Hyperoxia upregulates the NO pathway in alveolar macrophages in vitro: role of AP-1 and NF-κB

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EXPOSURE TO PURE OXYGEN leads to lung injury characterized by endothelial and epithelial cell damage, alveolar edema, type II cell proliferation, and an influx of granulocytes and macrophages (8). Although Joseph Priestly, who discovered the O2 molecule in 1774, already foresaw its “adverse effects on the healthy state of the body” (13), the exact mechanisms of O2 toxicity still remain to be elucidated. The current concept of O2 toxicity is ascribed to the formation of reactive oxygen species (ROS) such as superoxide (O2−), hydroxyl radical (OH·), and hydrogen peroxide (H2O2) (19). If the delicate balance between intracellular oxidants and antioxidants is disturbed, ROS may contribute to tissue damage by lipid peroxidation, inactivation of enzymes, or DNA oxidation. Oxidative stress can also alter the intracellular hemeostasis by influencing gene expression (41).

To further understand the exact mechanism of O2 toxicity, recent studies have focused on the regulation of gene expression by antioxidants and oxidants. ROS are known to be implicated in the stress response of cells by activating stress response genes such as heme oxygenase-1 (32-kDa heat shock protein) (32) and tumor necrosis factor (TNF)-α (18, 34). Two well-defined transcription factors influenced by the intracellular redox state are nuclear factor (NF)-κB and activator protein (AP)-1 (40, 43). Antioxidants such as pyrrolidine dithiocarbamate and N-acetyl-L-cysteine (NAC) have been shown to inhibit NF-κB and AP-1 activation (28, 38). However, activating effects of antioxidants have also been reported (38).

A stress response gene that may contribute to tissue injury is inducible nitric oxide (NO) synthase (iNOS). The protective effects of iNOS activation after hyperoxia have been discussed (6, 15). Two NF-κB and two AP-1 binding sites have been identified in the human (7) and mouse (50) iNOS promoters. The iNOS product NO, a simple gas, is known to be involved in a variety of physiological as well as pathophysiological processes such as signal transduction, vascular resistance, platelet aggregation, and destruction of microbes and tumor cells (1, 31). In resting cells, iNOS is not detectable, but induction through the stimulation of cells with lipopolysaccharide (LPS) or interferon (IFN)-γ is well established (12, 30, 31).

An important source of NO formation by iNOS in the lung is the alveolar macrophage (AM). The cytotoxic effects of activated macrophages on epithelial cells mediated by NO in vitro have been reported (17). Several studies indicated that hyperoxia-induced oxidative stress can enhance NO production. Haddad et al. (16) reported that nitrotyrosine formation through peroxynitrite was elevated in patients with acute respiratory distress syndrome and rats exposed to 100%
O₂. Arkovitz et al. (2) showed that AMs from immature rats exposed to hyperoxia in vivo produced higher levels of NO than those from control animals. Cuchiaro et al. (9) found, however, that hyperoxia induces iNOS in the lung without an increase in NO concentration in the exhaled air.

We hypothesized that hyperoxia can elevate the generation of ROS, which, in turn, activate redox-sensitive transcription factors such as NF-κB and AP-1 and thus upregulate the NO pathway. In the present study, we show that hyperoxia-induced oxidative stress increases NO production and iNOS expression only in activated rat AMs. Moreover, our data suggest that increased levels of intracellular ROS under hyperoxia activate NF-κB and AP-1. Inhibition of iNOS induction by the antioxidants PDTC and NAC indicates an important role for ROS in the intracellular signaling under normoxic as well as under hyperoxic conditions.

MATERIALS AND METHODS

AM donors. Male Sprague-Dawley rats (250–300 g) were obtained from Charles River (Sulzfeld, Germany) and were given 3 days at the animal holding facilities of the institute to recover from transport stress. The animals were kept on a 12:12-h light-dark cycle in a conventional nonbarrier rodent housing unit. Tap water and standard rodent laboratory diets (ssniff, Soest, Germany) were supplied ad libitum.

Cell isolation and processing. AMs were obtained by bronchoalveolar lavage as described earlier (12). After anesthesia with an intraperitoneal injection of pentobarbital sodium (30 mg/kg body wt), the trachea was cannulated, the thorax was opened, and the lungs were mobilized. Bronchoalveolar lavage was performed in situ 10 times, each with 1 ml of sterile nonpyrogenic phosphate-buffered saline (PBS) solution (Serva, Heidelberg, Germany). The pooled samples were centrifuged at 300 g for 10 min, and the cell pellet was washed twice and resuspended in DMEM (GIBCO BRL, Eggenstein, Germany) supplemented with 2 mM l-glutamine (Seromed, Berlin, Germany), 0.16 mg/ml of gentamicin (Curaasan, Kleinostheim, Germany), and 10% heat-inactivated fetal bovine serum (GIBCO BRL). The total number of cells was assessed with a standard hemacytometer (Coulter Electronics, Krefeld, Germany). To identify the cellular populations, air-dried cytospun smears (500 rpm for 5 min) were stained with May-Gru¨nwald-Giemsa. The preparations contained 97–100% AMs as characterized by morphological criteria. The cell viability proved to be >95% by trypan blue exclusion. The cells were adjusted to 1 × 10⁶/ml, plated in 96-well (0.2 × 10⁶ AMs/well) or 6-well (4 × 10⁶ AMs/well) flat-bottomed cell culture plates (Nunclon Delta, Nunc, Roskilde, Denmark), and incubated for 2 h at 37°C in 5% CO₂ and 21% O₂ to adhere. Subsequently, the AMs were washed twice with 37°C DMEM to remove nonadherent cells and covered with fresh DMEM. For flow cytometric analysis, the cells were incubated in suspension in polypropylene tubes (0.25 × 10⁶ AMs/tube; Greiner, Frickenhausen, Germany).

The AMs were treated with either Escherichia coli LPS serotype 055:B5 (100 ng/ml) purchased from Sigma Chemie (Taufkirchen, Germany), rat recombinant IFN-γ (100 U/ml) from Innogenetics (Ismaning, Germany), or a combination of both. PDTC at a concentration of 50 ng/ml and NAC at a concentration of 30 mM (both Sigma, Deisenhofen, Germany) served as antioxidants and were added 1 h before stimulation. The plates were incubated at 37°C in 5% CO₂ and either 21 or 85% O₂ for up to 48 h. Most in vitro studies have applied O₂ concentrations between 90 and 100% O₂ that cannot be reached in vivo because of saturated water vapor pressure and P CO₂ in the alveolar space. Therefore, we used an O₂ concentration of 85% in vitro to resemble in vivo conditions for respiration with high O₂ concentrations.

Flow cytometric analysis. Viability, necrosis, and apoptosis were assessed by flow cytometry with a FACSort flow cytometer (Becton Dickinson, Heidelberg, Germany). For AM staining, a phosphatidylserine detection kit (DPC Biemann, Bad Nauheim, Germany) that allowed distinction between apoptosis and necrosis was used. Apoptotic cells expose phosphatidylserine, bind annexin V, and are recognized by their green fluorescence. Necrotic cells do not exclude propidium iodide and are detected by their red fluorescence (49). According to the manufacturer’s protocol, the cells were washed and resuspended in calcium buffer. Ten microliters of annexin V-FITC were added and incubated for 20 min before 10 µl of propidium iodide were added. After 10 min on ice, fluorescence distribution properties were recorded for 1 × 10⁴ events, and percentages of viable, necrotic, and apoptotic cells were determined.

Assessment of NO production. NO release was determined spectrophotometrically by evaluating the accumulation of its oxidation reaction product, nitrite, in the culture medium with the Griess reaction (11). All samples were analyzed in triplicate. Briefly, 50 µl of the AM supernatants were incubated with 50 µl of Griess reagent (1% sulfanilamide, 0.1% N-1-naphthylethelenediamine, and 2.5% phosphoric acid) in a 96-well microtiter plate for 10 min at room temperature. The absorbance of the reaction product at 550 nm was measured with a microplate reader (Salzburger Labortektronik, Salzburg, Austria), and concentrations were calculated with a standard nitrite calibration curve.

Protein determination. Protein concentrations were measured with the bicinchoninic acid assay (42). Briefly, 200 µl of bicinechonic acid reaction solution were added to 20 µl of cell lysates and incubated at 37°C for 30 min. All samples were analyzed in duplicate. Extinction at 562 nm was measured in a microplate reader (Salzburger Labortektronik), and protein concentrations were calculated with BSA (Sigma) as a standard.

Western blot analysis. Western blot analysis was performed as previously described (12). In brief, 4 × 10⁶ cells were incubated for 4 h, and the cytoplasmic protein was isolated with the method of Dignam et al. (10). The cells were scraped into hypotonic buffer [10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride (PMSP), and 1 mM dithiothreitol (DTT)], incubated on ice for 15 min, and centrifuged at 3,300 g for 10 min (4°C). The supernatants with the cytoplasmic protein were collected, and protein concentrations were determined. Samples (30 µg protein/lane) were separated on a 7.5% sodium dodecyl sulfate-polyacrylamide gel for 1 h. Electrophoretic protein transfer to nitrocellulose membranes (Serva, Heidelberg, Germany) was carried out overnight, and nonspecific binding was blocked with 1.5% BSA in buffer (100 mM Tris and 150 mM NaCl, pH 7.4) for 1 h at room temperature. The membranes were incubated with a polyclonal rabbit iNOS antibody (1:2,000; Dianova, Hamburg, Germany) for 2 h at room temperature and washed with a 1% PBS-Tween solution. After application of alkaline phosphatase-conjugated goat anti-rabbit IgG (1:2,000; Sigma) for 1 h, the nitrocellulose membranes were washed again and developed with 5-bromo-4-chloro-3-indole phosphate/nitro blue tetrazolium in alkaline phosphatase developing buffer (100 mM NaCl, 100 mM Tris, and 5 mM MgCl₂, pH 9.5). A high molecular mass kit (Dianova) served as a molecular mass standard.
RT-PCR. RT-PCR was performed as described by Jesch et al. (20). After 12 h of incubation at 37°C in the absence and presence of LPS and/or IFN-γ under either 21 or 85% O2, total RNA was extracted with a ribonuclease protection kit (RNeasy Kit, QIAGEN, Hilden, Germany). cDNA was prepared from 2 μg of RNA by adding 14.3 μl of a cDNA synthesis mixture containing 1.5 μM p(dT)15 primer, 0.5 mM deoxynucleotide triphosphate mixture, 32 U of RNase inhibitor (all from Boehringer Mannheim, Mannheim, Germany, 5× first-strand buffer, 0.1 M DTT, and 400 U of Moloney murine leukemia virus RT (all from Gibco BRL). The reaction mixture was incubated at 37°C for 60 min and subsequently heat inactivated for 10 min at 95°C. For amplification with PCR, a DNA thermal cycler (RoboCycler, Stratagene, Heidelberg, Germany) was used. The PCR buffer contained 100 mM Tris-HCl (pH 8.3); 1.5 mM MgCl2; 50 mM KCl; 200 μM each dATP, dGTP, dCTP, and dTTP; 400 nM each sense and antisense primers; and 1 U of Taq DNA polymerase (Boehringer Mannheim). Oligonucleotide primers for iNOS were 5'-ACAGAAAGCGCATCAGGATTC (sense) and 5'-TGCATAACCAGTACCCGAG-3' (antisense; MWG Biotech, Ebersberg, Germany). These correspond to murine macrophage iNOS and also react with rat cDNA (29). The reaction mixture was topped with light mineral oil and denatured for 1 min at 94°C. Amplification was done for 27 and 35 cycles as published earlier (20) (denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 40 s). Amplified PCR products were analyzed on a 1% agarose gel (Promega, Madison, WI) stained with ethidium bromide and visualized by ultraviolet illumination. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with the oligonucleotide sequences 5'-TCCCTCAAGATTGTG (antisense) and 5'-AGATCCACAACGGATACC-ATT-3' (forward; MWG Biotech) served as an internal control. Electrophoretic mobility shift assay. Nuclear and cytoplasmic protein fractions were separated with the method of Dignam et al. (10) with modifications. In brief, the cells were scraped into hypotonic buffer (10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 0.2 mM PMSF, and 1 mM DTT) and high-salt buffer (20 mM HEPES, 25% glycerol, 1.5 mM MgCl2, 0.2 mM PMSF, and 1 mM DTT) and centrifuged at 3,300 g for 10 min. The supernatants were discarded, and the pellets were washed with hypotonic buffer and centrifuged again. The supernatants were discarded, and the pellet was resuspended in low-salt buffer (20 mM HEPES, 25% glycerol, 1.5 mM MgCl2, 20 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 1 mM DTT) and centrifuged on ice for 15 min, and centrifuged at 3,300 × g for 10 min (4°C). The supernatants with the cytoplasmic protein were removed, and the pellets were washed with hypotonic buffer and centrifuged again. The supernatants were discarded, and the pellet was resuspended in low-salt buffer (20 mM HEPES, 25% glycerol, 1.5 mM MgCl2, 20 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 1 mM DTT) and centrifuged (20 mM HEPES, 25% glycerol, 1.5 mM MgCl2, 1.2 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 1 mM DTT) in aliquots to get a final concentration of 0.4 M KCl. After 30 min of incubation on ice and 30 min of centrifugation at 25,000 g at 4°C, the supernatants with nuclear protein were collected and the protein concentrations were quantified. NF-κB and AP-1 that had been activated and translocated to the nucleus were detected from the nuclear protein extracts by electrophoretic mobility shift assay (EMSA) (24). Ten micrograms of nuclear protein were incubated with 2 μl of 32P-labeled oligonucleotide for 30 min on ice. Oligonucleotide sequences for NF-κB were 5'-AGTTGAGGGACTTTCCAGGC-3' (sense) and 5'-GGCTGGAAAGTCCCTCACA-CT-3' (antisense) and for AP-1 were 5'-CCGTTGAGTGGTACCCGAGA-3' (sense) and 5'-TCCGGCTGACTCATCAAGGC-3' (antisense; MWG Biotech). For specificity controls, specific or nonspecific unlabeled probe was applied in excess. Electrophoresis was performed for 2 h with 250 V with a 6% polyacrylamide gel. Densitometric analysis. For densitometric analysis of immunoblots and RT-PCR gels, BIO-1D V.96 software (Vilber Lourmat, Marne La Vallée, France) was used. The results are presented as a percentage of the maximal value. Detection of intracellular ROS. To detect intracellular ROS, 2',7'-dichlorofluorescin diacetate (DCFH-DA) (MobiTec, Göttingen, Germany) was used. DCFH-DA diffuses into the cell and is hydrolyzed by intracellular esterases to polar 2',7'-dichlorofluorescin. This nonfluorescent fluorescein analog is trapped in the cells and can be oxidized to highly fluorescent 2',7'-dichlorofluorescin by intracellular oxidants, mainly H2O2 or hydroxyl radicals (47). The cells were cultured to adhere and incubated with 10 μM DCFH-DA for 30 min. Baseline fluorescence was measured, and the antioxidants (NAC and PDTC) and the stimuli (LPS and IFN-γ) were added in the concentrations described in Cell isolation and processing. After 2 h of incubation under 21 or 85% O2, fluorescence was measured with a fluorometer (FLUOstar, BMG LabTechnologies, Offenburg, Germany). The results are shown as a percentage of the baseline value. The addition of 1 μM H2O2 served as an internal positive control. Statistical analysis. The results are presented as arithmetic means ± SE. The data were analyzed with Friedman analysis of variances on ranks followed by Dunnett’s test versus the control. Results among different O2 concentrations were compared with the Wilcoxon signed rank test. A P value < 0.05 was considered significant.

RESULTS

Cell viability after 24 h of hyperoxia. On the basis of the observation that apoptosis and necrosis may contribute to cell death during hyperoxia (3), we determined the percentages of apoptotic and necrotic AMs after 24 h to see whether changes in cell viability might influence the results (Table 1). In unstimulated cells after incubation under normoxia or hyperoxia for 24 h, viability was unchanged. Treatment with LPS and/or IFN-γ as well as preincubation with PDTC or NAC increased apoptosis and necrosis in AMs. A discrete but significant effect of hyperoxia on cell viability was only detectable after stimulation with IFN-γ alone.

NO production in activated AMs under hyperoxia. To investigate whether hyperoxia induced NO release in AMs or enhanced NO formation after stimulation, the cells were incubated with 100 U/ml of IFN-γ, 100 ng/ml of LPS, or a combination of both in either 21 or 85% O2 for 24 h (Fig. 1). In unstimulated cells, no NO formation was detectable under normoxia or hyperoxia (<0.5 nmol nitrite/mg protein). Treatment with LPS or IFN-γ independently resulted in a significant NO release after 24 h under normoxic conditions (LPS, 165.6 ± 11.5 nmol/mg protein; IFN-γ, 198.6 ± 12.3 nmol/mg protein; LPS plus IFN-γ, 221.0 ± 8.5 nmol/mg protein). The combination of LPS and/or IFN-γ and hyperoxia resulted in a significant increase in NO release (LPS, 235.6 ± 17.7 nmol/mg protein; IFN-γ, 237.2 ± 12.7 nmol/mg protein; LPS plus IFN-γ, 360.4 ± 18.5 nmol/mg protein) compared with that in normoxic cells.

Time course of NO production under hyperoxic conditions. Figure 2 illustrates the time course of NO formation under hyperoxic conditions. AMs were stimulated with 100 ng/ml of LPS plus 100 U/ml of IFN-γ for increasing periods of time (12, 24, and 48 h). There was a time-dependent formation of nitrite by rat AMs.
that reached maximal levels at 48 h under 21% O₂ (12 h, 106.7 ± 9.4 nmol/mg protein; 24 h, 218.8 ± 10.2 nmol/mg protein; 48 h, 268.0 ± 14.4 nmol/mg protein). Hyperoxia did not affect nitrite concentrations after 12 h but compared with normoxic exposure, induced a significant increase in nitrite levels after 24 and 48 h (12 h, 113.7 ± 11.2 nmol/mg protein; 24 h, 368.2 ± 35.0 nmol/mg protein; 48 h, 358.2 ± 21.0 nmol/mg protein). Maximal enhancement of NO production through hyperoxia was reached at 24 h.

Effect of hyperoxia on iNOS expression. To investigate the expression of the iNOS protein, immunoblots of the cytoplasmic protein fraction were performed after 12 h under 21 or 85% O₂ (Fig. 3A). In unstimulated AMs, no specific reactivity was found (Fig. 3A, lane 1). In line with NO levels, hyperoxia did not induce iNOS expression in unstimulated cells (Fig. 3A, lane 2). Treatment with 100 ng/ml of LPS, 100 U/ml of IFN-γ, or the combination caused the appearance of a band with a molecular mass of 125–130 kDa (Fig. 3A, lanes 3, 5, and 7, respectively). Hyperoxia enhanced iNOS protein expression in stimulated cells (Fig. 3A, lanes 4, 6, and 8). To quantify the differences in iNOS protein expression, the results were analyzed densitometrically (Fig. 3B). In the presence of LPS and IFN-γ, iNOS protein expression was significantly increased by hyperoxia compared with normoxia.

With RT-PCR techniques, iNOS mRNA was analyzed in AMs after normoxic or hyperoxic incubation. There was no iNOS mRNA present in unstimulated cells under normoxia and hyperoxia (Fig. 4A). When cells were cultured for 12 h in the presence of LPS and IFN-γ, a PCR product of the predicted size of 741 bp was detected after 25 cycles under 21 and 85% O₂. Densitometric analysis showed that hyperoxic exposure caused a significant increase in iNOS expression in activated AMs by 37% compared with normoxic conditions (Fig. 4B). GAPDH was used as a normalization control.

Activation of NF-κB and AP-1 under hyperoxia. We extended our studies on the redox-sensitive transcription factors NF-κB and AP-1. Nuclear translocation of NF-κB has been thought to play a key role in the activation of the iNOS gene (21). NF-κB was detected by EMSA after 4 h of incubation (Fig. 5A). In normoxic unstimulated cells, only small amounts of NF-κB were

![Fig. 1. Effect of hyperoxia on nitric oxide (NO) production assayed as its stable reaction product nitrite with the Griess reaction. Alveolar macrophages (AMs) were incubated with 100 ng/ml of lipopolysaccharide (LPS), 100 U/ml of interferon (IFN)-γ, or a combination of both for 24 h under 21 or 85% O₂. Values are means ± SE of 12 experiments performed in triplicate. *P < 0.05 compared with 21% O₂.](Image 60x124 to 301x293)

![Fig. 2. Influence of hyperoxia on the time course of NO formation after incubation with 100 ng/ml of LPS plus 100 U/ml of IFN-γ. AMs were incubated for 12, 24, or 48 h under 21 or 85% O₂. Values are means ± SE of 6 experiments performed in triplicate. *P < 0.05 compared with 21% O₂.](Image 324x106 to 565x289)
detectable in the nucleus. Hyperoxia without further stimulation dramatically increased NF-κB activation. Stimulation with LPS and IFN-γ led to NF-κB activation, which was enhanced by hyperoxic conditions. AP-1 was weakly activated in unstimulated cells under 21% O₂ (Fig. 5B). Hyperoxia alone resulted in an increase in AP-1 binding activity. Stimulation of AMs with LPS and IFN-γ under normoxic conditions had no impact on AP-1 activity. Simultaneous treatment of the AMs with LPS and IFN-γ and hyperoxia again resulted in a strong augmentation of AP-1 binding activity compared with that in normoxia-exposed cells.

Influence of antioxidants on NO release. To explore the role of ROS in NO production, the antioxidants NAC and PDTC were added 1 h before stimulation. After 24 h in the presence of NAC, NO production was strongly reduced under normoxia (control, <0.5 nmol/mg protein; LPS, 15.4 ± 0.8 nmol/mg protein; IFN-γ, 23.1 ± 1.2 nmol/mg protein; LPS plus IFN-γ, 96.3 ± 11.5 nmol/mg protein) and hyperoxia (control, <0.5 nmol/mg protein; LPS, 19.3 ± 11.5 nmol/mg protein; IFN-γ, 38.5 ± 3.8 nmol/mg protein; LPS plus IFN-γ, 119.4 ± 27 nmol/mg protein). Preincubation with PDTC completely inhibited NO release (all groups, <0.5 nmol/mg protein) under normoxic as well as hyperoxic conditions.

Influence of antioxidants on iNOS expression and activation of NF-κB and AP-1. To examine whether the effect of antioxidants on NO production was a direct effect based on its radical scavenger properties or whether iNOS induction was inhibited, immunoblots were performed (Fig. 6A). After treatment with LPS and/or IFN-γ, the antioxidant NAC inhibited iNOS protein expression. Interestingly, inhibition by NAC was stronger under hyperoxic conditions. In the presence of PDTC, no iNOS protein was detectable after incubation with either 21 or 85% O₂. RT-PCR products of iNOS mRNA were analyzed after 35 cycles (Fig. 6B). The higher number of amplification cycles masks the differences between O₂ concentrations but shows that there was some iNOS mRNA detectable in the presence of NAC. In line with the findings for NO production and iNOS protein expression, no iNOS mRNA was detectable in the presence of PDTC in both normoxia- and hyperoxia-exposed cells. Expression of the house-
keeping gene GAPDH was unchanged (data not shown).

To further understand whether the inhibition of iNOS induction is based on changes in the activation of the transcription factors NF-κB and AP-1, EMSAs were run after 4 h of incubation and pretreatment with NAC and PDTC. NAC led to a dramatic decrease in NF-κB activity that seemed to be more prominent under hyperoxic conditions. AP-1 activity was enhanced by NAC under normoxia and inhibited under hyperoxia. PDTC is known to inhibit NF-κB activation (38). Its influence on AP-1, however, remains controversial (28, 37). Here, we demonstrated that in rat AMs, PDTC completely inhibited activation of NF-κB and AP-1 under normoxia as well as under hyperoxia (Fig. 6C).

**Intracellular production of ROS.** To see whether ROS indeed participate in the upregulation of the NO pathway under hyperoxia and whether the effects of NAC and PDTC are due to their antioxidative capacity, intracellular ROS were measured with DCFH-DA (Fig. 7). Under stimulation with LPS and IFN-γ, hyperoxia led to a significant increase in ROS production compared with normoxia (21% O2, 160.4 ± 12.9% of baseline; 85% O2, 190.2 ± 19.1% of baseline). In the LPS- and/or IFN-γ-stimulated cells, NAC and PDTC caused a significant suppression of ROS production under normoxia as well as under hyperoxia (NAC in 21% O2, 105.8 ± 2.1% of baseline; NAC in 85% O2, 108.1 ± 2.8% of baseline; PDTC in 21% O2, 118.1 ± 2.5% of baseline; PDTC in 85% O2, 115.1 ± 2.6% of baseline).

**DISCUSSION**

Oxidative stress caused by exposure to elevated O2 concentrations, an important therapy for hypoxicemic patients, may result in hyperoxic lung injury (8). High levels of NO produced by iNOS may contribute to...
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tissue damage. For various pathophysiological conditions in the lung, such as intestinal ischemia-reperfusion-induced pulmonary vascular dysfunction (46) and LPS-induced lung injury (33), the beneficial effects of iNOS inhibition have been shown, indicating that NO formation by iNOS mediates deleterious effects in lung tissue. However, the beneficial effects of NO produced by iNOS in the lung are also discussed (6, 15).

In this study, we focused on the influence of hyperoxia on NO formation, iNOS mRNA and protein expression, and possible regulatory mechanisms through transcription factors in primary cultured AMs from rats. We chose AMs as a target for our study because AMs are exposed directly to elevated O2 concentrations in the alveoli (30). In addition, AMs are a major source of NO produced by iNOS in the lung (14), and overproduction of NO by AMs might be the culprit of hyperoxic lung injury. Berg et al. (4) showed that AM depletion in rats resulted in prolonged survival under hyperoxia. Survival times of adult rats exposed to 100% O2 are only 60–72 h (8). Despite species differences between rats and humans (20), it has been shown that human AMs are capable of expressing iNOS (22, 45). Also, NF-κB and AP-1 binding sites have been described in the human iNOS promoter (7).

Our findings indicate that hyperoxia alone does not suffice to induce iNOS expression (Figs. 3 and 4) and NO formation (Figs. 1 and 2) in rat AMs. However, the response to inflammatory stimuli such as LPS and/or IFN-γ, which increased iNOS expression and NO formation in AMs (Figs. 1, 3, and 4), was significantly enhanced by the application of hyperoxia (Figs. 1–4). Activation of the transcription factors NF-κB and AP-1 was already observed during hyperoxia alone. Stimulation with LPS and IFN-γ activated NF-κB, but surprisingly, they had no influence on AP-1. Coapplication of hyperoxia further enhanced activation of both transcription factors (Fig. 5). Incubation of AMs with the antioxidants PDTC and NAC before stimulation resulted in inhibition of the NO pathway. PDTC completely inhibited activation of transcription factors, iNOS expression, and NO formation under both 21 and 85% O2 (Fig. 6), suggesting that ROS play an important role in iNOS induction under normoxic as well as under hyperoxic conditions. NAC, although in a much higher concentration, caused a weaker inhibition. Interestingly, on the protein and transcription factor level, inhibition by NAC seemed to be even greater under hyperoxic conditions. Measurement of intracellular ROS revealed that there is increased ROS production under hyperoxia and that NAC and PDTC can serve as potent antioxidants.

Our data are consistent with a previous study (25) demonstrating that rat AMs treated with LPS and/or IFN-γ produce NO in a time-dependent manner. Hyperoxia alone failed to induce NO production in unstimulated cells, but in the presence of LPS and IFN-γ, elevated O2 concentrations led to a significant increase in NO formation. Enhancing effects of hyperoxia after stimulation with LPS have also been shown for TNF-α and interleukin-1β release in human AMs (34) and TNF-α and macrophage inflammatory protein-1α gene and protein expression in mouse AMs (48).

Here, we report that enhanced NO production correlates with elevated iNOS protein concentrations, suggesting that it is not the increased enzyme activity but the accumulation of iNOS protein that causes increased NO formation under hyperoxia. O2 is a cosubstrate for NO synthesis (7), but, obviously, O2 is not a limiting factor for iNOS activity under normoxia. This is in line with the finding that NOs in intact tissue are exposed to saturating concentrations of O2 under normoxic conditions, as expected from the Michaelis-Menten constant values for O2 (36).

Our data indicate that LPS and IFN-γ activate NF-κB under normoxic conditions. Oxidative stress induced by H2O2 has been shown to activate NF-κB, an essential transcription factor for the iNOS induction as proven by truncation of the iNOS promoter (39, 23). In our experimental setting, NF-κB was strongly activated through incubation with 85% O2 in unstimulated cells. In stimulated cells that also produce NO, activation under hyperoxic conditions was less despite the combination of stimuli, indicating a negative feedback mechanism on NF-κB via NO that has already been suggested in hepatocytes (44). In contrast to the findings by Camhi et al. (5), who found increased AP-1 activity after stimulation with LPS in RAW 264.7 cells, in our setting, AP-1 activity was not induced by stimulation with LPS or IFN-γ but was dramatically increased by hyperoxia as shown by Lee et al. (26).

The O2 concentration-dependent activation of AP-1 and NF-κB and iNOS induction in rat AM primary culture on stimulation with LPS and/or IFN-γ suggests that hyperoxia can influence intracellular signaling directly or indirectly via ROS. To further investigate the involvement of ROS, we used the antioxidants NAC and PDTC. NAC acts as a glutathione precursor and has just recently been shown to be efficient in protecting the lungs of patients with acute respiratory distress syndrome (35). In our experimental setting, application of NAC in vitro downregulated the NO pathway under normoxia as well as under hyperoxia. There was only little iNOS mRNA, iNOS protein, and NO detectable in the presence of NAC. The impact on transcription factors, however, was diverse. Inhibition of NF-κB and AP-1 by NAC seemed to be stronger under hyperoxic conditions. Under normoxia, even enhancing effects of NAC on AP-1 activity have been found. A concentration-dependent activating or inhibitory effect of NAC on NF-κB has already been shown (27). The effects, however, are not yet fully understood. PDTC is a radical scavenger and an iron chelator and has also been described as a potent NF-κB inhibitor (38). Its iron-chelating properties reduce the production of OH- radicals by inhibiting the Fenton reaction. In our experimental setting, 50 μM PDTC completely inhibited the activation of NF-κB and AP-1 as described before (28, 37, 38, 40). Concomitantly, iNOS mRNA transcription, protein expression, and NO production were not detectable in the presence of PDTC under both normoxic and hyperoxic conditions. PDTC
is known to be toxic in higher concentrations. The PDTC concentrations used in our experiments did reduce viability to ~70%. Because this cannot explain the almost complete inhibition of the NO pathway, our result must be attributed to the antioxidative capacity of PDTC. To further investigate the role of oxidants and antioxidants, DCFH-DA was used to detect intracellular ROS. We show that hyperoxia significantly increases intracellular ROS production after stimulation with LPS and IFN-γ, indicating that the increased NO production under hyperoxia is a result of enhanced ROS production, with subsequent activation of NF-κB and AP-1. In the presence of NAC and PDTC, intracellular levels of ROS were suppressed dramatically under normoxia as well as under hyperoxia. Therefore, we conclude that despite possible nonspecific actions of NAC and PDTC, these molecules act as antioxidants in our experimental setting. Our results indicate that ROS are necessary for intracellular signaling in the NO pathway after treatment with LPS or IFN-γ in normoxia-exposed cells. Moreover, increased production of ROS in hyperoxia-exposed cells resulted in augmentation of NF-κB and AP-1 activation and, subsequently, enhanced iNOS gene transcription, protein expression, and NO production after stimulation with LPS and IFN-γ. Hyperoxia alone, although activating NF-κB and AP-1, was not sufficient to induce the NO pathway. Therefore, other transcription factors such as NF-interleukin-6 and signal transducer and activator of transcription that are activated by stimulation with LPS or IFN-γ must also play a role in iNOS induction.

In summary, we report that hyperoxia increased NO production and iNOS mRNA and protein expression in rat AMs after stimulation with LPS and/or IFN-γ in vitro. Enhanced NF-κB and AP-1 activation participates in iNOS upregulation under hyperoxia, but other transcription factors are involved. ROS are implicated in the intracellular signaling under normoxic conditions. Hyperoxia leads to an increase in intracellular ROS formation. PDTC and NAC can inhibit the NO pathway through their antioxidative capacity. To further shed light on the role of NO in hyperoxic lung injury, in vivo studies with selective iNOS inhibitors or iNOS-deficient mice are essential.

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