present to protect the cell against oxidative damage. The ubiquitous tripeptide glutathione is one of the most important nonenzymatic intracellular oxidant scavenging agents. Its reduced (GSH) and oxidized (GSSG) states provide the major redox buffer within the cell (7,8). Among the many intracellular antioxidant enzymes, which include the superoxide dismutase system (including the metalloproteases and catalases), are the two related cytosolic enzymes, glutathione peroxidase and glutathione reductase. Glutathione peroxidase is active in the reduction of the oxygen intermediate  $H_2O_2$  to water and in the breakdown of the harmful products of lipid peroxidation (9-11). Reduced glutathione which is used as a cofactor in these

reactions is first reduced to GSSG, and is then recycled by the enzyme glutathione reductase to replenish the free radical scavenging pool (8,10). When these antioxidant defenses are challenged with supra-physiological concentrations of O<sub>2</sub>, excess ROI are generated by the incomplete reduction of oxygen and its metabolites (2,12,13). This oxidative stress can cause substantial injury and cell death through the oxidation of essential lipids, proteins and nucleic acids (14,15). The lungs, which receive the highest O<sub>2</sub> exposure, face the greatest threat of oxidative damage. Suttner et al. recently provided evidence of protein oxidation and cell membrane disruption in their study of pulmonary cells exposed to 95 % O<sub>2</sub> for 48 hours (16).

The pathophysiological manifestations of O<sub>2</sub> toxicity include the loss of microvascular integrity resulting in widespread edema and alveolar congestion (1,17-19,60), hemorrhage and hyaline membrane formation (1,20,21), pulmonary inflammation (22-24), and cell death (14,16,18,21). The perivascular effusion and interstitial edema are both pronounced and widespread. The migration of leukocytes out of the blood vessels and into the tissue during inflammation often gives rise to interstitial edema. However, recent studies have reported evidence of pulmonary edema in the setting of hyperoxia well before (17) and even in the absence (25,26) of leukocyte infiltration. These data suggest that the loss of vascular integrity may be caused, at least in part, by endothelial cell toxicity and death. The accumulation of fluid in the interstitial and alveolar spaces has serious

implications for lung function. The thickening of the alveolar-capillary wall caused by this edema impairs the diffusion of gas molecules into and out of the circulating blood (1). Fluid congestion in the alveolar space disrupts the phospholipid surfactant layer lining the alveoli, decreasing its compliance, and potentiating collapse (27). Matthews and co-workers measured a marked decrease in lung compliance and a significant loss of surfactant in mice exposed to 95 % O<sub>2</sub> for 72 hours (27).

The development of pulmonary inflammation is another important feature of hyperoxic lung injury. The increased expression of inflammatory mediators and the associated cellular infiltration induced by hyperoxia have been well described in the literature. The secretion of pro-inflammatory cytokines and cell surface adhesion molecules such as TNF- $\alpha$  (29,30), IL-1 $\beta$  (29,30), ICAM-1 (28), and CD40 (20), has been observed in rodent models of hyperoxic lung injury. These agents promote the recruitment and infiltration of inflammatory cells into the lung parenchyma and alveolar spaces (18,22,23,28,29). In addition to causing the loss of endothelial and epithelial wall integrity, the accumulation of neutrophils significantly increases the oxidative load presented to the pulmonary cells (8,31). The release of toxic ROI, proteases, and arachidonic acid metabolites by the invading neutrophils can cause extensive local cellular damage (9,32) and can exacerbate the lung injury.

In addition to the circulating leukocytes, the pulmonary mast cells are also

important effectors of the inflammatory response. The preferential location of these cells in epithelial membranes places them right at the air/lung interface where they can be quickly activated by oxidative stress. Activated mast cells release a variety of pro-inflammatory agents such as cytokines, histamine, leukotrienes and prostaglandins (33). Furthermore, mast cell proteases have recently been shown to mediate inflammation. These proteases actually constitute the majority of the proteins exocytosed from activated mast cells, but the biological substrates of each of these enzymes have not all been identified (33). The proteases can stimulate neighboring cells to produce large amount of cytokines (34,35) and can directly contribute to the breakdown of the blood/endothelial barrier (36). The role of the mast cell in hyperoxia-induced pulmonary inflammation has not yet been elucidated.

Cell death plays an integral role in the development of hyperoxic lung injury. The significant breakdown of the alveolar capillary barrier is indicative of microvascular endothelial and alveolar epithelial cell death. Crapo and co-workers examined rat lungs using morphometric and biochemical techniques and clearly demonstrated endothelial cell death followed by epithelial cell death during prolonged exposure to hyperoxia (60). Most of the recent literature on hyperoxic lung injury now provides evidence of cell death as an important event in the pathology of this injury (14,16,18,21,29,37,38). The mechanism of cell death involved in hyperoxic lung injury is of great significance. The necrotic lysis of pulmonary cells and the concomitant release of their contents can exacerbate the

oxidative stress on neighboring cells by promoting an inflammatory response (39). Apoptotic cell death, although a more favorable alternative to necrosis can nevertheless lead to a serious loss of critical cell mass. Both apoptotic and necrotic cell death have been reported in in vivo models of hyperoxic lung injury, although apoptotic death appears to be the predominant type of cell death described. Despite recent advances in the laboratory, little is available clinically to protect the lungs of patients from the devastating effects of O<sub>2</sub> toxicity.

## **2.0 Nitric oxide**

### **2.1 Nitric Oxide: A Biological Mediator**

It wasn't until the late 1980s that the role of nitric oxide (NO) as an important biological mediator was realized. It had long been proposed that the nitrovasodilators which mimicked the effects of the endothelium-derived relaxing factor (EDRF) were acting through the release of NO (40,41) and in 1987 Palmer et al. published seminal findings which indicated that NO and EDRF were, in fact, the same (42). With the recent advances in the understanding of endothelial cell mediated vascular relaxation it is now known that NO is not the only EDRF, but appears to be the predominant relaxing agent (43). Nitric oxide is synthesized in mammalian cells from the amino acid L-arginine by the enzyme nitric oxide synthase (NOS) (44). Three different isoforms of the NOS enzyme have been identified, each with distinct cell specificities, activities and regulatory factors (45). Endothelial NOS (eNOS) and neuronal NOS (nNOS), named for their localization within those specific cell types, are constitutively expressed enzymes which play a continuous role in cellular homeostasis. They are largely activated by elevations in the intracellular calcium ion content to increase the production of NO. The third NOS isoform, inducible NOS (iNOS), was originally identified in macrophages but has since been localized in a wide variety of cell types. This isoform is not sensitive to fluctuations in intracellular calcium but rather is induced by inflammatory molecules such as endotoxins and cytokines. These inflammatory mediators

upregulate the transcription and translation of iNOS and provide sustained NO synthesis (46). The levels of NO produced by this isoform are up to three orders of magnitude higher than those produced by the constitutive NOS enzymes (45).

Nitric oxide serves a multitude of diverse physiological functions. As a highly reactive free radical, NO reacts quickly with available metal and thiol groups within proteins, altering their stability and enzymatic activity. Many of its key regulatory roles are derived from its smooth muscle relaxing capacity (47). Nitric oxide stimulates the production of cyclic GMP in smooth muscle cells which promotes the sequestration of calcium in the intracellular stores and inhibits the contraction of muscle fibers (5,41,48). Nitric oxide is continuously released by vascular endothelial cells to maintain basal vasodilation through the relaxation of the surrounding smooth muscle (49). Reducing vascular resistance is central to maintaining adequate blood flow to meet the metabolic demands of each tissue and organ, but also plays an integral role in the function of many physiological systems. NO is pertinent to the maintenance of renal blood flow and juxtaglomerular filtration in the kidney (50,51), the coordinated relaxation of the stomach and intestinal wall during digestion (52), and in the maintenance of pulmonary blood flow to provide adequate ventilation perfusion matching in the lung (49). In addition to smooth muscle relaxation, NO acts as an inflammatory mediator, a neurotransmitter, and as an intracellular signal (5). New insights into the vast biochemical roles of NO has caused scientists to reconsider the theory that cell signaling occurs through specific

“lock and key” type specific receptor ligand interactions (53). Nitric oxide signals through the direct chemical reaction with cellular substrates by changing the structure and stability of proteins and important signaling molecules. The extraordinary quantity of data currently emerging in this relatively new field suggest that scientists have only begun to understand the complex nature of this molecule.

## **2.2 Inhaled Nitric Oxide**

The clear understanding that NO is the biochemical mediator of vascular relaxation raised to numerous new therapeutic possibilities. Frostell et al. demonstrated the use of inhaled nitric oxide to successfully reverse pulmonary vasoconstriction in lambs (54). Since then, inhaled nitric oxide has been investigated clinically and scientifically and was used effectively to improve pulmonary perfusion in patients suffering from pulmonary hypertension and ARDS (55-58). The successful use of inhaled NO to reverse pulmonary hypertension in the injured lung is clinically very important, since the use of circulating pharmacological vasodilators has been less than ideal for the treatment of pulmonary hypertension. Although these drugs are effective at lowering pulmonary vascular resistance, they also lower the systemic resistance, which is not always desirable (55,59). Moreover, circulating vasodilators enhance the blood flow to all regions of the lung, including any atelectatic and under-ventilated areas, which worsens the mismatch of ventilation and perfusion in the diseased lung. Conversely, inhaled nitric oxide increases the pulmonary blood flow to the areas of

the lung that are well ventilated, thereby promoting the oxygenation of blood and reducing the shunting of deoxygenated blood back to the heart (54,55). Furthermore, the short half life of molecular NO limits its biochemical effects to the pulmonary vasculature and, therefore, inhaled NO will not lower the systemic vascular resistance. The improvement of lung hemodynamics through the use of inhaled NO has exciting implications for hyperoxic therapy. The associated improvements in gas exchange could allow doctors to curtail their use of high fractional inspired O<sub>2</sub> in the treatment of respiratory distress. However, the combined effects of inhaled NO and hyperoxia on oxidative lung injury is currently a subject of intense debate. Numerous clinical trials have been reported on the used of inhaled NO in conjunction with high concentrations of O<sub>2</sub>, in the treatment of ARDS, and some controversy has ensued.

### **2.3 Nitric Oxide Toxicity**

Despite its exciting therapeutic potential, NO is a highly reactive free radical with a significant toxic capacity. Nitric oxide can react with superoxide to form several other redox-active derivatives (59,61,62). Some of these species, such as the nitroxyl anion (NO<sup>-</sup>) have clinically beneficial properties similar to those of EDRF, while others such as peroxynitrite (ONOO<sup>-</sup>) are powerful oxidants with enormous toxic potential and do not promote vasodilation (61,63-66). The rate constants for the oxidation of protein and non-protein thiols by peroxynitrite are three orders of magnitude higher than those for hydrogen peroxide (61). The

interactions with superoxide are of particular significance since inhaled NO is routinely administered to patients inhaling hyperoxic gas mixtures. Consequently, much attention is being focused on the possibility that NO may exacerbate hyperoxic lung injury (24,67). It appears, that the cytotoxic or cytoprotective effects of NO depend upon several factors, including the redox status of the cellular environment, the timing and method of NO administration, and, most importantly, the concentration used. In view of the data above, the barrage of clinical data is difficult to interpret. There is a lack of consistency among the studies with respect to administered doses, the inclusion criteria for subjects, and, more importantly, the comparison to proper controls. Therefore, the heterogeneity of patients and outcomes need to be interpreted with great caution.

In general, the scientific literature presents extensive and convincing data that low dose inhaled NO significantly protects against oxidant induced lung injury, although there are some reports which do not concur. There are a number of studies which dispute the cytoprotective role of NO and present evidence of pulmonary surfactant dysfunction (24,63,68), inflammation (24,68), cytotoxicity and cell death (61,67,69), however, such findings are generally restricted to the use of high concentrations of NO gas ( $\geq 80$  ppm) and to the in vitro use of chemical NO donors which generate a single burst of very high NO levels as opposed to the sustained low levels clinically administered through inhalation. Together, the data seem to indicate that high dose NO can have great cytotoxic potential, in contrast

to the reported cytoprotective effects of NO administered in low doses. Similar dose response effects have been observed with endogenously produced NO. For example, Li and co-workers recently reported that the conservative levels of NO generated by the endothelial cells are cytoprotective and anti-apoptotic while the high levels of NO induced during inflammatory injury can cause cytotoxicity (70,71).

Some of the cytotoxic effects reported with the use of high dose exogenous NO may be attributable to the generation of nitrogen dioxide (NO<sub>2</sub>). NO<sub>2</sub> is rapidly and spontaneously synthesized through the reaction of NO with O<sub>2</sub> and has been considered up to twenty times more toxic than NO (61,72). The interaction of these gases and the subsequent generation of NO<sub>2</sub> can become problematic in the gasing chambers commonly used in the laboratory, most specifically when high doses of inhaled NO are used. If the gas exchange in the experimental setting is not adequate, the generation of NO<sub>2</sub> could cause extensive cellular injury. Great care is taken in the clinical setting to minimize the contact of NO with O<sub>2</sub>.

## **2.4 Nitric Oxide Protection**

There is increasing experimental evidence that, while high concentrations of inhaled NO are likely to be injurious to tissues, the low, clinically relevant, concentrations are not only harmless but are in fact, quite beneficial. Recent studies have shown that in the range of 5 to 20 ppm, NO has a protective effect against hyperoxia-induced pulmonary injury (73-75). The precise mechanisms by

which inhaled NO protects against the damaging effects of prolonged exposure to hyperoxia are not clearly understood, but are likely to be multifaceted. NO appears to possess both anti-inflammatory and antioxidant properties, and can lessen alveolar and capillary wall damage caused by oxygen toxicity. Rats exposed to 95% O<sub>2</sub> in combination with 10-20 ppm NO had significantly less fluid accumulation in the alveolar and pleural spaces, and less leakage of labeled albumin from the vasculature compared to control animals which received 95% O<sub>2</sub> alone (73,75). Low dose inhaled NO has also been shown to significantly reduce the accumulation of inflammatory cells in the interstitial and alveolar spaces during oxidative lung injury (76-79). This anti-inflammatory effect may be attributable to the ability of NO to inhibit the vascular adhesion and transmigration of leukocytes (77-79), possibly by blocking the synthesis of inflammatory cytokines (79,80) or cell-surface adhesion molecules such as ICAM-1 (81). Moreover, in addition to attenuating their transmigration, NO significantly modulates neutrophil viability and oxidative function (82,83). Neutrophils collected from ARDS patients during NO inhalation produced significantly less H<sub>2</sub>O<sub>2</sub>, superoxide, and inflammatory cytokines (83,84) than neutrophils from patients who did not receive NO. There were no significant differences in the quantity of cells assayed from each patient group, indicating that the data truly reflect neutrophil dynamics and not cell numbers. The functional down regulation of the neutrophil respiratory burst could have a beneficial effect by significantly reducing the oxidative load to the lung tissue. However, the consequences of altering neutrophil viability are less clear. There is in vitro data

which indicates that neutrophils cultured in the presence of 20 ppm NO die by apoptosis (66,69,85). The safe elimination of neutrophils from the lung parenchyma through apoptotic cell death will reduce the release toxic cellular byproducts to the surrounding tissue and thereby protect the surrounding lung tissue from further damage. However, if NO were to induce neutrophil cell death via necrotic mechanisms, the concomitant release of ROI would only potentiate the inflammatory lung injury. Further research is required to ascertain the fate of these cells in vivo.

At the molecular level, the protective effect of NO may be ultimately attributable to its ability to alter gene expression through the modulation of redox sensitive transcription factors (86,87). Pulmonary inflammation (81) and hyperoxic lung injury (88,89) are both associated with an increase in the activity of the redox sensitive transcription factor Nuclear Factor Kappa B(NF- $\kappa$ B). NO is capable of inhibiting the DNA binding activity of NF- $\kappa$ B (86,90), and, since the genes for several pro-inflammatory cytokines and adhesion molecules contain binding sites for NF- $\kappa$ B in their promoter regions (91,92), this inhibition may attenuate the inflammatory response and its attendant injury. More specifically, since NF- $\kappa$ B activity has been associated with hyperoxia induced apoptosis (88), NO may confer protection against hyperoxic lung injury through the attenuation of NF- $\kappa$ B activation and apoptotic cell death. The effects of NO on cell death have only recently been explored. A number of in vitro studies have furnished data demonstrating that NO

confers protection against stress-induced apoptosis in cultured endothelial (93), hepatic (94), and Jurkat cells (95), and can prevent FAS induced apoptosis in cultured leukocytes (96). Since apoptosis has recently been implicated in hyperoxic lung injury (14, 17, 18, 29), the potential for NO to protect the lung in this capacity is very exciting. To date, no studies have explored the ability of inhaled NO to modulate transcriptional activity in the lung or to block hyperoxia induced pulmonary apoptosis.

### **3.0 Apoptosis**

#### **3.1 Programmed Cell Death**

Cell death is an integral part of many physiological processes including growth and differentiation, tissue remodeling, and host defense (97,98). Apoptosis is a genetically programmed active process which involves a tightly regulated sequence of intracellular biochemical events. This type of cell death allows the safe removal of unwanted cells without the spillage of pro-inflammatory cellular materials, thus averting secondary damage to the surrounding tissue and inflammatory responses (39,98). During apoptosis, the nuclear and cytoplasmic contents of the dying cell are condensed, fragmented and packaged into small membrane-bound vesicles which are engulfed by neighboring cells and macrophages (97,98). The importance of apoptotic cell death in the pathophysiology of injury and disease has been extensively investigated. Although apoptosis is a less injurious form of cell death, when compared to necrosis which involves the swelling and lysis of clusters of contiguous cells, apoptosis nevertheless can result in a significant loss of critical cell mass. A more complete understanding of the molecular events which control apoptosis could, therefore, give rise to a number of therapeutic possibilities for its prevention.

The morphological features of apoptosis have been known for some time but the molecular events and signals involved continue to be elucidated. Several

hallmarks for the identification of apoptotic cells have emerged. First, there is the characteristic pattern of DNA fragmentation. This is a stepwise process involving the condensation of nuclear chromatin (pyknosis), followed by the internucleosomal fragmentation of the DNA (karyohexis). The cleavage of DNA in this manner gives rise to a range of fragments, with each a multiple of 200 base pairs (97,98). The separation of these fragments by agarose gel electrophoresis results in a characteristic "ladder" formation. The associated nuclear blebbing and fragmentation can often be observed microscopically.

The second hallmark involves biochemical events central to the execution of apoptosis. The activation of a unique family of enzymes called caspases has been recognized as a reliable indicator of programmed cell death (99,100). Caspases are cysteine-dependant, aspartate-specific, proteolytic enzymes that participate in the signaling, transduction, and execution of apoptotic mechanisms (101,102). To date, at least ten caspases have been identified and grouped into three functional subfamilies (100,103). The caspase-3 subfamily, which includes caspases 3, 6 and 7, has a number of identified roles within the effector phase of apoptosis. Caspase-3 has been extensively studied and is known to be involved in the activation of DNA cleaving nucleases, and in the proteolytic cleavage and inactivation of DNA repair enzymes (104-106). Caspase-3 is constitutively expressed in the form of a 32 kDa cytosolic protein (pro-caspase-3) which is inactive in its native state. Through autologous or heterologous proteolysis, pro-

caspase-3 is cleaved to produce 12 and 17 kDa subunits which combine in tetrameric form to produce the active enzyme (102,103). Although some examples of caspase-independent programmed cell death have been reported in the literature (107,108), the activation of these proteases is generally considered a definitive marker of apoptosis. The mitochondria have recently emerged as important mediators of apoptotic cell death (109-112). The transient opening of pores in the mitochondrial inner membrane, and the subsequent leakage of cytochrome c into the cytosol, has been reported to precede the activation of caspase-3, and is thought to play a key role in the activation of the execution caspases (111,113-115,120). Studies have shown that the micro-injection of purified cytochrome c into cells can initiate the cascade of apoptotic events (121). The mechanisms of the membrane permeability changes have not been fully elucidated. However, the oxidation of essential membrane-bound proteins (113), and the activation of caspases may play a role in this process (116). Among the numerous known targets of the caspases is the Bcl-2 family of proteins which are involved in the antioxidant protection of mitochondrial membrane integrity (117-119). The biochemical complexity of apoptosis presents considerable opportunity for therapeutic intervention. The interruption or prevention of apoptosis through the blocking of signal transduction or the inhibition of critical enzymes appears to be both pharmacologically feasible and scientifically exciting.

### **3.2 The Role of Oxidants In Apoptosis**

Reactive oxygen intermediates are among the numerous known triggers of apoptosis (122,123). Activation of the apoptotic cell death program by oxidants has been well described in a number of experimental models. Apoptosis has been observed following the direct application of ROI (124,125), after treatments known to generate ROI within the cells (126,127), and also following the inhibition of intracellular antioxidants (123,128). Researchers have recently also implicated oxidants in the triggering of pulmonary apoptosis. For example, Kazzaz and co-workers provided evidence of programmed cell death in a model of cultured alveolar epithelial cells following treatment with H<sub>2</sub>O<sub>2</sub> and paraquat (38). Similar studies have demonstrated H<sub>2</sub>O<sub>2</sub>-induced apoptosis in pulmonary fibroblasts (129), and tracheobronchial epithelial cells (130). Similarly, Aoshiba et al. caused intracellular thiol depletion and subsequent apoptosis in a model of pulmonary fibroblasts cultured in cysteine-free medium (130). Moreover, supplementation with exogenous GSH in this study was successful in preventing apoptosis induced by H<sub>2</sub>O<sub>2</sub> (130). These data are of particular relevance because they present strong evidence that hyperoxic lung injury may be associated with apoptosis. Otterbein and co-workers were the first to demonstrate apoptosis in their in vivo model of hyperoxic lung injury (14). Since then, reports from other laboratories have supported these findings (17,21,29,37,131). Together, these data suggest that the significant loss of pulmonary cells associated with hyperoxia-induced apoptosis may be the cause of the loss of microvascular integrity and pronounced edema

observed in this model of lung injury. Paradoxically, Kazzazz et al. argued that hyperoxia does not directly causes apoptosis in the lung (38). They found that prolonged exposure to hyperoxia caused necrosis rather than apoptosis in their model of cultured alveolar epithelial cells. These data, however, need to be interpreted with caution. The cells used in these studies (A549) were grown from a human carcinoma cell line which is well known for its inability to undergo apoptosis and for the general poor resemblance of these cells to healthy, in vivo, alveolar epithelial cells. Moreover, a more recent study showed that a primary culture of healthy human small airway epithelial cells did succumb to apoptosis under hyperoxic conditions, while their cancerous A549 counterparts did not (40).

It has been suggested previously that, in addition to the widely accepted role of ROI as triggers of apoptotic mechanisms, the generation of such species by the mitochondria was essential for the execution of apoptosis. This theory is supported by the large body of evidence that intracellular levels of ROI are increased during apoptosis (122,123,132). Such ROI generation has even been observed following the triggering of apoptosis by stimuli which themselves do not constitute oxidative stress, such as serum deprivation (133,134), and receptor mediated apoptosis (135,136). Lending further support to this is the fact that apoptosis can be inhibited by antioxidants such as glutathione (132), and N-acetylcysteine (137), and by Bcl-2, a protein with well-established antioxidant functions (138-139). This theory, however, has since been challenged by evidence that apoptosis can occur under

anaerobic conditions where ROI are not generated (141,142), and in cells that have no mitochondrial DNA and hence, no respiratory chain (143). Together, these findings indicate that, while ROI may not be requisite to the execution of apoptosis, oxidants are involved in the transduction of apoptotic signals in many models. Given the highly reactive and non-specific nature of such ROI as  $\text{OH}^\cdot$  and  $\text{O}_2^\cdot^-$ , it is not likely that they modulate specific proteins required for the execution of apoptosis but more likely, exert their effects by altering the redox status of the intracellular milieu.

The transcription and translation of numerous genes have been shown to be important events in the apoptotic process. It is therefore possible that the activation of redox-sensitive transcription factors during apoptosis represents a mechanism by which ROI might mediate their apoptotic effects. Interestingly, recent reports have shown the increased expression of several pro-apoptotic genes during hyperoxic lung injury. The expression of Bax and Bcl-x, two proteins intimately involved in the execution of apoptosis, is increased in models of hyperoxia-induced pulmonary apoptosis (21,37), as is the expression of p21, a protein more indirectly involved in apoptotic mechanisms (37). The expression of the tumor suppressor protein p53, a transcription factor known to upregulate the synthesis of genes responsible for cell cycle arrest and apoptosis, is also increased in the lungs of mice, and in primary cultures of airway epithelial cells following prolonged exposure to hyperoxia (31,37). However, p53 nul mice are not resistant to hyperoxia induced

apoptosis, indicating that other transcriptional mechanisms must also be involved. The activation of the transcription factors NF- $\kappa$ B and Ap-1 has been implicated in apoptosis (144-147), and is known to be caused by changes in the intracellular redox state (148-151). The role of these two transcription factors in hyperoxia-induced apoptosis has not yet been examined *in vivo*.

### **3.3 The Role of Nitric Oxide in Apoptosis**

Nitric oxide is frequently referred to in the literature as a double edged sword, and evidently lives up to its reputation with its conflicting roles in apoptosis. It has been shown to possess both pro and anti-apoptotic properties and, not surprisingly, this paradox seems to be both cell specific and dose dependant (152). High cellular concentrations of NO generated *in vivo* by iNOS, or synthesized *in vitro* through the use of chemical NO donors, often results in apoptotic cell death (67,85,153-155). This apoptosis is associated with the increased synthesis of the tumor suppressor p53, the activation of caspases, the internucleosomal fragmentation of DNA and the modulation of Bcl-2-like proteins (152,156). Apoptosis in these models is largely attributable to the ONOO<sup>-</sup> associated oxidative stress caused by the interaction of NO with O<sub>2</sub><sup>-</sup>. However, a recent study with cultured cardiomyocytes indicates that cGMP mediated mechanisms may be involved in the induction of apoptosis by NO. The authors showed that 8-bromo-cGMP, a stable cGMP analog, could mimic the apoptosis inducing effects of NO (154). It is important to note here that these studies used millimolar concentrations

of chemical NO donors and 8-bromo-cGMP which far exceed physiologically relevant NO concentrations.

In contrast to its well-characterized pro-apoptotic effects, NO has been reported to have anti-apoptotic effects. The apoptosis suppressing effects are usually associated with low doses of exogenously administered NO or the low physiological levels generated in vivo through the constitutive NOS enzymes. In fact, the anti-atherosclerotic effects of eNOS are thought to be mediated in part by the role of NO in the prevention of endothelial cell apoptosis (157,158). It seems that NO can protect against apoptosis through a variety of biochemical mechanisms. In addition to the well described antioxidant effects of NO, it possesses several distinct anti-apoptotic mechanisms which involve the nitrosylation of required proteins and the activation of cGMP mediated signal transduction pathways. Moreover, it has been proposed that NO can interrupt apoptotic process at a number of stages. The first anti-apoptotic mechanism outlined was the inhibition of caspase activity through the S-nitrosylation of the active cysteine residue conserved in all caspases (159,160). The caspase modulating effects of NO, however, are not restricted to the inactivation of active caspases. There is now evidence that NO can also inhibit the proteolytic activation of several pro-caspases (95,161), and prevent the increase in permeability of the mitochondrial membranes, and the associated release of cytochrome c into the cytosol (161,162). Studies have shown that NO can block the caspase-induced

cleavage of Bcl-2 which normally protects the integrity of the mitochondrial membrane (163). The protection of mitochondrial stability can therefore be an important step in blocking the signaling of downstream apoptotic events. Since cytochrome c has been implicated in caspase-3 activation, mitochondrial preservation may be a mechanism whereby NO prevents the proteolytic processing and activation of the execution caspases. Cyclic GMP mediated mechanisms have also been implicated in the anti-apoptotic effects of NO (154,164). The activation of guanylyl cyclase and the subsequent increases in cGMP production decrease the levels of intracellular  $Ca^{2+}$ , which is one of the many known apoptotic signals (112,126,165). Nitric oxide may also modulate the activity of transcription factors involved in the expression of apoptotic proteins, in particular those that are redox sensitive. The activation of both the transcription factors NF- $\kappa$ B and AP-1 is both subject to modulation by NO (87,93,166,167), and each has been implicated in apoptosis (93,144,145,159). DeMeester and co-workers reported decreased NF- $\kappa$ B activation, and the concomitant inhibition of apoptosis by NO in a model of endotoxin stimulated endothelial cells (93). No changes in NF- $\kappa$ B binding activity or apoptosis were detected in this model following treatment with 8-bromo-cGMP, indicating that the NO effects were not cGMP mediated. There is, however, little available information on the molecular effects of NO in the lung. The effects of inhaled NO on hyperoxia-induced apoptosis in the lung have not been investigated.

## **4.0 Transcriptional Modulation**

The activation of redox sensitive transcription factors has been clearly implicated in the etiology of oxidative lung injury and ARDS. Increases in the DNA binding activity of both NF- $\kappa$ B (168-171), and AP-1(172,173), have been documented in models of lung injury. Both of these transcription factors can be activated by the administration of oxidants and through receptor mediated mechanisms. Interestingly, NF- $\kappa$ B can be inhibited by antioxidants, suggesting that not only are ROI one of the possible triggers of its activation but, they may in fact be required for induction (124,171,174). Nitric oxide has recently been shown to inhibit the activity of both NF- $\kappa$ B (86,166,175) and AP-1 (87,167) in vitro. However, the ability of inhaled NO to modulate the activity of these transcription factors in the lung has not been investigated.

### **4.1 Nuclear Factor Kappa-B**

NF- $\kappa$ B is an ubiquitously expressed transcription factor with a well defined role as a key mediator of gene expression in inflammatory injury. It is composed of homo and heterodimers of the Rel family of proteins which includes p50 and p65 (7,176,177). There is a conserved Rel domain in each of these proteins which functions in dimerization, as well as DNA binding. In its native state, NF- $\kappa$ B is sequestered and inhibited in the cytoplasm by the protein, inhibitory kappa-B (I $\kappa$ B). It is activated in response to a number of stimuli including ultraviolet irradiation,

endotoxins, cytokines and oxidants. During activation, I $\kappa$ B is phosphorylated and subsequently degraded, allowing NF- $\kappa$ B to migrate to the nucleus where it can bind to specific DNA sequences and initiate transcription. The inhibitory effects of reductants may therefore be caused by the suppression of I- $\kappa$ B phosphorylation (175,178). Increased activation of NF- $\kappa$ B has been reported in various models of oxidative lung injury. Increased DNA binding activity of this transcription factor has been also implicated in ozone-induced lung injury (170,173), ARDS (168), endotoxin mediated lung injury (169), and, most recently, in hyperoxic lung injury (89). NF- $\kappa$ B regulates the expression of a number of genes involved in immune function and inflammation such as interleukin-8, vascular adhesion molecule-1 and ICAM-1 and, therefore, can exacerbate lung injury through the promotion of the infiltration of inflammatory cells (81,92,179).

The role of NF- $\kappa$ B in apoptotic cell death is currently the subject of intense investigation. There are extensive data which suggest that NF- $\kappa$ B plays a pro-active role in the transduction of apoptosis. Numerous studies have reported increased binding activity of NF- $\kappa$ B during, and immediately prior to, apoptosis induced by a number of stimuli, and, have demonstrated that the inhibition of NF- $\kappa$ B activation through antioxidants (180), or pharmacological agents (181), can attenuate apoptosis. Moreover, binding sites for NF- $\kappa$ B have been identified in the promoter regions of pro-apoptotic genes such as interleukin-1 $\beta$  converting enzyme (ICE), one of the early caspases involved in the transduction of apoptotic signals (182).

There is, however, also strong evidence in the literature that NF- $\kappa$ B may protect cells from apoptotic cell death under certain conditions. In vitro studies involving the overexpression of I $\kappa$ B and experiments with transgenic NF- $\kappa$ B knockout mice indicate that cells are more susceptible to apoptosis in the absence of NF- $\kappa$ B activity (126,183,184). It is important to remember, however, that the multiple potential dimers of the various NF- $\kappa$ B subunits are not redundant. Rather, each is capable of initiating the transcription of a multitude of genes with diverse cellular functions and cell specificities. It is therefore possible that the activation of one NF- $\kappa$ B isoform may inhibit apoptosis, while another could strongly promote it. Moreover, transcription factors interact with DNA in large complexes in combination with other requisite proteins and co-factors. The transcription of specific genes and the ultimate fate of the cell may depend not only upon the activation of specific NF- $\kappa$ B dimers, but also upon the relative balance of NF- $\kappa$ B with other cell signals.

#### **4.2 Activator Protein-1 (AP-1)**

The transcription factor AP-1 is a complex of two protein subunits derived from the proto-oncogenes c-Fos and c-Jun. These subunits can combine to form a c-Jun/c-Jun homodimer or a c-Jun/c-Fos heterodimer. As with NF- $\kappa$ B, the activation of AP-1 occurs independently of protein synthesis and involves post-translational processing of the subunit proteins. It was proposed by Boyle et al. that a nuclear protein phosphatase dephosphorylates the c-Jun moiety of each dimer, thereby activating the transcription factor (140). A conserved cysteine residue in

each of the two subunits has been identified as central to the regulation of the DNA binding activity of AP-1 and consequently, mutations in which the cysteine is replaced by a serine residue show constitutive DNA binding (185). The highly reactive thiol groups of the cysteine residues make this transcription factor very sensitive to intracellular redox modulation. The data from numerous studies indicate that AP-1 activity is promoted by reducing agents and is strongly inhibited by oxidants (151,185,186). However, following prolonged exposure to strong oxidizing conditions AP-1 binding can be upregulated through alternate cell signaling pathways (151,187).

#### **4.3 Specificity Protein 1 (Sp1)**

The transcription factor Sp1 is also modulated by the redox environment within the cell and, like AP-1, is subject to such modulation through a cysteine residue located within the DNA binding domain. Sp1 is known to induce the transcription of a wide variety of genes, many of them encoding for constitutive housekeeping proteins. While the pathological implications of the activation of this transcription factor are not clear, it may nonetheless provide important information about the intracellular redox status.

#### **4.4 Nitric oxide and Redox Sensitive Transcription Factors**

Recently, evidence has been presented in the literature which indicates that NO can inhibit the activation of both NF- $\kappa$ B and AP-1. Zeiher et al. showed that the

inhibition of NO synthesis in cultured human endothelial cells leads to the activation of the transcription factor NF- $\kappa$ B and, furthermore, that the administration of exogenous NO inhibited its binding activity (166). In a similar model of cultured endothelial cells, Peng and co-workers determined that the mechanism of NF- $\kappa$ B inhibition by NO involved the induction and stabilization of I $\kappa$ B mRNA (175). They were unable to mimic the effects of NO with 8-bromo-cGMP, indicating that these effects are not mediated by cGMP but, more likely, are redox modulated. Since the constitutive expression of NO by eNOS has been implicated in endothelial cell proliferation (157), it seems likely that NO may attenuate endothelial cell apoptosis by inhibiting the activation of NF- $\kappa$ B. In a recent study, DeMeester and co-workers presented data which lends support to this theory. They showed that NO attenuates apoptosis in endotoxin stimulated endothelial cells, and showed a concomitant decrease in the binding activity of NF- $\kappa$ B (93).

Nitric oxide has also been used to inhibit the activity of the transcription factor AP-1. In vitro studies have shown that the DNA binding activity of the purified AP-1 subunits c-Fos and c-Jun are potently inhibited by the addition of NO (167). The authors also showed that these effects could be reversed by the addition of the antioxidant dithiothreitol (DTT), which suggests that the mechanism involved is the S-nitrosylation of the conserved cysteine residues situated in the DNA binding domains of both proteins.

## **Summary and Statement of Problem**

Hyperoxic gas mixtures are frequently administered to mechanically ventilated, critically ill patients with pulmonary hypertension and acute respiratory distress syndrome in order to increase oxygen tension in arterial blood and tissues. Inhaled NO, a potent and selective pulmonary vasodilator, is also delivered via the ventilator circuit, frequently in combination with hyperoxia, to further improve arterial oxygenation in these patients. However, both hyperoxia and NO have the potential to cause substantial cellular damage. Prolonged inhalation of hyperoxic air can cause serious lung injury, primarily as a result of the production of ROI whereas NO itself is a highly reactive free radical with significant toxic potential. Consequently much attention is being focused presently on the possibility that NO may exacerbate lung injury. Paradoxically, though, recent studies have shown that low dose NO has a protective effect against hyperoxic pulmonary injury.

The precise mechanisms by which NO protects against hyperoxia are not clearly understood, but are likely to be multifaceted. NO appears to possess both anti-inflammatory and antioxidant properties, and can lower the alveolar and capillary wall damage caused by oxygen toxicity. The anti-inflammatory effects may be attributable to the ability of NO to inhibit the vascular adhesion and transmigration of leukocytes possibly by blocking the synthesis of inflammatory cytokines or cell-surface adhesion molecules such as ICAM-1. Recent studies have suggested that hyperoxia can cause apoptosis in lungs. Since NO can protect

against apoptosis caused by pro-inflammatory agents in cultured endothelial cells, it may therefore also be able to effect similar pulmonary protection in vivo, possibly by directly inactivating caspases, the proteolytic enzymes responsible for apoptotic cellular degradation.

At the molecular level, the protective effect of NO may be ultimately attributable to its ability to alter gene expression through the modulation of redox sensitive transcription factors. Pulmonary inflammation and hyperoxic lung injury are both associated with an increase in the activity of the redox sensitive transcription factor NF- $\kappa$ B. NO is capable of inhibiting the nuclear activity of NF- $\kappa$ B and since the genes for several pro-inflammatory cytokines and adhesion receptors contain binding sites for NF- $\kappa$ B in their promoter regions, this inhibition may attenuate the inflammatory response and its attendant cell injury. Moreover, since hyperoxia-induced apoptosis is associated with an increase in NF- $\kappa$ B activity, the inhibition of this transcription factor by NO may also block this form of cell death.

To date no studies have explored the ability of inhaled NO to block hyperoxia-induced pulmonary apoptosis. The principal objective of this study was therefore to investigate the ability of inhaled NO to attenuate hyperoxic injury in an in vivo rat model, and to examine the possible involvement of apoptosis, the pro-apoptotic enzyme caspase-3 and the redox-sensitive transcription factors in this process.

## Hypothesis and Principal Objectives

In view of the data above, the hypothesis underlying this study was that inhaled NO will protect against hyperoxia-induced lung injury through the attenuation of apoptosis and that this protection is mediated through the inactivation of caspases and the modulation of redox-sensitive transcription factors. Four principal objectives were investigated in these studies using a rat model of hyperoxia and NO inhalation:

- 1. Determine the involvement of apoptosis in hyperoxic lung injury and the ability of NO to protect against that apoptosis.**
- 2. Determine the ability of NO to protect against the increased vascular leak in hyperoxic lung injury.**
- 3. Determine the involvement of ROI in hyperoxic lung injury and the effect of NO.**
- 4. Determine the involvement of the redox-sensitive transcription factors NF- $\kappa$ B, AP-1 and Sp1 in hyperoxic lung injury and the effects of NO on these factors.**

## Methods

All reagents were products of Sigma Chemical (St.Louis, MO) unless otherwise indicated.

**Animal Protocol:** All animal protocols were performed in accordance with the guidelines of the Canadian Council on Animal Care, and received institutional approval. No animals died during the exposure to experimental gases. Male Sprague Dawley rats weighing 250-300 g (Charles River, St-Constante, PQ) were placed in 4.9 liter airtight plastic chambers with free access to food and water. The chambers were washed and bedding refreshed each day throughout the experiment. Oxygen and NO (Vitalaire Health Care, Ottawa, ON) were mixed prior to entry into the chambers and were delivered through a humidified circuit at a constant flow rate of 10 l/min. This rapid flow was employed to minimize the transit time of NO in the chambers and thereby lower the generation of toxic nitrogen dioxide (NO<sub>2</sub>). Oxygen, NO, and NO<sub>2</sub> concentrations were monitored continuously using in-line analyzers at the outport of the chambers (Pac II Drager, Germany and Miniox, Catalyst Research, Owings Mills MD). The oxygen concentration in the hyperoxic groups was stable at 97.0±1.1 %, and the mean NO and NO<sub>2</sub> concentrations were 20.7±0.7 ppm and 0.3±0.05 ppm respectively. A total of six experimental groups of rats were used. Three groups inhaled either I) >95% O<sub>2</sub>, II) >95%O<sub>2</sub> plus 20 ppm NO, or III) room air only for 24 h, while the remaining three groups inhaled the same gas mixtures for 60 h. Following treatment, the animals

were immediately sacrificed under pentobarbital anesthesia and the lungs were harvested in one of two ways: i) to measure intracellular biochemical parameters, the residual vascular blood was removed by perfusing the lungs with 50 ml of cold phosphate buffered saline (PBS) through a cannula which was placed through the right ventricle and into the pulmonary artery. The lungs were then excised, rapidly chilled on ice, and were processed as described below. ii) For histologic examination, the lungs were fixed by intratracheal instillation of buffered neutral 10% formalin at 20 cm H<sub>2</sub>O pressure, were paraffin embedded, and each lung was cut into 4 µm midcoronal sections.

### **Apoptosis**

**In Situ End Labeling (ISEL):** Coronal sections from the left lung of each animal were double labeled using the ISEL protocol for the visualization of fragmented DNA (188,189), as well as with Hoechst 33258, a general nuclear stain. Briefly, the lung sections were deparaffinized with toluene, rehydrated in serial dilutions of ethanol and washed thoroughly with PBS. The sections were permeabilized with methanol acetone, (1:1), rinsed, and incubated for ten minutes at room temperature with 20 µg/ml proteinase K. The sections were washed for 20 minutes with distilled water and then incubated in the dark for thirty minutes with Hoechst bis benzamide 33258 (0.05 µg/ml in water). The sections were quickly rinsed with PBS and incubated with a buffered solution containing 2 mM copper cobalt, 10 µM b-16-dUTP (Boehringer Mannheim, Laval, PQ) and 25 units of terminal

transferase enzyme (Boehringer Mannheim, Laval, PQ), protected from the light in a humidified 37°C chamber for one hour. The sections were rinsed again with PBS to stop the reaction and were incubated in the dark with a 4x sodium citrate saline solution containing 2.5 µg/ml avidin-FITC (fluorescein isothiocyanate-avidin) for thirty minutes. The sections were given a final wash with PBS prior to coverslipping in "anti-fade" solution (1 mg/ml p-phenylenediamine, 90 % glycerol in PBS). Positive controls for the ISEL label were prepared by incubating lung sections with 5 U/ml DNase just prior to the terminal transferase treatment. ISEL and Hoechst stained cells in the alveolar walls were visualized using a Zeiss Axiophot fluorescence microscope, and were counted in each experimental group in a blinded fashion at 400 x magnification. A total of approximately 125 cells were counted in 20 randomly selected fields from the left lung of each rat and apoptotic cells were expressed as a percentage of the total number of lung cells in the corresponding field.

**Agarose Gel Electrophoresis of DNA:** Low molecular weight DNA was extracted from freshly isolated perfused rat lung as follows: The tissue was homogenized on ice using a Polytron homogenizer at 10 000 rpm for 30 s in an equal volume ratio of a cold buffer containing 10 mM Tris-HCl, 10 mM EDTA, 75 mM NaCl, (pH 8.0). The crude homogenates (100 µl) were incubated for fifteen minutes at room temperature with 1.25 ml of a lysis buffer containing 10 mM Tris-HCl, 10 mM EDTA, 75 mM NaCl and 1% SDS (pH 8.0), and were then centrifuged

at 13 000 xg for fifteen minutes. The supernatant containing the fragmented DNA was gently separated from the viscous pellet of intact DNA and was incubated at 60°C with 100 µg/ml proteinase K for one hour. The DNA was then precipitated overnight in 50% ethanol, 0.5M NaCl at -20°C. Subsequently, the DNA precipitate was pelleted following centrifugation at 13 000 xg at 4°C and air dried for 30 minutes at room temperature. The pellet was then resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and the DNA was purified with phenol/chloroform (1:1) and chloroform washes. The ethanol precipitation was repeated and the purified DNA was pelleted and dried as above. The DNA pellet was resuspended in 45 µl of TE buffer and incubated with 100 µg/ml RNase at 37°C for thirty minutes. The concentration of DNA was determined spectrophotometrically at 260 nm and the purity assessed using 260/280 nm = 1.7 to 2.0 as the acceptable range. DNA (10 µg) was subjected to electrophoresis on 1.5% agarose gels at 100 volts in TAE buffer containing 400 mM Tris-HCl, 300 mM acetic acid, and 20 mM EDTA (pH 8.0). Subsequently, the gels were stained with 0.5 µg/ml ethidium bromide then photographed over an ultraviolet light source.

**Western Blotting:** The content of pro-caspase-3 and ICAM-1 in the lung tissues was determined using standard Western blotting techniques with antibodies selective for these proteins. The lung tissue was homogenized on ice for 45 seconds using a Polytron homogenizer at 10 000 rpm in eight volumes of 10 mM HEPES (pH 7.9) containing 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.1% Nonidet P-40, 0.5 mM

DTT, 0.5 mM PMSF, 0.5 mM spermidine, 0.15 mM spermine, and 5  $\mu\text{g}/\text{m}\ell$  each of aprotinin, leupeptin and pepstatin. The homogenate was incubated on ice for fifteen minutes and centrifuged at 35 000 x g at 4°C for fifteen minutes. The protein concentrations in the supernatants were determined using the Bio Rad protein assay (Bio Rad Laboratories, Hercules, CA). Aliquots of the supernatant (15  $\mu\text{g}$  protein) were subjected to electrophoresis on 12% polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked overnight in a Tris- buffered saline and tween (TBST) solution containing 5% skim milk. The membranes were incubated for two hours at room temperature in a solution of TBST with 2% skim milk containing the appropriate primary antibody. For pro-caspase-3, a 1/2000 dilution of the 0.2  $\mu\text{g}/\mu\ell$  stock solution of the polyclonal rabbit antibody was prepared (MF, Merck Frosst, Pointe Claire, PQ) and for ICAM-1, a 1/500 dilution of the 0.2  $\mu\text{g}/\mu\ell$  stock solution of the monoclonal mouse antibody was used (Cedarlane Laboratories, Hornby, ON). The membranes were given three five-minute washes with TBST and were then incubated for thirty minutes at room temperature with a horseradish peroxidase (HRP) conjugated secondary antibody appropriate for the primary antibody used (goat anti-rabbit IgG for caspase-3 and goat anti-mouse IgG for ICAM-1, both from Bio-Rad laboratories, Hercules, CA). The membranes were washed thoroughly in TBST (at least 1 h) and protein band chemiluminescence was visualized on X-ray film according to the manufacturer's instructions (NEN Life Science Products, Boston, MA). The intensity of the bands was quantified with a densitometer and Molecular Analyst Software

(Bio Rad Laboratories, Hercules, CA).

### **Pulmonary Inflammation and Edema**

**Lung Histology:** Coronal sections from the left lung of each animal were stained with hematoxylin-phloxin-saffron (HPS) or toluidine blue. All histologic evaluations were performed by a pathologist (Dr. J.P. Veinot), who was blinded to the experimental groups. HPS-stained slides were scored for perivascular and interstitial edema on a scale ranging from 0 (no edema) to 4 (severe edema). Neutrophils were counted in twenty randomly selected fields in each of the HPS stained sections using 250 x magnification. Only the neutrophils in the alveolar spaces and lung parenchyma were included in the tally. Those located within the capillaries were excluded. Mast cells were counted in the same fashion using the toluidine blue stained sections. Peribronchial and bronchial mast cells of the larger bronchi were excluded.

### **Redox modulation**

**Glutathione and Protein Sulfhydryl Content:** Freshly isolated lungs (200-300 mg) were homogenized on ice using a Polytron homogenizer at 10 000 rpm for 30 s in two volume of cold PBS. The homogenate was centrifuged at 35 000 x g for 15 min at 4°C. The supernatants were adjusted to a final protein concentrations of 5 mg/ml with PBS, and, were analyzed for total thiol (TSH), glutathione (GSH), and protein sulfhydryl (PSH) content using DTNB [5,5' dithiobis(2-nitrobenzoic acid)].

For total thiol content, the reaction mixture consisted of supernatant, 1 mM DTNB, and 6M guanidine-HCl (pH 8.2). The mixture was incubated for 30 min, protected from the light, and was then analyzed spectrophotometrically at a wavelength of 412 nm against 6M guanidine-HCl and DTNB blanks. The TSH content was calculated using the molar extinction coefficient of  $13\,600\text{ cm}^{-1}\text{ }\mu\text{M}^{-1}$  and all values were reported as nmol of thiol per mg protein. To measure GSH, the protein was precipitated out from the supernatants with ice cold 5% trichloroacetic acid (TCA) and pelleted by centrifugation at  $20\,000\times g$  for 5 min at  $4^{\circ}\text{C}$ . The pellets were discarded and the protein-free supernatants were adjusted to pH 8.2 with NaOH and reacted with 1 mM DTNB alone. Absorbance at 412 nm was measured and PSH content was calculated as above. Protein sulfhydryl content was determined by subtracting the GSH content from the TSH.

**Glutathione Peroxidase and Reductase Assays:** Lung tissue was homogenized and centrifuged as described for the Western blots, and the activity of both glutathione peroxidase and glutathione reductase were measured as previously described (190). To measure glutathione peroxidase,  $20\text{ }\mu\text{l}$  of the lung supernatants were incubated for 10 min at  $37^{\circ}\text{C}$  in 50 mM  $\text{KPO}_4$  buffer containing 0.24 U glutathione reductase, 1 mM reduced glutathione (GSH), and 1 mM  $\text{NaN}_3$ . Nicotinamide adenine dinucleotide phosphate (NADPH) (0.15 mM) was then added to the mixture and a baseline absorbance reading was taken at 340 nm. To begin the reaction, 0.15 mM  $\text{H}_2\text{O}_2$  was added to the solution. The oxidation of glutathione

and the concomitant consumption of NADPH was measured spectrophotometrically at a wavelength of 340 nm against a blank containing no lung supernatant. Readings were taken every minute for a duration of fifteen minutes. The enzymatic activity was calculated using the molar extinction coefficient for NADPH ( $6.22 \times 10^3 \text{ cm}^{-1} \mu\text{M}^{-1}$ ). One unit of glutathione peroxidase activity was defined as the content that could oxidize 1.0  $\mu\text{mol}$  of GSH in one minute.

The activity of glutathione reductase in the lung extracts was measured in a similar fashion. Lung supernatant (20  $\mu\text{l}$ ) was incubated in 50 mM KPO<sub>4</sub> buffer containing 0.15 mM NADPH and 1 mM oxidized glutathione (GSSG). The reduction of GSSG and the concomitant oxidation of NADPH was measured spectrophotometrically at 340nm against a blank containing no lung supernatant. Readings were taken each minute for a duration of fifteen minutes and the enzymatic activity was calculated using the molar extinction coefficient for NADPH. One unit of glutathione reductase activity was described as that which can reduce 1.0  $\mu\text{mol}$  of GSSG in one minute.

### **Transcription Factor Analysis**

**Electrophoretic Mobility Shift Assay:** Nuclear pellets were obtained from lung tissue with the aid of sucrose gradient centrifugation using modifications of previously published protocols (191). Briefly, 400 mg of lung tissue were homogenized on ice using six slow strokes of a Teflon pestle homogenizer at 1 000

rpm in eight volumes of 0.25 M sucrose containing 10 mM HEPES (pH 7.6), 25 mM KCl, 1 mM EDTA, 10% glycerol, 0.15 mM spermine, and 0.5 mM spermidine. The homogenate was filtered through a 45 µm nylon sieve and layered over a 10 ml cushion of 2 M sucrose containing 10 mM HEPES (pH 7.6), 25 mM KCl, 1 mM EDTA and 10% glycerol. The homogenate was centrifuged at 100 000 x g at 4°C for 1 h, the supernatant was decanted, and the pelleted nuclei were resuspended in 40 µl of a cold lysis buffer containing 20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF, 0.5 mM spermidine, 0.15 mM spermine, and 0.5 µg/ml each of aprotinin, leupeptin and pepstatin. The suspension was incubated on ice for 45 min and centrifuged at 20 000 x g at 4°C for 10 min. The supernatant containing the nuclear proteins was collected and diluted 1:1 with a buffer containing 20 mM HEPES (pH 7.9), 50 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM DTT, 0.5 mM PMSF, 0.5 mM spermidine, 0.15 mM spermine, and 5 µg/ml of each of aprotinin, leupeptin and pepstatin. The protein concentration of the nuclear extracts was determined using the Bio Rad protein assay (Bio Rad Laboratories, Hercules, CA).

For the EMSA assays, double-stranded consensus oligonucleotides for NF-κB, AP-1 and Sp1 (Promega, Madison, WI) were radiolabelled with γ[<sup>32</sup>P]ATP (Amersham, Arlington Heights, IL). Five µg of nuclear protein were first incubated for 10 min at room temperature with 5 µg poly-d[I-C] (Boehringer Mannheim, Laval, PQ) in DNA binding buffer containing 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 0.2 mM

EGTA, 100 mM KCl, 5% glycerol and 2 mM DTT. Labelled probe (2 ng) was then added and the reaction mix incubated for an additional 20 min at room temperature in a final volume of 20  $\mu$ L. The reaction mixture was subjected to electrophoresis on a 5% native polyacrylamide gel, and the dried gel was exposed to X-ray film. The intensity of the bands was quantified with a densitometer and Molecular Analyst software (Bio Rad Laboratories, Hercules, CA). The unit composition of NF- $\kappa$ B was determined with supershift assays. Antibodies (2  $\mu$ g) to either p50 or p65 (Santa Cruz Biotechnologies, Inc. Santa Cruz, CA) were added to the reaction mixture and incubated for 20 min prior to the addition of poly-d[I-C]. The specificity of the bands for NF- $\kappa$ B, AP-1, and Sp1 were determined using cold probe competition. The unlabelled probe (100 ng) was added to the reaction mixture and incubated for 10 min prior to the addition of the radiolabelled probe.

**Statistical Analysis:** All data represent the mean  $\pm$  standard deviation in measurements from 3-7 rats from each experimental group. The differences between the groups were analyzed using one-way analysis of variance (ANOVA) and post hoc Tukey tests. Values of  $p < 0.05$  were considered to be statistically significant.

## Results

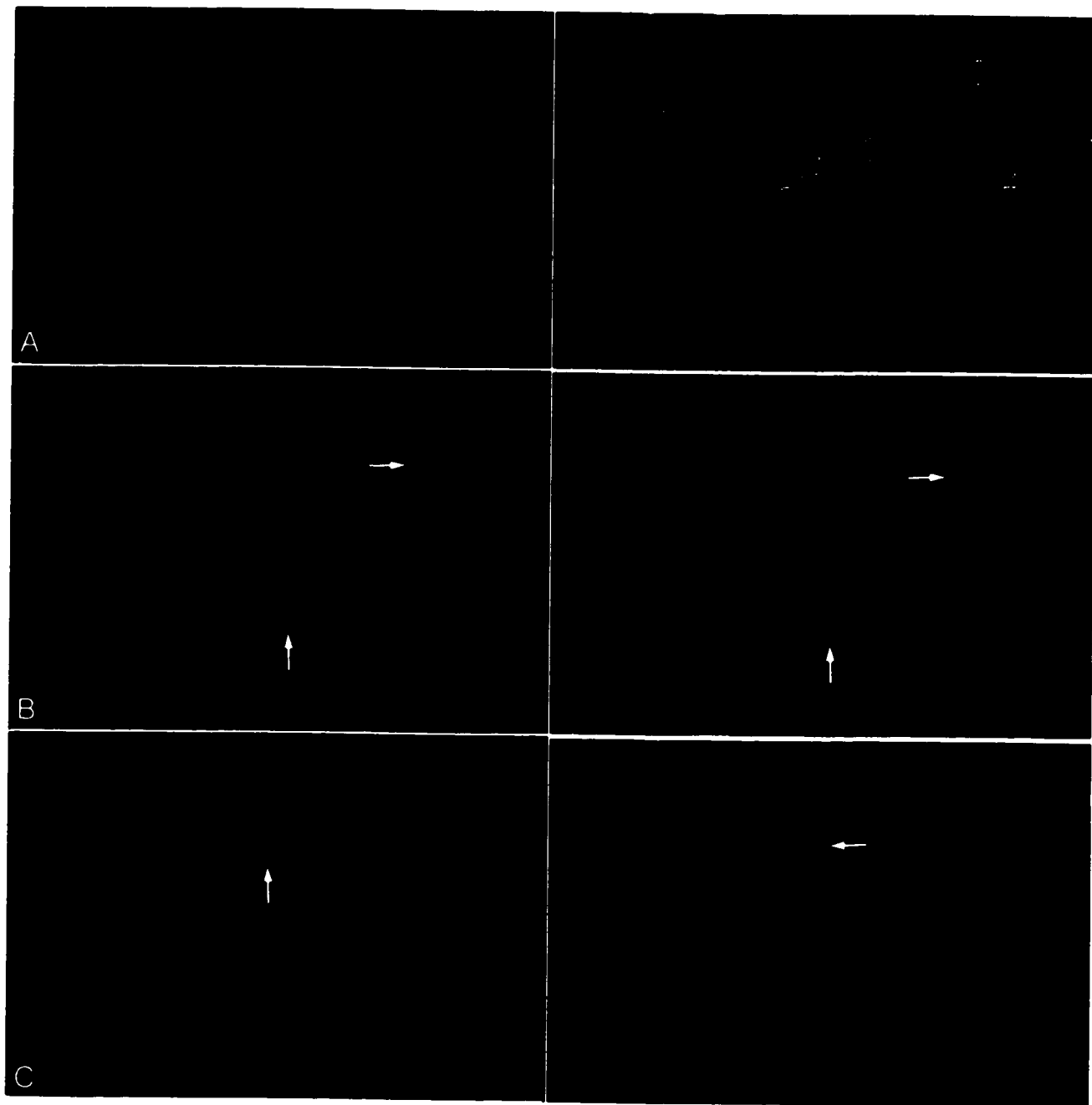
### **Apoptosis**

**ISEL Labeling:** In-situ end labeling of fragmented DNA in lung sections from rats exposed to 60 h hyperoxia revealed numerous labeled nuclei in the alveolar cells whereas control lungs showed very few ISEL-positive nuclei (Figure 1). The inclusion of NO in the hyperoxic gas lowered the number of ISEL-positive cells (Figure 1). Quantitative analysis of the percent labeled nuclei showed a significant increase after 60 h hyperoxia when compare to control (Figure 2). Only  $0.5 \pm 0.1\%$  (n=4) of the nuclei in the control lungs showed evidence of DNA fragmentation. This is likely the result of cell death involved in the natural cycling and turnover of pulmonary cells. Following 60 h exposure to hyperoxia, the percentage of nuclei in the rat lungs containing fragmented DNA was increased to  $11.5 \pm 3.9\%$  (n=6) of the total number. This is indicative of a significant loss of cells in response to the hyperoxic stress. However, the presence of NO in the hyperoxic air significantly lowered the percentage ISEL-positive cells (Figure 2). Those rats breathing both hyperoxia and 20 ppm NO for 60 h showed evidence of DNA fragmentation in only  $4.2 \pm 2.2\%$  (n=4) of the total nuclei. Statistical analysis of these data showed no significant difference in the number of ISEL positive nuclei counted in the control vs. hyperoxia plus NO groups at the 60 h time point. There was no significant increase in ISEL-positive cells in either the hyperoxia alone or the hyperoxia plus NO groups after only 24 h (data not shown).

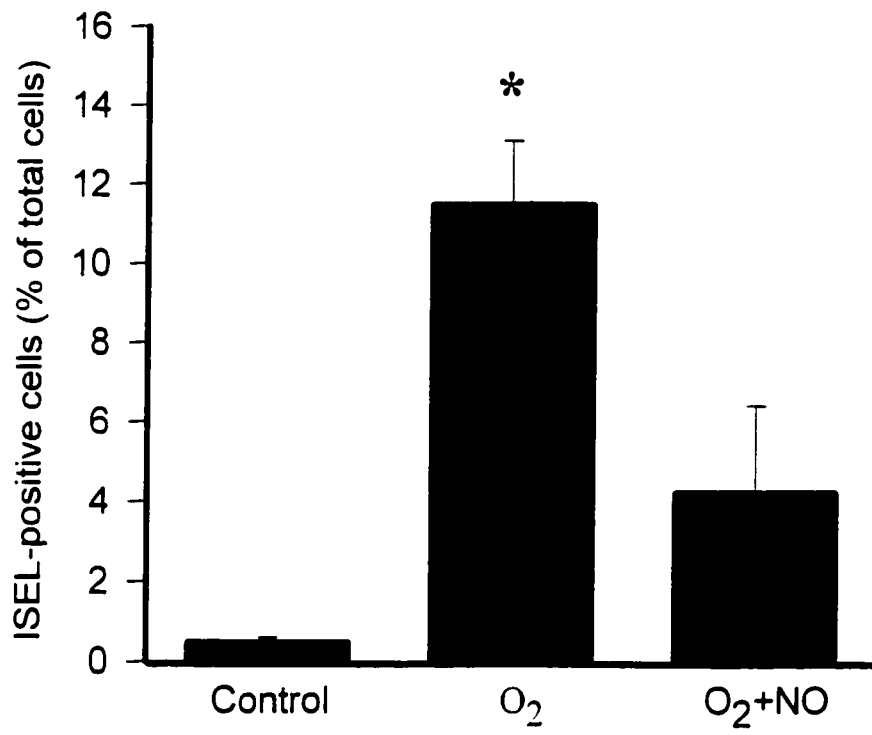
**Agarose Gel Electrophoresis of DNA:** DNA extracted from the lungs of rats exposed to 60 h hyperoxia showed the “laddering” typical of apoptotic cells, whereas lungs from rats exposed to room air showed no laddering (Figure 3). DNA from rats treated with hyperoxia plus NO showed greatly diminished laddering compared to the hyperoxia group (Figure 3). There was no detectable laddering in any experimental group after only 24 h (not shown). No attempt was made to quantify the DNA fragmentation using this technique.

**Caspase-3 Activation (Western blot):** The enzymatically active subunits of caspase-3 could not be detected using this Western blotting technique, however, the intracellular 32kDa pro-enzyme was detectable. High constitutive levels of pro-caspase-3 were detected in the cytosolic extracts from control rat lungs (Figure 4). Hyperoxia for 60 h caused a decline in the level of this protein, whereas the addition of NO to the hyperoxic gas prevented this decline. Quantitative analysis of the relative intensities of these immunoblot bands showed a statistically significant decrease in pro-caspase-3 levels in the 60 h hyperoxic lungs (n=6), when compared to controls (n=6). The inclusion of NO significantly protected against this decrease (n=6). No significant changes in the intensity of the 32 kDa band were observed in the hyperoxia or hyperoxia + NO groups after only 24 h (data not shown).

**Figure 1: ISEL of Fragmented DNA.** ISEL (left panel) and Hoechst (right panel) staining of histologic sections of rat lungs exposed to (A) room air; (B) 60 h hyperoxia; (C) 60 h hyperoxia with 20 ppm NO. Arrows indicate representative cells containing fragmented DNA (original magnification 400x).



**Figure 2: Quantitative Analysis of ISEL.** The percentage of ISEL positive lung cells in lung sections from control rats (control, n=4), rats exposed to 60 h hyperoxia (O<sub>2</sub>, n=6) and 60 h hyperoxia with 20 ppm NO (O<sub>2</sub>+NO, n=4). \* p<0.001 vs. control and O<sub>2</sub> + NO

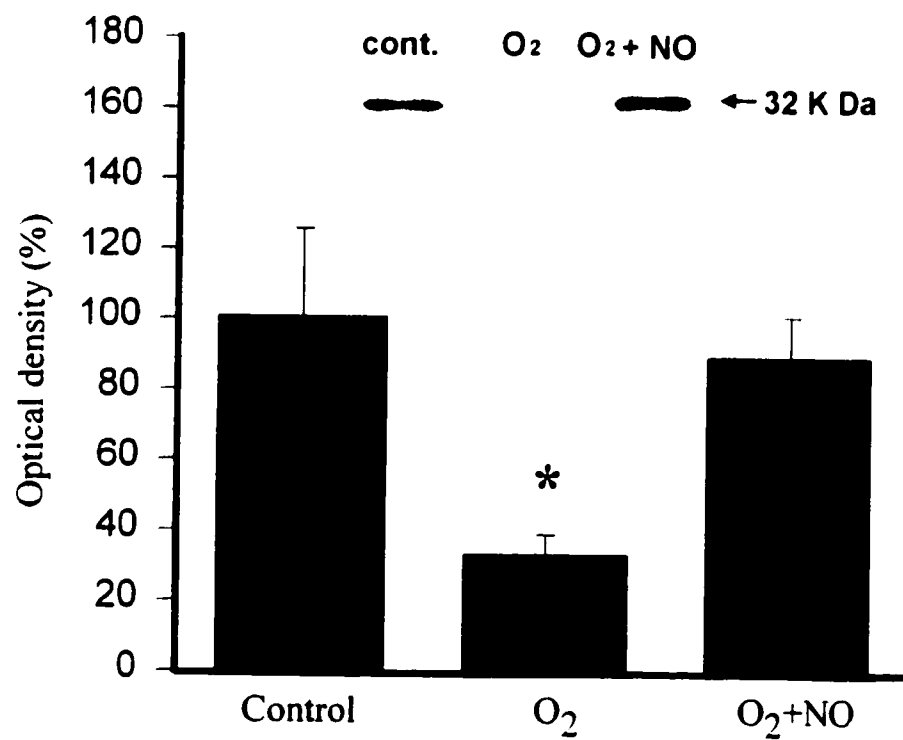


**Figure 3: Gel Electrophoresis of DNA.** Agarose gel electrophoresis of DNA extracted from rat lungs exposed to 60 h room air (Lane 1), 60 h hyperoxia (Lane 2), showing "laddering" characteristic of internucleosomal fragmentation of DNA, and 60 h hyperoxia plus 20 ppm NO (Lane 3), showing little or no laddering. Lane 4 contains DNA extracted from irradiated thymocytes and was used as a positive control.

1 2 3 4



**Figure 4: Western Immunoblotting for Pro-Caspase-3 (CPP32) with Densitometric Analysis.** Cytosolic extracts (20  $\mu$ g protein) from the lungs of rats exposed to room air (control, n=6); 60 h hyperoxia ( $O_2$ , n=6), showing a significant decrease in caspase-3 pro-enzyme; and 60 h hyperoxia with 20 ppm NO ( $O_2$ +NO, n=6) were subjected to Western blotting (inset) and, were then analyzed for their content of pro-caspase-3 by densitometric techniques. \* p=0.023 vs. control and  $O_2$  + NO



## **Pulmonary Inflammation and Edema**

**Lung Histology:** Examination of lung sections at the end of 60 h of hyperoxia revealed pronounced perivascular and interstitial edema (Figure 5). The edema was substantially decreased in the presence of NO (Figure 5). Statistical analysis of the edema scores in the lungs exposed to hyperoxia, showed them to be significantly greater than controls (Figure 6). Each of the control sections examined had a score of 0 for edema (n=5), indicating that there was no evidence of vascular leak in the lungs of these animals. In contrast, rats exposed to 60 h hyperoxia had a score of  $2.6 \pm 0.7$  (n=6) out of a maximum of 4.0, indicating significant perivascular edema in the lungs of these animals. The inclusion of NO in the hyperoxic gas significantly lowered the edema scores to  $1.5 \pm 0.4$  (n=4)(Figure 6). Three of the six lungs examined after 60 h of hyperoxia showed alveolar changes indicative of diffuse alveolar damage (DAD). None of the lungs in the control or hyperoxia + NO groups showed evidence of DAD (not shown).

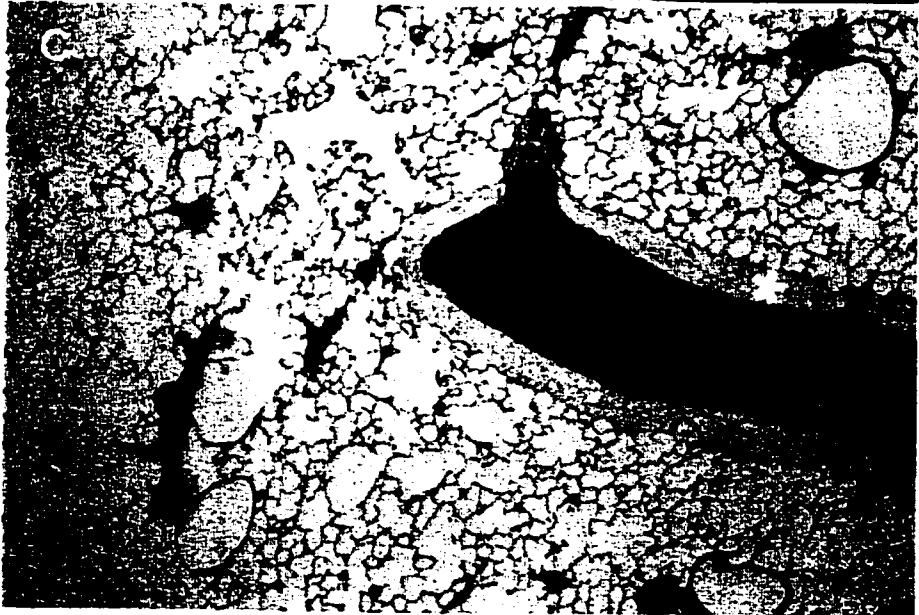
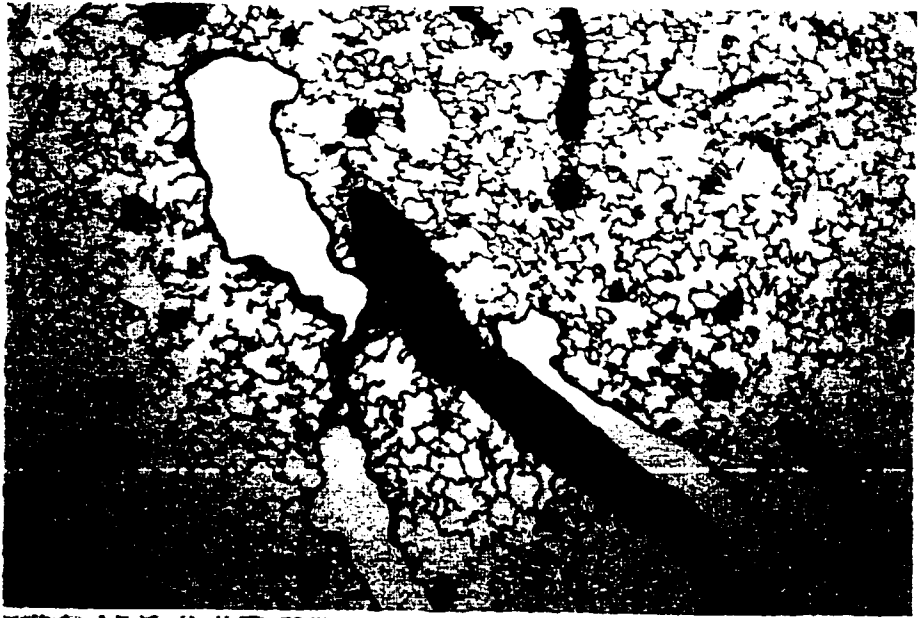
Hyperoxia also produced features of mild inflammation, with a small but statistically significant increase in neutrophils and mast cells when compared to room air controls (Figure 7). These cells were counted in randomly selected fields of identical size in each lung section and are expressed as average number of cells per microscopic field. In the lungs of rats exposed to 60 h of hyperoxia,  $1.8 \pm 0.7$  mast cells were counted per field (n=6) compared to only  $0.9 \pm 0.3$  cells per field

(n=5) in lungs of rats breathing room air alone (Figure 7). The numbers of neutrophils counted in the hyperoxic lungs increased in a similar fashion to  $2.9 \pm 0.8$  cells per microscopic field (n=6) from  $0.8 \pm 0.4$  per field (n=5) in the control lungs (Figure 7). Although the numbers of these inflammatory cells showed a statistically significant increase following hyperoxic exposure, they are nevertheless only indicative of a very mild leukocyte infiltration. The inclusion of NO in the hyperoxic gas mixture did not attenuate the numbers of inflammatory cells (Figure 7). Evaluation of those cells revealed  $3.7 \pm 0.8$  neutrophils and  $2.0 \pm 0.6$  mast cells per microscopic field (n=4). No obvious alveolar inflammation or injury were observed in any of the groups after only 24 h (not shown).

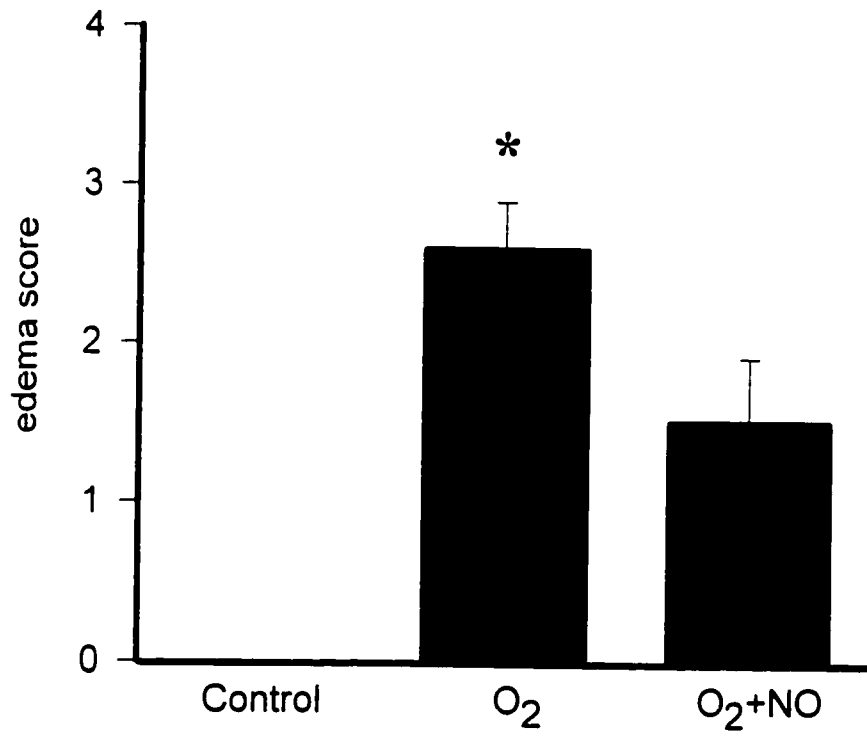
**ICAM-1 Analysis:** The cytosolic content of the cytokine ICAM-1 was barely detectable in extracts from control lungs but increased dramatically in lung tissue following 60 h hyperoxia (Figure 8, inset). The presence of NO in the hyperoxic gas, however, attenuated this increase. Quantification of the protein bands showed that the increase in ICAM-1 caused by 60 h hyperoxia (n=5) was significantly greater than both the room air control (n=4) and hyperoxia + NO (n=5) groups (Figure 8). The ICAM-1 protein appears as a doublet band on the autoradiograph. The distinction between the two bands is not clear but is presumed to represent two different phosphorylated states of the protein.

**Figure 5: Histologic Analysis:** Tissue sections (5  $\mu\text{m}$ ) from the lungs of control animals (Panel A), rats exposed to 60 h hyperoxia (Panel B), and hyperoxia with 20 ppm NO (Panel C) were stained with Hematoxylin Phloxyn Safran (HPS).

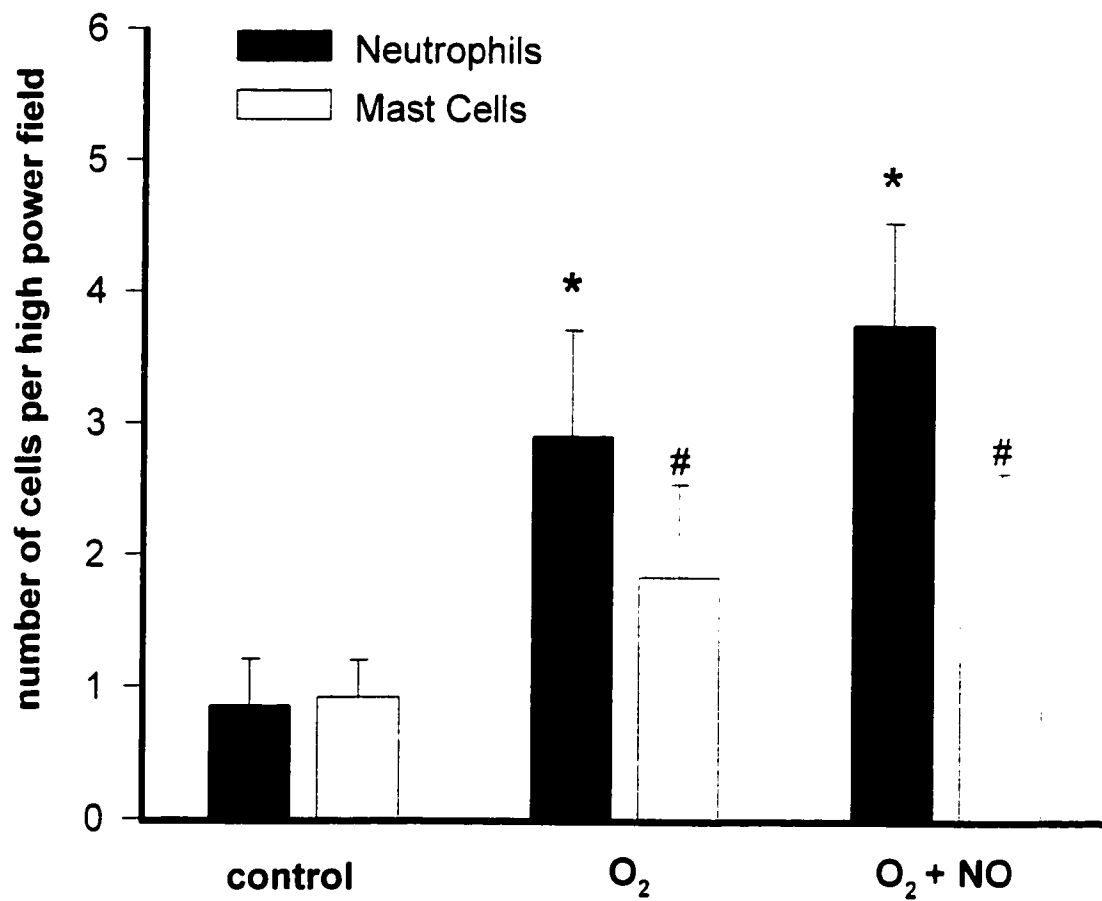
\* indicates location of perivascular edema (40x magnification).



**Figure 6: Scoring of Interstitial Edema.** Histologic lung sections from rats exposed to room air only (control, n=5), 60 h hyperoxia (O<sub>2</sub>, n=6) and 60 h hyperoxia with 20 ppm NO (O<sub>2</sub>+NO, n=4) were scored for the severity of interstitial edema. Scores ranged from 0 for no edema to 4 for severe edema. \* p<0.05 vs. control and O<sub>2</sub> + NO.

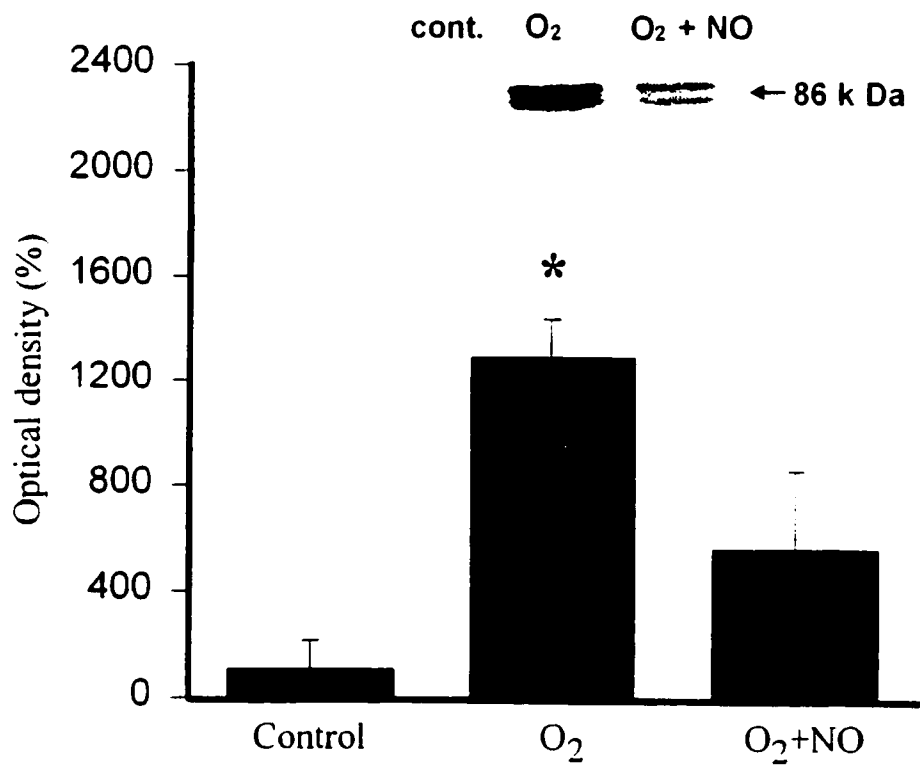


**Figure 7: Quantification of Inflammatory Cells.** Neutrophils and mast cells were counted in randomly selected fields at 250x magnification in the HPS stained sections from rats exposed to 60 h room air (control, n=5), 60 h hyperoxia (O<sub>2</sub>, n=6) and 60 h hyperoxia with NO (O<sub>2</sub>+NO, n=4). \* p <0.001 vs. control for neutrophils and p<0.05 vs. control for mast cells.



**Figure 8: Western Immunoblotting for ICAM-1 with Densitometric Analysis.**

Cytosolic extracts (20 µg protein) from the lungs of rats exposed to room air (control, n=4); 60 h hyperoxia (O<sub>2</sub>, n=5), and 60 h hyperoxia with 20 ppm NO (O<sub>2</sub>+NO, n=5) were analyzed for their content of ICAM-1 (Inset). \* p< 0.001 vs. control and O<sub>2</sub> + NO.



## **Redox Modulation**

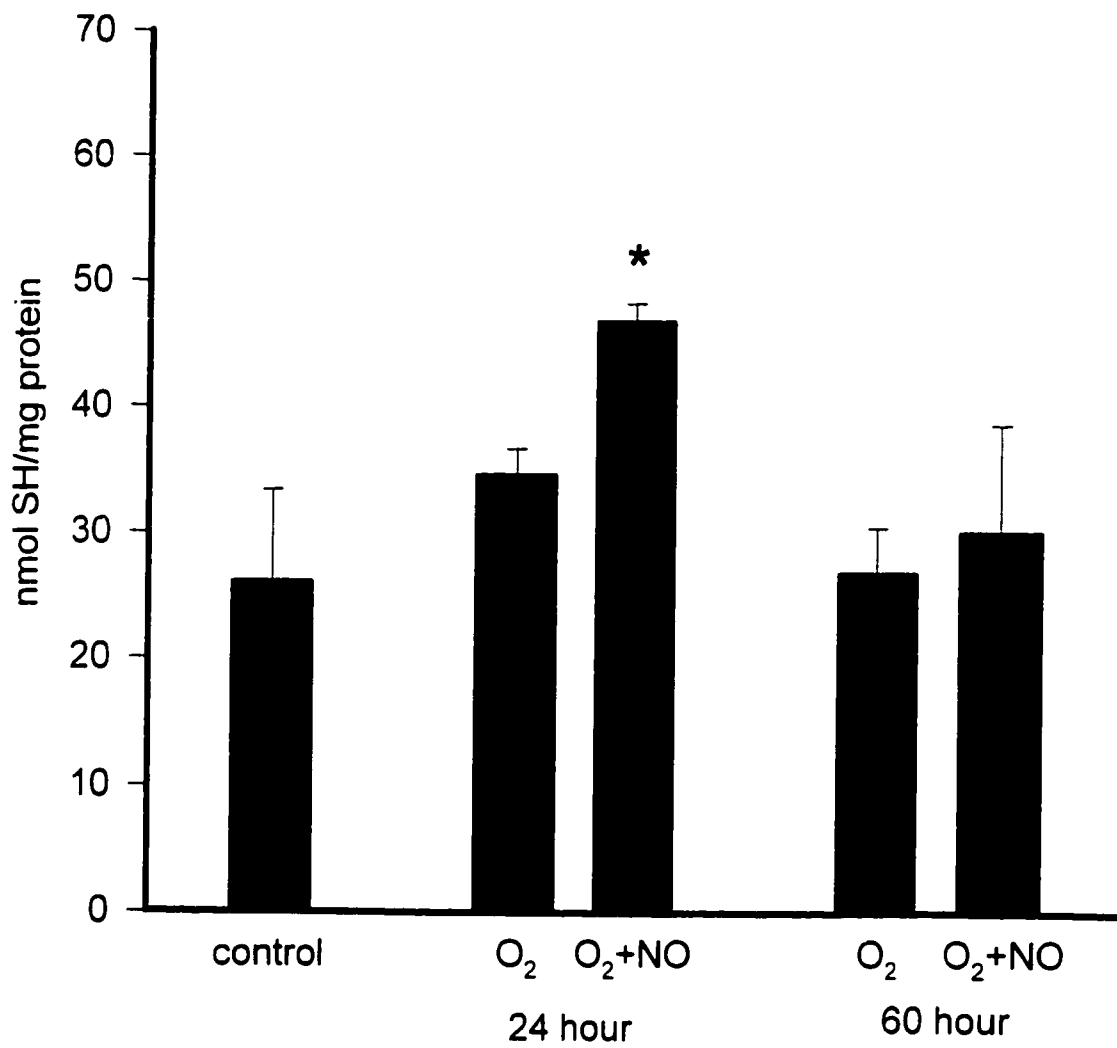
**Glutathione and Protein Sulfhydryl Content:** Exposure to either 24 or 60 h of hyperoxia alone did not result in a significant change in the cytosolic glutathione content ( $34.7 \pm 2.0$  [n=4] and  $26.9 \pm 3.5$  [n=6] nmol/mg protein, respectively) with respect to control ( $26.2 \pm 7.2$  nmol/mg protein, n=11) (Figure 9). However, the inclusion of NO resulted in a significant increase in GSH after 24 h ( $46.9 \pm 1.4$  nmol/mg protein, n=4,  $P < 0.05$ ) with respect to both control and hyperoxia (Figure 9). By 60 h, the GSH content in the hyperoxia + NO group returned to control levels ( $30.2 \pm 8.4$  nmol/mg protein, n=6).

The PSH values did not change significantly from control values ( $60.0 \pm 6.3$  nmol/mg protein, n=11) after 24 h with hyperoxia alone ( $56.0 \pm 3.2$ , n=4) or hyperoxia with NO ( $56.8 \pm 1.1$ , n=4) (Figure 10). However, at 60 h the PSH levels in the hyperoxic group were significantly lower than control ( $36.8 \pm 8.2$  nmol/mg protein, n=6) (Figure 10). The NO containing group showed a clear, but statistically non-significant, restoration of PSH at 60 h ( $43.3 \pm 10.8$  nmol/mg protein, n=6) (Figure 10).

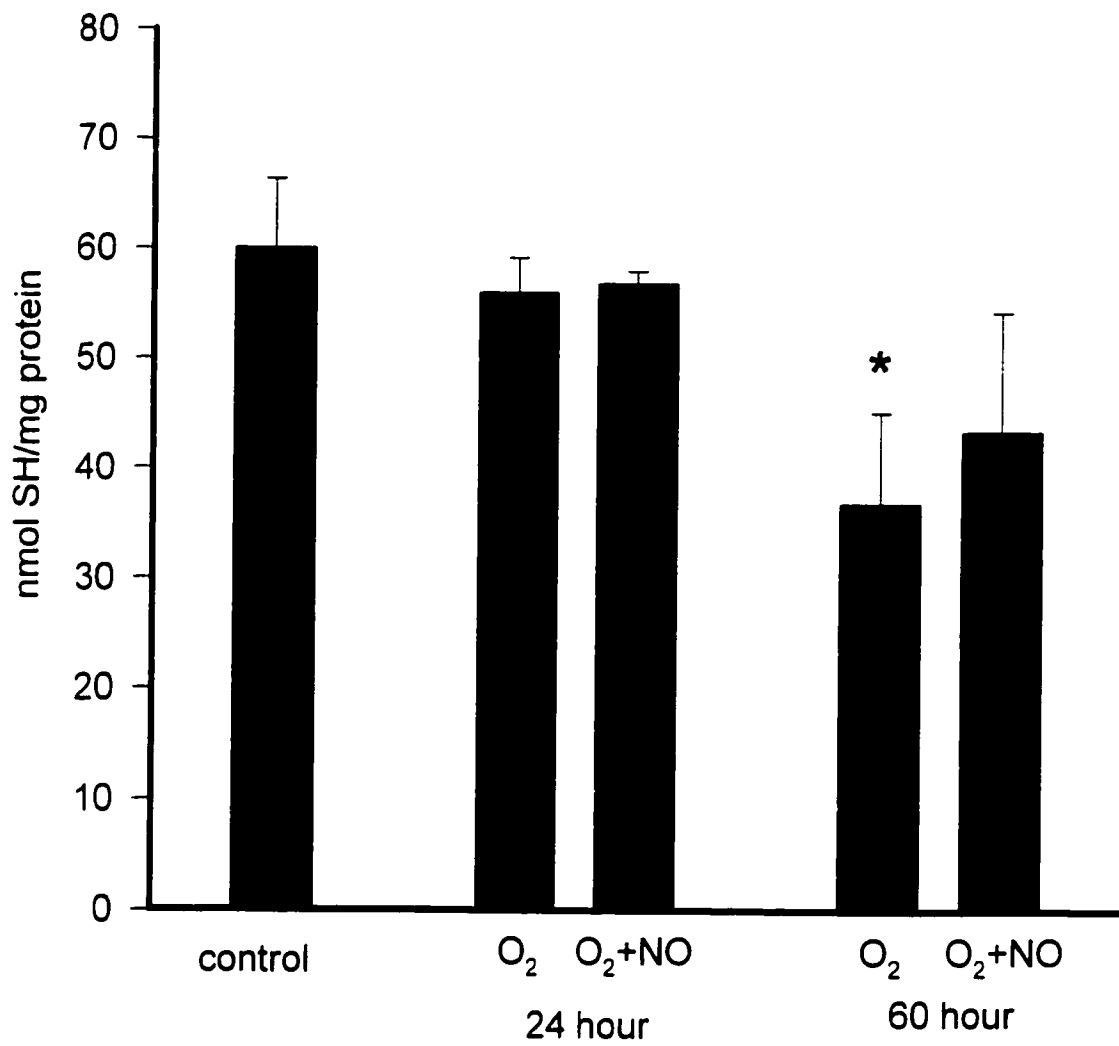
**Glutathione Reductase and Glutathione Peroxidase Activity:** After 60 h of hyperoxia, the glutathione reductase activity decreased significantly from control values ( $46.7 \pm 10.3$  vs.  $64.8 \pm 12.8$  mU/mg protein respectively, n=6), but in the

presence of NO the activity remained at control levels ( $71.5 \pm 25.3$  mU/mg protein,  $n=6$ )(Figure 11). No significant changes in the activity of glutathione peroxidase were observed after 60 h (control,  $153 \pm 14$  mU/mg protein,  $n=6$ ) in either the hyperoxia or hyperoxia + NO groups ( $131 \pm 7$  and  $132 \pm 14$  mU/mg protein respectively).

**Figure 9: Glutathione Sulfhydryl Content.** The concentration of cytosolic glutathione was measured in lung extracts from rats exposed to room air (control, n=11), hyperoxia for 24 (n=4) and 60 h (n=6) durations ( $O_2$ ), and hyperoxia with 20 ppm NO for 24 (n=4) and 60 h (n=6) durations ( $O_2+NO$ ). \* p= 0.01 vs. all other groups.

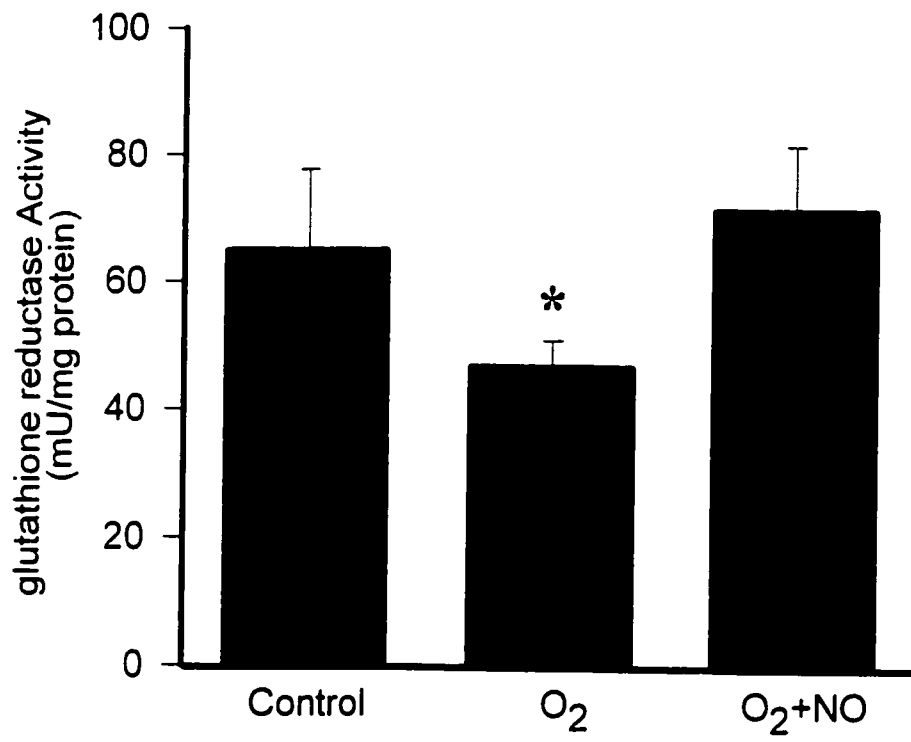


**Figure 10: Protein Sulfhydryl Content.** The concentration of cytosolic protein sulfhydryls was measured in lung extracts from rats exposed to room air (control, n=11), hyperoxia for 24 (n=4) and 60 h (n=6) durations ( $O_2$ ), and hyperoxia with 20 ppm NO for 24 (n=4) and 60 h(n=6) durations ( $O_2$ +NO). \* p= 0.02 vs all other groups.



**Figure 11: Glutathione Reductase Activity.** The enzymatic activity of glutathione reductase was determined in cytosolic extracts from rats exposed to room air (control, n=6), 60 h hyperoxia (O<sub>2</sub>, n=6), and 60 h hyperoxia with 20 ppm NO (O<sub>2</sub>+NO, n=6).

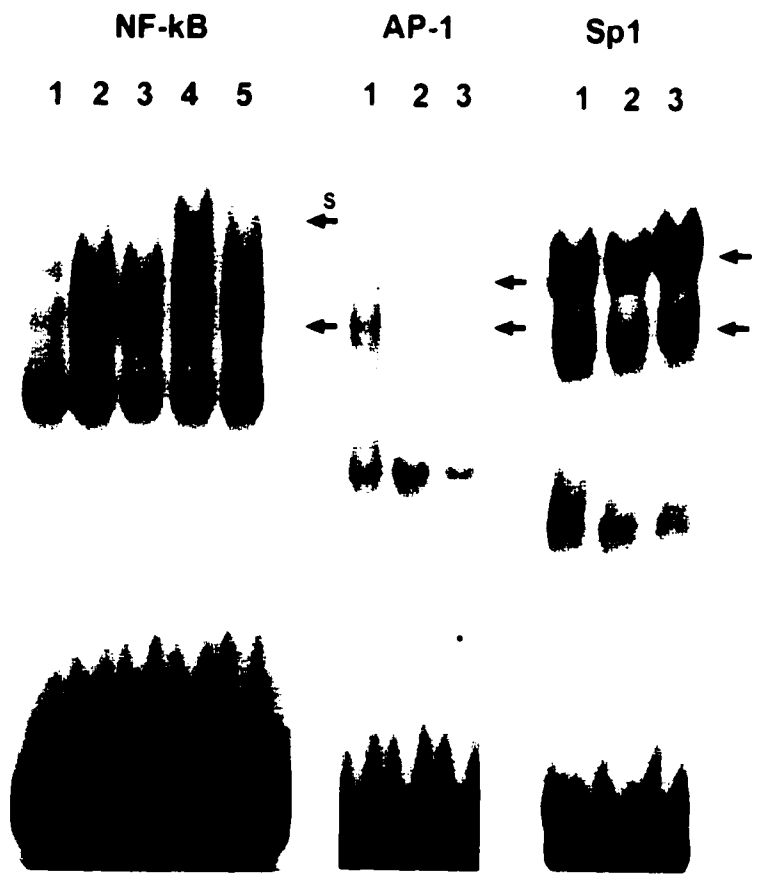
\* p < 0.05 vs control and O<sub>2</sub> + NO by unpaired T test.



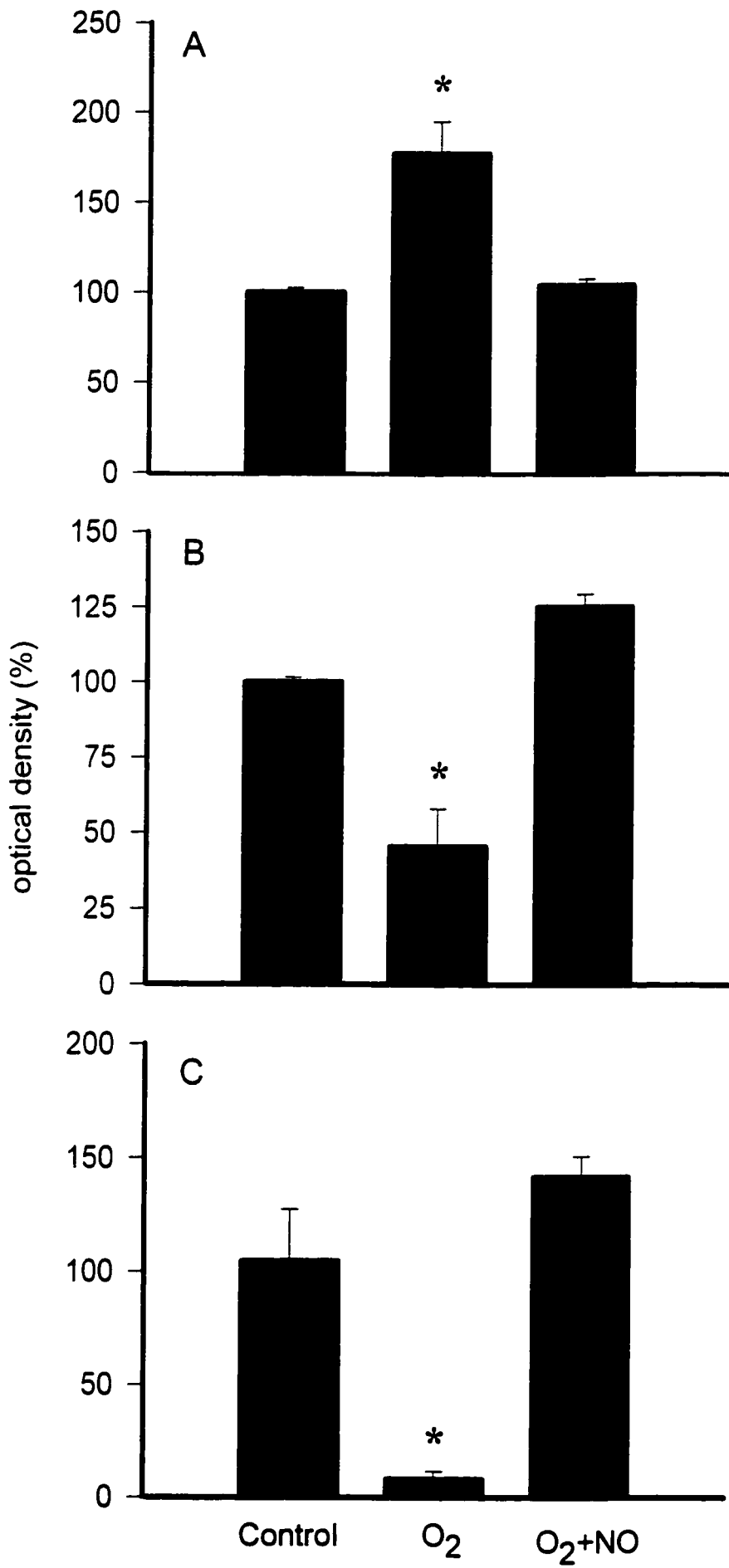
## **Transcriptional Modulation**

**EMSA:** Hyperoxia for only 24 h caused large alterations in the nuclear activity of the redox sensitive transcription factors NF- $\kappa$ B, AP-1 and Sp1 when compared to room air controls. These changes were abolished when NO was added to the hyperoxic gas (Figure 12). Supershift analysis showed that the NF- $\kappa$ B band was retarded by the inclusion in the reaction mix of the antibody to the p50 subunit, but not the antibody to the p65 subunit, suggesting that the hyperoxia-inducible isoform of NF- $\kappa$ B contained p50 but not p65 (Figure 12). Quantitative analysis of band intensity showed that NF- $\kappa$ B DNA binding activity increased significantly when compared to controls or hyperoxia + NO (n=4) (Figure 13). In contrast to the NF- $\kappa$ B changes, 24 h hyperoxia caused a significant decrease in the DNA binding activity of Sp1 when compared to control, and this decrease was blocked by the inclusion of NO (n=4) (Figures 12 and 13). The changes shown by AP-1 followed the same trend as Sp1 (n=4) (Figures 12 and 13). After 60 h of hyperoxia, the 24 h changes in the binding activity of NF- $\kappa$ B and Sp1 were no longer significantly different from room air controls or the hyperoxia + NO group. However, 60 h of exposure to hyperoxia caused a dramatic ( $548 \pm 41\%$ , n=3) increase in AP-1 binding activity with respect to control, which was not blocked by the addition of NO ( $522 \pm 47\%$ , n=3) (data not shown).

**Figure 12: Electrophoretic Mobility Shift Assay.** EMSA of the transcription factors NF- $\kappa$ B, AP-1 and Sp1 was performed with the nuclear extracts from control (Lane 1.), 24 h hyperoxia alone (Lane 2), and 24 h hyperoxia with 20 ppm NO (Lane 3). The specific bands for each transcription factor (as determined by competition with cold probe, not shown) are indicated with arrows and were selected for the densitometric analysis illustrated in Figure 13. Supershift analysis of the NF- $\kappa$ B band was performed with the antibodies to p50 (Lane 4) and p65 (Lane 5). The shifted band (s) is indicated in Lane 4.



**Figure 13: Densitometric Analysis of EMSA Bands.** The cumulative intensity of all the specific bands for each transcription factor (shown in Figure 7) was determined for NF- $\kappa$ B (Panel A), Sp1 (Panel B) and AP-1 (Panel C). \*  $p < 0.01$  vs control and O<sub>2</sub> + NO for each transcription factor. (n=3 for all groups)



## Discussion

### **Apoptosis**

This study shows for the first time that inhaled NO protects against hyperoxia-induced apoptosis in rat lung, and the data suggest that this protective effect may be occurring through the attenuation of caspase-3 activation, as well as through NO-induced modulation of redox sensitive transcription factors. The occurrence of apoptosis in the hyperoxic lungs was inferred from two lines of evidence. First, the data show that DNA fragmentation occurs in the hyperoxic lungs as demonstrated by both increased ISEL labeling and the presence of DNA “ladders” in agarose gels. These two indications of apoptotic nuclear disintegration remain the two most reliable indexes of programmed cell death. It should be noted that, since some examples of DNA fragmentation have been reported recently in apparently necrotic cells (192), such fragmentation data should be interpreted with caution (29). However, these anomolous cases remain rare and, may actually result from interrupted apoptosis (see below). Second, the constitutive level of pro-caspase-3 found in the control lungs was greatly decreased after the hyperoxic exposure. In most tissues, apoptosis is accompanied by a rapid conversion of the inactive pro-caspase-3 to the enzymatically active caspase-3 through proteolytic cleavage (102). The active subunits subsequently play a critical role in the degradative process of apoptosis. The significant decrease in the pro-caspase-3 content in our study is strongly suggestive of caspase-3 activation and provides

further support for the presence of apoptosis in our model of hyperoxia. Moreover, similar observations of ISEL positive staining and DNA laddering have recently been reported in hyperoxic lungs together with other experimental evidence of apoptosis including the upregulation of the pro-apoptotic proteins p53, p21 and Bax (37), and electron microscopic evidence of apoptotic bodies (21,29,193). Together these data provide clear and convincing evidence that hyperoxia can induce apoptosis in lungs. Although we did not determine the cell types undergoing apoptosis in the hyperoxic lungs, the data suggest that they were normal alveolar constituents, rather than invading inflammatory cells. We base this assumption on the fact that approximately 12% of the cells were apoptotic in the hyperoxic lungs whereas histologic examination showed only 1% to be leukocytes. Furthermore, other studies of hyperoxic lung injury have identified the cells undergoing apoptosis as a mixture of both microvascular endothelial and alveolar epithelial cells (21,29,37,193).

The inclusion of 20 ppm NO in the hyperoxic gas provided strong protection against injury in our model. The ability of inhaled NO to protect against hyperoxia-induced injury in vivo has been demonstrated previously (73,75) and the accumulated data suggest that only low concentrations of NO are protective while high concentrations can exert a synergistically injurious effect with hyperoxia (68,75). This study, therefore, confirms the beneficial effects of low concentrations of inhaled NO with hyperoxia, and suggests that this protection may be through the

attenuation of cell death. Inhaled NO caused a sharp decrease in apoptosis as illustrated by the dramatic decrease in ISEL-positive cells and DNA ladders. However, NO also abolished the hyperoxia-induced decrease in cytosolic pro-caspase-3 content, suggesting that it blocked the activation of caspase-3. In view of a recent report which shows that apoptotic DNA fragmentation may be triggered by active caspase-3 (106), it is possible that the NO-induced inhibition of apoptosis was caused largely by the attenuation of caspase-3 activation. Nitric oxide can inhibit caspase-3 by preventing the proteolytic processing of the pro-enzyme (161) or by blocking the active site of the activated enzyme (160,164). Since the activity of caspase-3 was not measured in this model, it cannot be assumed that no activation of caspase-3 had taken place. It is therefore possible that some caspase-3 activation had indeed occurred, and that NO may have provided protection through inactivation of this enzyme by means of S-nitrosylation of the conserved cysteine residue located in the active site (164). Further research is required to elucidate the precise mechanism of NO mediated inhibition of caspase-3 activity in this model.

The ability of NO to block apoptosis is being increasingly recognized as an important feature of this remarkable molecule. For example, a study with particular relevance to the present investigations has shown that NO can attenuate apoptosis in cultured endothelial cells exposed to a variety of pro-inflammatory agents (194). The data presented here, however, are the first to show that this apoptosis blocking

effect of NO can extend to lungs in vivo. In view of the previously demonstrated correlation between NO concentration and its effects in lungs (195), it bears emphasizing that the protection observed in this study occurred at low, clinically relevant concentrations in contrast to the cytotoxic effects of NO previously reported with the use of high concentrations of this gas, often derived from pharmacological agents (67,154).

### **Pulmonary Inflammation and Edema**

In addition to its ability to inhibit apoptosis in the present study, NO also showed significant protection against hyperoxia-associated vascular leak, as shown by the significant decrease in the development of edema. Similar evidence of NO protection against perivascular effusion have been reported in other studies (73,75,77,196). This vascular leakage, the most pronounced morphological change associated with prolonged exposure to hyperoxia, has significant implications for pulmonary function. The attenuation of alveolar and interstitial fluid congestion by the inhalation of NO could cause a marked improvement in gas exchange and clinical outcome. This protection by NO may involve several mechanisms, including the quenching of hyperoxia-generated reactive oxygen intermediates, inhibition of cytokine and adhesion molecule synthesis (197), and the attenuation of endothelial cell apoptosis (93,194). These studies are the first to show that NO provides protection against both inflammation and apoptosis. Whether this combined protection is achieved through the same signaling mechanisms and pathways

remains to be elucidated.

It is presently generally assumed that the role of apoptosis is to eliminate injured cells in a manner which avoids cell membrane rupture and the attendant release of the pro-inflammatory intracellular contents. The presence of inflammation is therefore assumed to be indicative of necrotic cell death (39), suggesting that the apoptotic and inflammatory responses in the hyperoxic lungs may have different etiologies, and that the protective effects of NO may therefore reflect the different mechanisms of protection. However, there are increasing indications that apoptosis and necrosis share a common mechanism and that necrosis occurs only when the apoptotic machinery is disrupted in some way. Therefore, to a significant extent, tissue inflammation may ensue from the interruption of apoptosis in injured cells. This interruption, which may be caused by relentlessly increasing injury or ATP depletion, may compel the cells to die necrotically (198,199), and may therefore account for the occasional observation of necrotic cells with apoptotic DNA fragmentation (192). It is therefore possible that in the initial stages of our hyperoxic lung model, injured alveolar cells begin an apoptotic mechanism which is subsequently arrested by the ever increasing oxidative stress. As the injury increases in these arrested cells, they may die by necrosis and initiate an inflammatory response. The inhibition of inflammation by NO in the hyperoxic lung may therefore stem from its effective inhibition of the onset of apoptosis and, hence, the prevention of the necrotic phase.

The data collected in the present study clearly show decreased cell death and drastically improved microvascular integrity in the animals inhaling NO along with the hyperoxic gas. The relatively mild inflammation observed in these studies and the significant protection against vascular leak offered by NO indicate that there is not likely to be any significant necrotic cell death in this injury model. Together these data suggest that the loss of microvascular integrity associated with this apoptosis may be the cause of the pronounced interstitial and alveolar edema observed in this model of lung injury.

Nitric oxide, surprisingly, did not significantly reduce the infiltration of inflammatory cells in this study. However, it is important to consider the low severity and the marginal physiological relevance of the observed inflammation when interpreting these data. Although there were greater numbers of infiltrated leukocytes cells in the hyperoxic lungs when compared to controls, the cellular inflammatory response was graded, at its worst, only as mild. Despite the statistically significant difference in inflammatory cell numbers between the groups, the physiological significance of these small numbers of cells is not clear. The absence of a substantial cellular infiltration in the lungs following 60 h of hyperoxia, a time when there is considerable apoptosis, supports the notion that apoptotic cell death is a defense mechanism which wards off inflammation. The accumulating oxidative stress associated with the continued exposure to O<sub>2</sub> causes serious inflammation by 72 h (27-29), thus supporting the theory of interrupted apoptosis

as a trigger of inflammation. The relentless build up of intracellular oxidants may have overwhelmed the apoptotic machinery resulting in cell lysis and subsequent inflammation.

### **Redox Modulation**

Hyperoxia is known to cause lung injury through the production of reactive oxygen intermediates (2,13). The intracellular redox status of the cell, therefore, is an important index of hyperoxic lung injury. The significant decrease in protein sulfhydryl content in cytosolic extracts prepared from the lungs of rats following 60 h exposure to hyperoxia is an important finding from the perspective of tissue pathology. It indicates that the oxidative stress associated with hyperoxia has caused protein modification. This could have profound implication for protein stability and enzymatic activity and may constitute one of the mechanisms of cellular injury in this model. The clear but statistically non-significant restoration of protein sulfhydryl content following the inclusion of NO in the hyperoxic gas is highly suggestive of the anti-oxidant properties of low dose inhaled NO.

Glutathione is the most important ubiquitous antioxidant defense in place to protect cells from the damaging effects of ROI (132). It is an abundant and low molecular weight molecule easily mobilized and readily available to quench oxidant radicals. The oxidation of glutathione in the cells of hyperoxic lungs could, therefore, be an important indicator of the oxidative load to those cells. In our

investigation, however, we were unable to detect any significant decrease in the intracellular content of GSH in the lungs exposed to hyperoxia. These findings are supported by the studies of Van Klaveren and co-workers who also reported no measurable depletion of GSH in lung homogenates extracted from rats in their model of hyperoxia (193). However, these results may merely reflect the efficiency of GSH replenishment. The temporary depletion of GSH, if any, may have occurred within the initial stages of the hyperoxic exposure, and the cellular compensatory mechanisms may have returned these levels to normal by 60 hr, the time the tissue was examined in these studies. The intracellular concentration of GSH represents a dynamic balance between the rate of de novo synthesis of GSH, its oxidation by ROI, and or the recycling of GSSG. During intense oxidative stress the consumption of GSH can increase significantly, causing the intracellular levels to decline temporarily. However, the recycling of oxidized glutathione (GSSG) by glutathione reductase occurs rapidly to maintain the normal concentrations of this potent reducing agent (7). Furthermore, the de novo synthesis of GSH by the enzymes glutathione synthase and gamma-glutamyl cysteine synthase can be quickly upregulated to replenish the depleted stores (132). In fact, some models of hyperoxia have been shown to increase GSH levels in the lungs (3,200), presumably as a result of overcompensation to the oxidative stress. However, in this model no significant increase in GSH in the hyperoxic lungs was observed. The enzymatic synthesis of new GSH, however, is also sensitive to negative feedback by the increasing intracellular concentrations and is dampened to prevent excessive

production (132).

As a highly reactive free radical, NO is likely to interact with numerous cellular targets, altering the intracellular redox status in the process (62,201). However, its particularly pronounced reactivity with the cysteine sulfhydryl group suggests that the protective actions of NO may be associated with alterations in the cellular thiol redox status (202). In the present investigation, the significant increase in the cytosolic glutathione levels in the 24 h NO-treated lungs is strongly suggestive of such redox modulation. Similar NO-induced increases in the levels of GSH have been observed previously (203), and raise the possibility that it may provide a mechanism by which NO blocks hyperoxia-induced thiol depletion. It is of significance that NO appeared to protect glutathione reductase from hyperoxia-induced inactivation, thereby providing additional antioxidant protection to the tissue, and possibly contributing to the elevated GSH in the NO-treated lungs. Interestingly, it has been reported that the glutathione cycle can protect cells from apoptosis induced by oxidative stress (8,129,130). Moreover, studies have shown that the overexpression of glutathione peroxidase mimics the anti-apoptotic effects of Bcl-2 (130P). The augmented expression of these enzymes has been associated with increased resistance to hyperoxic lung injury (29,204,205). However, in itself, the increase in GSH levels in the lungs is difficult to interpret. The temporal and quantitative changes in cellular GSH in response to hyperoxic stress are complex and cell specific (206). In fact, studies have reported that the antioxidant capacities

of the different lung cells vary profoundly. Furthermore, given the tight regulation of the GSH levels within the cell, there may only be a small window of opportunity to observe changes in the redox state of glutathione, and the timing of specimen collection is therefore critical in these studies. It is therefore more likely that the redox effects of NO are expressed at a more fundamental level. Redox-sensitive transcription factors, of which NF- $\kappa$ B is a prominent member, represent one such potential target of NO redox modulation, one that is currently being examined with increasing interest.

### **Transcriptional Modulation**

The remarkable sensitivity of NF- $\kappa$ B to redox modulation by oxidants in general, and NO in particular, make it a likely target for NO modulation in this model of hyperoxic lung injury. While the role of NF- $\kappa$ B in apoptotic cell death pathways remains controversial, its contribution to the development of inflammatory injury is fairly well established. NF- $\kappa$ B is widely recognized as a potent inducer of pro-inflammatory proteins (92,166,207). It is of particular relevance here that hyperoxic lung injury has previously been associated with an increase in NF- $\kappa$ B binding activity in vivo (88). Pardo and coworkers measured increased activation of NF- $\kappa$ B in rats exposed to hyperoxia and described mild inflammatory cell infiltration and extensive edema. The data collected in the present study confirm the reports of Pardo and others who have demonstrated that prolonged exposure to hyperoxia causes extensive pulmonary injury, and verify that this injury is associated with

increased nuclear binding activity of NF- $\kappa$ B. This study, however, shows for the first time that the protective effect of inhaled NO on hyperoxic lung injury is accompanied by decreased activation of NF- $\kappa$ B. Moreover, the ability of NO to prevent against the activation of this transcription factor resulted in the down-regulation of the synthesis of the adhesion molecule ICAM-1. This intercellular adhesion molecule, which appears to be intimately involved in pulmonary inflammation (208), is known to be under NF- $\kappa$ B regulation (81) and its synthesis can be induced by oxidative stress and redox reactions (209). Similar redox linked inhibition of ICAM-1 synthesis by NO in other models of injury has been observed previously (81). The modulation of this redox sensitive transcription factor presents one likely mechanism whereby NO protects against the inflammation associated with this injury.

Apoptosis is an active process requiring de novo synthesis of so called "death" proteins and consequently much attention is being focused on the identification and characterization of the transcription factors involved in this process, and the potential for therapeutic interventions. NF- $\kappa$ B is one of the primary regulators of programmed cell death, although the complex mechanisms in which it is involved are not fully understood. Despite extensive research, this transcription factor has defied all attempts to generalize its function. There is considerable evidence that NF- $\kappa$ B can both promote or inhibit apoptosis depending upon the system, cell types, and the nature of the stimulus. It seems clear that NF- $\kappa$ B is

activated in the lung following prolonged exposure to hyperoxia (88,89). Of particular relevance to the present investigation is a study by Li et al. who show that NF- $\kappa$ B is activated in alveolar epithelial cells exposed to hyperoxia and that it fails to protect against cell death (89). These data indicate that NF- $\kappa$ B does not play a cytoprotective role in this model. It should be noted that those findings were obtained from an in vitro model of transformed cells and, therefore, may not accurately reflect the cell death mechanisms of those cells in their natural environment. However, the findings of our in vivo model confirm that the DNA binding activity of NF- $\kappa$ B is increased during hyperoxic lung injury and that this increase is associated with significant apoptotic cell death.

In view of the lack of functional redundancy among the various NF- $\kappa$ B subunits, it may be important to examine which of the subunits make up the constituents of the dimer(s) that are activated. NF- $\kappa$ B is composed of homo and heterodimers of the Rel family of proteins which includes p50 and p65. It is possible that the activation of one NF- $\kappa$ B dimer might inhibit apoptosis while another could strongly promote it. The supershift assays performed in these experiments indicate that p50 is the dominant subunit activated in this model. Interestingly, the majority of the knockout and mutant studies which have demonstrated an anti-apoptotic role for NF- $\kappa$ B have modified the p65 or Rel A subunit (210,211). In contrast, p50 knockout animals do not appear to have increased sensitivity to apoptotic stimuli and, moreover, show greater resistance to inflammatory stimuli than their wild type

counterparts (211). A recent study of focal cerebral ischemia in mice revealed that p50 knockouts were much more resistant to ischemic damage and cell death than wild type animals (144). The knockouts sustained markedly reduced DNA fragmentation and nuclear condensation, supporting that the p50 subunit of NF- $\kappa$ B promotes apoptosis in that model (144). The regulation of gene expression by NF- $\kappa$ B and its effects on the ultimate fate of the cells are far too complex to be explained by the simple composition of this transcription factor. Rather, the implications of NF- $\kappa$ B activation depend heavily upon the availability of required co-factors and on the relative levels of other cellular signals. Further research is required to identify which genes are induced by NF- $\kappa$ B in hyperoxic lung injury and what the effects of those new proteins are.

Interestingly, the apoptosis blocking effect of NO seems to be associated with NF- $\kappa$ B. In an in vivo model of cardiac ischemia and reperfusion, Kupatt and coworkers showed that NO protects against endothelial cell injury and showed a strong correlation between decreased NF- $\kappa$ B activation and the attenuation of apoptosis in that model (81). Our in vivo data provide strong support for these observations. The attenuation of apoptosis observed in our lung injury model was accompanied by large decrease in the activation of NF- $\kappa$ B when low dose inhaled NO was included in hyperoxic gas. The experimental evidence that NF- $\kappa$ B can promote apoptotic cell death is becoming increasingly sound and it is therefore reasonable to anticipate an attenuation of apoptosis through the inhibition of NF-

$\kappa$ B activation. These data strongly suggest that the modulation of NF- $\kappa$ B by NO is an important mechanism of its protection against pulmonary apoptosis. Together, these findings indicate that hyperoxic lung injury increases the expression of NF- $\kappa$ B-modulated genes, possibly those involved in inflammation and apoptosis, and that NO can reverse these effects by inactivating NF- $\kappa$ B. The fact that ICAM-1 was found to be greatly increased in our hyperoxic lungs, and that this effect was greatly inhibited by NO, support this hypothesis.

Interesting changes in the nuclear activity of the redox-sensitive transcription factor AP-1 was also observed. AP-1 is known to regulate the expression of numerous genes (212) and has generally been shown to be pro-apoptotic (213). It is of particular relevance here that AP-1 is responsive to redox modulation (185,214), and its upregulation has been linked in vitro with oxidant-induced apoptosis in lung epithelial cells (215). Our data show that the activation of AP-1 is significantly decreased in the hyperoxic lungs at 24 h and that this effect was reversed by NO. These data suggest that hyperoxia-induced pulmonary apoptosis is accompanied by the early down regulation of this transcription factors from their normal constitutive levels. The redox regulation of this transcription factor dictates that the conserved cysteine residues in the Jun and Fos subunits of AP-1 must be reduced in order for it to actively bind DNA, and that oxidation of those thiols results in a loss of binding activity (167,185). However, following relentless exposure to oxidative stress, AP-1 binding activity can be stimulated by MAP kinase dependant

pathways (151,187). In light of these findings, it is reasonable to expect the activity of AP-1 to be inhibited during the early hyperoxic stress, and that this inhibition may be prevented by the inclusion of NO in the hyperoxic gas because of the ability of NO to lessen the oxidative load to the cells. The significance of this NO-induced restoration of AP-1 activity remains to be elucidated but it may merely reflect the ability of NO to block the generation of transcription factor modulating ROI in the cell at this early time point. This suggests once again that redox-dependent alterations in transcriptional regulation may underlie the protective effects of NO. The dramatic increase in the nuclear activity of AP-1 detected following 60 hr of hyperoxia was not altered by the addition of NO. The intense oxidative stress at this late time point may have exceeded the antioxidant capacity of NO.

The nuclear activity of Sp1 was also significantly reduced following 24 h exposure to hyperoxia. This transcription factor is known to be involved in the regulation of a number of genes, many with constitutive housekeeping roles. Although it has been described as a redox-sensitive protein, its role in the pathology of oxidative injury is not known. Hence, the significance of the early decline in its constitutive activity level and the NO-induced restoration are not clear. In the absence of additional information, it is difficult to predict which AP-1 and Sp1 inducible genes may be involved in the hyperoxia induced apoptosis.

In summary, these studies confirm that hyperoxia can cause pronounced vascular leak and apoptosis in rat lungs, and show for the first time that inhaled NO can reverse both these effects at the clinically relevant low concentration of 20 ppm. These data further suggest that the protective effect of NO is attributable to its redox-modulating properties, and that it may exert these effects by inhibiting the activation of caspase-3 or by altering the activity of redox-sensitive transcription factors. These findings therefore provide a possible mechanism for the previously observed ameliorative effects of inhaled NO, when administered in conjunction with hyperoxia, and should therefore provide a platform for future investigations of the molecular mechanisms involved.

These findings outline an exciting new protective role for inhaled NO which has great clinical relevance. The inhibition of hyperoxia-induced apoptosis in the lung significantly protects against the loss of microvascular and alveolar integrity. These protective effects can potentially lead to improvements in pulmonary function and matching of ventilation to perfusion and improve the overall outcome of patients with pulmonary hypertension and ARDS. The use of low dose inhaled NO may allow doctors to reduce the exposure of their patients to high concentrations of oxygen and moreover, to protect the lungs against hyperoxia-induced injury during the course of treatment. The data collected in this study provides important scientific information regarding the biochemical and molecular effects of a clinically relevant concentration (20 ppm) of inhaled NO in a model of lung injury. This study

will help clinicians to make informed decisions regarding the use of inhaled NO in the care of their patients.

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