Brief Exposure to 95% Oxygen Alters Surfactant Protein D and mRNA in Adult Rat Alveolar and Bronchiolar Epithelium

Adegboyega O. Aderibigbe, Ronald F. Thomas, Robert R. Mercer, and Richard L. Auten, Jr.

Department of Pediatrics (Neonatal Medicine), Duke University Medical Center, Durham, North Carolina; and National Institute of Occupational Safety and Health, Morgantown, West Virginia

Surfactant protein D (SP-D), which has structural homology to C-type lectin binding regions, may play a role in host defense and has no known surfactant function. Because other surfactant proteins have been shown to be increased after prolonged periods of hyperoxia, we sought to evaluate the early effects of hyperoxia (95% O_2) on expression of SP-D in the adult male rat lung. Animals were exposed to air or to 12, 36, or 60 h of 95% O_2. Northern blot analysis of total lung RNA revealed marked SP-D mRNA increases at 12 h 95% O_2 compared with air-exposed controls, with decreasing expression to near that of air-exposed animals by 60 h. Semiquantitative *in situ* RNA hybridization demonstrated parallel results, with increased numbers of labeled alveolar epithelial (AE) and bronchiolar epithelial (BE) cells at 12 h and increased intensity of labeled alveolar cells, compared with air-exposed controls. After 60 h of exposure to 95% O_2, mRNA label intensity in AE and BE was decreased to levels near those seen in air-exposed animals. In contrast, Western blotting showed a decline in total lung SP-D with 95% O_2 exposure, beginning at 12 h and continuing at 36 and 60 h, respectively. Semiquantitative immunohistochemistry demonstrated a decline in AE labeling parallel to the total lung Western blot results, but labeled total BE cell numbers increased ($P = 0.10$). Hyperoxia had differential effects on SP-D abundance in AE and BE cells, and therefore may influence the availability of SP-D to bind microbial pathogens in the airways depending on cell type and location. **Aderibigbe, A. O., R. F. Thomas, R. R. Mercer, and R. L. Auten, Jr. 1999. Brief exposure to 95% oxygen alters surfactant protein D and mRNA in adult rat alveolar and bronchiolar epithelium. Am. J. Respir. Cell Mol. Biol. 20:219–227.**

Surfactant protein D (SP-D) has been isolated with lavaged lung surfactant but has no known role in reducing surface tension in the lung. Its molecular structure and cDNA sequence are highly homologous to SP-A, particularly in the carbohydrate recognition domain common to all C-type lectins (1). Evidence for a role for SP-D in lung host defense includes its enhancement of oxygen radical-mediated killing in rat alveolar macrophages (2, 3), significant binding to *Escherichia coli* and other gram-negative bacteria in isolated bronchoalveolar lavage (4–6), and significant calcium-dependent binding of the carbohydrate domains of *Pneumocystis carinii* (7). SP-D may provide protection against pulmonary influenza A infection (8–12), although it does not appear to opsonize influenza A for alveolar macrophages in the rat (13).

Hyperoxia has been used as a model of acute and chronic lung injury. Data on effects on gene expression following relatively brief oxygen exposures are limited. The earliest events in oxygen-mediated toxicity are as yet unknown, and may include alterations in host defense, as has been shown with more prolonged oxygen exposure (14). Responses to prolonged acute hyperoxia appear to be differentially regulated among the surfactant proteins (15–17). Previous studies have, in general, examined the effects of exposures > 24 h, and $F_{I_{O_2}} > 80\%$.

Because of the potentially important role of SP-D in lung defense, we postulated that lung injury might alter the abundance of SP-D, thereby potentially increasing pulmonary susceptibility to pathogens. Our experiments were designed to test the hypothesis that 95% O_2 exposure-mediated lung injury would reduce SP-D abundance.

Materials and Methods

Reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. Adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were exposed to 95% O_2 and 5% air or to air alone in sealed

(*Received in original form February 25, 1998 and in revised form May 27, 1998*)

Address correspondence to: R. L. Auten, DUMC Box 3179, Durham, NC 27710. E-mail: auten@acpub.duke.edu

Abbreviations: alveolar epithelium, AE; bronchiolar epithelium, BE; ethylenediamine tetraacetic acid, EDTA; phosphate-buffered saline-1% Tween-20, PBS-T; sodium dodecyl sulfate, SDS; surfactant protein D, SP-D; saline sodium citrate, SSC; Tris-buffered saline, TBS; ultraviolet, UV.

Plexiglas cages 60 × 45 × 25 cm. CO_2 was removed with a soda lime trap, and oxygen concentrations in the chambers were monitored with a continuous oxygen sensor (Servomex 572, Norwood, MA). Exposures were interrupted at 12 h intervals for < 10 min to clean cages and replenish food and water supplied *ad libitum*. Six animals were exposed to each condition: air or 95% O_2 for 12, 36, and 60 h. Animals were killed after exposure with sodium pentobarbital 160 mg/kg intraperitoneally. For RNA and protein isolation lungs from six animals per treatment condition, for a total of 24, were excised and flash-frozen in liquid nitrogen. For immunohistochemistry and *in situ* RNA hybridization, an additional six animals per treatment condition, for a total of 24, were used. Lungs were inflated and fixed *in situ* with 10% phosphate-buffered formalin at 20 cm H_2O inflation pressure. Tracheae were ligated and lungs and trachea were removed *en bloc*, fixed overnight, paraffin embedded, and sectioned at 4 to 6 μm.

Northern Blots

Frozen lungs were weighed, pulverized under liquid nitrogen, and thoroughly mixed. Total RNA was isolated by homogenizing 1 g (approximately 10% of the entire lung) of tissue per animal, with a Tissue Mizer (Tekmar, Cincinnati, OH) homogenizer in guanidinium isothiocyanate 1 M buffer as previously described (18). Total RNA was extracted in acid-equilibrated phenol and phenol:chloroform and precipitated with ethanol and sodium acetate. Yields and purity were determined by ultraviolet (UV) spectrophotometry at 260 and 280 nm, and yields per lung were calculated using the weights of the excised frozen lung for each animal. Ten-microgram samples of total lung RNA from air- and hyperoxia-exposed animals were denatured in formaldehyde and formamide and electrophoresed in formaldehyde-agarose 1.2% in 1× 3-(*N*-Morpholino)propanesulfonic acid buffer for 2 to 3 h (19). Equal loading was verified by staining with ethidium bromide 0.5 μg/ml in ammonium acetate 0.3 M for 20 min, followed by longwave UV transillumination. Gels were rinsed in diethylpyrocarbonate-treated water, and RNA was capillary blotted onto charged Nylon membranes (Magna, Westboro, MA) overnight, which were then UV crosslinked with 150 mJ. Hybridization conditions were as follows: 5× saline sodium citrate (SSC); 1× Denhardt's solution; 10% dextran; 0.5 mg/ml salmon sperm DNA; 10 mM Tris-HCl, pH 7.5; 1 mM ethylenediamine tetraacetic acid (EDTA), pH 7.5; and SP-D cDNA probe (gift of James Fisher [20]), labeled by nick translation according the manufacturer's instructions (BRL, Gaithersburg, MD) to an activity of 10^6 precipitable cpm/ml of ^{32}P α deoxyadenosine triphosphate. Membranes were hybridized overnight at 45°C, and washed in 2× SSC 0.5% sodium dodecyl sulfate (SDS) twice at room temperature followed by a wash in 0.1× SSC, 0.5% SDS at 62°C. Autoradiography was performed with Kodak XAR 5 film (Kodak, Rochester, NY) for 1 to 3 d at −70°C with an intensifying screen (Cronex; DuPont, Wilmington, DE). Membranes were reprobed with an 18S RNA cDNA probe, nucleotides 890 to 1108 (21), labeled as above, using the same hybridization conditions, to control for symmetry of RNA sample loading and transfer.

Western Blotting

Rabbit antirat SP-D (gift of Jo Rae Wright and Samuel Hawgood) was used for the immunoblots and immunohistochemistry and has previously been characterized (22, 23). Pulverized frozen rat lung was sonicated and briefly homogenized on ice using a microhomogenizer (KONTES, Vineland, NJ) in sample buffer, final concentration 1% SDS, 20% glycerol, 2% β-mercaptoethanol, 10 mM Tris-HCl, pH 6.8. Protein concentrations were determined in triplicate using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Fifty microgram samples from six animals at each exposure condition were analyzed using 10% SDS–polyacrylamide gel electrophoresis gels, with visible (Novex, San Diego, CA) and biotinylated (MW Protein Standards, Broad Range; Bio-Rad) protein size standards. Gels were blotted onto nitrocellulose (ECL; Amersham, Arlington Heights, IL) between two sheets of 3 MM filter paper (Whatman, Clifton, NJ) in a transfer tank using 192 mM glycine, 20% methanol, 25 mM Tris-HCl, pH 8.0, under constant 300 mA current, with stirring at 4°C for 3 h. Transfer was verified by staining the gel and the nitrocellulose (after detection) with Coomassie blue (24), as well as an additional nitrocellulose membrane between the transfer membrane and the filter paper on the anode side. Membranes were blocked for 2 h in 5% low-fat milk in phosphate-buffered saline-0.1% Tween-20 (PBS-T), then washed three times for 10 min in PBS-T. Membranes were incubated in rabbit antirat SP-D 1:1,000 in PBS-T for 1 h, washed three times for 10 min each in PBS-T, then detected with biotinylated goat-antirabbit serum 1:1,000 in PBS-T for 1 h (Vector, Burlingame, CA). Membranes were again washed three times in PBS-T and then incubated for 45 min in avidin-horseradish peroxidase conjugate 1:1,000 in PBS-T (Bio-Rad). These were detected using the ECL system according to the manufacturer's instructions (Amersham). Autofluorograms were exposed for 0.5 to 1.5 min using BioMax film (Kodak).

In Situ Hybridizations

These were performed as previously described with the following modifications (25). Paraffin-embedded 4-μm sections were deparaffinized in xylene, rehydrated, and treated with Proteinase K 1 μg/ml for 0.5 h at 37°C. Sections were hybridized with antisense 11-digoxigenin uridine triphosphate-labeled cRNA riboprobes transcribed from the SP-D cDNA according to the manufacturer's directions (Boehringer Mannheim, Indianapolis, IN) (26). The SP-D cRNA transcript was subjected to alkaline hydrolysis to reduce the transcript length to an average of 0.15 kb as determined by agarose gel electrophoresis. Probe and carbonate buffer were added to achieve a final concentration of 0.1 M carbonate buffer, pH 10.2 (40 mM $NaHCO_3$, 180 mM Na_2CO_3), and incubated at 60°C for 20 to 25 min according the method of Cox and colleagues (27). Hybridization and detection employed 0.15 M NaCl; 0.1 M Tris-HCl, pH 7.5 (buffer 1); 2% blocking reagent in buffer 1 (buffer 2); 0.1 M NaCl; 0.1 M Tris-HCl; and 0.05 M $MgCl_2$, pH 9.5 (buffer 3) (Boehringer Mannheim). Hybridization conditions were as follows: 5× SSC, 1× Denhardt's solution; 50% formamide; 10% dextran sulfate; 10 mM Tris-HCl, pH 7.5; 1 mM

EDTA, pH 7.5; 1% blocking reagent; 0.5 μg/ml cRNA probe, 100 μl/section encircled by a hydrophobic film (PAP pen; Research Products International, Mount Prospect, IL). After overnight hybridization in moist chambers containing 5× SSC/50% formamide at 45°C, sections were washed in 2× SSC for 10 min followed by RNase A 10 μg/ml, RNase T1 0.01 U/ml in 0.5 M NaCl; 10 mM Tris-HCl, pH 7.5; and 1 mM EDTA, pH 7.5 for 30 min at 37°C (Boehringer Mannheim). Sections were washed in RNase buffer an additional 30 min at 37°C. A high-stringency wash was performed in 400 ml 0.1× SSC at 60°C for 15 min. Detection was performed by blocking the sections with buffer 2 for 1 h, adsorbing away the buffer from the edge of the section, then incubating sections with antidigoxigenin antibody-alkaline phosphatase conjugate 1:500 in buffer 2, 100 μl/section overnight at 4°C. The antibody was washed off in two 10-min washes in buffer 1, followed by a 2-min incubation in buffer 3. Excess buffer was adsorbed from near the edge of the section, which was then covered with 200 μl of substrate (nitroblue tetrazolium 337.5 μg/ml, 5-bromo-4-chloro-3-indolyl phosphate toluidinium 175 μg/ml in buffer 3) and incubated at room temperature in the dark for 2 h. Controls included sections hybridized with sense 11-digoxigenin-labeled RNA probes and sections that were treated with RNase A as above before hybridization. Color development was stopped in Tris-HCl 10 mM, pH 7.5, EDTA 5 mM, pH 7.5, and mounted in Gelvatol prior to photomicroscopy. Experiments were performed on multiple lung sections of six different animals for each exposure condition. Photomicrographs were done with an Olympus Vanox-S AH-2 microscope (Olympus America, Melville, NY).

Immunohistochemistry

Immunohistochemistry was performed as previously described (17). Sections from air- and hyperoxia-exposed animals were dewaxed in xylene for 10 min twice, then rehydrated through graded ethanols and equilibrated in Tris-buffered saline (TBS). The sections were treated with 0.1% trypsin; 0.1% $CaCl_2$; 20 mM Tris-HCl, pH 7.5, for 5 min at room temperature, then washed in TBS for 5 min. The sections were treated with 0.3% hydrogen peroxide in methanol for 30 min, followed by 10-min washes in TBS and 0.05% Triton X-100 in TBS, followed by 20 min in 3% bovine serum albumin (Boehringer Mannheim) in TBS at room temperature. Excess buffer was adsorbed from near the sec-

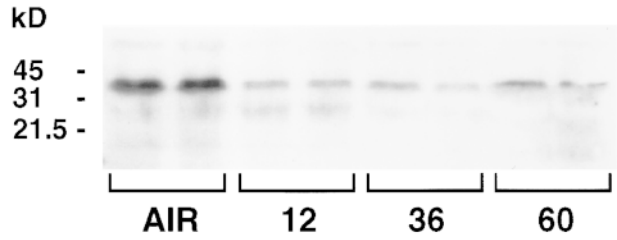

Figure 2. Representative Western blot of lung tissue homogenate isolated from air- and oxygen-exposed animals. SP-D is decreased at 12, 36, and 60 h of hyperoxia compared with control (AIR).

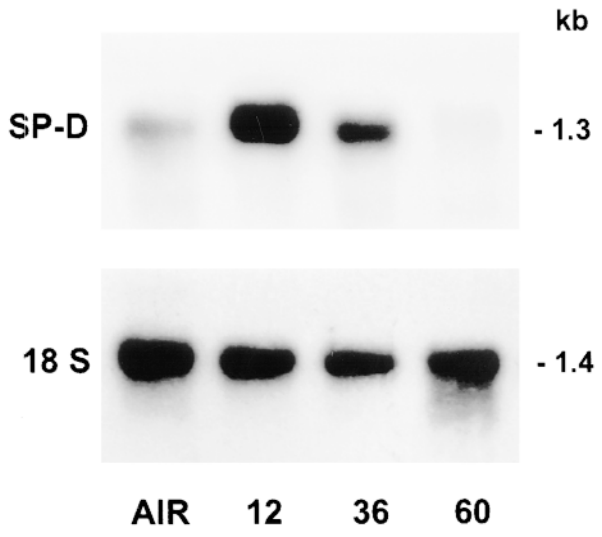

Figure 1. Representative Northern hybridization showing SP-D mRNA signal in whole-lung total RNA isolated from air- and oxygen-exposed animals. SP-D mRNA is increased at 12 and 36 h, returning to near control (AIR) by 60 h, whereas there is no change in 18S mRNA abundance.

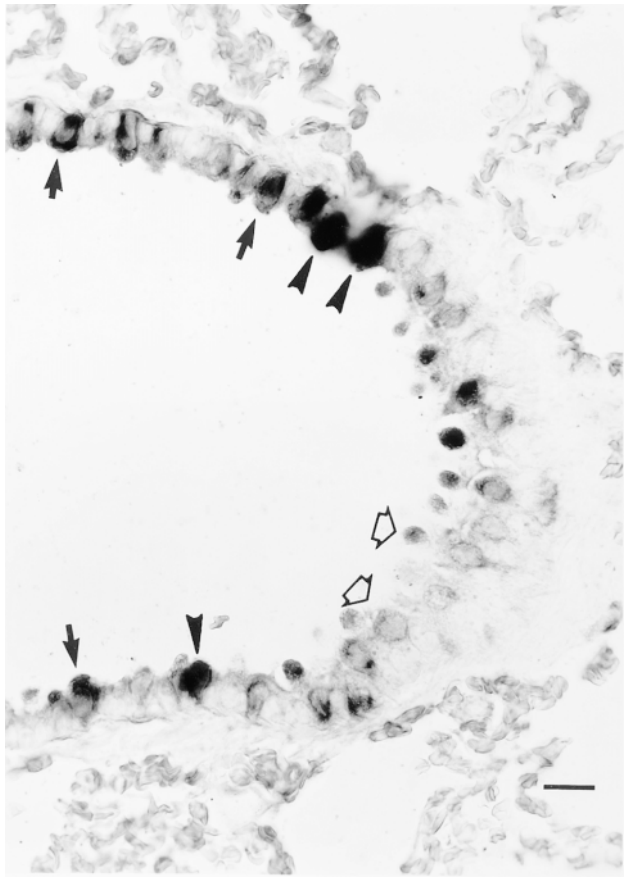

Figure 3. Grading of label intensity. *In situ* hybridization of SP-D cRNA antisense probe to airway, showing low- (*arrowhead*), medium- (*solid arrow*), and high- (*open arrow*) intensity labels.

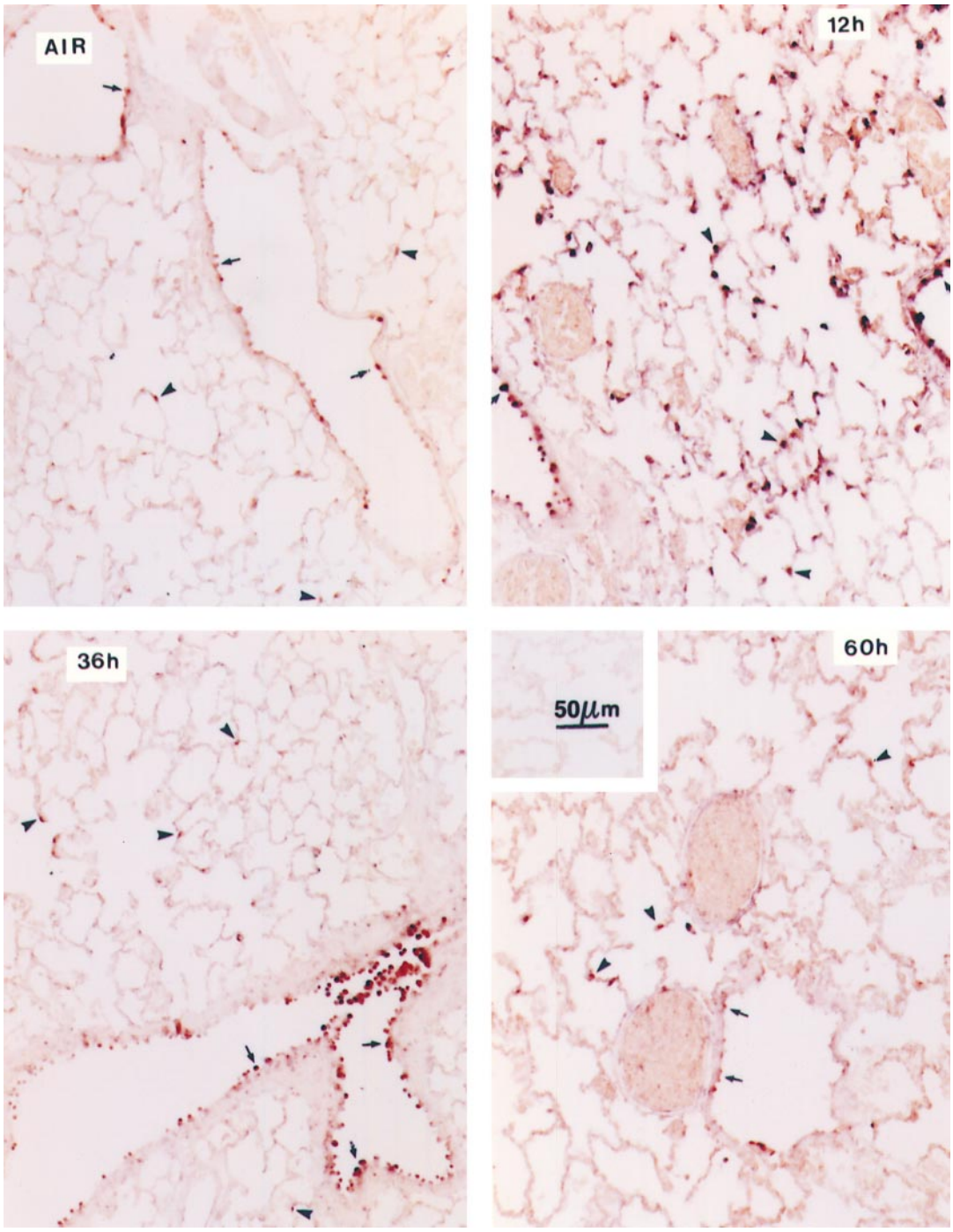

Figure 4. Effects of hyperoxia on *in situ* hybridization of SP-D. Purplish precipitate localizes the SP-D mRNA to AE (*arrowhead*) and BE (*arrow*). The RNase-pretreated assay control is shown in the *inset* of the 60 h photograph. *Bar* = 50 μm.

tion edge, and sections were encircled with a hydrophobic film (PAP Pen; Research Products International) before overlaying with 100 μl of rabbit antirat SP-D 1:1,000 in TBS. Antibody had been preadsorbed with an equal volume of rat serum overnight at 4°C before centrifugation at 14,000 × *g* for 10 min. Control slides were similarly treated throughout, omitting the primary antibody. Sections were then detected using the Vectastain ABC Elite kit (Vector Labs, Burlingame, CA), rinsed in TBS, and incubated with biotinylated goat antirabbit IgG, according to the manufacturer's instructions. Sections were rinsed in TBS, incubated for 1 h in Vectastain ABC reagent, rinsed in TBS, and incubated at room temperature for 2 to 10 min in 0.05% diaminobenzidine tetrahydrochloride; 0.01% hydrogen peroxide; 0.05 M Tris-HCl, pH 7.2. After color development, sections were rinsed in distilled water, dehydrated through

graded ethanol, and mounted with Permount (Fisher, Pittsburgh, PA) before photomicroscopy.

Cell Counting

The number of alveolar epithelial type II and nonciliated bronchiolar epithelial Clara cells as well as the distribution of labeled mRNA for SP-D and the immunohistochemical labeling for SP-D was determined by standard morphometric methods that have been described in detail previously (28, 29–31). Labeled cells were counted using an eyepiece-counting graticule in 50 randomly selected high-power fields (magnification: ×400). The section area counted per high-power field was 0.025 mm^2. Gunderson's unbiased counting rules were used to eliminate size-dependent edge effects in counting cells that crossed the boundaries of the counting field (32). Calculations of the total cell numbers per lung were based on these determinations of cell number per field, a section thickness of 4 μm, and the average or mean caliper diameter of 9.6 μm for Clara cells and 9.4 μm for type II cells as previously described (29–31). In both immunohistochemistry and *in situ* RNA hybridization experiments, labeled cells were classified according to intensity. Low-intensity cells demonstrated a signal just discernible above background, medium-intensity cells demonstrated homogeneous staining across the entire diameter of the cell but with preservation of visible intracellular detail, and high-intensity cells were homogeneously labeled at sufficient intensity to obliterate intracellular detail, as demonstrated in Figure 3. Unlabeled type II cells and Clara cells were also counted. The total number of cells and the number of type II and Clara cells in each condition were determined by counting unlabeled cells on a similar number of hematoxylin- and eosin-stained sections.

Statistical comparisons were made using analysis of variance, with significance determined to be $P < 0.05$. Calculations were done on a Macintosh computer (Apple Computer, Cupertino, CA) using Excel (Microsoft, Seattle, WA).

Results

Northern and Western blotting. SP-D mRNA in whole lung was increased at 12 and 36 h 95% O_2. Message returned to near air-control levels by 60 h, as determined by Northern hybridization shown in Figure 1, with no significant change in 18S RNA. The total RNA recovered per lung was relatively constant except for 60 h 95% O_2: air = 21.7 ± 4.4 μg/lung; 12 h = 20.3 ± 4.0; 36 h = 26.7 ± 4.2; 60 h = 36.6 ± 5.1 (mean ± SD). The increase in recovered RNA was not statistically significant compared with the amount recovered at 36 h, but approached significance when compared with air and 12 h 95% O_2, $P = 0.07$.

In contrast to the increased SP-D mRNA, there was a decline in immunodetectable SP-D in lung homogenates by 12 h 95% O_2, which persisted at 36 and 60 h 95% O_2, as shown in Figure 2, demonstrating a prompt effect of 95% O_2 on SP-D. The total protein recovered per lung did not differ significantly: air = 35.9 ± 9.7 mg/lung; 12 h = 37.5 ± 6.8; 36 h = 44.8 ± 8.0; 60 h = 43.5 ± 7.8 (mean ± SD).

Semiquantitative in situ *hybridization and immunohistochemistry.* Because local effects of hyperoxia on SP-D may differ by cell type and location, we performed semiquantitative measurements of SP-D mRNA and SP-D immunolabeling signal classified by numbers of labeled alveolar epithelium (AE) or bronchiolar epithelium (BE) cells. Positive cells were classified by intensity as shown in Figure 3. Based on the morphometric measurements, a total of 158 million type II AE cells and 15.5 million Clara cells were present in air-exposed rats. Values of 125 million AE cells and 17.3 Clara cells have previously been reported in rats of comparable size (31). Measurements of total numbers of AE and BE cells in hyperoxia-exposed animals did not differ significantly from control, so changes in SP-D mRNA abundance likely reflect changes in cell-specific expression rather than loss of SP-D expressing cells during hyperoxia exposure.

In situ RNA hybridizations (Figure 4) demonstrate SP-D

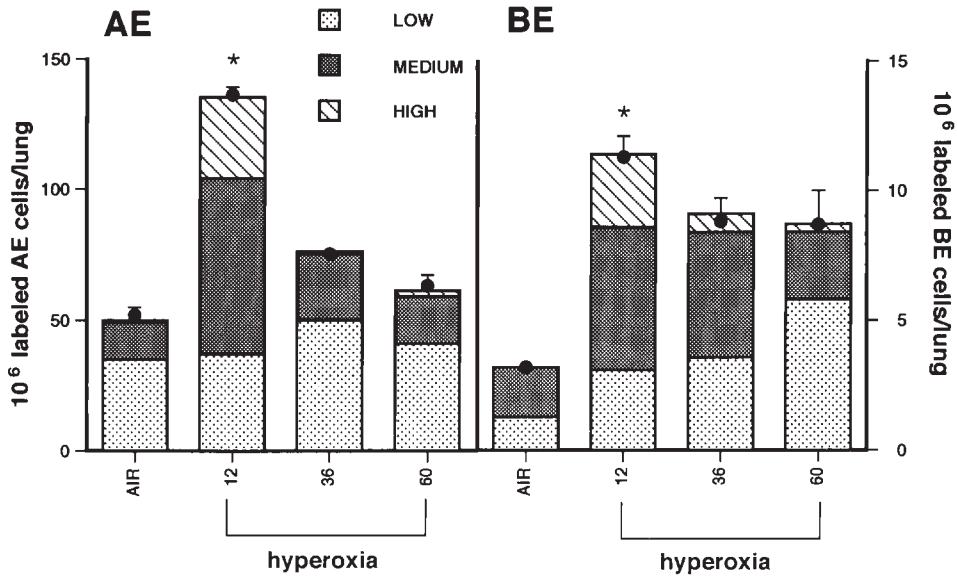

Figure 5. Semiquantitation of SP-D *in situ* hybridization signal, cell number, and intensity grade. Results are expressed in terms of mean labeled cell number and labeled cell number at each intensity grade per lung ± SEM. *$P <$ 0.05 versus control cells.

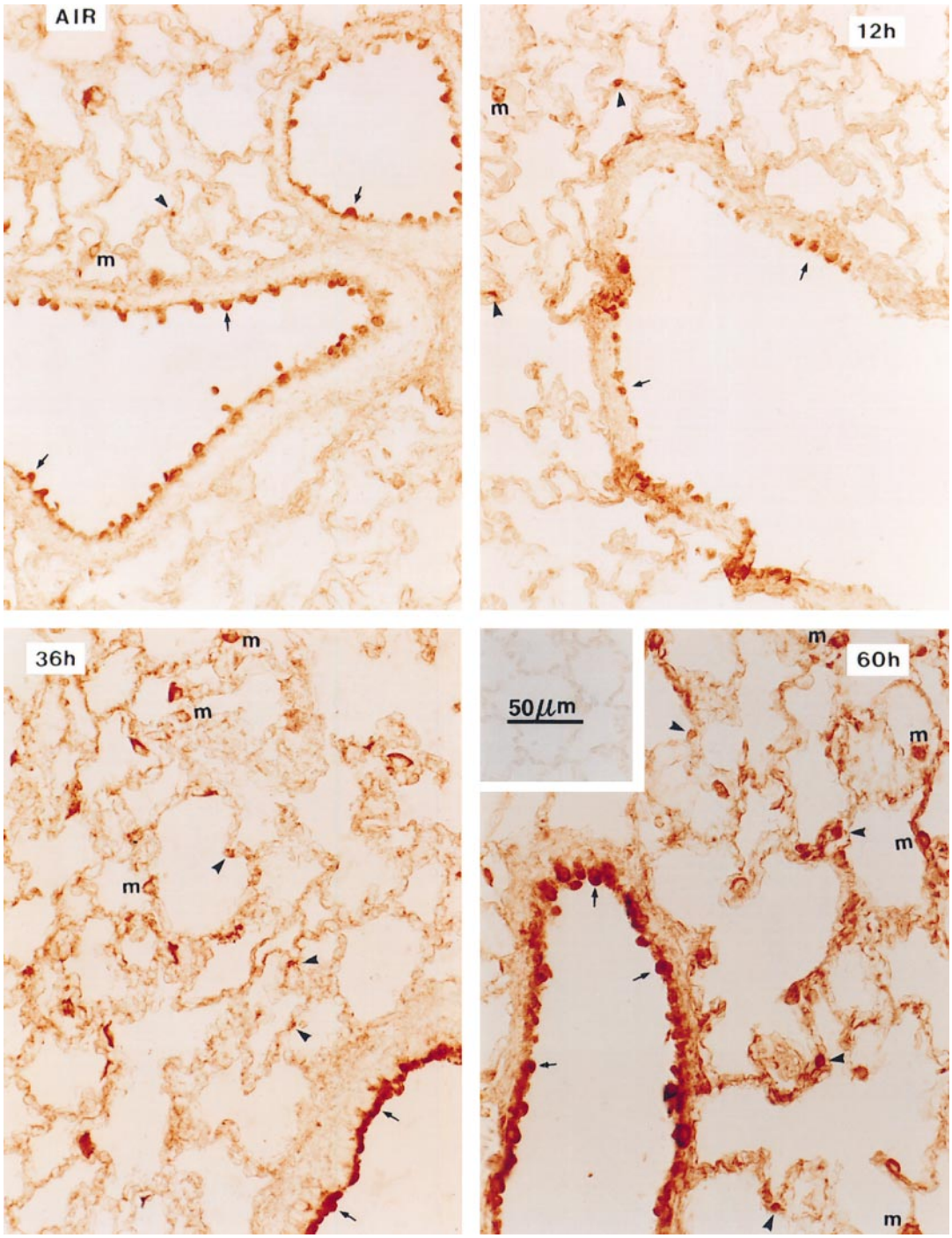

Figure 6. Immunohistochemistry. Brownish precipitate localizes SP-D in AE (*arrowhead*) and BE cells (*arrow*), as well as macrophages (m). Section detected without primary antibody is shown in the *inset* of the 60 h photograph. *Bar* = 50 μm.

mRNA in AE and BE as previously shown (33). The overall effect on AE cells paralleled the Northern blot results. By 12 h of hyperoxia, SP-D was increased in both cell types, with a nearly threefold increase in the number of labeled AE cells, as shown in Figure 5. Label intensity was also increased in AE cells. At 36 h of hyperoxia, labeled AE and BE cell numbers declined from the 12 h levels and were reduced to near control levels by 60 h of hyperoxia. The effects on label intensity in AE cells also paralleled the Northern blot results. By 60 h of hyperoxia, the SP-D mRNA signals in both AE and BE cells were reduced to levels near air control. There was no hybridization signal seen in the sense cRNA-hybridized assay controls or in RNase-pretreated assay controls (*see inset*, Figure 4).

Immunohistochemistry similarly paralleled the Western blot results, with significantly decreased numbers of la-

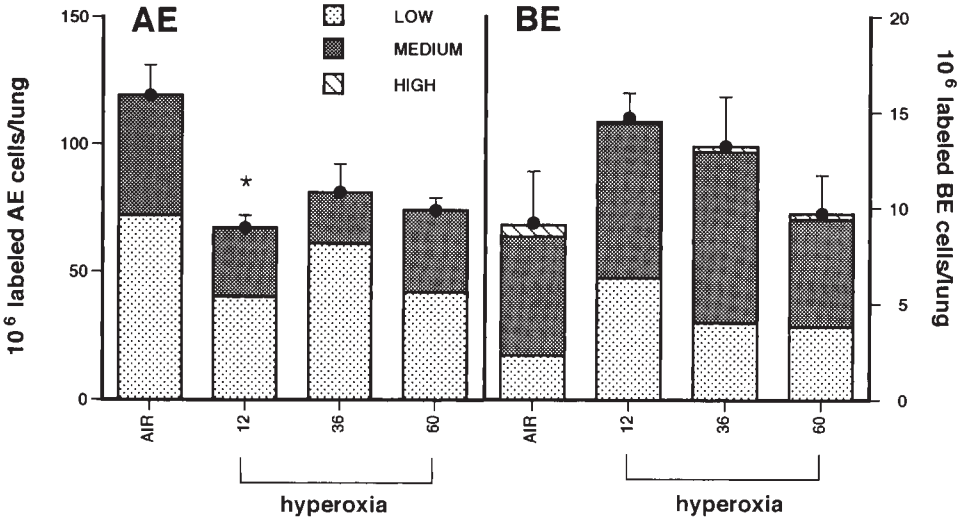

Figure 7. Effects of hyperoxia on SP-D immunohistochemistry signal, cell number, and intensity grade per lung ± SEM. $*P < 0.05$ versus control cells.

beled AE cells of both low and medium intensity, at each level of hyperoxia exposure as shown in Figures 6 and 7. There were modestly increased numbers of BE cells after 12 h of hyperoxia, predominantly of low-intensity cells, although total numbers did not achieve statistical significance ($P = 0.10$), with declines in labeled cell number to air control levels by 60 h. High-intensity immunolabeled BE but not AE cells were seen. High-intensity labeling was also seen in macrophages, but these were not counted.

Discussion

We studied the effects of 95% O_2 on expression of SP-D and its mRNA at exposure durations of 12, 36, and 60 h compared with air exposure because we previously determined that there were decreases in SP-A expression following 12 h 95% O_2 (34). Both SP-D and SP-A play roles in lung response to bacterial and viral pathogens. If hyperoxia results in significant sustained depression of SP-D expression, it may adversely affect pulmonary defense against microbial invasion, particularly in lung injury conditions necessitating supplemental oxygen treatment.

We found that SP-D was depressed after 12 h of 95% O_2 in whole lung through its effect on AE cell expression, as shown by the parallel responses in Western blots and immunohistochemistry results. This was sustained throughout the exposure up to 60 h. Alveolar-capillary leak due to hyperoxia-induced lung injury might have led to loss of immunodetectable SP-D from epithelium into the airways, and to subsequent phagocytosis and clearance by alveolar macrophages, leading to decreased SP-D detected in whole lung. Further studies using pulse labeling of SP-D may elucidate the fate of newly synthesized and secreted SP-D in acute lung injury states. This may be particularly important because a decrease in SP-D detected in whole lung occurred when the SP-D mRNA in whole lung was increased. If newly synthesized SP-D were cleared from airways by alveolar macrophages and reached the circulation, then the amount of immunodetectable SP-D in the lung might underestimate the SP-D translated from newly transcribed SP-D mRNA. This possibility is suggested by observations that increased SP-D has been detected in the circulation in clinical lung injury states (35). There may also be a lag between elevations in SP-D mRNA abundance and increased SP-D translation, as has been shown with manganese superoxide dismutase following hyperoxia exposure (36). Clarifying the nature of the dose-response of SP-D expression to hyperoxia may require even shorter exposures, and at different $F_{I_{O_2}}$.

Hyperoxia appears to affect specific SP-D mRNA accumulation at 12 and 36 h 95% O_2, as SP-D mRNA was increased but total RNA and 18S RNA signal were unchanged. It is possible that there were global effects of 95% O_2 exposure on mRNA as a proportion of total RNA, which might affect the results, but this was not directly tested because the focus of our experiments was on cell-specific effects.

The suppressive effects of hyperoxia on SP-D were primarily in AE as shown in Figures 5 and 7. In contrast, immunolabeled BE cells tended to increase at 12 and 36 h of hyperoxia, followed by a decline at 60 h, in parallel with the *in situ* RNA hybridization results, although this did not achieve statistical significance ($P = 0.15$). The coordinate increases in BE SP-D immunolabeling and *in situ* RNA abundance are consistent with increased mRNA transcription and consequent SP-D synthesis in BE cells. We speculate that decreased SP-D secretion or metabolism, or uptake in BE cells could also account for the apparent increase in immunolabeling, both in number and intensity, although uptake of SP-D by BE cells has not been demonstrated to our knowledge. This differential effect of injury on SP-D abundance in AE and BE cells has also been seen in radiation injury (37). Similar noncoordinate effects of hyperoxia have been noted in SP-A expression in AE and BE cells (17). The effects of hyperoxia on SP-D mRNA *in situ* hybridization signal in AE cells did not parallel the effects on immunolabeling in contrast with the pattern seen in BE cells, possibly because of increased secretion of SP-D from AE into airways and clearance from the lung compartment as discussed previously.

The apparent differential effects of hyperoxia on AE

and BE immunolabeling may be attributable to oxidative effects on the carbohydrate-binding domain, the anti-SP-D epitope, which might alter antibody detection. Oxidative stress has had similar effects on the carbohydrate-binding domain in SP-A (38–41). In addition, secretory pathways for SP-D differ between the cell types, which may affect antigen exposure or preservation with oxidant stress. In BE cells, SP-D is prominently labeled in secretory granules (42), but in AE cells the endoplasmic reticulum is most prominently labeled (43).

Precise cellular quantitation using immunologic detection of SP-D or labeled SP-D mRNA hybrids can be problematic. Both methods exhibited sufficiently low background in these experiments to permit counting positively labeled cells using the criteria listed in Materials and Methods. The intensity grading system is arbitrary, so firm conclusions about the overall amount of mRNA or protein per cell cannot be made. The overall effects of hyperoxia on SP-D expression in lung were corroborated by whole-lung studies. Image analysis of nonisotopically labeled *in situ* hybridization may permit more accurate quantitation in the future (44), but the methodology is not yet well established. The principal strength of the *in situ* detection in the present studies is to demonstrate and distinguish the relative contribution of the different cell types to the injury response. This may be of particular importance for understanding localized effects on lung immunity in injury states, as it may influence the clinical manifestation of pulmonary infection.

The pattern of SP-D responses in lung injury models is variable. We found an acute rise in SP-D mRNA after brief hyperoxia exposure, as was noted after acute lipopolysaccharide administration (23). In other studies, proinflammatory stimulus with cytokines and endotoxin did not increase SP-D immunolabeling in intact lung (45), nor did they increase SP-D mRNA expression in fetal lung explant (46). Keratinocyte growth factor may be a required cytokine for SP-D induction, particularly in isolated cell culture (47).

The role of SP-D in pulmonary host defense includes opsonization, ligand binding, and macrophage signaling (8, 48, 49). Although it shares substantial homology with SP-A, particularly in the lectin domain (50, 51), it demonstrates differential ontogeny in fetal development (46) and responses to hormonal regulation and injury response. The depressive effects of 95% O_2 exposure on SP-D protein in alveolar cells, which developed after only 12 h of hyperoxia and which were sustained throughout the exposure duration, may alter the capacity of the lung to opsonize a number of pathogens or signal alveolar macrophage clearance of pathogens, particularly in lung injury states requiring high levels of oxygen therapy such as pneumonia or adult/acute respiratory distress syndrome. This may be particularly relevant in patients colonized with *Pseudomonas aeruginosa* (or other gram-negative bacteria [4]), or in patients with influenza (52).

In summary, these studies indicate that brief hyperoxia exposure decreases SP-D abundance in adult rat lung, accompanied by alterations in SP-D gene expression. The responses to brief hyperoxia are differentially regulated in BE and AE, and may lead to alterations in SP-D-mediated host defense following hyperoxia exposure, particularly in alveoli. Studies of the infectivity and clearance of microbial SP-D ligands *in vivo* following hyperoxia may help determine the significance of this effect.

Acknowledgments: The authors thank Jo Rae Wright and Samuel Hawgood for providing polyclonal antirat SP-D antibodies. They also thank Dr. James Fisher for the gift of rat SP-D cDNA, and Dr. Wright and Dr. Judith Voynow for the critical review of this manuscript. This study was supported in part by a grant from Wyeth Pediatrics.

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