Brief 95% O_2 exposure effects on surfactant protein and mRNA in rat alveolar and bronchiolar epithelium

THOMAS F. ALLRED,[1] ROBERT R. MERCER,[2]
RONALD F. THOMAS,[1] HUI DENG,[1] AND RICHARD L. AUTEN[1]
[1]*Division of Neonatal Medicine, Department of Pediatrics, Neonatal-Perinatal Research Institute, Duke University Medical Center, Durham, North Carolina 27710; and* [2]*Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, West Virginia 26505*

Allred, Thomas F., Robert R. Mercer, Ronald F. Thomas, Hui Deng, and Richard L. Auten. Brief 95% O_2 exposure effects on surfactant protein and mRNA in rat alveolar and bronchiolar epithelium. *Am. J. Physiol.* 276 (*Lung Cell. Mol. Physiol.* 20): L999–L1009, 1999.—In acute lung injury, a disturbed surfactant system may impair gas exchange. Previous evaluations of hyperoxia effects on surfactant proteins (SPs) followed exposures >1–2 days. To evaluate the effects of brief exposure to hyperoxia on the SP system, we exposed adult male rats to 95% O_2 or air for 12, 36, and 60 h. SP-A, -B, and -C mRNAs were analyzed by Northern blot and semiquantitative in situ hybridization (ISH). SP-A and -B were analyzed in whole lung homogenates, lung lavage fluid, and fixed tissue by semiquantitative immunohistochemistry (IHC). All SP mRNAs were diminished at 12 h and rose to or exceeded control by 60 h as determined by Northern blot and ISH. These effects were seen mainly in the intensity of ISH signal per cell in both type II and bronchiolar epithelial (Clara) cells and to a lesser extent on numbers of positively labeled cells. SP-B declined to 50% of control in lavage at 12 h, but no changes in total lung SP-A and -B were seen. The number of SP-A positively labeled cells did not change, but SP-A label intensity measured by IHC in type II cells showed parallel results to Northern blots and ISH. The response of SP-A in Clara cells was similar. SP-B immunolabeling intensity rose in both type II and Clara cells throughout the exposure. SP-C ISH intensity fell at 12 h and was increased to two times control by 60 h of hyperoxia. Sharp declines in SP expression occurred by 12 h of 95% O_2 and may affect local alveolar stability.

lung injury; in situ hybridization; hyperoxia

PULMONARY SURFACTANT is necessary to maintain alveolar stability. The complex effects of hyperoxia on the surfactant system are of particular interest since supplemental O_2 is necessary therapy in a variety of lung injury syndromes. Hyperoxia-associated effects on pulmonary surfactant have been shown to stimulate surfactant protein (SP) and SP mRNA accumulation (23) and inhibit accumulation of surfactant phospholipids (16, 20). Effects of hyperoxia have depended on dose, species, and cell type (12). Responses to hyperoxia appear to be differentially regulated among the SPs (21). Previous studies have, in general, examined the effects of exposures >24 h and fraction of inspired O_2 ($F_{I_{O_2}}$) >80%.

We sought to determine the pattern of SP and mRNA accumulation beginning with relatively brief periods of 95% O_2 exposure for 12 h as well as for longer 36- and 60-h exposures. If hyperoxia adversely affects the surfactant system after relatively brief exposures, then alterations of alveolar stability within the pulmonary microenvironment may occur, with potential effects on serum protein transudation, inflammation, and the propagation of lung injury. Such changes might not be observable at the whole lung level or with evaluations of pulmonary lavage: regional variations of surfactant function would tend to be obscured. Disturbances in surfactant processing after even brief periods of hyperoxia may have important implications for the potential therapeutic role of surfactant therapy in conditions requiring high ambient O_2 therapy. The therapeutic role of exogenous surfactant in acute lung injury syndromes such as adult respiratory distress syndrome is being increasingly recognized (13).

We therefore evaluated the effects of 95% O_2 on adult male rat lung SP-A and SP-B accumulation determined by immunohistochemistry and SP-A, -B, and -C mRNA accumulation determined by in situ RNA hybridization and compared them with similar determinations by Western and Northern blotting. The results indicate that all three SP mRNAs are significantly affected after 12 h of 95% O_2, with effects on SP-A and SP-B protein accumulation largely paralleling the alterations in mRNA as measured by immunohistochemistry and in situ hybridization.

METHODS

Reagents were obtained from Sigma (St. Louis, MO) unless otherwise specified. Adult male Sprague-Dawley rats, average weight 350 ± 15 g (Charles River Laboratories, Wilmington, MA), were exposed to 95% O_2 and 5% air or to air alone in sealed plastic cages 60 × 45 × 25 cm. CO_2 was removed with a soda lime trap, and O_2 concentrations in the chambers were monitored with a continuous O_2 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. Animals were exposed to air or to 12, 36, or 60 h of 95% O_2, four animals under each condition. Animals were killed with 160 mg/kg ip pentobarbital sodium after exposure. Lungs were quickly removed and inflation fixed as previously described (1).

Northern blots. Lungs were obtained from an additional four animals per treatment group, weighed, flash-frozen, pulverized under liquid nitrogen, and thoroughly mixed. Total RNA was isolated by homogenizing 1 g (~10% of the entire lung) of tissue per animal with a Tissue Mizer (Tekmar, Cincinnati, OH) homogenizer in 1 M guanidinium isothiocyanate buffer as previously described (30). Total RNA was extracted in acid-equilibrated phenol and phenol-chloroform, precipitated with ethanol and sodium acetate. Yields and

purity were determined by ultraviolet (UV) spectrophotometry at 260 and 280 nm. Samples (10 μg) of total lung RNA from air- and hyperoxia-exposed animals were denatured in formaldehyde and formamide and electrophoresed in 1.2% formaldehyde-agarose in 1× MOPS buffer for 2–3 h (26). Equal loading was verified by staining with 0.5 μg/ml ethidium bromide in 0.3 M ammonium acetate for 20 min, followed by long-wave UV transillumination. Gels were rinsed in diethyl pyrocarbonate-treated water, and RNA was capillary blotted onto charged Nylon membranes (Magna, Westboro, MA) overnight, which were then UV cross-linked with 150 mJ.

Hybridization conditions. Hybridization conditions were 5× saline-sodium citrate, 1% Denhardt's solution, 10% dextran, 0.5 mg/ml salmon sperm DNA, 10 mM Tris·HCl (pH 7.5), 1 mM EDTA (pH 7.5), and SP-A, -B, or -C cDNA labeled by nick translation according the manufacturer's instructions (GIBCO BRL, Gaithersburg, MD) to an activity of 10^6 precipitable counts·min^{-1}·ml^{-1} of [α-^{32}P]dATP. DNA templates encoded rat SP-A [nucleotides (nt) 1–774], SP-B (nt 413–817), and SP-C (nt 54–646; kind gift of M. Steele) cloned into pGEM vectors (Promega, Madison, WI). Orientation was confirmed by DNA sequence analysis. Membranes were hybridized overnight at 45°C and washed in 2× SSC-0.5% SDS two times at room temperature followed by a wash in 0.1× SSC-0.5% SDS at 62°C. Autoradiography was performed with Kodak X-AR 5 film (Kodak, Rochester, NY) for 1–3 days at −70°C with an intensifying screen (Cronex; DuPont, Wilmington, DE). Membranes were reprobed with 18S RNA cDNA probe, with nt 890–1108 (31) labeled as above, using the same hybridization conditions, to evaluate reproducible RNA sample loading and transfer.

Western blots. Rabbit anti-rat SP-A and SP-B (gifts of Jo Rae Wright and Samuel Hawgood) were used for the immunoblots and immunohistochemistry and have previously been characterized (8, 9, 35). After air or hyperoxia exposure, an additional four animals per condition were anesthetized as above and had tracheal lavage with 15 ml of 0.9% NaCl and 1 mM EDTA after removal of the anterior chest. Lavage was done five times slowly and then snap-frozen for later analysis. Protein concentrations were determined in triplicate using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Because recovered lavage volumes were similar (13 ± 1 ml), 30-μl lavage samples from four animals at each exposure condition were analyzed using 15% SDS-PAGE. Similar experiments were done with whole lung homogenates in a final concentration of 1% SDS, 20% glycerol, 2% β-mercaptoethanol (omitted for SP-B Western blots), and 10 mM Tris·HCl (pH 6.8), using 100 μg protein/well. 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), and 0.1% SDS 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. Membranes were blocked for 2 h in 5% nonfat milk in PBS-0.1% Tween 20 (PBS-T) and then were washed three times for 10 min in PBS-T. Membranes were incubated in a 1:5,000 dilution of primary antibody in PBS-T for 1 h, washed three times for 10 min each in PBS-T, and then detected with a 1:5,000 dilution of biotinylated goat-anti-rabbit serum in PBS-T for 1 h (Vector, Burlingame, CA). Membranes were washed again three times in PBS-T and then incubated for 45 min in a 1:5,000 dilution of avidin-horseradish peroxidase conjugate in PBS-T (Bio-Rad). They were detected in diaminobenzidine-$NiCl_2$ according to the manufacturer's directions (DAB Kit; Vector).

Quantification. Autoradiograms and immunoblots were scanned with a flat-bed scanner (Astra 1200, UMAX) and were analyzed with National Institutes of Health Image version 1.54 using a Power PC Macintosh computer. Equal band areas were quantified for comparisons, and data were normalized to the control air-exposed values.

In situ hybridizations. In situ hybridization was performed on paraffin-embedded sections as previously described, with some modifications (1). Sections were hybridized with antisense 11-digoxigenin UTP-labeled cRNA riboprobes transcribed from cDNAs for SP-A, -B, and -C noted above. DNA templates were linearized before cRNA in vitro transcription, which was performed according to the manufacturer's directions (Boehringer Mannheim, Indianapolis, IN; see Ref. 3). Labeled cRNA transcripts were subjected to alkaline hydrolysis to reduce the transcript length to an average of 0.15 kb. Hybridization and detection employed 0.15 M NaCl and 0.1 M Tris·HCl (pH 7.5; *buffer 1*), 2% blocking reagent in *buffer 1* (*buffer 2*), and 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 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, and 0.5 mg/ml cRNA probe at 100 μl/section encircled with a PAP Pen (Research Products International, Mt. Prospect, IL). After overnight hybridization on racks in sealed moist chambers containing 5× SSC and 50% formamide at 45°C, sections were washed in 2× SSC for 10 min followed by treatment with 10 μg/ml RNase A and 0.01 U/ml RNase T1 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. Sections were removed from enzymes and washed in fresh buffer for an additional 30 min at 37°C. A high-stringency wash was performed in 400 ml of 0.1× SSC at 60°C for 15 min. Detection was performed by incubating the sections with *buffer 2* for 1 h, adsorbing away the buffer from the edge of the section, and then incubating sections with 1:500 anti-digoxigenin antibody-alkaline phosphatase conjugate in *buffer 2*, 100 μl/section, overnight at 4°C. Antibody was removed with three 15-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 (337.5 μg/ml nitro blue tetrazolium and 175 μg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidinium in *buffer 3*; Boehringer Mannheim) and incubated at room temperature in the dark for 1–6 h. Controls included sections hybridized with sense 11-digoxigenin-labeled RNA probes and sections that were treated with RNase A (50 μg/ml) for 1 h before hybridization. After color development, reactions were stopped in 10 mM Tris·HCl (pH 7.5) and 5 mM EDTA (pH 7.5) and were mounted in aqueous medium (ProbeMount; Innovex) before photomicroscopy. Experiments were performed on random lung sections of four different animals for each exposure condition.

Positive controls. To assess the availability of target mRNA in tissue sections to probe, sections were dewaxed, rehydrated, and treated with proteinase K (see *In situ hybridizations*). They were hybridized with a digoxigenin-labeled oligothymidine probe prepared by 3′-end labeling according to the manufacturer's instructions Boehringer Mannheim; see Ref. 3). This was done to take advantage of the polyadenylated 3′ sequences of mRNA. The probe length was an average of 38 bases determined by acrylamide gel electrophoresis. Random sections were prehybridized in a moist chamber at 37°C for 2 h in the following conditions: 2× SSC, 1× Denhardt's solution, 36% formamide, 10 mg/ml BSA, 50 mM Na_2HPO_4/NaH_2PO_4 (pH 7.0), 50 mM dithiothreitol, 0.25 mg/ml yeast tRNA, 0.5 mg/ml salmon sperm DNA (which was denatured

at 95°C for 5 min just before being added to the hybridization buffer), and 5 μg/ml oligonucleotide probe. Assay controls included sections that were pretreated with RNase A and sections that were cohybridized with 1,000-fold excess unlabeled probe to demonstrate signal specificity. Positively labeled cells were compared with adjacent sections stained with methyl green to determine the labeling index.

Immunohistochemistry. Immunohistochemistry was performed to evaluate the cellular distribution and abundance of SP-A and SP-B, but not of SP-C, since antibodies to the mature peptide were not available. Paraffin-embedded sections were dewaxed, rehydrated, and processed as previously described (12). Sections were rinsed in PBS for 10 min and treated with 0.1% trypsin and 0.1% $CaCl_2$ in 20 mM Tris·HCl (pH 7.5) for 15 min. Two washes in PBS for 10 min each were followed by treatment with 0.3% hydrogen peroxide in methanol for 30 min at room temperature to quench endogenous peroxidase activity. Slides were washed for 10 min in PBS, 10 min in 0.05% Triton X-100 in PBS, and 20 min in 1% gelatin in PBS. Sections were carefully dried by adsorbing excess buffer from near the section edge, encircled with a PAP pen, and incubated overnight with rabbit anti-rat primary antibody to SP-A at a dilution of 1:1,000 and SP-B at a dilution of 1:500 in PBS. Antibody had been preadsorbed with an equal volume of rat serum overnight at 4°C, followed by centrifugation at 13,000 *g* for 10 min to remove nonspecific antigen-antibody complex. Antibody was washed off sections in two 5-min PBS washes. Labeling was detected using the avidin-biotin complex (ABC) Vectastain Elite kit (Vector) according to the manufacturer's instructions. Biotinylated goat anti-rabbit secondary antibody (5 μl) and goat serum (15 μl) were diluted in 1 ml of PBS and applied to sections for 1 h. Secondary antibody was washed two times in PBS for 15 min followed by incubation in ABC avidin-horseradish peroxidase complex for 30 min, according to the manufacturer's instructions. After being washed two times for 5 min in PBS, sections were covered with 0.05% diaminobenzidine and 0.01% hydrogen peroxide in 50 mM Tris·HCl (pH 7.2) for 2–10 min. Color development was stopped with tap water, and sections were dehydrated through graded ethanols and mounted with Permount (Fisher) without counterstaining before photomicroscopy with an Olympus Vanox-S AH-2 microscope.

Cell counting. Lung sections were cut from blocks oriented to include upper and lower lobes. Random sections from sequential coronal blocks were chosen to sample all areas of the lung. At least 10 sections per lung were counted. The number of alveolar epithelial type II and nonciliated bronchiolar epithelial Clara cells as well as the distribution of mRNA for SP-A, -B, and -C and the immunohistochemical labeling for SP-A and SP-B were determined by standard morphometric methods (33) that have been described in detail previously (18, 19, 29). Positive 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 (6) unbiased counting rules were used to eliminate size-dependent edge effects in counting cells that crossed the boundaries of the counting field. 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 and 9.4 μm for type II cells as previously described in detail (18, 19, 29). The extrapolated number of labeled cells per lung was then averaged among the animals in each treatment condition, yielding mean labeled cell number vs. treatment. 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 Fig. 1. Unlabeled type II cells and Clara cells were also counted. Type II cells were counted if they met the following criteria: located in the corners of alveoli and possessing cytoplasmic inclusions. The cells were counted at high-power magnification (×1,000). Clara cells were counted if they were nonciliated cuboidal epithelial cells present along bronchioles. 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.

RESULTS

On the basis of the morphometric measurements, a total of 158×10^6 type II cells and 15.5×10^6 Clara cells were present in air-exposed rats. Values of 125×10^6 type II cells and 17.3×10^6 Clara cells have previously been reported in comparable-sized rats (19). Measurements of total numbers of type II and Clara cells throughout the 12-, 36-, and 60-h 95% O_2 exposures did not differ significantly from control. The average number of total lung cells per high-power field counted did not significantly change with O_2 exposure. Semiquantitative immunohistochemistry and in situ RNA hybridization results are therefore expressed in terms of labeled cell number per lung and labeled cell number at each intensity grade per lung, as shown in Fig. 1.

Northern blots. There was decreased SP-A mRNA signal beginning at 12 h of exposure to 95% O_2. Message in whole lung was increased at 60 h over that of air-exposed controls, as shown in Fig. 2. SP-B mRNA declined at 12 h but exceeded air-exposed controls by 60 h of 95% O_2 exposure. The effects on total lung SP-B mRNA were parallel to SP-A mRNA, although more pronounced. The decrease in SP-C mRNA at 12 and 36 h of 95% O_2 exposure was even more pronounced. There were no changes in 18S mRNA expression.

Western blotting. Measurements of SP-A and SP-B were made in whole lung homogenates and in lavage fluid. Lavage fluid total protein increased significantly at 36 and 60 h of 95% O_2 exposure, but the air and 12-h 95% O_2 levels were not significantly different. The immunoreactive SP-A detected in lavage fluid was not

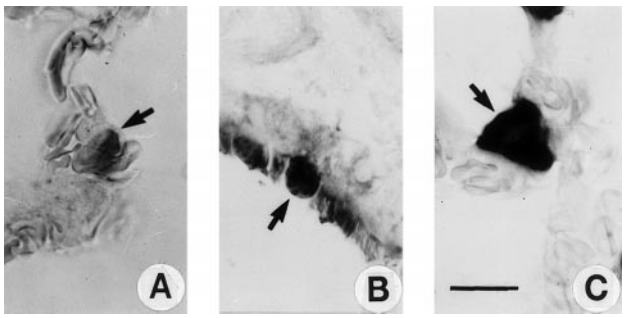

Fig. 1. Grades of cell signal intensity. Positive in situ hybridization signal for surfactant protein (SP) A mRNA at low (*A*), medium (*B*), and high (*C*) label intensity. Bar = 10 μm. Arrows indicate cells staining positively at each intensity grade.

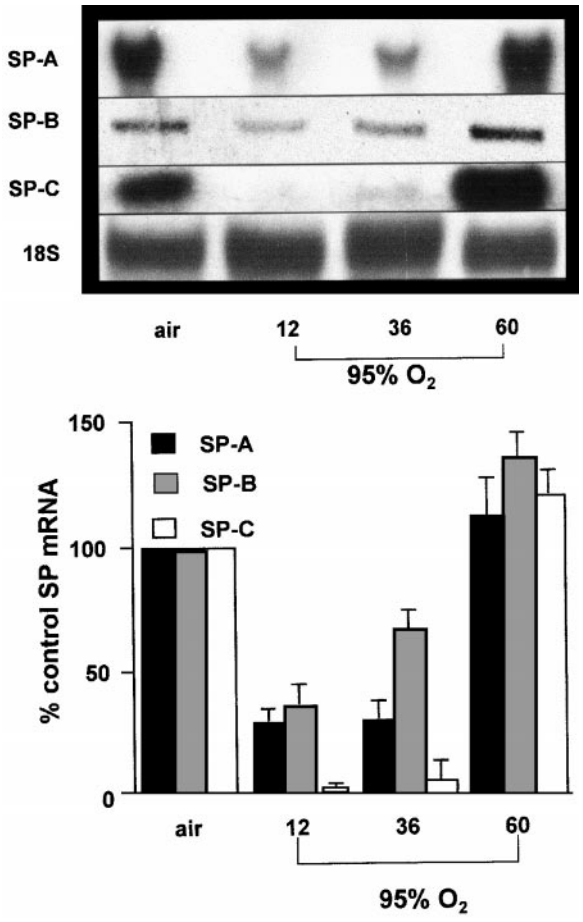

Fig. 2. Effect of hyperoxia on SP-A, -B, and -C and 18S mRNAs in whole lung. Northern blots employed 10 μg total RNA/lane. Autoradiograms were scanned, image analyzed, and normalized to air-exposed controls. Results are means ± SE.

changed significantly, as shown in Fig. 3, nor was there an appreciable effect on whole lung SP-A (data not shown). The effects on detected SP-B in lavage fluid demonstrated a decline to 50% of control by 12 h of 95% O_2 exposure, with a return to control levels at 36 h and a near twofold increase at 60 h. SP-B did not change in whole lung homogenates (data not shown).

In situ hybridizations. With O_2 exposure, there were significant increases in type II cell numbers expressing SP-A mRNA at 60 h, as shown in Fig. 4. However, by 12 h, the number of alveolar cells expressing high-intensity signal was reduced compared with air controls. The number of type II cells expressing medium intensity remained unchanged, and the number expressing high intensity rose with continued O_2 exposure to over 4-fold higher than in air-exposed controls and over 16-fold higher than with 12 h of hyperoxia. Representative photomicrographs are shown in Fig. 5.

In contrast to type II cell expression of SP-A mRNA, Clara cell numbers expressing SP-A mRNA were significantly reduced at 36 h of hyperoxia, as shown in Fig. 4, but returned to higher levels by 60 h. Low-intensity and medium-intensity labeled Clara cells were relatively unchanged with exposure, but high-intensity SP-A mRNA Clara cells rose threefold with 60 h of 95% O_2 exposure.

The number of high-intensity SP-B-labeled type II cells declined significantly at 12 h and then rose to levels exceeding control by 60 h, as summarized in Fig. 4. In contrast, the SP-B mRNA-positive Clara cells declined in number and intensity at 12 h of exposure to 95% O_2, and the proportion of high-intensity cells rose to eight times air control. Representative photomicrographs are shown in Fig. 5.

SP-C, which is only expressed in type II cells in adult rats, showed a general rise in the number of type II cells positively labeled with increasing O_2 exposure. There was a significant decline in the number of high-intensity grade cells after 12 h of hyperoxia, which

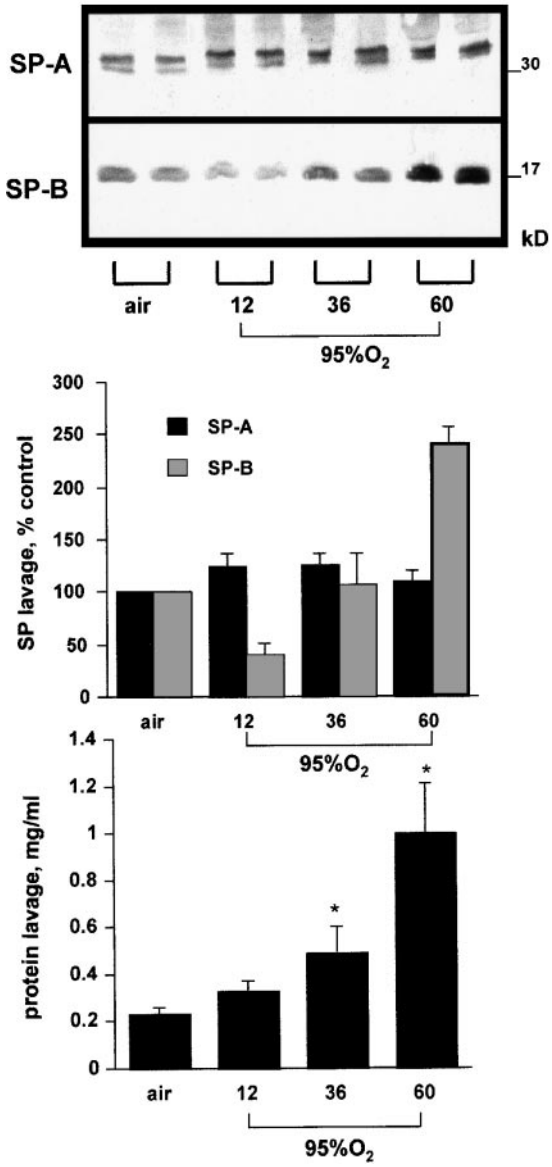

Fig. 3. Effect of hyperoxia on SP-A, SP-B, and total protein measured in lung lavage fluid. Lavage fluid per animal (30 μl) was analyzed on SDS-PAGE and immunodetected. Each lane is from a different individual. Immunoblots were scanned and analyzed with the same methods used for the Northern blots. Results are means ± SE. *$P <$ 0.05 vs. air.

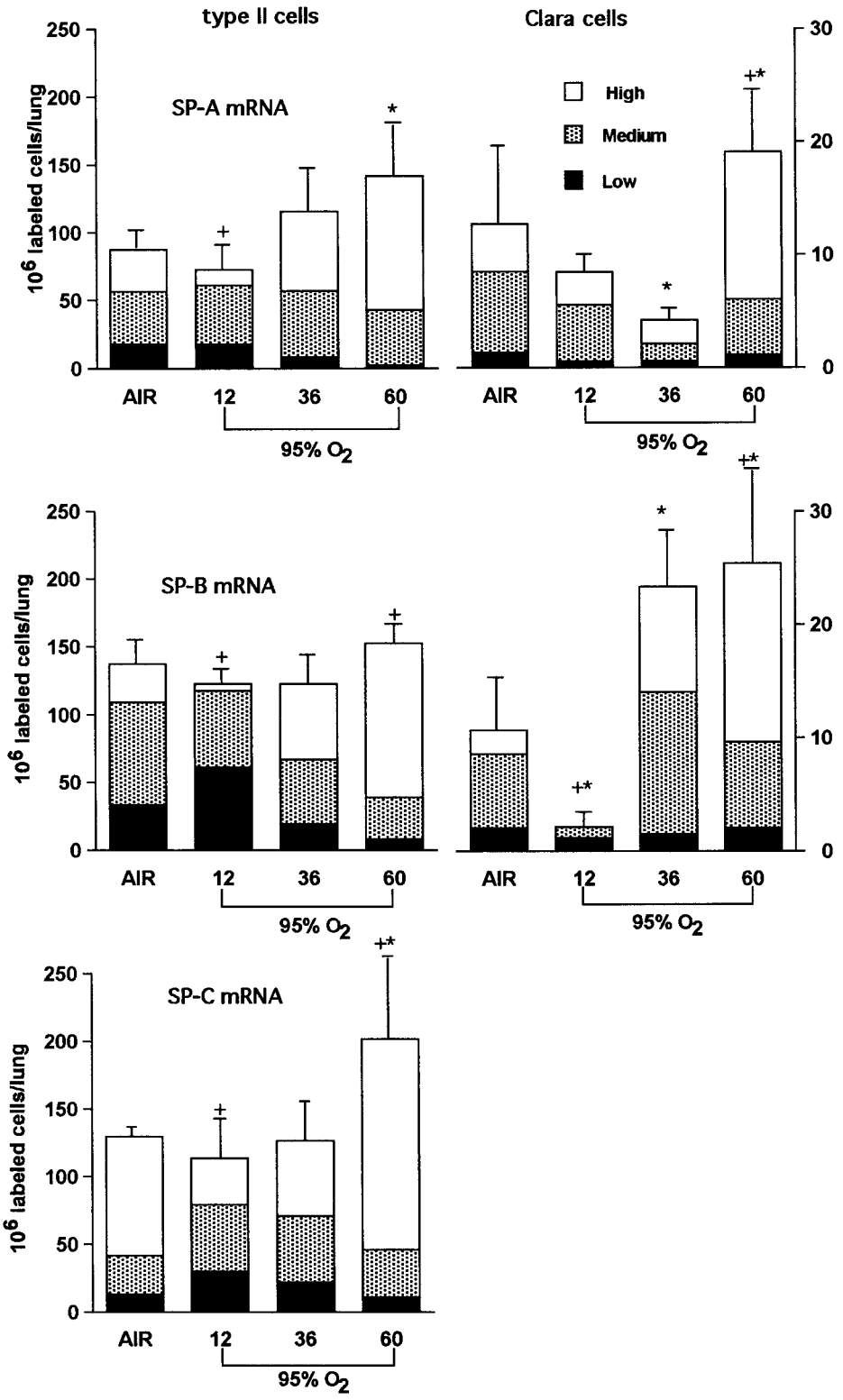

Fig. 4. Effects of hyperoxia on cells positively labeled for SP-A, -B, and -C by in situ hybridization. Total number of positively labeled type II and Clara cells per lung (means ± SE) vs. exposure condition is shown. Stacked bars represent number of cells at each intensity grade. $^*P < 0.05$ vs. air control. $^+P < 0.05$ vs. air control for high-intensity grade cells.

then rose to exceed air controls by 60 h by twofold. The number of low- and medium-intensity cells was not changed significantly.

Positive controls for the in situ hybridization employed a probe for the polyadenylated tail of mRNA to assess the overall availability of mRNA to detection at each exposure condition. There was uniformly intense ubiquitous cellular labeling, as shown in Fig. 6, which was absent when sections were pretreated with ribonuclease or cohybridized with excess unlabeled oligonucleotide probe. Comparisons with adjacent methyl green-stained sections showed labeling of polyadenylated tails in >90% of cells, with >90% demonstrating high-grade intensity labeling.

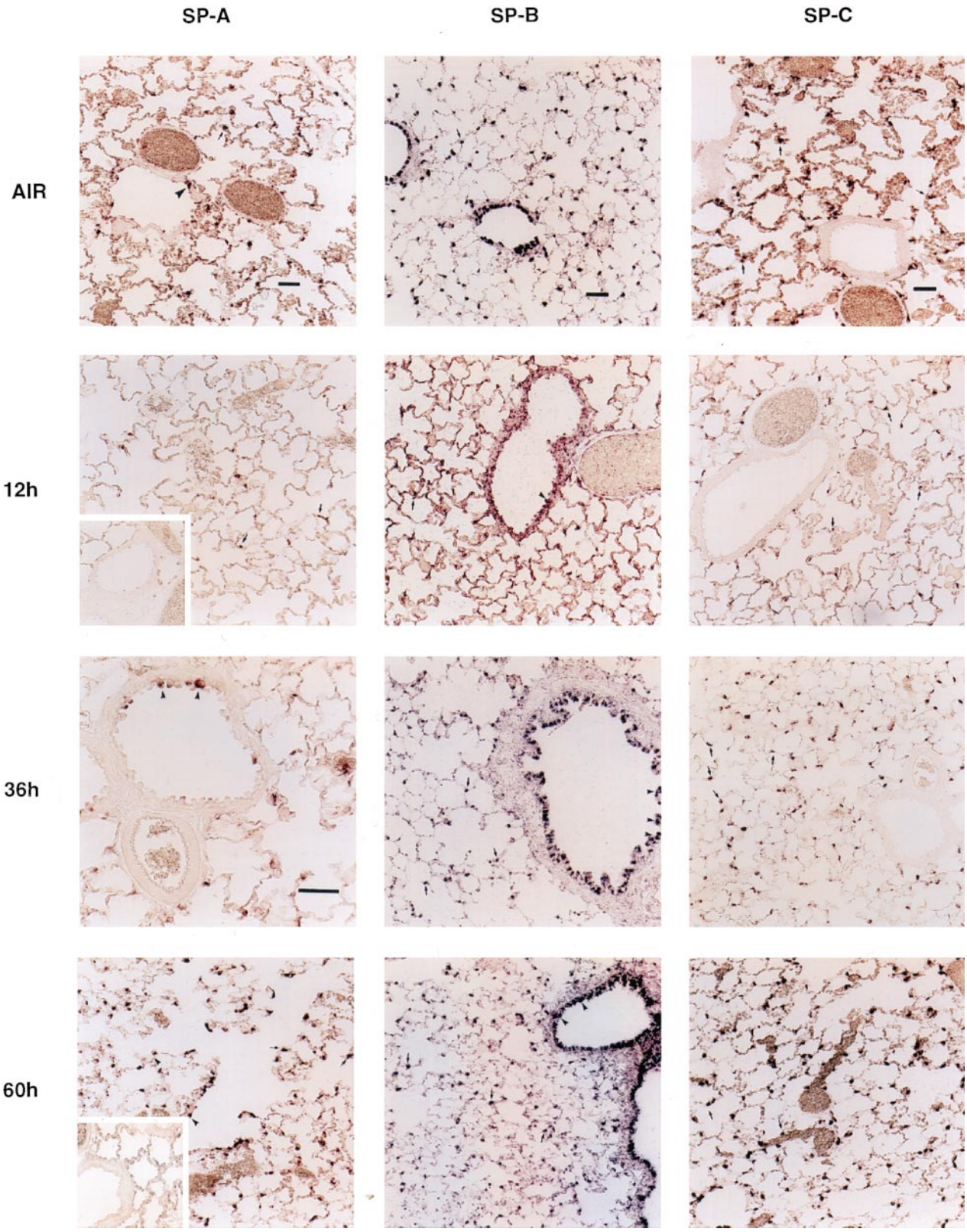

Fig. 5. In situ hybridization for SP-A, -B, and -C mRNAs. *Insets* show Clara cell detail. *Inset* at 60 h shows negative assay control. Bars = 50 μm.

Immunohistochemistry. There were no statistically significant changes in anti-SP-A-labeled type II cell number with hyperoxia, although a trend toward reduction was seen at 12 h of hyperoxia. This is in agreement with the whole lung and lavage Western blot results. There was a significant decline in labeling intensity at 12 h of 95% O_2 exposure in type II cells, followed by an increase by 60 h of 95% O_2, summarized

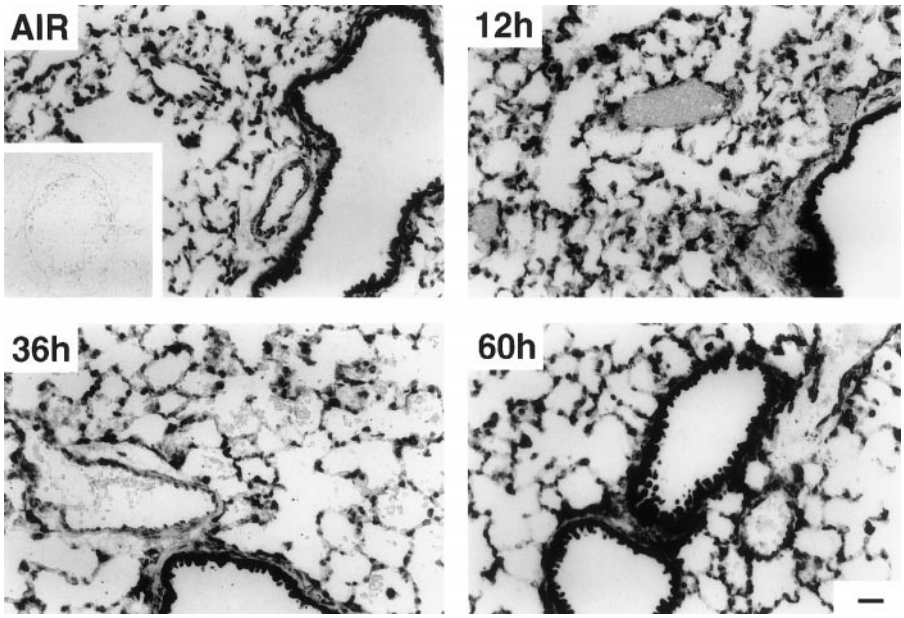

Fig. 6. In situ hybridization for polyadenylated mRNA. *Inset* shows negative assay control. Bar = 50 μm.

in Fig. 7 and demonstrated in Fig. 8. The changes in intensity were parallel to the changes in RNA measured by Northern blotting and in situ hybridization. The proportion of high-intensity labeled cells rose sharply by 60 h of hyperoxia. SP-A-labeled Clara cell numbers tended to decline, but labeling intensity rose steadily with increasing exposure duration, as shown in Fig. 7.

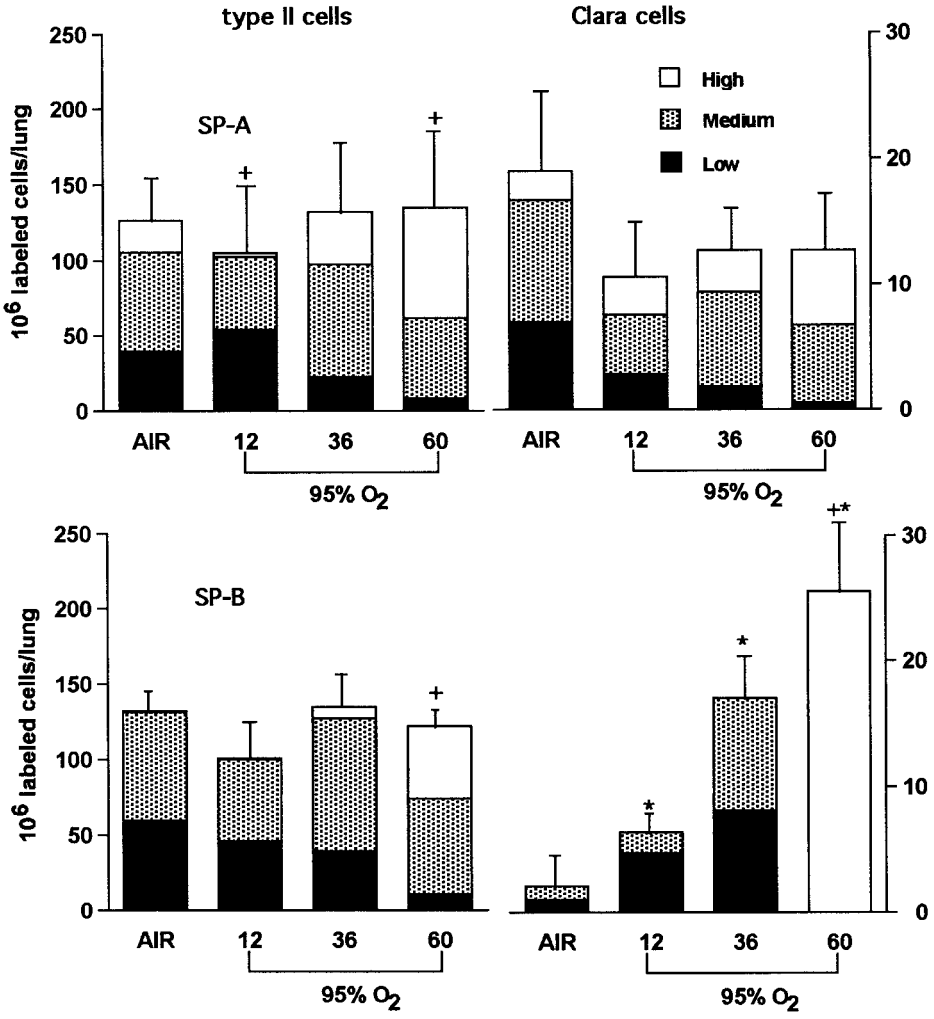

Fig. 7. Effects of hyperoxia on cells positively labeled for SP-A and -B by immunolabeling. Total number of positively labeled type II and Clara cells per lung (means ± SE) vs. exposure condition is shown. Stacked bars represent number of cells at each intensity grade. *$P < 0.05$ vs. air control. $^+P < 0.05$ vs. air control for high-intensity grade cells.

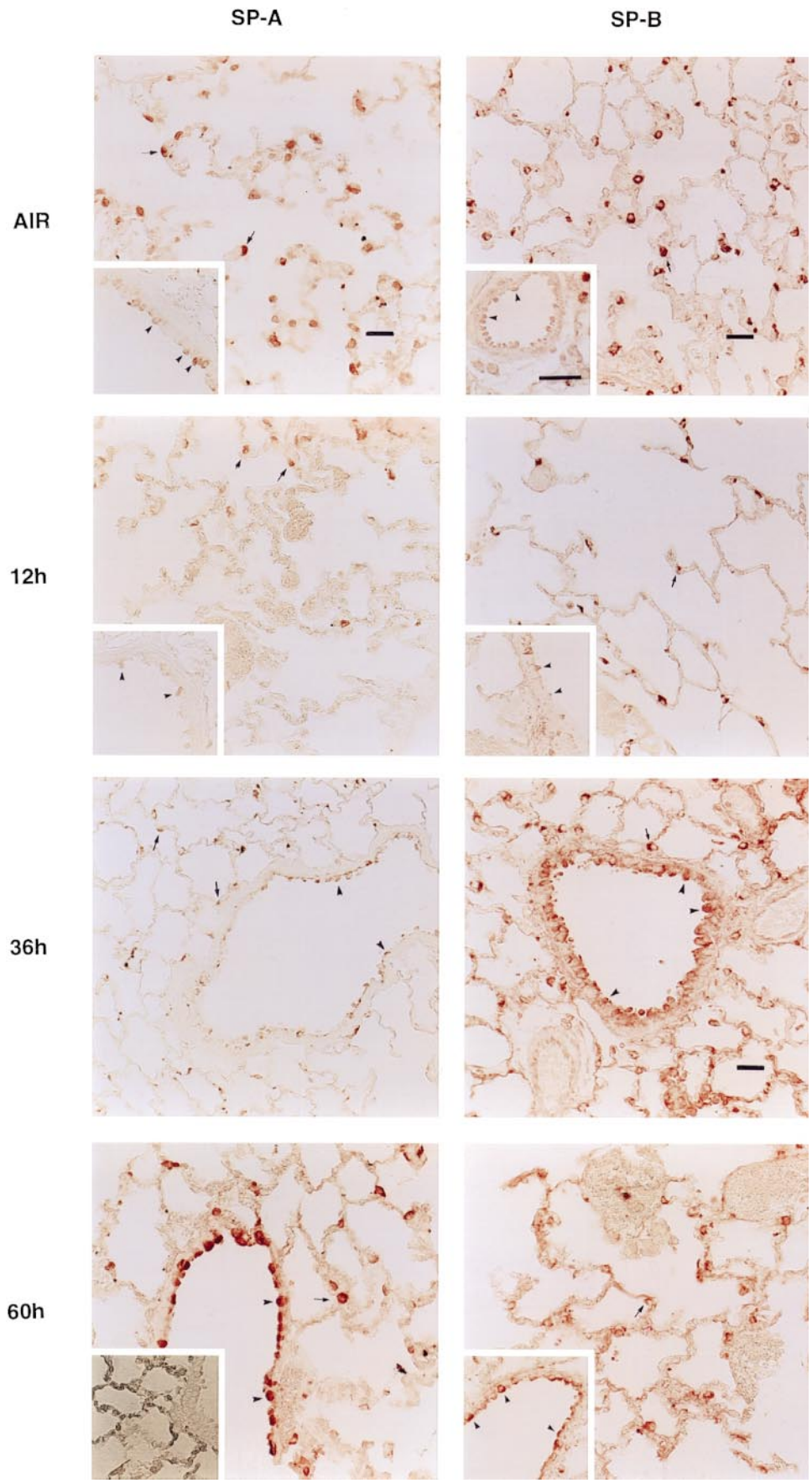

Fig. 8. Immunolabeling for SP-A and -B. *Insets* show Clara cell detail. *Inset* at 60 h shows negative assay control. Bars = 50 μm.

SP-B labeling of type II cells increased in intensity but not in number. There was a tendency toward a decline in the number of labeled type II cells at 12 h of 95% O_2 parallel to the changes in lavage SP-B. Labeling of Clara cells increased in both intensity and number, as summarized in Fig. 7.

DISCUSSION

The response of the surfactant system to hyperoxia is complex: the SP genes appear to be regulated differentially after exposure to hyperoxia. The degree of induction or suppression after hyperoxia differed among SP-A, -B, and -C and between cell types, with the degree of either suppression or induction being most prominent in the bronchiolar epithelial cells rather than in alveolar epithelium. Differential cellular responses of the SP genes to hyperoxia have been reported previously in O_2-tolerant rabbits as well (12). The abundance and composition of surfactant lipids recovered by lavage after O_2 exposure appear to differ by species (20) and exposure duration.

Previous reports by a number of investigators have shown increased surfactant apoprotein abundance after exposure to hyperoxia for periods generally exceeding 24 h. Minoo et al. (20) reported a biphasic response in adult hamsters after 100% O_2 exposure, showing an initial decrease in SP-B mRNA after 48 h, with an accompanying increase in SP-A and -C mRNA. Minoo et al. also reviewed the species differences that exist in the surfactant responses to O_2-induced injury. In addition to species differences, differential response of SP genes to hyperoxia and cell type-specific responses have been reported (12, 34). We demonstrate that relatively brief exposures of 12 h of 95% O_2 in adult rats can reduce SP-A, -B, and -C mRNA accumulation in both alveolar and bronchiolar epithelial cells and that changes in SP-A and -B, as detected by immunohistochemistry, in general parallel the changes in mRNA, although the increases in SP-B intensely labeled cells lagged behind the increases in SP-B mRNA. The Northern blot analysis of SP-A, -B, and -C mRNA expression in total lung RNA paralleled the findings of the in situ hybridization experiments. Western blot analysis of SP-B recovery in lavage fluid paralleled the findings of the in situ hybridization and immunolabeling experiments. The whole lung Western blotting studies did not reveal significant variation in immunodetectable SP-A or SP-B. SP-A in lavage fluid did not change, nor were there significant changes in the number of SP-A-immunolabeled type II cells. Elevations of SP-A in lavage fluid have been observed after more prolonged exposures to 85% O_2 (23). Hyperoxia effects on lavage fluid SP-A and -B presumably reflect effects on secretion as well as on synthesis, although these phenomena were not tested directly. SP-B in lavage fluid declined in parallel to the decline in SP-B-immunolabeled type II cells.

Labeled type II cells outnumbered Clara cells by nearly 10-fold and account for the preponderant effects on total lung SP content. The effects of the early depression of SP-A, -B, and -C mRNAs followed by increases are uncertain. Biophysical effects were not measured in these studies. SP-C, which is believed to be synthesized exclusively in type II cells or precursors to these cells (14), serves to confer, in part, the adsorption and dynamic surface tension-lowering properties necessary to pulmonary surfactant, in cooperation with SP-B (7, 9, 27). The reduced accumulation of SP-C mRNA appeared to be due to reduced signal per cell rather than decreases in the number of cells positively labeled. Whether the reduced levels of SP-C mRNA expression after brief hyperoxia exposure result in low levels of SP-C is unknown. Recently developed chemical methods to detect SP-C may succeed where more common methods for protein detection of the mature 3.5-kDa peptide have failed (25), which may help quantify SP-C in lavage fluid. Investigators have recently been able to detect the SP-C propeptide (4, 15, 32), but antibodies to mature rat SP-C will be necessary to obtain spatial information about SP-C abundance in lung injury states.

Reduction in SP-B and -C levels may contribute to diminished surfactant function and hence alveolar collapse in the microenvironment (7). We did not test this hypothesis directly. One of the potential strengths of in situ analysis would be to demonstrate alterations in gene expression in close proximity to morphologically evident injury. We focused these studies on early hyperoxia effects on SP expression, and hence no attempt was made to evaluate morphological evidence for injury, or inflammation, that occurs late in the hyperoxia exposure (2). The relevance of hyperoxia effects on the surfactant system may be greater when hyperoxia is added to underlying lung injury.

The intensity grading system is relative, so firm conclusions about the exact amount of mRNA or protein per cell cannot be made. We did not attempt to subtract background intensity from nonexpressing cells, which may affect the quantitation, particularly in low-expressing cells. Image analysis of nonisotopically labeled in situ hybridization and immunolabeling may permit more accurate quantitation in the future, but the methodology is not yet well established. To determine that the effects on SP mRNA were specific rather than general effects on cellular RNA or on RNA accessibility to probe, we performed positive control in situ hybridizations using a probe for the polyadenylated tail of mRNA at each exposure condition. So-called housekeeping genes, such as actin or glyceraldehyde-3-phosphate dehydrogenase, may have unpredictable cell-specific responses to alterations in cellular redox states expected with hyperoxia treatment, depending on cell type and location, and may be unsuitable as a uniformly positive control. However, because all mature mRNAs have polyadenylated tails, they should hybridize with polythymidine oligonucleotide probes, which we found to be the case in these experiments.

The overall effects of hyperoxia on SP-A, -B, and -C in situ expression in lung were compared with whole lung measurements of protein and RNA. The principal strength of the in situ detection in the present studies is to demonstrate and distinguish the relative contribu-

tion of the different cell types to the hyperoxia response and whether the spatial pattern of expression is affected. This may be of particular importance to understand localized effects on lung immunity or potential biophysical effects in evolving injury states, since it may influence the clinical manifestations of lung injury. For example, in species with narrow-caliber distal airways where Clara cells comprise a larger proportion of airway cells (28), SP production in Clara cells may play a more significant role in maintaining stability of small airways.

In other models of prolonged O_2 exposure, diminished surfactant function, decreased SP production (5, 10, 11, 17, 20), and decreased surfactant phospoholipids (16) have been reported. Some of these physiological effects are undoubtedly attributable to inhibitory effects of proteins, lipids, and fluid, which are transudated into alveoli in O_2-induced injury (5, 10, 11, 17). In addition, decreased surfactant lipid synthesis likely contributes to this sequence of events (16, 20, 22, 24).

The biphasic response of SP gene expression, namely inhibition, as shown by these studies, followed by induction, as shown here and by others studying more prolonged exposures to hyperoxia, suggests that O_2-induced changes in gene expression may depend on different thresholds for different genes, depending on cell type, species, $F_{I_{O_2}}$, and duration. We speculate that unanticipated disturbances of SP abundance after a brief exposure to high ambient O_2 may potentially reduce alveolar stability in the microenvironment and contribute to the pathophysiology of O_2-induced lung injury at an earlier point than has been recognized previously. Hyperoxia effects on SP-A in particular may alter the host defense against pathogens known to bind to SP-A.

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Address for reprint requests and other correspondence: R. L. Auten, Box 3179, Duke Univ., Durham NC 27710 (E-mail: auten@duke.edu).

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