Inhaled nitric oxide protects against hyperoxia-induced apoptosis in rat lungs

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Howlett, Clare E., J ames S. Hutchison, J ohn P. Veinot, Aaron Chiu, Pradeep Merchant, and Henry Fliss. Inhaled nitric oxide protects against hyperoxia-induced apoptosis in rat lungs. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L596–L605, 1999.—Inhaled nitric oxide (NO), frequently administered in combination with hyperoxic gas mixtures, was recently shown to protect against the injurious consequences of prolonged hyperoxia. We investigated the possibility that this protective effect is attributable to the ability of NO to block pulmonary apoptosis. We show that rats exposed to 100% O2 for 60 h develop severe lung injury consisting of pronounced vascular leak and alveolar apoptosis as inferred from the presence of positive terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling and DNA ladders in agarse gels and a decrease in constitutive pro-caspase-3 levels. However, the inclusion of NO (20 parts/million) 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 nuclear factor-κB, activator protein-1, and Sp1 after 24 h, lowered intercellular adhesion molecule-1 levels, and increased glutathione content. We therefore show for the first time, that NO can protect against both hyperoxia-induced apoptosis and inflammation. The data suggest that this protection may occur at the transcriptional and caspase-activation levels.

DNA fragmentation; nuclear factor-κB; activator protein-1; Sp1; intercellular adhesion molecule-1; caspase-3

HYPEROXIC GAS MIXTURES are frequently administered to mechanically ventilated, critically ill patients with pulmonary hypertension and acute respiratory distress syndrome to increase oxygen tension in arterial blood and tissues. Inhaled nitric oxide (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 (15, 24). However, both hyperoxia and NO have the potential to cause substantial cellular damage. Prolonged inhalation of hyperoxic air can cause serious lung injury (9), primarily as a result of the production of reactive oxygen intermediates (40), whereas NO is itself a highly reactive free radical with significant toxic potential (46). Consequently, much attention is presently being focused on the possibility that NO may exacerbate hyperoxic lung injury (33). However, recent studies have also shown that, when administered at low concentrations [10–20 parts/million (ppm)], NO has a surprisingly protective effect against hyperoxic pulmonary injury (32).

The precise mechanisms by which inhaled 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 O2 toxicity (32). The anti-inflammatory effects may be attributable to the ability of NO to inhibit the vascular adhesion and transmigration of leukocytes (5), possibly by blocking the synthesis of inflammatory cytokines (45) or cell surface adhesion molecules such as intercellular adhesion molecule (ICAM)-1 (25). Recent studies (34, 47) have suggested that hyperoxia can cause apoptosis in lungs. Because NO can protect against apoptosis caused by proinflammatory agents in cultured endothelial cells (43), 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 (23).

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 (31). Pulmonary inflammation (26) and hyperoxic lung injury (35) are both associated with an increase in the activity of the redox-modulated transcription factor nuclear factor-κB (NF-κB). NO is capable of inhibiting the nuclear activity of NF-κB (25, 31), and because the genes for several proinflammatory cytokines and adhesion receptors contain binding sites for NF-κB in their promoter regions (29), this inhibition may attenuate the inflammatory response and its attendant cell injury. Moreover, because hyperoxia-induced apoptosis is associated with an increase in NF-κB activity in vivo (35), 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 the present 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 proapoptotic enzyme caspase-3, and redox-sensitive transcription factors in this process. We show, for the
first time, that NO can attenuate hypoxia-induced apoptosis in rat lungs and that this protection is associated with an inhibition of the activation of caspase-3 and NF-κB.

METHODS

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 exposure to the experimental gases. Male Sprague-Dawley rats weighing 250–300 g (Charles River, St-Constante, PQ) were placed in plastic chambers with free access to food and water. O2 (100%) and NO (Vitalaire Health Care, Ottawa, ON) were mixed immediately before entry into the chambers and were delivered through a humidified circuit at a 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 concentration of nitrogen dioxide (NO2). O2, NO, and NO2 concentrations were monitored continuously by means of in-line analyzers at the outlet of the chambers (Pac II, Drager, Lubeck, Germany; Miniox, Catalyst Research, Owings Mills, MD). O2 concentration in the hyperoxic groups was stable at 97.0 ± 1.1%, and the mean NO and NO2 concentrations were 20.7 ± 0.7 and 0.3 ± 0.05% ppm, respectively. Six experimental groups of rats were used. Three groups inhaled either 1) >95% O2, 2) >95% O2 plus 20 ppm NO, or 3) room air for only 24 h. The remaining three groups inhaled the same gas mixtures for 60 h. After treatment, the animals were immediately killed under pentobarbital sodium anesthesia, and the lungs were harvested in one of two ways. 1) To measure intracellular biochemical parameters, the residual vascular blood was first removed by perfusing the lungs with 50 ml of cold phosphate-buffered saline (PBS) through a cannula placed through the right ventricle into the pulmonary artery. The lungs were then excised rapidly, chilled on ice, and processed for nuclear and cytosolic fractions as described in Western blot and Electrophoretic mobility shift assay. 2) For histological examination, the lungs were fixed by intratracheal instillation of buffered 10% formalin at 20 cmH2O pressure, paraffin embedded, and cut into 4-µm midcoronal sections.

Lung histology. Lung coronal sections were stained with hematoxylin-phloxin-saffron (HPS) or toluidine blue. All histological evaluations were performed by a pathologist blinded to the experimental groups. HPS-stained slides were scored for perivascular and interstitial edema on a scale of 0 (no edema) to 4 (severe edema). Neutrophils and macrophages were counted in 20 randomly selected fields in each of the HPS-stained sections with ×250 magnification. Only 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 with the toluidine blue-stained sections. Peribronchial and bronchial mast cells of the larger bronchi were excluded.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling. Lung coronal sections were double labeled with the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) protocol as well as with Hoechst 33258, a general nuclear stain, for the visualization of fragmented DNA in apoptotic cells as previously described by Fliss and Gattinger (12). Positive controls for the TUNEL test were prepared by incubating lung sections with DNase (12). TUNEL- and Hoechst-stained cells in the alveolar walls were visualized with a Zeiss Axiophot fluorescence microscope and counted in each experimental group in a blinded fashion at ×400 magnification. Apoptotic cells were counted in 20 random fields from the left lung of each rat and are expressed as a percentage of the total lung cells in the corresponding fields.

Agarose gel electrophoresis of DNA. Fragmented DNA was extracted from perfused lungs with phenol-chloroform, subjected to electrophoresis on 1.5% agarose gels, and stained with ethidium bromide as previously described by Fliss and Gattinger (12).

Western blot. The content of caspase-3 procenzyme and ICAM-1 in the lung tissues was determined with standard immunoblotting techniques. The lung tissue was homogenized on ice for 45 s with a Polytron homogenizer at 10,000 rpm in 8 volumes of 10 mM HEPES (pH 7.9) containing 10 mM KCl, 1.5 mM MgCl2, 0.1% Nonidet P-40, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM spermidine, 0.15 mM spermine, 5 µg/ml of leupeptin, 5 µg/ml of aprotinin, and 10% glycerol. The homogenate was incubated on ice with 5 µg of poly(dI-dC) (Boehringer Mannheim, Montreal, PQ) or ICAM-1 (Cedarlane Laboratories, Hornby, ON) followed by goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA). Protein band chemiluminescence was visualized on film according to the manufacturer's instructions (NEN Life Science Products, Boston, MA) and quantified with a densitometer and Molecular Analyst software (Bio-Rad Laboratories).

Electrophoretic mobility shift assay. Nuclear pellets were obtained from lung tissue with the aid of sucrose gradient centrifugation with modification of a previously published protocol (27). Briefly, 400 mg of lung tissue were homogenized on ice with six slow strokes of a Teflon pestle homogenizer at 1,000 rpm in eight volumes of buffer containing 0.5 M sucrose, 10 mM HEPES, pH 7.6, 25 mM KCl, 1 mM EDTA, 10% glycerol, 0.15 M 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 and 0.5 mM spermidine. The homogenate was centrifuged at 100,000 g at 4°C for 1 h. The supernatant was discarded, and the pellet nuclei were gently resuspended in 40 µl of a lysis buffer containing 20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF, 0.5 mM spermidine, 0.15 mM spermine, and 5 µg/ml each of aprotinin, leupeptin, and pepstatin. The suspension was incubated on ice for 45 min and centrifuged at 20,000 g at 4°C for 10 min. Supernatant containing the nuclear protein 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 each of aprotinin, leupeptin, and pepstatin. Protein concentrations were determined with the Bio-Rad protein assay.

For the electrophoretic mobility shift assay, double-stranded consensus oligonucleotides for NF-κB, activator protein (AP)-1, and Sp1 (Promega, Madison, WI) were radiolabeled with γ-32P]ATP (Amersham, Arlington Heights, IL). Five micrograms of nuclear protein were first incubated for 10 min at room temperature with 5 µg of poly(dl-dC) (Boehringer Mannheim, Montreal, PQ) in DNA binding buffer (20 mM HEPES, pH 7.9, 0.2 mM EDTA, 0.2 mM EGTA, 100 mM KCl, 5% glycerol, and 2 mM DTT). Labeled probe (0.2 ng) was then added, and the reaction mixture was incubated for an additional 20 min in a final volume of 20 µl. The reaction...
mixture was subjected to electrophoresis on a 5% polyacrylamide gel, and the dried gel was exposed to X-ray film. The intensity of the bands was quantitated with a densitometer and commercially available software (Molecular Analyst, Bio-Rad Laboratories). The subunit composition of NF-κB was determined with supershift assays. Antibodies (2 µg) to either p50 or p65 (Santa Cruz Biotechnology, Santa Cruz, CA) were added to the incubation mixture and incubated for 20 min before the addition of poly(dI-dC).

Determination of glutathione and protein sulfhydryl contents. Freshly isolated lungs were homogenized on ice as in Western blot in two volumes of cold PBS, and the homogenate was centrifuged at 35,000 g for 15 min at 4°C. The glutathione (GSH) and protein sulfhydryl (PSH) contents in the supernatant were determined with 5,5′-dithio-bis(2-nitrobenzoic acid) as previously described by Fliss and Ménard (13).

Enzyme assays. GSH peroxidase and GSH reductase activities were measured in lung extracts as previously described by Fliss and Ménard (13).

Statistical analysis. All data are means ± SD of measurements from 3 to 7 individual rats from each treatment group. Differences between the groups were analyzed with one-way analysis of variance and post hoc Tukey’s test. Values of P < 0.05 were considered to be significant.

RESULTS

Effects of hyperoxia and NO on histology. Examination of lung sections at the end of 60 h of hyperoxia revealed pronounced perivascular and interstitial edema (Fig. 1). Analysis of the edema scores showed them to be significant compared with those in control lungs (data not shown). The inclusion of NO in the hyperoxic gas significantly lowered the edema scores compared with hyperoxic levels (P = 0.02). Three of the six lungs examined after 60 h of hyperoxia showed alveolar changes indicative of diffuse alveolar damage. None of the lungs in the control or hyperoxia plus NO group showed evidence of diffuse alveolar damage. Hyperoxia also produced features of mild inflammation (n = 6), with a small but significant increase in neutrophils and mast cells compared with room air control lungs (n = 5). Inclusion of NO in the hyperoxic gas mixture (n = 4) did not attenuate the number of inflammatory cells (data not shown). No obvious alveolar inflammation or injury was observed in any of the groups after only 24 h.

TUNEL. In situ end labeling of fragmented DNA in lung sections from rats exposed to 60 h of hyperoxia revealed numerous labeled nuclei in the alveolar cells, whereas control lungs showed very few or no TUNEL-positive nuclei (Fig. 2). The inclusion of NO in the hyperoxic gas mixture lowered the number of observed TUNEL-positive nuclei (Fig. 2). Quantitative analysis of the percent labeled nuclei showed a significant increase after 60 h of hyperoxia compared with that in room air control lungs (Fig. 3). However, the presence of NO in the hyperoxic air significantly lowered the percentage of TUNEL-positive cells (Fig. 3). No TUNEL-positive cells were detected in any of the experimental groups after only 24 h (data not shown).

Agarose gel electrophoresis of DNA. DNA extracted from the lungs of rats exposed to 60 h of hyperoxia showed the “laddering” typical of apoptotic cells, whereas lungs from rats exposed to room air showed no ladder (Fig. 4). DNA from rats treated with NO plus hyperoxia showed greatly diminished laddering compared with that in the hyperoxia group (Fig. 4).
was no detectable laddering in any of the experimental groups after only 24 h (data not shown).

Western blot. High constitutive levels of the 32-kDa procaspase-3 were detected in cytosolic extracts from control rat lungs. Hyperoxia for 60 h caused a decline in the level of this protein, whereas the addition of NO to the hyperoxic gas mixture prevented this decline (Fig. 5, inset). Quantitative analysis of the relative intensities of these immunoblot bands showed a significant decrease in procaspase-3 levels in the 60-h hyperoxic lungs compared with that in the control lungs (Fig. 5). The inclusion of NO significantly protected against this decrease (Fig. 5). No significant changes in the intensity of the 32-kDa band were observed in the hyperoxia or hyperoxia plus NO group after only 24 h (data not shown). The content of ICAM-1 increased dramatically in lung tissue after 60 h of hyperoxia compared with that in control lungs, but the presence of NO in the
Hyperoxic gas significantly attenuated this increase (Fig. 6).

Electrophoretic mobility shift assay. Hyperoxia for only 24 h caused large alterations in the nuclear activity of the redox-sensitive transcription factors NF-κB, AP-1, and Sp1 compared with those in room air control lungs. These changes were abolished when NO was added to the hyperoxic gas (Fig. 7). Supershift analysis showed that the NF-κB band was retarded by inclusion in the reaction mixture of the antibody to the p50 subunit but not to the antibody to the p65 subunit, suggesting that the hyperoxia-inducible isoform of NF-κB contained p50 but not p65 (Fig. 7). Quantitative analysis of band intensity showed that NF-κB activity increased significantly compared with that in the control or hyperoxia plus NO group (Fig. 8). In contrast, 24 h of hyperoxia caused a significant decrease in the nuclear activity of Sp1 compared with that in the control group, and this decrease was blocked by the inclusion of NO (Fig. 8). The changes shown with AP-1 followed the same trend as those with Sp1 (Fig. 8). After 60 h of hyperoxia, the 24-h changes in the binding activity of NF-κB and Sp1 were reversed and were no longer significantly different from those in the room air control or hyperoxia plus NO group (data not shown). However, 60 h of exposure to hyperoxia caused a dramatic increase (548 ± 41%) in AP-1 binding activity with respect to control. This increase was not blocked by the addition of NO (522 ± 47%).

GSH and PSH contents. Exposure to either 24 or 60 h of hyperoxia did not result in a significant change in the cytosolic GSH content (34.7 ± 1.2 and 26.9 ± 1.4 nmol/mg protein, respectively; n = 6) with respect to control value (26.2 ± 2.2 nmol/mg protein; n = 6). However, the inclusion of NO resulted in a significant increase in GSH after 24 h (46.9 ± 0.8 nmol/mg protein; n = 6; P < 0.05) with respect to both the control and hyperoxic values. By 60 h, the GSH content in the hyperoxia plus NO group returned to control levels (30.2 ± 3.5 nmol/mg protein; n = 6). PSH values did not change significantly from the control values (60.6 ± 1.9 nmol/mg protein; n = 4) after 24 h with either hyperoxia or NO. However, at 60 h, PSH in the hyperoxic group (36.8 ± 3.4 nmol/mg protein; n = 6) was significantly lower than that in the control group (58.0 ± 3.1 nmol/mg protein; n = 6; P < 0.05). The NO group showed a pronounced but not significant protection of PSH at 60 h (43.3 ± 4.4 nmol/mg protein; n = 6).

Enzyme assays. After 60 h of hyperoxia, the GSH reductase activity decreased significantly from the control values, but in the presence of NO, the activity remained at the control level (Fig. 9). No significant changes in the activity of GSH peroxidase were observed after 60 h (control, 153 ± 14 mU/mg protein; n = 6) in either the hyperoxic or NO group (131 ± 7 and 132 ± 14 mU/mg protein, respectively).

DISCUSSION

Our study shows, for the first time, that inhaled NO protects against hyperoxia-induced apoptosis and sug-
gests that the 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, we show that DNA fragmentation occurs in the hyperoxic lungs as demonstrated by both increased TUNEL-positive cells and the presence of DNA ladders in agarose gels. These two hallmarks of apoptotic nuclear disintegration remain the two most reliable indexes of programmed cell death. It should be noted that because some examples of DNA fragmentation have been reported recently in apparently necrotic cells (16), such fragmentation data should be interpreted with caution (47). However, those anomalous cases remain rare and, moreover, may actually result from interrupted apoptosis (see below). Second, we show that the constitutive level of procaspase-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 procaspase-3 to the enzymatically active caspase-3 through proteolytic cleavage (7). The active caspase subsequently plays a critical role in the degradative processes of apoptosis. The significant decrease in the procaspase-3 content in our study is therefore strongly suggestive of caspase-3 activation and provides further support for the presence of apoptosis in our model of hyperoxia. Additional support is provided by the fact that apoptosis (TUNEL-positive cells) was observed recently in other in vivo studies of hyperoxic rat lungs (21, 34) and that oxidants can cause apoptosis in cultured lung epithelial cells (19). It therefore appears very likely 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 ~12% of the cells were apoptotic in the hyperoxic lungs, whereas histological examination showed only 1% to be leukocytes.

The inclusion of NO at 20 ppm in the hyperoxic gas mixture provided strong protection against injury in our model. A similar ability of inhaled NO to attenuate
hyperoxia-induced injury in vivo has been previously demonstrated (32), and the accumulated data suggest that only low concentrations of NO are protective in vivo, whereas high concentrations can exert a synergistically injurious effect with hyperoxia (38). Our study therefore confirms the beneficial effect of low concentrations of inhaled NO with hyperoxia and suggests that this protective action may be attributable to the attenuation of programmed cell death. In our studies, NO caused a sharp decrease in apoptosis as illustrated by the dramatic decrease in TUNEL-positive cells and DNA ladders. However, NO also abolished the hyperoxia-induced decrease in cytosolic procaspase-3 content, suggesting that it blocked the activation of caspase-3. In view of a recent report (10) that showed that apoptotic DNA fragmentation may be triggered by active caspase-3, it is possible that the NO-induced inhibition of apoptosis was caused largely by the attenuation of caspase-3 activation. However, because we did not measure caspase-3 activity in this model, we are unable to demonstrate that no activation of caspase-3 had taken place. It is therefore also 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 (23).

The ability of NO to modulate apoptosis is being increasingly recognized as an important feature of this remarkable molecule. For example, a study (43) with particular relevance to the present investigation has shown that NO can attenuate apoptosis in cultured endothelial cells exposed to a variety of proinflammatory agents. To the best of our knowledge, our data 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 (39), it bears emphasizing that the protection observed in our study occurred at low, clinically relevant concentrations. In contrast, a previous study (33) that found significant toxicity with NO was done with higher concentrations of this gas, often derived from pharmacological agents.

In addition to its ability to inhibit apoptosis in our study, NO also provided significant protection against hyperoxia-associated vascular leak as shown by the significant decrease in the development of edema. This protection by NO may involve several mechanisms, including the quenching of hyperoxia-generated reactive oxygen intermediates, inhibition of cytokine and adhesion molecule synthesis (6), and the attenuation of endothelial apoptosis (43). We therefore show, for the first time, 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

![Fig. 8. Densitometric analysis of EMSA bands from 24-h control, O2, and O2+NO lungs (n = 3 rats). Cumulative intensity of all specific bands for each transcription factor (see Fig. 7) was determined for NF-κB (A), Sp1 (B), and AP-1 (C) as described in METHODS. NF-κB was significantly increased after 24-h exposure to O2 compared with control and O2+NO groups. Both AP-1 and Sp1 show significant decreases in binding activity after 24-h exposure to O2 compared with control and O2+NO groups. *P < 0.05 vs. control and O2+NO.](http://ajplung.physiology.org/)

![Fig. 9. Glutathione reductase activity in response to O2 and NO. Lung homogenates (n = 6 rats) were assayed as described in METHODS. *P < 0.05 vs. control and O2+NO groups.](http://ajplung.physiology.org/)
is presently generally assumed that the role of apoptosis is to eliminate injured cells in a manner that avoids cell membrane rupture and the attendant release of the proinflammatory intracellular contents. The presence of inflammation is therefore assumed to be indicative of necrotic cell death (30), 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 different mechanisms of protection. However, there are increasing indications that, to a significant extent, tissue inflammation may ensue from the interruption of apoptosis in injured cells. Such interruption, which may be caused by relentlessly increasing injury or ATP depletion, may compel the cells to die necrotically (11, 12) and may account for the occasional observation of necrotic cells with apoptotic DNA fragmentation (16). It is therefore possible that in the initial stages of our hyperoxic lung model, injured alveolar cells begin an apoptotic mechanism that is subsequently blocked by the continuously accumulating oxidative injury. The inhibition of inflammation by NO in the hyperoxic lung may therefore stem from its effective inhibition of apoptosis.

As a highly reactive free radical, NO is likely to interact with numerous cellular targets, altering the intracellular redox status in the process (14). 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 (22). In our studies, the significant increase in the cytosolic GSH levels in the 24-h NO-treated lungs is strongly suggestive of such redox modulation. Similar NO-induced increases in the levels of GSH, an important cellular oxidant scavenging agent, have been observed before (48) 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 GSH reducatase from hyperoxia-induced inactivation, thereby providing additional antioxidant protection to the tissue and possibly contributing to the elevated GSH in the NO-treated lungs. 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 (20). In fact, hyperoxia alone is known to increase GSH levels in the lungs (8), although in our model, we did not observe a significant increase in GSH in the hyperoxic lungs. 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-κB is a prominent member, represent one such potential target of NO redox modulation, one that is currently being examined with increasing interest.

NF-κB is normally located in the cytosol complexed with its inhibitor IκB (3). On activation, IκB is degraded proteolytically, freeing NF-κB to translocate to the nucleus. NF-κB is known to regulate a wide variety of genes involved in immune function and inflammation. However, recent data suggest that it is also directly involved in the regulation of apoptosis (1). It is remarkably sensitive to redox modulation by oxidants in general and NO in particular, presumably at the site of essential sulfhydryls (44) and therefore represents a particularly likely target for NO modulation in our hyperoxic lung model. The role of NF-κB in apoptosis remains controversial. In some tissues, it appears to prevent apoptosis (4), whereas in others, it plays a proapoptotic role (28). It is of particular relevance here that hyperoxic injury has previously been shown to be associated with an increase in NF-κB activity in vivo (35). Moreover, the apoptosis-blocking effect of NO appears to be linked to the inhibition of NF-κB (25). Our in vivo data provide strong support for these observations. We detected a large increase in NF-κB activity in the hyperoxic lungs that was significantly lowered by the addition of NO.

Our findings therefore suggest that hyperoxic lung injury increases the expression of NF-κB-modulated genes, possibly those involved in inflammation and apoptosis, and that NO can reverse this effect by inactivating NF-κB. The fact that ICAM-1 was found to be greatly increased in our hyperoxic lungs and that this effect was greatly inhibited by NO supports this hypothesis. ICAM-1 is a cell adhesion molecule intimately involved in pulmonary inflammation (17). It is known to be under NF-κB regulation (25) and can be induced by oxidative stress and redox reactions (2). The increased expression of this protein by hyperoxia and its inhibition by NO in our study is therefore strongly suggestive of the involvement of redox-modulated NF-κB. Similar redox-linked inhibition of ICAM-1 synthesis by NO in other models of injury has been previously observed (25).

We also observed interesting changes in the nuclear activity of two other transcription factors, AP-1 and Sp1. AP-1 is known to regulate the expression of numerous genes (42) and has generally been shown to be proapoptotic (18). It is of particular relevance here that AP-1 is responsive to redox modulation (36), and its upregulation has been linked to transition-induction apoptosis in lung epithelial cells (19). Interestingly, NO-generating agents were shown to cause apoptosis and a concomitant increase in AP-1 activity (19). However, the concentration of NO may have been high in this in vitro model. Sp1, another redox-sensitive transcription factor (41), is also known to have a link to apoptosis (37). Our data surprisingly show that the activity of both AP-1 and Sp1 was decreased significantly 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 downregulation of these transcription factors from their normal constitutive levels. NO, as part of its antiapoptotic effect, reversed this decline in activity, suggesting once again that redox-dependent alterations in transcriptional regulation may underlie the protective effects of NO. The significance of the NO-induced restoration of AP-1 and Sp1 activity remains to be elucidated, as is the late (60-h) increase in AP-1
activity in both the hyperoxia and hyperoxia plus NO groups. In the absence of additional information, it is difficult to predict which AP-1 or Sp1-inducible genes may be involved in the hyperoxia-induced pulmonary apoptosis.

In summary, our data confirm that hyperoxia can cause pronounced apoptosis in rat lungs and show that inhaled NO can reverse both these effects at the clinically relevant low concentration of 20 ppm. Our 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.

We thank Judy MacGregor and the Respiratory Therapy Department of the Children's Hospital of Eastern Ontario (Ottawa) for assistance with the experimental apparatus. These studies were supported by Marathon Events for Charity (Ottawa, Ontario), by the Children's Hospital of Eastern Ontario Research Institute, by the Ottawa Hospital General Campus Research Institute, and by the Ontario Thoracic Society.

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Received 21 December 1998; accepted in final form 7 May 1999.

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