Protein nitration in rat lungs during hyperoxia exposure: a possible role of myeloperoxidase

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EXPOSURE TO A HIGH CONCENTRATION of oxygen leads to lung injury characterized by epithelial and endothelial damage and edema (13). Hyperoxia causes oxidative stress in the lung, evident by increased generation of free radicals. This, in turn, disturbs the delicate balance of redox status in the cells, leading to cell injury and death (16, 24, 34). Reactive oxygen and nitrogen radicals such as superoxide (O2·−), hydroxyl radical (·OH), and peroxynitrite (ONOO−) are known to attack all the biomolecules, causing tissue damage by means of lipid peroxidation, protein oxidation, and DNA damage (6, 17, 21). Several recent reports have demonstrated that oxidative stress induces posttranslational modifications of proteins, including nitration (2, 6). Nitration of tyrosine at 3-carbon is the major modification that occurs in proteins. This posttranslational modification may alter the protein structure. Such change may result in gain or loss of protein function, depending on the conformation and active site of the protein (27, 30, 32). Nitration of proteins could be caused by two mechanisms: ONOO− or myeloperoxidase (MPO). ONOO− is formed from a diffusion-limited reaction of nitric oxide (NO) and O2·−. The oxidation of NO metabolite nitrite by MPO forms NO radical (NO•−), a strong oxidant capable of nitrating tyrosine residues in proteins (10).

Nitrated proteins have been detected in many diverse lung diseases, including acute lung injury and bronchopulmonary dysplasia (BPD) (4, 19, 27). Plasma levels of 3-nitrotyrosine were directly related to the amount of supplemented oxygen in patients with BPD (4). Nitration of plasma proteins, such as ceruloplasmin, transferrin, α1-antichymotrypsin, and β-chain fibrinogen, was observed in patients suffering from acute respiratory distress syndrome (18). Increased nitrotyrosine expression was found in bronchial epithelial cells and alveolar interstitium after hyperoxia exposure for 72 h (27). Although several investigators observed the nitrations of proteins during hyperoxia (4, 9, 23, 26, 28), no attempt was made to identify those nitrated proteins. Furthermore, little is known regarding the mechanisms of hyperoxia-mediated protein nitration in lungs.

The present study aimed to investigate the effect of exposure time of hyperoxia on lung injury and to correlate these effects with protein nitration in the lungs of rats. We assessed lung injury by bronchoalveolar lavage fluid (BALF) protein, cell counts, pleural effusions, and histopathology. We detected the formation of nitrotyrosine in hyperoxic lung tissues by Western blot and immunohistochemistry and identified two of the nitrated proteins. Furthermore, we evaluated the role of neutrophils in protein nitration during hyperoxia by analyzing nitrite and MPO levels and by coculturing neutrophils isolated from hyperoxia-exposed lungs with a rat lung epithelial L2 cell line.

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Materials. Guaiacol, trypan blue, nitrate reductase, N-(1-naphthyl)ethylenediamine dihydrochloride, sodium dithionite, sodium nitrite, sodium nitrate, NaNDFP, 2,4-dinitrophenylhydrazine, formaldehyde, sulfuramidine, Tween 20, rat IgG, N-propyl galate, glycerol, hematoxylin and eosin, leupeptin, aprotinin, phenylmethylsulfonyl fluoride (PMSF), and anti-actin antibodies were purchased from Sigma (St. Louis, MO). Membrane inserts (Millicell-CM insert, 0.4 μm) were purchased from Millipore (Bedford, MA). L2 cells were obtained from American Type Culture Collection (Rockville, MD). MEM was from ICN (Costa Mesa, CA). Fetal bovine serum (FBS), trypsin-EDTA, penicillin, streptomycin, and Ham’s F-12K medium were from GIBCO (Grand Island, NY). Nitrocellulose membrane was obtained from Schleicher & Schuell (Keene, NH). Anti-p53 and anti-MPO antibodies and protein G-agarose beads were from Santa Cruz Biotechnology (Santa Cruz, CA). An enhanced chemiluminescence (ECL) detection system was obtained from Amersham Biotech (Piscataway, NJ). Horseradish peroxidase (HRP)-conjugated anti-mouse or rabbit IgG, DC protein assay kit, and Affi-Gel 10 agarose beads were purchased from Bio-Rad (Hercules, CA). FITC-conjugated anti-goat IgG and Cy3-conjugated anti-mouse IgG were from Jackson Immunoresearch Laboratories (West Grove, PA). Unconjugated and agarose-conjugated monoclonal anti-nitrotyrosine antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Antibodies against surfactant protein A (SP-A) and t1α were raised in rabbits using synthetic peptides corresponding to the 83–94 amino acids of rat SP-A (H3N-CNWYPGEPR-T-COOH; Affi-Gel 10) and 94 amino acids of rat t1α (H3N-C T S D H D H K E H E S T-COOH; Affinity Bioreagents, Golden, CO). These antibodies were purified by affinity chromatography using the corresponding peptides in our laboratory. The affinity column was prepared by using the SP-A and t1α peptides conjugated with agarose beads (Affi-Gel 10) according to the manufacturer’s instructions.

Animal exposure. Pathogen-free male Sprague-Dawley rats (250–275 g) were housed for 1 wk before use. The Oklahoma State University Animal Care and Use Committee approved the use and care of animals used in this study. Rats were placed in a sealed Plexiglas chamber (90 × 45 × 45 cm) and exposed to >95% oxygen for 48, 60, and 72 h. During the experiments, 2 rats died during a 72-h exposure time. The flow rate was maintained at 8 l/min using a flow meter (Vacu-Med, Ventura, CA). The oxygen concentration was continuously monitored with an oxygen analyzer (Vacu-Med). Animals had free access to food and water. Soda lime was used in the chamber to remove CO2. The control rats were kept at room air.

Tissue collection. At different exposure times, animals were anesthetized with intraperitoneal injections of ketamine (40 mg/kg) and xylazine (8 mg/kg). A tracheotomy was performed. Rats were ventilated with a rodent ventilator and perfused with 50 mM PBS (pH 7.2). Lungs were lavaged with 5 ml of normal saline. This was repeated four times until the final lavage volume became 20 ml. Tissue samples were immediately frozen in liquid nitrogen.

Differential cell counts. Bronchoalveolar lavage (BALF) was centrifuged at 250 g for 10 min. The supernatant (BALF) was used for biochemical analysis as described in Measurement of nitrate alone and total nitrate/nitrite in BALF and lung homogenate. The cell pellet was resuspended in 1 ml of saline. The cell viability was determined with trypan blue exclusion assay. In brief, 10 μl of cells were mixed with 10 μl of 0.4% trypan blue and loaded onto a hemocytometer. Viable cells excluded the dye. Both live and dead cells were counted, from which the percentage of dead cells was calculated. For differential cell counts, the cells were cytospun on a slide (Cytofuge 2, Stat Spin) and stained with modified Giemsa stain. A total of 200 cells/slide were counted. Activated macrophages, which show the morphology of “spreading,” were counted separately to determine the percent of activated macrophages.

MPO enzyme assay. MPO activity in the lung homogenate was assayed according to Klebanoff et al. (25). Briefly, 20 μl of lung homogenate was mixed with 980 μl of MPO assay solution. The latter was prepared fresh before use by mixing 107.6 ml H2O, 12 ml 0.1 M sodium phosphate buffer (pH 7.0), 0.192 ml guaiacol, and 0.4 ml 0.1 M H2O2. The generation of tetra圭aicol was measured spectrophotometrically at 470 nm, and the change of optical density (OD) per minute was calculated from the initial rate. The MPO activity was then calculated from the formula: units/ml = ΔOD/min × 45.1 and expressed as U/mg protein. One unit of the enzyme is defined as the amount that consumed 1 μmol of H2O2/min.

Measurements of nitrite alone and total nitrite/nitrate in BALF and lung homogenate. Nitrite alone and total nitrate and nitrite (NO2−/NO3−) were measured with Griess reagents (35). To measure NO2− level, 100 μl of Griess reagent containing 0.5% sulfanilamide and 0.05% N-(1-naphthyl)ethylene diamine dihydrochloride were added to 100 μl of BALF and incubated for 10 min. Absorbance was measured at 550 nm, and concentration was determined using NaNO2 and NaNO3 standards. For measurement of total NO2−/NO3−, NO3− was converted to NO2− before Griess reaction. BALF sample (50 μl) was incubated with 40 μl of 50 mM phosphate buffer, pH 7.5, at room temperature for 2 h in the presence of 0.2 U/ml of nitrate reductase. Ten microliters of 0.2 mM NADPH were added and incubated for an additional 45 min at room temperature. For estimation of NO2− alone and total NO2−/NO3− in the lung tissues, lavaged lungs were homogenized in 10 mM Tris buffer (pH 7.4) containing 1% Triton X-100, 1 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. The homogenate was centrifuged at 2,500 g for 20 min, and the supernatant was further centrifuged at 100,000 g for 1 h. The resulting clear lysate was used for estimating NO2− alone and total NO2−/NO3− levels using Griess reagent.

Histopathological analysis. In a parallel experiment at different time exposures, rats were killed with intraperitoneal injections of ketamine (40 mg/kg) and xylazine (8 mg/kg). The trachea was cannulated, and the lungs were inflated (1 min at 25-cmH2O gauge pressure) with 4% formaldehyde in PBS. After 24 h of immersion, the lungs were washed in PBS, dehydrated in graded alcohol and xylene, and embedded in paraffin (60°C). Paraffin-embedded lungs were sectioned (4 μm) and placed on glass slides (Fisher Scientific, Pittsburgh, PA). The slides were deparaffinized with xylene and rehydrated with graded alcohol and PBS. The sections were stained with hematoxylin and eosin.

Western blot for nitrotyrosine. Frozen lung tissue was homogenized in lysis buffer (10 mM Tris·HCl, pH 7.5, 1% Triton X-100, 1 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin), and protein concentration was determined using the DC protein assay kit. Thirty micrograms of protein were solubilized in SDS sample buffer (62.5 mM Tris·HCl, pH 6.8, containing 2% SDS, 10% glycerol, and 0.01% bromphenol blue), separated on 12% SDS-PAGE, and transferred onto a nitrocellulose membrane at 100 mA for 2 h. The membrane was stained with Ponceau S stain to check the efficiency of transfer of proteins and blocked overnight with 5% dry milk in 100 mM.
Tris-buffered saline containing 0.1% Tween 20 (TBST). The membrane was then washed twice in TBST (each for 5 min) and incubated with anti-nitrotyrosine antibodies (1:1,000) for 3 h at room temperature (40°C). The membrane was washed three times (5 min each) in TBST and then incubated with HRP-conjugated anti-mouse IgG (1:2,500) for 1 h. After being washed three times (5 min each), the blot was developed with ECL reagents and exposed to X-ray film to visualize the protein bands. For control, the blot was incubated with 20 mM sodium dithionite (Na₂S₂O₄) overnight before being blocked with 5% dry milk.

**Immunoprecipitation and identification of nitrated proteins.** Lung tissue homogenates (250 µg) were preclarified for 1 h with 15 µl of protein G-agarose beads, centrifuged at 1,000 g for 10 min, and incubated with 5 µg of agarose-conjugated monoclonal anti-nitrotyrosine antibody overnight at 4°C with shaking. The samples were centrifuged at 1,000 g for 20 min, and agarose-bound immune complex was washed three times with lysis buffer and finally dissolved in 50 µl of SDS sample buffer.

Nitrated proteins were separated on 12% SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. Nitrotyrosines were detected with polyclonal antibodies raised against 2% N-hydroxy-4-nitrotyrosine and subsequently incubated with polyclonal anti-SP-A, anti-t1-actin antibodies, or mouse monoclonal p53 antibodies at dilutions of 1:1,000. Immunoreactivity was detected as described above by incubation with HRP-conjugated anti-mouse or anti-rabbit IgG (1:2,500) and further detected with ECL reagents.

**Immunohistochemistry.** Nitrotyrosine and MPO expression in lung tissue were measured by immunohistochemistry (as described in Ref. 2). Paraffin-embedded tissue sections were de waxed with xylene and rehydrated. Sections were rinsed in PBS (pH 7.4) for 10 min. The slides were then treated with 0.05% Triton X-100 in PBS and blocked with 10% FBS in PBS for 20 min. Sections were carefully dried by absorbing excess buffer and incubated simultaneously overnight at 4°C with primary antibodies against MPO and nitrotyrosine at 1:200 and 1:1,000 dilutions, respectively. After being washed three times in PBS, the sections were incubated for 1 h with FITC-conjugated anti-goat IgG and Cy3-conjugated anti-mouse IgG at a dilution of 1:2,500 at 4°C. The sections were washed three times in PBS and drained off. We added antifade mounting media (5% N-propyl galate and 80% glycerol in PBS, made inhouse) and placed a coverslip. The slides were examined using a Nikon Eclipse E600 fluorescence microscope.

**Isolation of neutrophils and macrophages.** For coculture studies, lavage cells were collected from control animals or 72 h-exposed animals. Macrophages were separated from other cells using rat IgG panning. The lavage cells were incubated for 1 h in a 100-mm bacteriological dish coated with 3 mg of rat IgG. Macrophages attached to the dish and were released from the plate using 1× trypsin-EDTA. The purity of macrophages was >95%, and the remaining cell contamination was lymphocytes. The unattached cells contained neutrophils, red blood cells, and lymphocytes. Red blood cells were lysed with hypotonic buffer, and the resulting cell suspension contained ~90% neutrophils and ~10% lymphocytes.

**Coculture.** L2 cells (a rat lung epithelial cell line) were seeded onto the outer side of the Millicell-CM insert, placed in eight-well chamber plates at 0.5 × 10⁶ cells/well, and allowed to adhere to the membrane for 4 h in Ham's F-12K medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 5% CO₂ and 37°C. The insert was then inverted and placed in the well so that the attached cells would remain at the bottom of the insert and merge in Ham's F-12K medium (without FBS). Neutrophils or macrophages at 0.5 × 10⁶ in MEM (without FBS) were added to the inside of the insert to prevent direct contact of the cells. The coculture was carried out for 6 h in the presence or absence of sodium nitrite (5 mM). In one set of experiments, 5 µg of anti-MPO antibodies were added to the neutrophil-L2 coculture in the presence of sodium nitrite. Lavage cells isolated from air-exposed animals were used as controls. L2 cells cultured alone were used as a negative control. After 6 h, L2 cells were washed with Ham's F-12K medium and lysed in Tris-buffer (pH 7.5) containing 1% Triton X-100, 1 mM EDTA, 1 mM PMSF, 10 µg/ml aprotinin, and 10 µg/ml leupeptin. Equal amounts of proteins were loaded onto SDS-PAGE, and Western blot analysis was performed for detection of nitrated proteins.

**Statistical analysis.** Data are expressed as means ± SE. Statistical analyses were performed by ANOVA, followed by Dunn's or Student's t-test using the software Sigma Stat 2.03. Significance was assigned at P < 0.05.

**RESULTS**

**Lung injury during hyperoxia.** Earlier studies have shown that lung injury occurs after hyperoxia exposure (5, 37). We first assessed biochemical and histopathological changes in the hyperoxia-exposed lungs with an emphasis on the relationship between the severity of lung injury and exposure time. This provided the basis to correlate hyperoxia-induced lung injury and protein nitration. As shown in Fig. 1A, the total cell number recovered from bronchoalveolar lavage was increased approximately two- to fourfold in hyperoxia-treated animals compared with animals in normal air. Neutrophils and macrophages increased significantly after a 60- and 72-h exposure to hyperoxia. However, the change was not significant at a 48-h exposure. Cell viability of bronchoalveolar lavage was decreased significantly to 82.6 ± 4.5, 79.8 ± 3.3, and 76.3 ± 4.0 for 48, 60, and 72 h of exposure from the control value of 90.0 ± 4.5 (P < 0.05 vs. control for all exposure times, n = 6 animals). Macrophages, when activated, showed a typical morphology of spreading with pseudopodia and granules in the cytoplasm. Activated macrophages increased at all time intervals of hyperoxia exposure compared with the control. However, the percent of activated macrophages slightly decreased from 48 to 72 h (control, 20.7 ± 1.8%; 48 h, 77.5 ± 3.9%; 60 h, 74.0 ± 3.6%; and 72 h, 66.0 ± 1.9%; P < 0.05 vs. control for all exposure times, n = 6 animals). Exposure to hyperoxia increased the amount of proteins recovered in the lavage fluid from 3.6 ± 0.3 mg (control) to 4.6 ± 0.2 mg (48 h), 9.2 ± 2.4 mg (60 h), and 6.6 ± 0.7 mg (72 h; P < 0.05 vs. control for all exposure times, n = 6 animals). Pleural effusions were found at 60 h of exposure (3.9 ± 0.5 ml), and higher volumes were found at 72 h (5.7 ± 0.8 ml). No pleural fluid was found in the control animals or in animals exposed for 48 h.

Histopathological analysis of lung sections revealed some interesting results that showed exposure between 48 and 60 h dramatically changes the severity of lung injury (Fig. 1B). Infiltration of phagocytic cells was found at all exposure time points, with the maximum at 72 h. A mild injury was observed at 48 h with no edema and hemorrhage, but after exposure for 60 h,
Fig. 1. Differential bronchoalveolar lavage cell count and hematoxylin and eosin staining of lungs from control (con) and hyperoxia-exposed rats. A: differential cell counting of alveolar lavage cells. Total lavage cells were cytospun and stained with Giemsa stain, and different cell types were counted on the basis of morphology and nuclear stain. A total of 200 cells were counted per slide, each prepared in duplicate. Data are means ± SE (n = 6 animals). *P < 0.05 vs. control. mac, Macrophages; lym, lymphocytes; neut, neutrophils. In y-axis, E + 06 or E + 07 = 10⁶ or 10⁷. B: lung sections were stained with hematoxylin and eosin. Control, air-exposed rats. Arrows point to normal alveoli. At 48 h (48-h hyperoxia exposure), infiltration of phagocytic cells was observed with minimum damage to the epithelium. Arrows illustrate increased cellularity in the alveolar space. At 60 h (60-h hyperoxia exposure), lungs show infiltration of phagocytic cells, hemorrhage, expansion of perivascular regions, interstitial proteinacious material accumulation, and type II pneumocyte hyperplasia. Arrows denote expansion of perivascular regions with proteinacious material. At 72 h (72-h hyperoxia exposure), lungs show hyperplasia of type II pneumocytes, thickening of alveolar septa, infiltration of phagocytic cells, hemorrhage, and interstitial edema with increased proteinacious material. Arrows illustrate thickening of alveolar septa with infiltrated cells and interstitial edema. Results are representative of at least 3 different experiments.

Severe interstitial edema and expansion of peribronchial and perivascular spaces were found. Proteinacious material accumulated in the alveolar interstitium at 60- and 72-h exposure times. Airway, alveolar epithelial, and endothelial damage was clearly seen at 60- and 72-h exposure times. These results indicate that the exposure time between 48 and 60 h is critical, and this could be used as a time point for the onset of edema, which worsens the effects of injury.

Nitrate/nitrite levels. One of the mechanisms for protein nitration is oxidation of NO₂ by MPO. Exposure to hyperoxia increased NO₂ and total NO₂/NO₃ levels in BALF compared with the control groups (Fig. 2). These results are in concurrence with the increased levels of NO₂ and total NO₂ and NO₃ in the lung tissue. MPO was used as a specific marker for neutrophil infiltration into the lung. MPO activity was increased twofold in 48- and 60-h exposed rats and fourfold after a 72-h exposure (Fig. 3A). MPO protein level as determined by Western blot analysis showed a similar pattern (Fig. 3B).

Immunohistochemistry for nitrotyrosine and MPO. Immunohistochemistry was performed to identify the tissue localization of nitrotyrosine and MPO after hyperoxia. The number of cells for nitrotyrosine staining increased in the lungs of hyperoxia-exposed rats (Fig. 4). Nitrotyrosine staining was found in alveolar epithelial cells and infiltrated cells and showed a diffused staining in the alveolar interstitium and epithelial lining of lung sections. At 48 h, the nitrotyrosine staining colocalized with the MPO staining (Fig. 4B). However, at 60 and 72 h, colocalization of nitrotyrosine and MPO was only observed in some of the epithelial cells and infiltrated cells (Fig. 4, C and D). These results suggest that infiltrated phagocytic cells may contribute, at least partly, to tissue protein nitration.

Western blot for nitrotyrosine. Western blot analysis showed that several proteins with a molecular mass range between 29 and 66 kDa were nitrated in the lung tissue after hyperoxia exposure (Fig. 5A). The protein nitration increased markedly between 48 and 72 h of exposure, corresponding to the lung injury (Fig. 1). Treatment of the blot with Na₂S₂O₄, which converts nitrotyrosine to aminotyrosine, abolished the nitrated bands, although the bands corresponding to 29 and 52 kDa did not completely disappear (Fig. 5B). The possible reason for this could be due to the unavailability of these modified nitrated groups for reduction by Na₂S₂O₄.

Immunoprecipitation and identification of nitrated proteins. SP-A and t1α antibodies were raised in rabbits using synthetic peptides and were affinity purified.
The purified SP-A antibodies recognized two bands with molecular masses of 32 and 36 kDa in lung tissue. Preincubation of the antibodies with the peptide used for generating the antibodies eliminated the bands (Fig. 6A). The purified t1α/H9251 antibodies recognized a major 39-kDa band and a weaker 76-kDa band (Fig. 6D). The higher-molecular-mass band may be a dimer because it also disappeared after being preblocked with the SP-A peptide. Nitrated proteins were immunoprecipitated with agarose-conjugated monoclonal anti-nitrotyrosine antibodies and further probed with antibodies against SP-A, t1α/H9251, β-actin, and p53. The results showed two bands at molecular masses of 32 and 36 kDa for SP-A (Fig. 6B) and a band at 36 kDa for t1α (Fig. 6). The nitrated SP-A and t1α were significantly increased in the hyperoxia-treated groups (Fig. 6, C and F). Maximum nitration of both proteins was found in the animals exposed for 72 h. The t1α level was lower at 60 h than 48 h. β-Actin and p53 blots did not show any positive bands at all conditions, indicating a lack of nitration of these proteins during hyperoxia exposure (data not shown).

Coculture. To determine the roles of neutrophils and macrophages in protein nitration of alveolar epithelial cells, we cocultured the L2 cell line (a rat lung epithelial cells) with neutrophils or macrophages. To avoid direct contact and cross contamination, we cultured two types of cells on either side of the membrane insert. A faint 66-kDa protein was nitrated when L2 cells were cocultured with the macrophages isolated from normal rats. This band was not present in L2 cells alone (Fig. 7, lanes 1 and 2). Coculture of L2 cells with the macrophages isolated from hyperoxic lungs resulted in a marked increase of the 66-kDa protein and an appearance of an additional 30-kDa protein (Fig. 7, lanes 3 and 4). The 66-kDa band was also increased when L2 cells were cocultured with the neutrophils isolated from hyperoxic lungs. Inclusion of NO2 in the medium resulted in two additional nitrated bands (52 and 30 kDa; Fig. 7, lanes 5 and 6). The addition of anti-MPO antibodies to the culture medium containing NO2 during neutrophil-L2 coculture decreased the nitration of these proteins (Fig. 7, lane 7).

**DISCUSSION**

Despite several studies that have documented the cytotoxic effects of hyperoxia exposure, supplemental oxygen is inevitable in the treatment of patients suffering from pulmonary and cardiovascular diseases. Protein modifications by oxidation and nitration have been shown earlier as mechanisms of hyperoxia-mediated lung injury (4, 28). However, the specificity and
pathway of protein nitration during hyperoxia were not known. The present study identified two proteins, SP-A and t1α, specific for alveolar epithelial type II and type I cells that were nitrated during hyperoxia. Evidence including colocalization of nitrotyrosine with MPO, increased MPO activity and NO₂ level after hyperoxia treatment, and protein nitration caused by coculture of L2 cells with neutrophils indicated a possible role of MPO in protein nitration.

Hyperoxia exposure caused lung inflammation that was evident by infiltration and activation of macrophages and neutrophils. Our results showed that total bron-
Alveolar lavage cell counts increased as a function of exposure time. Neutrophils and macrophages accounted for most of the increases. In addition to producing highly reactive oxygen and nitrogen radicals, these cells produced proinflammatory cytokines such as IL-1, interferon-γ, macrophage-inflammatory protein-2, IL-4, and IL-5, etc., leading to inflammation in the lung (33, 36).

The present study also showed an increase in protein content in BALF and pleural effusions during hyperoxia exposure, representing the damage in alveolar epithelium and endothelium. These results are in agreement with earlier studies (1, 31). On the basis of our histology results, interstitial edema, damage to endothelium, expansion of peribronchial and perivascular spaces, and hemorrhage occur at 60 h, but not at 48 h, of exposure. These results suggest that exposure time between 48 and 60 h is critical for changes in the severity of lung injury. These results are supportive of pleural effusions, which were found at 60 and 72 h but not at 48 h. The leak of pleural fluid indicates severe damage to the vascular endothelium. Although the pleural effusion volume at 72 h was higher than at 60 h, the BALF protein content decreased. This could be due to increased permeability in the endothelium and thickening of alveolar septa with proliferation of alveolar type II epithelial cells and fibroblasts. The latter reduces leakage of proteins into the alveolar space.

A recent study has demonstrated that mice exposed to hyperoxia showed increased levels of 3-nitrotyrosine in the lung (28). The possibilities for this protein nitration could be either a direct reaction with ONOO− or caused by the oxidation of nitrite by MPO. MPO, in the presence of nitrite and HOCl, produces reactive NO, which leads to protein nitration (15). Brennan et al. (8) recently used MPO-deficient mice to study the role of MPO in protein nitration. They found that MPO is responsible for protein nitration in vivo in some, but not all, acute inflammation models. Our results concur with previous results demonstrating increased MPO activity in the lung (12, 34). The levels of total nitrite
and nitrated or nitrite alone in the lungs and BALF were increased during hyperoxia exposure for 60 and 72 h. On the basis of these results, we speculate that MPO may have a role in protein nitration during hyperoxia. Immunohistochemical analysis clearly demonstrated the colocalization of MPO and nitrotyrosine in alveolar epithelial and interstitial space, suggesting a correlation between MPO and nitrotyrosine. However, at 60- and 72-h hyperoxia exposure, only a partial colocalization was observed for MPO and nitrotyrosine. This could be due to the diffusion of reactive oxygen species or migration of infiltrated phagocytic cells. The diffusive immunoreactivity of MPO in the epithelium indicates the transcytosis of MPO into the epithelium, which occurs during the endocytotic process associated with pulmonary surfactant metabolism (22). MPO could also be incorporated into the epithelium because of the affinity between the cationic MPO and heparan sulfate-rich epithelial surface (3, 14).

Western blot for nitrotyrosine clearly demonstrated an increase in protein nitration of lung tissue during hyperoxia. Our results support the earlier studies by Lorch et al. (28), who have demonstrated that exposure to 100% oxygen for 72 h increased nitrotyrosine immunostaining in the alveolar regions of the mouse lung. The distinct pattern of nitrotyrosine staining in the alveolar epithelial lining suggests the nitration of specific proteins in the epithelium. When nitrated proteins were immunoprecipitated with monoclonal anti-nitrotyrosine antibodies and probed with antibodies against SP-A, t1α, p53, and β-actin, SP-A and t1α, but not p53 and β-actin, were found to be nitrated. Although the physiological relevance of SP-A nitration in this model was not known, several in vitro studies demonstrated that SP-A nitration decreased its ability to aggregate lipids (20), to enhance the binding to Pneumocystis carinii to alveolar macrophages (39), and to bind to mannose receptor (38). Nitration of t1α increased at all exposure times. T1α is a plasma membrane protein expressed on type I pneumocytes. The functions of t1α are not known. Ma et al. (29) demonstrated that t1α does not serve as a water channel in alveolar epithelium; however, its localization on the apical membrane creates the possibility of a role in fluid transport (7). The nitration of t1α might cause alterations in the membrane and thus may lead to membrane damage. This was further supported by the presence of t1α in the BALF at 60 and 72 h of exposure time, an indication of alveolar epithelial damage (data not shown). Chazotte-Aubert et al. (11) demonstrated that NO produced in inflamed tissues could nitrate p53 protein and thus play a role in carcinogenesis. Earlier studies have shown a continuous staining of alveoli with nitrotyrosine, a possible nitration of structural proteins (23). However, our results did not show nitration of p53 and β-actin in rat lungs during hyperoxia.

A coculture study was performed to demonstrate the contribution of neutrophils or macrophages to protein nitration. In this system, neutrophils or macrophages and target cells (L2 cells) were cultured on two sides of a membrane insert. The two types of cells were separated by a membrane and did not come in contact with each other directly. However, they were in close proximity to each other. When L2 cells were cocultured with neutrophils, an increase in protein nitration was found. The nitration further increased when nitrite was included in the coculture, indicating the active role of myeloperoxidase. Isolated macrophages from hyperoxic lungs also caused L2 cell protein nitration; however, the extent of nitration was less compared with the neutrophils. Further addition of 5 μg of anti-MPO antibodies reduced the protein nitration, thus indicating the contribution of myeloperoxidase in protein nitration. Although more nitration was observed when L2 cells were cocultured with neutrophils, the possibility for a significant contribution of macrophages in protein nitration cannot be ruled out, because BALF cells in hyperoxia-exposed rats are composed of 60–70% macrophages, 15–20% neutrophils, and 10–15% lymphocytes.

The results of the present study indicate that activation of MPO through infiltrated neutrophils, in part, contributes to protein nitration, and SP-A and t1α may be two targets for protein nitration in the alveoli, thus leading to epithelial damage. Further identification of other nitrated proteins will provide a therapeutic approach to prevent free radical-mediated lung damage.

**DISCLOSURES**

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