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Effect of Dexamethasone on Pulmonary Surfactant Metabolism in Hyperoxia-Treated Rat Lungs

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ABSTRACT. We have examined the effect of dexamethasone on the metabolism of pulmonary surfactant in normal and hyperoxia-treated rats. The relative abundance of the surfactant-specific apoprotein A (SP-A) mRNA in lung tissues and the contents of disaturated phosphatidylcholine (DSPC) and SP-A were measured in bronchoalveolar lavage fluids and in lung tissues in 4-wk-old rats exposed to room air or >90% oxygen for 7 d with or without simultaneous treatment with dexamethasone (0.5 mg/kg body wt for 7 d). The relative abundance of the SP-A mRNA was marginally increased by hyperoxia (1.3-fold over controls). Dexamethasone increased the relative abundance of the SP-A mRNA to a level comparable to that with hyperoxia treatment (1.5-fold over controls). In lavage fluids, the contents of DSPC and SP-A were increased by 4- and 6-fold over controls by hyperoxia, respectively, but they were increased only by 2-fold by dexamethasone. In lung tissues, the contents of DSPC and SP-A were increased by 3- and 2-fold over controls by hyperoxia, respectively. These values in lung tissues in the air-exposed rats were not significantly increased by dexamethasone. In hyperoxia-treated rats, dexamethasone did not significantly affect the relative abundance of the SP-A mRNA level and the contents of DSPC and SP-A in lavage fluids and lung tissues. These results indicate that mechanisms other than increased synthesis of SP-A are involved in hyperoxia-induced SP-A accumulation and that dexamethasone does not affect the abnormal accumulation of pulmonary surfactant induced by hyperoxia. (*Pediatr Res* 29: 173–177, 1991)

Abbreviations

SP-A, surfactant-specific apoprotein A
DSPC, disaturated phosphatidylcholine
BPD, bronchopulmonary dysplasia
SSC, sodium chloride, sodium citrate

Therapeutic oxygen given to premature infants having respiratory distress syndrome has adverse effects on lungs and may give rise to a chronic lung disorder known as BPD (1). Multiple factors are involved in the pathogenesis of BPD, *i.e.* deficiency of pulmonary surfactant, mechanical stress, immaturity of lung antioxidant enzymes, and imbalance between protease and antiprotease in the airway (2). Accumulating evidence has suggested that high oxygen concentration in the airway plays a major role in the development of BPD (3–6). Prolonged exposure of animals to hyperoxia has been associated with progressive injury to cell components of the blood gas barrier, leading to increased alveolar and pulmonary vascular permeability, pulmonary edema, atelectasis, and eventually death from hypoxemia or respiratory failure (7). It has been reported that hyperoxia increased the airway content of SP-A, which is the most abundant nonserum protein in pulmonary surfactant with a mol wt between 26 000 and 36 000, and the SP-A mRNA in adult rats (8, 9). Dexamethasone has been successfully used for the treatment of BPD (10–12); it has been also shown to increase the SP-A mRNA level and the amount of SP-A both *in vivo* and *in vitro* (13, 14). We conducted this study to clarify the mechanisms of hyperoxia-induced SP-A accumulation in the airway by comparing the effect of dexamethasone on the SP-A metabolism with that of hyperoxia. The results were further analyzed to determine the possible preventive effect of dexamethasone on the acute oxygen toxicity represented by the abnormal accumulation of pulmonary surfactant.

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MATERIALS AND METHODS

Materials. Horseradish peroxidase was purchased from Sigma Chemical Co. (St. Louis, MO). Sphingomyelinase, choline oxidase, and phospholipase D were kindly provided by Toyo Jozo (Shizuoka, Japan). Rabbit anti-rat SP-A polyclonal antibody and affinity-purified SP-A were generous gifts of Drs. Y. Kuroki, D. R. Voelker, and R. J. Mason (Department of Medicine, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO). Rat SP-A cDNA was cloned and sequenced as previously described (15). The plasmid containing rat α-tubulin cDNA (pILαTI) was kindly provided by Dr. S. Farmer (Boston University, Boston, MA). Sodium 2-hydroxy-3,5-dichlorobenzensulfonate (HDCBS) was obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI).

Animals and experimental design. Four-wk-old male Wistar rats were divided into the air group and the hyperoxia group. The former were maintained in room air. The latter were placed in a Plexiglas chamber (40 cm × 42 cm × 50 cm) under continuous oxygen flow at 5 L/min. The concentration of oxygen was greater than 90% and that of carbon dioxide was less than 0.5%. Some rats were injected i.p. with a serial dose of dexamethasone (0.5 mg/kg/d) every day during the study. An equal volume of saline was given to others in a similar fashion. The study design was approved by the animal care committee at Kobe University School of Medicine.

Lavage collection and preparation of lung tissues. After a 7-d exposure period, rats received a lethal i.p. injection of pentobarbital (50 mg/kg). Leaving the thorax undisturbed, the tracheas were cannulated and connected to the syringe. The lungs were slowly inflated at 30 cm H_2O pressure with cold PBS, and this

volume was recorded as V_{30}. This pressure was maintained for 5 min before deflation. After a 5-min equilibration period, the lungs were lavaged 10 times with cold PBS with a volume equal to 80% of V_{30}. Fresh buffer was used for each lavage. Total recovered lavage fluids from each rat had to be at least 85% of the volume instilled to verify the analysis. The lungs were removed from the chest cavity immediately after lavage and separated into the right and left lobe. The former was placed in PBS containing 1% (vol/vol) Triton X-100 (1% Triton X-100/PBS) for determination of DNA, DSPC, and SP-A. The latter was placed in 4 M guanidinium isothiocyanate containing 0.1 M β-mercaptoethanol and 25 mM sodium acetate for preparation of RNA. Both were homogenized with a Polytron and stored at −80°C until the assays were performed.

Determination of DSPC. Lipids were extracted in chloroform/methanol (1/2) according to Bligh and Dyer (16). Samples of chloroform/methanol extract were taken to dryness under a nitrogen stream and reacted with osmium tetroxide. Then each sample was applied to an alumina column and DSPC was eluted with chloroform/methanol/H_2O/NH_4Cl (70/30/1/1) as described by Mason *et al.* (17). DSPC was determined by the enzymatic method essentially as described by Muneshige *et al.* (18). In brief, choline was released from DSPC in reaction with phospholipase D and subsequently hydrogen peroxide was generated in reaction with choline oxidase. Hydrogen peroxide generated was quantified spectrophotometrically at 510 nm after the addition of horseradish peroxidase, aminoantipyrine, and HDCBS.

Measurement of SP-A. The amount of SP-A was measured by a double sandwich ELISA using a rabbit anti-rat SP-A polyclonal antibody. The assay was capable of accurately measuring SP-A in concentrations of 1–20 ng/mL. Anti-SP-A IgG fraction (100 μg/mL in 0.1 M $NaHCO_3$) was incubated overnight at room temperature in wells of untreated 96-well microtiterplates (Dynatech Corp., Chantilly, VA). The wells were then incubated in PBS containing 1% (vol/vol) Triton X-100 and 3% (wt/vol) BSA (3% BSA/1% Triton X-100/PBS). After two washes with 1% Triton X-100/PBS, 100 μL of affinity-purified rat SP-A standard (0–20 ng in 5 mM Tris/HCl, pH 7.0) or various dilutions of samples were added to each well and allowed to incubate at 37°C for 90 min. After the wells were washed three times with 3% BSA/1% Triton X-100/PBS, 100 μL of biotinylated anti-SP-A IgG fraction were added to each well and allowed to incubate at 37°C for 90 min. Anti-SP-A IgG was biotinylated using a biotinylation kit from Amersham (Arlington Heights, IL). After four washes with 1% Triton X-100/PBS, 100 μL of diluted horseradish peroxidase-conjugated streptavidin (Amersham) were added to each well and allowed to incubate at 37°C for 30 min. After three washes with 1% Triton X-100/PBS, 100 μL of substrate solution {1 mM 2,2′-azino-di[3-ethyl-benzthiazoline sulfonate (6)] with 0.002% (vol/vol) hydrogen peroxide in citrate buffer} were added to each well. The reaction was stopped by the addition of 50 μL of 1.5 mM sodium azide in 0.1 M citric acid. The absorbance at 410 nm was recorded with a Microplate Autoreader (Bio Rad Laboratories, Richmond, CA).

Determination of DNA. DNA samples were prepared from 0.2 mL of the lung homogenate by precipitation with 10% perchloric acid followed by extraction with methanol and ether. The amount of DNA was then determined fluorometrically using diaminobenzoic acid as described by Setaro and Morley (19).

RNA analysis. Total RNA was extracted from lung tissues with 4 M guanidinium isothiocyanate containing 0.1 M β-mercaptoethanol and 25 mM sodium acetate. RNA was purified by centrifuging the lysate through a cushion of 5.7 M cesium chloride at 80 000 × *g* for 16 h at 22°C. The amount of RNA was quantitated by measurement of OD at 260 nm. For the Northern blot analysis, total RNA (20 μg) was size-fractionated by electrophoresis on a 1% agarose gel containing 3% formaldehyde and 0.5 μg/mL of ethidium bromide, and transferred to a nylon membrane sheet (Gene Screen from NEN Research Products, Boston, MA) by capillary action in 10 × SSC (1 × SSC; 0.15 M NaCl and 0.015 M sodium citrate). For dot blot analysis, 1 to 10 μg of total RNA in 10 × SSC were charged onto a nylon membrane sheet under vacuum.

After baking at 80°C for 3 h, the membrane was prehybridized at 42°C overnight in 5 × SSC, 0.5% SDS, 3 × Denhardt's solution (1 × Denhardt's solution; 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% BSA), 0.2 mg/mL salmon sperm DNA, and 45% formamide and then hybridized in the same solution that additionally contained 1.0×10^6 cpm/mL of a ^{32}P-labeled cDNA probe for rat SP-A. The probe was labeled using a random primer labeling kit (Amersham) with [α-^{32}P]deoxycytidine triphosphate. After a 24-h hybridization period, the membrane was washed twice in 2 × SSC at room temperature for 5 min, twice in 2 × SSC containing 0.1% SDS at 65°C for 30 min, and finally twice in 0.1 × SSC at room temperature for 30 min. Then the membrane was exposed to a Kodak X-Omat AR film with an intensifying screen at −70°C for 24 h. To quantify the relative amounts of the SP-A mRNA, the film was scanned at 550 nm using a Dual-wavelength TLC Scanner (CS-930, Shimadzu, Kyoto, Japan) and the area corresponding to each dot was computed. The area of each dot in a single sample was well correlated with the amount of total RNA charged. The average of the areas corresponding to 4 μg of total RNA recovered from control rats was expressed as 1 arbitrary unit and then the value of each sample was calculated and expressed as arbitrary units per 4 μg of total RNA. To assure the even loading of total RNA, the same membrane sheet was rehybridized with rat α-tubulin cDNA probe.

Purification of pulmonary surfactant. Pulmonary surfactant was isolated from the lavage fluids of the air group and the hyperoxia group according to the method described by Hawgood *et al.* (20). The pooled lung lavage fluids were spun at 150 × *g* for 15 min to remove contaminating cells and cell debris. The supernatant was then spun at 20 000 × *g* for 15 h at 4°C and the resulting pellet was dispersed in 5 mM Tris/HCl at pH 7.0 containing 100 mM NaCl and 1.64 M sodium bromide. After equilibration for 1 h, this suspension was spun at 100 000 × *g* for 4 h at 4°C. The pellicle was resuspended in the same buffer without sodium bromide and then spun at 100 000 × *g* for 1 h at 4°C. The pellet was resuspended in distilled water and subjected to electrophoresis.

Electrophoresis and Western blot analysis. Pulmonary surfactant-associated proteins were separated by SDS-PAGE as described by Laemmli (21) and transferred electrophoretically to a nitrocellulose sheet (Schleicher and Schuell, Inc., Keene, NH). The sheet was treated with PBS containing 3% (wt/vol) BSA (3% BSA/PBS) to block nonspecific binding, and subsequently reacted with biotinylated anti-SP-A IgG followed by horseradish peroxidase-conjugated streptavidin (Amersham). Diaminobenzidine was used as a substrate for the peroxidase reaction.

Statistical analysis. Data are expressed as mean ± SEM. Statistical analysis was performed with analysis of variance for multiple comparisons with the Statview statistical program.

RESULTS

Body weights, lung DNA contents, and lavage volume. The number of rats studied and their body weights, lung DNA contents, and V_{30} are shown in Table 1. One out of five rats treated with hyperoxia plus dexamethasone died during the study. Body weight gain of the dexamethasone-treated group, the hyperoxia group, and the hyperoxia plus dexamethasone-treated group was significantly less than that of the air group. Lung DNA contents were not significantly different among the four groups. V_{30} of the hyperoxia group and the hyperoxia plus dexamethasone-treated group was significantly less than that of the air group.

The relative amount of the SP-A mRNA. Rat SP-A cDNA probe hybridized with two species of mRNA, the sizes of which

Table 1. *Body weight, lung DNA content, and lavage volume*

	Body wt (g)											
	0 d	7 d			DNA (μg)				V_{30} (mL)			
Room air (n = 5)	77 ± 4	117 ± 4	*	*	48.8 ± 3.2	NS	NS	*	6.8 ± 0.4	NS		
Room air + dexamethasone (n = 5)	77 ± 4	92 ± 2			35.2 ± 1.6				5.8 ± 0.3			
Hyperoxia (n = 5)	75 ± 5	87 ± 7	NS		36.0 ± 2.8	NS			3.7 ± 0.2	NS		
Hyperoxia + dexamethasone (n = 4)	77 ± 2	80 ± 2			38.4 ± 2.2				4.1 ± 0.1			

* $p < 0.05$ when compared to the air group.

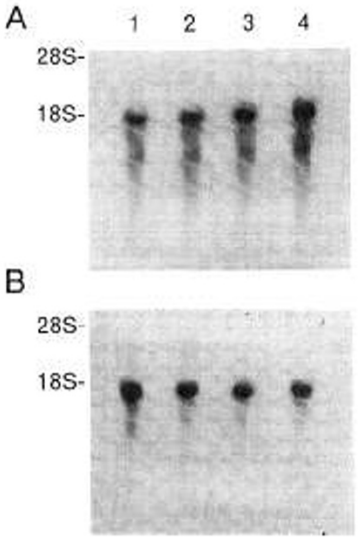

Fig. 1. The relative abundance of the SP-A and α-tubulin mRNA in lungs of air- or hyperoxia-exposed rats with or without treatment with dexamethasone. Total RNA was extracted from rat lungs in each group. Total RNA (20 μg) was electrophoresed, transferred to a nylon membrane sheet, and hybridized with the radiolabeled SP-A cDNA probe. The same membrane was hybridized with the α-tubulin cDNA probe after stripping off the SP-A cDNA. *A*, the SP-A mRNA; *B*, the α-tubulin mRNA. *Lane 1*, the air group; *lane 2*, the hyperoxia group; *lane 3*, the dexamethasone group; *lane 4*, the hyperoxia plus dexamethasone group.

were 1.6 and 0.9 kb, as described previously (22) (Fig. 1). The nucleotide sequences of the coding region of these two corresponding cDNA are the same, and the difference in the size between these two mRNA is attributed to the different lengths of the 3′-noncoding region (22). Which mRNA species is mainly translated into SP-A is not known. The relative abundance of the SP-A mRNA in the hyperoxia group, dexamethasone group, or hyperoxia plus dexamethasone group was higher than that in the air group (Fig. 1). The relative abundance of the two species of mRNA in lung tissues was not different among four groups. The relative abundance of the α-tubulin mRNA was not different among four groups. The relative abundance of the SP-A mRNA in each group was determined by dot blot analysis and is shown in Table 2. Hyperoxia increased the relative abundance of the SP-A mRNA by 1.3-fold over the control group. Dexamethasone increased the relative abundance of the SP-A mRNA by 1.5-fold over the control group. The relative abundance of the SP-A mRNA in the hyperoxia plus dexamethasone-treated group showed a 2.2-fold increase over that in the air group. The relative abundance of the α-tubulin mRNA was not significantly different in four groups.

Contents of SP-A and DSPC in lavage fluids and lung tissues. Table 3 shows the contents of SP-A and DSPC in lavage fluids. Hyperoxia increased the contents of SP-A and DSPC in lavage fluids by approximately 6- and 4-fold over the controls, respectively. Dexamethasone increased the contents of both SP-A and DSPC by approximately 2-fold over the controls. Dexamethasone did not significantly affect these values in the hyperoxia group. The contents of SP-A and DSPC in lung tissues in each group are shown in Table 4. Hyperoxia increased the contents of SP-A and DSPC in lung tissues by about 3- and 2-fold over the controls, respectively. In both the air and the hyperoxia groups, the effects of dexamethasone on the contents of SP-A and DSPC in lung tissues were not significant.

Table 2. *SP-A mRNA levels in lung tissues*

	Arbitrary unit*		
Room air (n = 10)	1.0 ± 0.1	†	†
Room air + dexamethasone (n = 5)	1.5 ± 0.1		
Hyperoxia (n = 10)	1.3 ± 0.1	NS	
Hyperoxia + dexamethasone (n = 4)	2.2 ± 0.5		

* The average of the values of the air group is expressed as 1.0 arbitrary unit.

† $p < 0.05$ when compared to the air group.

DISCUSSION

We confirmed the results reported by Nogee and Wispe (8) that hyperoxia increases the contents of DSPC and SP-A, major components of the pulmonary surfactant, both in lavage fluids and in lung tissues in rats. SP-A recovered from hyperoxia-treated rats was thought to be biologically similar to native SP-A in that they show the same mobility in SDS-PAGE (data not shown). The increase in the airway contents of DSPC and SP-A by hyperoxia could be attributed to either the increased synthesis and/or secretion or the decreased catabolism of the pulmonary surfactant. We have shown that hyperoxia increases the relative abundance of the SP-A mRNA by 1.3-fold over controls, whereas hyperoxia increased the SP-A content in lavage fluids and in lung tissues over the controls by 6- and 3-fold, respectively. Nogee *et al.* (9) reported that exposure to 85% oxygen increases the airway content of SP-A by 20-fold and SP-A mRNA by 10-fold in adult rats. The exact reason for the discrepancy between their results and ours is not known. However, one of the reasons for this may be the different age and strain of the rats used in our experiments. In our experience, significant numbers of adult rats (8 to 16 wk old) died during hyperoxia treatment, which is the reason we used young rats in our study. Yam *et al.* (23) also reported that all 4-wk-old or younger rats survived the 3-d oxygen exposure, but only 25% of rats older than 7 wk old were alive (23). Therefore, adult rat lungs may be more susceptible to hyperoxia than are young rat lungs, resulting in large increases in the SP-A content and the relative abundance of the SP-A mRNA. The rats younger than 4 wk old were not suitable for our study because it was difficult to recover lavage fluids correctly. Floros *et al.* (14) have reported that consistent results were not obtained using 28-d-old rats in similar experiments, probably because of the delicate hormone balance in this specific period. However, we could obtain fairly consistent results in terms of

Table 3. *Contents of SP-A and DSPC in lavage fluids*

	SP-A (μg/g body wt)			DSPC (nmol/g body wt)		
Room air (n = 11)	0.54 ± 0.08	*	†	2.90 ± 0.28	*	†
Room air + dexamethasone (n = 5)	1.14 ± 0.29			6.24 ± 0.78		
Hyperoxia (n = 11)	3.24 ± 0.95	NS		11.22 ± 2.57	NS	
Hyperoxia + dexamethasone (n = 4)	5.65 ± 3.05			10.34 ± 2.50		

* $p < 0.05$ when compared to the air group.
† $p < 0.01$ when compared to the air group.

Table 4. *Contents of SP-A and DSPC in lung tissues*

	SP-A (μg/μg DNA)			DSPC (nmol/μg DNA)		
Room air (n = 5)	4.4 ± 0.5	NS	*	30.5 ± 5.6	NS	*
Room air + dexamethasone (n = 5)	5.9 ± 0.9			31.3 ± 10.0		
Hyperoxia (n = 5)	14.4 ± 3.3	NS		55.4 ± 8.5	NS	
Hyperoxia + dexamethasone (n = 4)	14.1 ± 2.4			55.3 ± 3.6		

* $p < 0.05$ when compared to the air group.

the content of SP-A and the relative abundance of the SP-A mRNA (Tables 2–4). The exact reason for the discrepancy between their results and ours is not known. However, one possible reason could be the use of different protocols. Floros *et al.* examined the effects of oxygen treatment after a 24-h exposure (14), and we examined the effects of daily treatments for a total of 7 d.

Pulmonary surfactant is synthesized and secreted by alveolar type II cells (24). The increase in the SP-A mRNA level by hyperoxia might be attributed to the increase in the number of alveolar type II cells, the increase in the transcriptional rate of the SP-A gene, or a change in message stability. Young *et al.* (25) reported that rats exposed to 85% oxygen for 7 d had lungs that contained twice the number of alveolar type II cells compared with control rats (25). Because we have not measured the number of alveolar type II cells, the contribution of the increase in the number of alveolar type II cells to the increased airway content of pulmomary surfactant by hyperoxia is not known. Dexamethasone increases the relative abundance of the SP-A mRNA by 1.5-fold over the controls (Table 2) and increases the airway content of SP-A by 2-fold (Table 3). Dexamethasone does not significantly affect the content of SP-A in lung tissues (Table 4). From the comparison of these values between the hyperoxia group and the dexamethasone group, the increase in the content of SP-A both in lung tissues and lavage fluids in hyperoxia-treated rats cannot be fully explained by the increase in the relative abundance of the SP-A mRNA. Other mechanisms might be employed in the hyperoxia-induced large increase in the airway content of SP-A, such as the decreased catabolism of SP-A.

Accumulated evidence has shown that secreted pulmonary surfactant is taken up from the alveolar lumen by alveolar type II cells and reutilized for the synthesis of pulmonary surfactant (26). Furthermore, a significant portion of the pulmonary surfactant is taken up and metabolized by alveolar macrophages (26). We have previously reported that the function of alveolar macrophages recovered from rat lungs exposed to hyperoxia is significantly decreased when assessed by superoxide anion production (27). Therefore, impaired function of alveolar macrophages resulting in decreased uptake of the pulmonary surfactant might be one of the causes of the increase in the contents of DSPC and SP-A in lavage fluids of hyperoxia-treated rats. Because the primary site of acute oxygen toxicity has been considered to be plasma membranes (28), there is a possibility that hyperoxia impaired the uptake mechanism of SP-A by alveolar type II cells due to the damage of the plasma membrane. To examine these possibilities, however, the study of the clearance rate of surfactant in each cell type is required.

The effect of hyperoxia on the synthesis of DSPC is unknown. However, because the content of DSPC is also increased in lung tissues and lavage fluids to a similar extent as that of SP-A in hyperoxia-treated rats as shown in Tables 3 and 4, the same mechanism might be used to cause these changes in the amount of DSPC.

Dexamethasone has been successfully used for the treatment for BPD (10–12). Therefore, we examined the effect of dexamethasone on the pulmonary surfactant metabolism in hyperoxia-treated rats. Dexamethasone treatment does not significantly affect the contents of DSPC and SP-A in either lavage fluids or lung tissues in hyperoxia-treated rats as shown in Tables 3 and 4. Therefore, dexamethasone does not have preventive effect on the acute oxygen toxicity to lungs represented by the accumulation of pulmonary surfactant.

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