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Effects of Antenatal Dexamethasone on Antioxidant Enzymes and Nitric Oxide Synthase in the Rat Lung

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Abstract. We investigated the effects of prenatal dexamethasone (DEX) administration on antioxidant enzymes (AOEs) and nitric oxide synthase (NOS) in fetal and neonatal rat lungs. DEX (1 mg/kg, s.c., for 2 days) or vehicle alone was administered to pregnant rats, and the lungs of fetuses on days 19 and 21 of gestation and of 1- and 3-day-old neonates were examined. We measured protein levels of the AOEs manganese superoxide dismutase and copper-zinc superoxide dismutase (Mn SOD and Cu-Zn SOD), glutathione peroxidase (GSH-Px), catalase (CAT), and inducible and endothelial nitric oxide synthase (i-NOS and e-NOS). Mn SOD, GSH-Px, and e-NOS expression gradually increased with increasing gestational and postnatal age in the lungs of the control groups. Cu-Zn SOD, CAT, and i-NOS expression did not change with increasing gestational and postnatal age in the lungs of the control groups. DEX administration had significant effects on i-NOS and e-NOS protein and mRNA expression. The increased Mn SOD, GSH-Px, and e-NOS expressions during the perinatal period suggests that antenatal developmental changes in AOEs in the lungs of premature fetuses could be reduced by reactive oxygen species–mediated injury at birth. Furthermore, antenatal glucocorticoid treatment may accelerate the development of lungs via the two types of NOS.

Keywords: reactive oxygen species, antioxidant enzyme, dexamethasone, superoxide dismutase, nitric oxide synthase

Introduction

Many investigators have suggested that reactive oxygen species (ROS) and oxidative stress are involved in numerous disease states. Generally, ROS are electrically unstable molecules. ROS comprise superoxide ($O_2^{-\bullet}$), hydroxyradical (·OH), and hydrogen peroxide (H_2O_2). The toxicity of ROS and free radicals increases the damage caused by ischemia-reperfusion injury and infection and as a result produces oxidative stress. Oxidative stress causes damage due to an imbalance between oxidation and antioxidation. Recently, many investigators have suggested that the participation of ·OH causes cell damage in oxidative stress. Arroyo et al. reported that ·OH is generated during myocardial ischemia-reperfusion (1), and Das et al. reported that ·OH is generated in the myocardial mitochondrion (2). Furthermore, there were reports that dimethyl sulfoxide (DMSO), which is a radical scavenger, and dimethyl thiourea inhibited toxicity during myocardial ischemia-reperfusion (3). It is known that $O_2^{-\bullet}$ and H_2O_2 generate ·OH, and its toxicity has been confirmed.

Two major superoxide dismutases (SODs), copper-zinc superoxide dismutase (Cu-Zn SOD), which is present in the cytoplasm, and manganese superoxide dismutase (Mn SOD), which is present in mitochondria, remove $O_2^{-\bullet}$ and convert it to H_2O_2. Glutathione peroxidase (GSH-Px) and catalase (CAT) decompose H_2O_2 and inhibit the formation of ·OH. Nordström et al. (4) and Gupta et al. (5) reported that preventing the formation of ·OH protects cells from damage caused by oxidative stress.

Oxidative stress saturation in the perinatal period is a cause of neonatal respiratory dysfunction. The neonate

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can be considered a systemic ischemia-reperfusion model (6). The neonate undergoes oxidative stress due to the tissue damage from placental ischemia-reperfusion and saturation with higher concentrations of oxygen at birth.

Although the antioxidation system improves the environment with high oxygen concentrations in the late phase of viviparity, the system is less active in preterm infants compared with adults. Furthermore, preterm infants represent an environment of greater vulnerability to oxidative stress due to low vitamin E levels, high concentrations of oxygen due to treatment for respiratory disorders, and other problems of prematurity (7). Vitamin E and GSH-Px have similar and complementary physiologic roles in protecting cells from damage caused by endogenous peroxides (8). In addition, ROS are involved in the development of bronchoplumonary dysplasia (BPD), retinopathy of prematurity, and fragile erythrocyte membranes (7).

On the other hand, a relationship between nitric oxide (NO) and the ROS system has recently been reported (9). Poliandri et al. reported that NO protects against cell death by reducing oxidative stress (10). Therefore, it is possible that NO production affects ROS.

Currently, in clinical practice, glucocorticoids are administered to mothers at risk for premature delivery to accelerate fetal lung maturation and compensate for the lack of lung surfactants (11 – 14). However, the effects of antenatal glucocorticoid treatment on antioxidant enzymes (AOEs) in the lungs of premature neonates are unclear. In the present study, we investigated the effects of dexamethasone (DEX) treatment on antioxidants and nitric oxide synthase (NOS) in the lungs of fetal and neonatal rats.

Materials and Methods

Animals

Eight-week-old male and female Wistar rats were mated. The day on which the presence of a vaginal plug was confirmed after mating was counted as day 0 of gestation. Animals were housed in a room in which temperature (23 ± 1°C), humidity (55 ± 5%), and lighting (lights on from 06:00 to 18:00) were controlled. All studies were conducted according to the Guiding Principles for the Care and Use of Laboratory Animals of The Japanese Pharmacological Society, and approval was obtained from the Ethics Committee of the Institute of Experimental Animals of St. Marianna University Graduate School of Medicine.

DEX

DEX (1 mg/kg, s.c., in sesame oil; Wako Pure Chemical Industries, Ltd., Osaka) or vehicle alone was administered to pregnant rats on days 17 and 18 or 19 and 20 of gestation. Twenty-four hours after the second administration, pregnant rats were anesthetized with an injection of sodium pentobarbital (35 mg/kg, i.p.), and cesarean section was immediately performed. The lungs of the fetuses were removed and stored in a freezer at −80°C. Similarly, DEX (1 mg/kg, s.c.) or vehicle alone was administered to pregnant rats on days 20 and 21 of gestation, and then the lungs of the 1- and 3-day-old neonates were removed. Samples were stored in a freezer at −80°C. All rats delivered on day 22 of gestation.

Western blot analysis

Western blot analysis was performed as previously reported by Takeba et al., with slight modification (15), on samples from 83 rats. Each lung was homogenized in a four-fold volume of lung protein extraction buffer composed of (0.1% NP-40 in phosphate-bufferd saline) and then centrifuged at $14{,}000 \times g$ for 20 min at 4°C. Protein concentrations were determined by using a Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, USA). Protein samples (50 μg each) were boiled with gel loading buffer for 5 min, subjected to 10% SDS-PAGE, transferred to enhanced chemiluminescence (ECL) membranes (Hybond-ECL; GE Healthcare Bio-Sciences, Piscataway, NJ, USA), and blocked for 1 h at room temperature with Tris-buffered saline (TBS) – 0.1% Tween-20 (T-TBS) containing 5% skim milk. The membranes were then incubated for 2 h with the following anti-rabbit and anti-mouse antibodies: SOD-1, rabbit polyclonal antibody; SOD-2, rabbit polyclonal antibody; inducible NOS (i-NOS, NOS-2), rabbit polyclonal antibody; endothelial NOS (e-NOS, NOS-3), rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA); GSH-Px, rabbit polyclonal antibody (Abcam, Cambridge, UK); and catalase (CAT), mouse monoclonal antibody (Sigma, St. Louis, MO, USA) diluted 1:200 in TBS containing 1% skim milk. After five washes with 0.1% T-TBS, the membranes were incubated for 1 h with peroxidase-labeled anti-rabbit IgG and anti-mouse IgG antibody (Cappel, Aurora, OH, USA) diluted 1:1000 in T-TBS. After five washes with T-TBS, the immune complex was visualized with an ECL detection system (ECL plus Western Blotting Detection System; GE Healthcare Bio-Sciences) and quantified using the software program Image Gauge (FujiFilm, Tokyo).

Real-time PCR

Levels of i-NOS and e-NOS mRNA were determined with real-time PCR (LightCycler; Roche Diagnosis, Mannheim, Germany) as described previously (16). In

brief, total RNA was extracted from the lung tissue with an RNA extraction kit (RNAgents Total RNA Isolation System; Promega, Madison, WI, USA). A 2-μg sample of total RNA was reversed-transcribed with 100 U of Moloney murine leukemia virus reverse transcriptase (RETROscript kit; Ambion, Inc., Austin, TX, USA) in 20 μl of total reaction volume containing reverse-transcriptase buffer, random primer, dNTP, and RNase inhibitor. PCR was performed in 20 μl of total reaction volume containing 2 μg of cDNA, primers specific for i-NOS, e-NOS, and glyceraldehyde-3-aldehydro dehydrogenase; 4 mM $MgCl_2$: and LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics). The cycling protocol consisted of one cycle of 10 min at 95°C followed by 40 cycles of denaturation for 10 s at 95°C, annealing for 20 s at 59°C, and extension for 30 s at 72°C. The primers for i-NOS were 5'-GCTACACTTC CAACGCAACA-3' (sense) and 5'-TTCTTGGCGTGG ATGCTC-3' (antisense); those for e-NOS were 5'-TGACCCTCACCGATACAACA-3' (sense) and 5'-CGGGTGTCTAGATCCATGC-3' (antisense); and those for GAPDH were 5'-CTGAGTATGTCGTGG AGTCTA-3' (sense) and 5'-CTGCTTCACCACCTT CTTGAT-3' (antisense). Serial dilutions of the standard cDNA were also used for parallel amplifications. The threshold cycles (Ct) were calculated with LightCycler software (ver. 5.32). Standard curves were plotted with Ct-versus-log cDNA quantities, and the quantities of samples were determined from the standard curves. In addition, i-NOS and e-NOS mRNA levels were normalized to those of GAPDH in each sample.

Histologic examination

Immediately after the rats were killed, the lung was harvested, stored in saline on ice, and dissected from the surrounding tissues. Then the lung was fixed in 10% formalin neutral buffer solution, pH 7.4 (Wako Junyaku, Osaka). Sections of the lung were stained with hematoxylin and eosin.

Statistical analyses

Data are expressed as the mean ± S.E.M. Statistical significance was analyzed using ANOVA, followed by Dunnett's test (StatMate, ver. 3; Atomus, Tokyo). A value of $P<0.05$ was considered to represent a statistically significant difference between groups.

Results

Effects of DEX on Cu-Zn SOD protein expression

Cu-Zn SOD protein expression did not change significantly with increasing gestational and postnatal age in the lungs of control or DEX fetal and neonatal groups (Fig. 1).

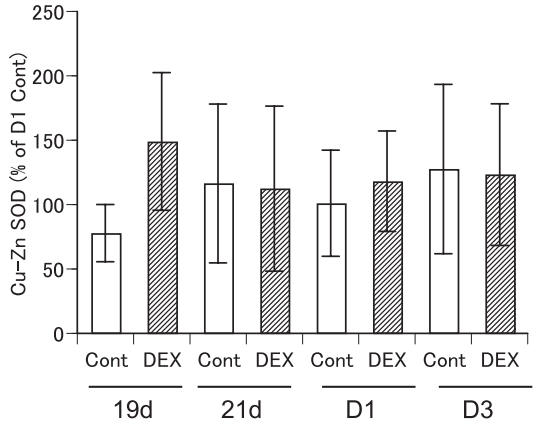

Fig. 1. Cu-Zn SOD in the lungs of fetal rats on days 19 (19d) and 21 (21d) of gestation and of neonatal rats on days 1 (D1) and 3 (D3) after birth. Values are each the mean ± S.E.M. of 4 – 6 rats. Cont indicates control groups (white column); DEX indicates dexamethasone groups (shaded column). DEX (1 mg/kg, s.c.) was administered to pregnant rats for 2 days. On day 19, the Cu-Zn SOD level was slightly increased in the DEX group. At other times no difference was found between control and DEX groups.

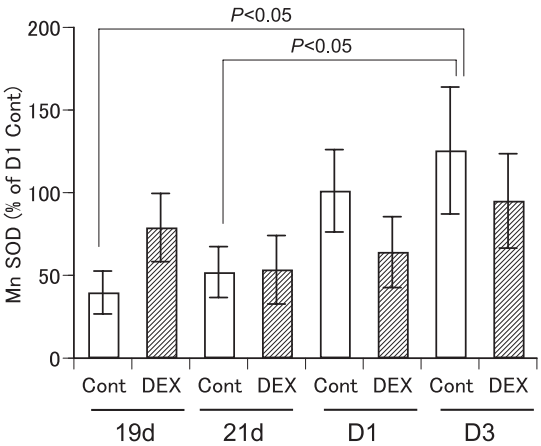

Fig. 2. Mn SOD in the lungs of fetal rats on days 19 (19d) and 21 (21d) of gestation and of neonatal rats on days 1 (D1) and 3 (D3) after birth. Values are each the mean ± S.E.M. of 6 – 9 rats. Cont indicates control groups (white column); DEX indicates dexamethasone groups (shaded column). DEX (1 mg/kg, s.c.) was administered to pregnant rats for 2 days. The Mn SOD level gradually increased with gestational and postnatal age. DEX increased the Mn SOD level on 19d, D1, and D3.

Effects of DEX on Mn SOD protein expression

Mn SOD protein expression gradually increased with increasing gestational and postnatal age in the lungs in the control groups. Mn SOD protein expression did not show any significant changes in fetal and neonatal lungs in the DEX groups (Fig. 2).

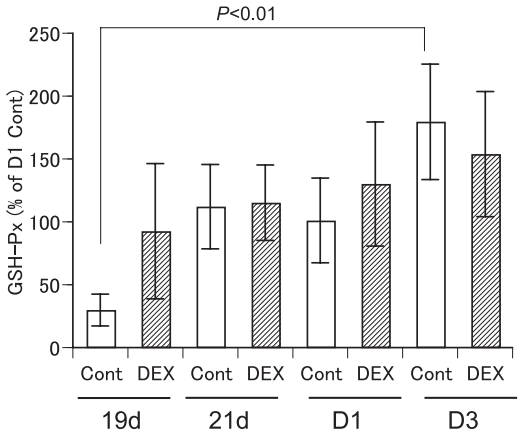

Fig. 3. GSH-Px in the lungs of fetal rats on days 19 (19d) and 21 (21d) of gestation and of neonatal rats on days 1 (D1) and 3 (D3) after birth. Values are each the mean ± S.E.M. of 4 – 6 rats. Cont indicates control groups (white column); DEX indicates dexamethasone groups (shaded column). DEX (1 mg/kg, s.c.) was administered to pregnant rats for 2 days. The GSH-Px level gradually increased with increasing gestational and postnatal age. On day 19, the GSH-Px level was slightly increased in the DEX group.

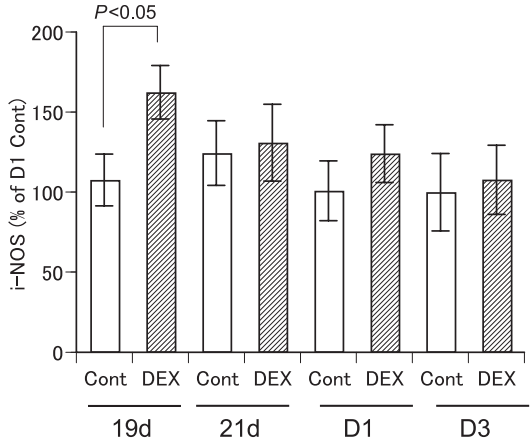

Fig. 5. i-NOS protein in the lungs of fetal rats on days 19 (19d) and 21 (21d) of gestation and of neonates on days 1 (D1) and 3 (D3) after birth. Values are each the mean ± S.E.M. of 5 – 12 rats. Cont indicates control groups (white column); DEX indicates dexamethasone groups (shaded column). DEX (1 mg/kg, s.c.) was administered to pregnant rats for 2 days. On day 19, the i-NOS level was significantly increased in the DEX treatment group.

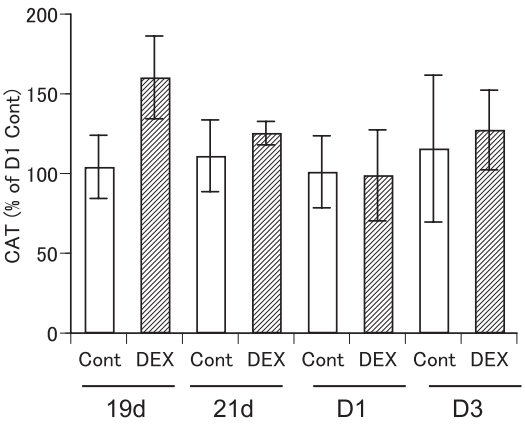

Fig. 4. CAT in the lungs of fetal rats on days 19 (19d) and 21 (21d) of gestation and of neonates on days 1 (D1) and 3 (D3) after birth. Values are each the mean ± S.E.M. of 4 – 6 rats. Cont indicates control groups (white column); DEX indicates dexamethasone groups (shaded column). DEX (1 mg/kg, s.c.) was administered to pregnant rats for 2 days. On day 19, the CAT level was slightly increased in the DEX group.

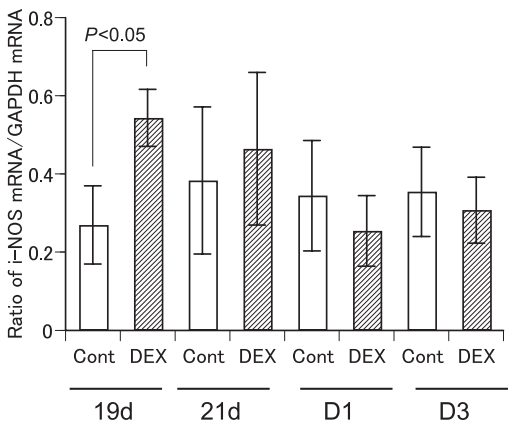

Fig. 6. i-NOS mRNA in the lungs of fetal rats on days 19 (19d) and 21 (21d) of gestation and of neonates on days 1 (D1) and 3 (D3) after birth. Values are each the mean ± S.E.M. of 5 – 12 rats. Cont indicates control groups (white column); DEX indicates dexamethasone groups (shaded column). DEX (1 mg/kg, s.c.) was administered to pregnant rats for 2 days. On day 19, the i-NOS mRNA level was significantly increased in the DEX group.

Effects of DEX on GSH-Px protein expression

GSH-Px protein expression gradually increased with increasing gestational and postnatal age in the lungs of control groups. GSH-Px protein expression did not change significantly in fetal and neonatal lungs in the groups administered DEX (Fig. 3).

Effects of DEX on CAT protein expression

No significant change was seen in CAT protein expression with increasing gestational and postnatal age in the fetal and neonatal control group or DEX group lungs (Fig. 4).

Effects of DEX on i-NOS protein expression

i-NOS protein expression showed no significant change with increasing postnatal age in the lungs in the control groups. However, DEX administration significantly increased i-NOS protein expression compared with the control groups on day 19 of gestation (Fig. 5).

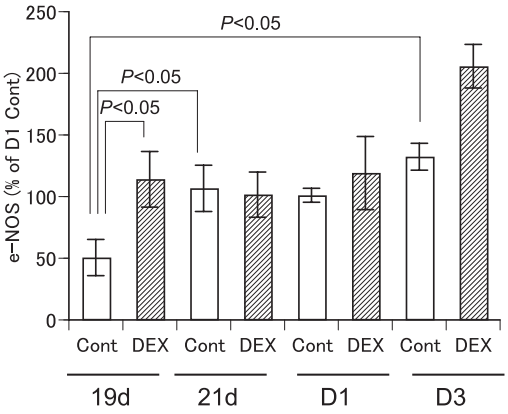

Fig. 7. e-NOS protein in the lungs of fetal rats on days 19 (19d) and 21 (21d) of gestation and of neonates on days 1 (D1) and 3 (D3) after birth. Values are each the mean ± S.E.M. of 3 – 12 rats. Cont indicates control groups (white column); DEX indicates dexamethasone groups (shaded column). DEX (1 mg/kg, s.c.) was administered to pregnant rats for 2 days. The e-NOS level gradually increased with increasing gestational and postnatal age. On day 19, the e-NOS level was significantly increased in the DEX group.

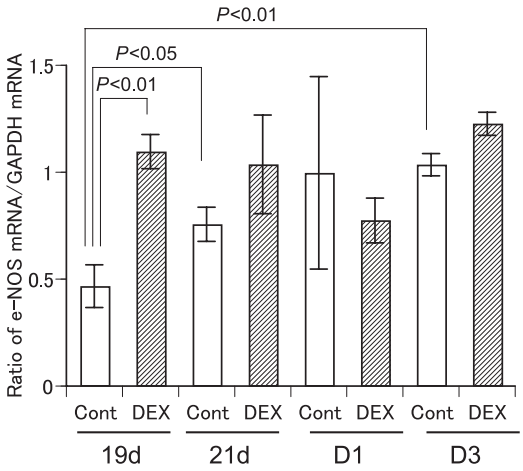

Fig. 8. e-NOS mRNA in the lungs of fetal rats on days 19 (19d) and 21 (21d) of gestation and of neonates on days 1 (D1) and 3 (D3) after birth. Values are each the mean ± S.E.M. of 3 – 12 rats. Cont indicates control groups (white column); DEX indicates dexamethasone groups (shaded column). DEX (1 mg/kg, s.c.) was administered to pregnant rats for 2 days. The e-NOS mRNA level gradually increased with increasing gestational and postnatal age. On day 19, the e-NOS mRNA level was significantly increased in the DEX group.

Effects of DEX on i-NOS mRNA expression

i-NOS mRNA expression showed no significant change with increasing postnatal age in control group lungs. Significantly increased i-NOS mRNA expression was observed on day 19 of gestation in the DEX fetal groups compared with control fetal groups (Fig. 6).

Effects of DEX on e-NOS protein expression

e-NOS protein expression gradually increased with increasing gestational and postnatal age in the lungs of control groups, and DEX administration significantly increased its expression on day 19 of gestation compared with fetal control groups (Fig. 7).

Effects of DEX on e-NOS mRNA expression

e-NOS mRNA expression gradually increased with increasing gestational and postnatal age in the lungs of the control groups, and the expression was significantly increased on day 19 of gestation in the fetal DEX compared with fetal control groups (Fig. 8).

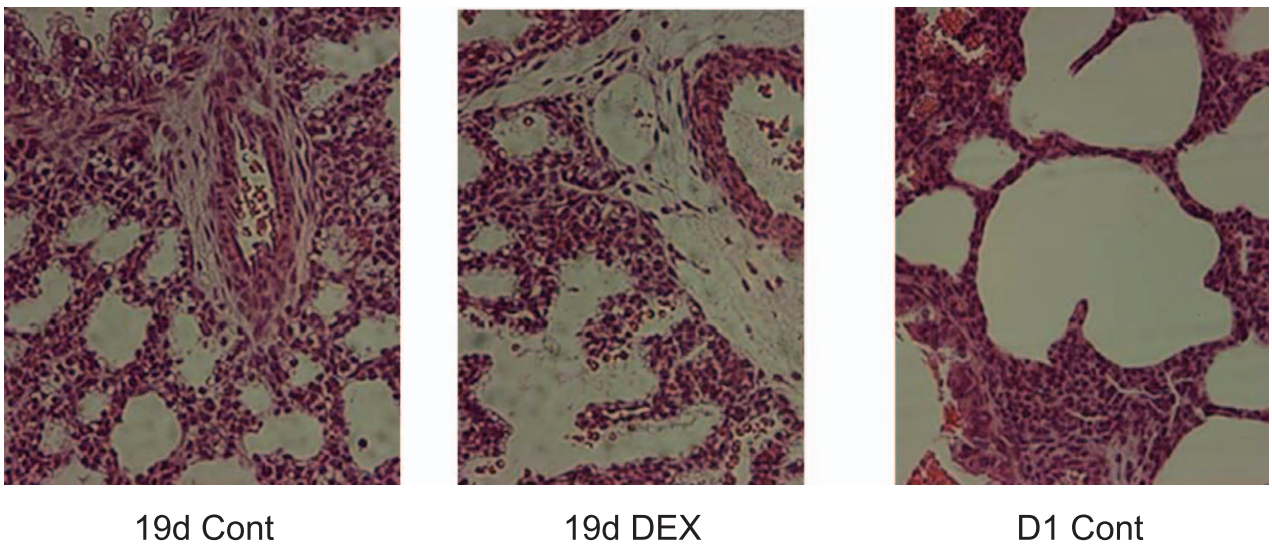

Fig. 9. Alveoli in the lungs of fetal rats on day 19 (19d) of gestation and of neonates on day 1 (D1) after birth. Cont indicates control groups; DEX indicates dexamethasone groups. DEX (1 mg/kg, s.c.) was administered to pregnant rats for 2 days. Hematoxylin and eosin staining, original magnification ×200.

Histologic examination

Fewer alveoli were detected on day 19 of gestation compared with lungs on postnatal day 1, which had larger alveoli and thus were more mature. DEX increased the size of alveoli on gestational day 19 compared with those in the fetal control groups (Fig. 9).

Discussion

Cu-Zn SOD is present in the cytoplasm, where it converts $O_2^{-\bullet}$ into H_2O_2. Because no AOEs are present in microsomes, $O_2^{-\bullet}$ is released into the extracellular membrane and removed by Cu-Zn SOD (17). Most studies have found that Cu-Zn SOD is not upregulated by cytokines and is induced by hyperoxia. McElroy et al. (18) and Strange et al. (19) reported that Cu-Zn SOD activity in the human lung did not change perinatally. In the present study, Cu-Zn SOD protein expression in the lung did not change during the perinatal period or after DEX administration. This suggests that Cu-Zn SOD does not play a role in the elimination of ROS during the development of fetal lungs near birth.

Mn SOD is present in the inner membrane of mitochondria where it removes $O_2^{-\bullet}$ and forms H_2O_2 (17). Mn SOD is induced by hyperoxia and by cytokines such as tumor necrosis factor-α, interferon-γ, interleukin (IL)-1, and IL-6 (20). Hayashibe et al. reported that Mn SOD protein concentrations in rat lung increased near birth (21). In the present study, Mn SOD expression in the lungs of day-3 neonates significantly increased compared with that in the lungs of 19- and 21-day fetuses. This suggests that Mn SOD is involved in the elimination of ROS during the development of the fetal lungs near birth.

In the cytoplasm, less H_2O_2 is decomposed by GSH-Px than by CAT (17). Furthermore, GH-Px decomposes H_2O_2 and converts it into H_2O in mitochondria (17). The mRNA expression and activity levels of intracellular GSH-Px are upregulated to some extent by hyperoxia and cytokines. In the present study, GSH-Px expression in the lungs of day-3 neonates significantly increased compared with that in 19-day fetuses. This suggests that GSH-Px plays a role in the reduction of H_2O_2 during the development of fetal lungs near the time of birth.

CAT is present in peroxisomes and decomposes high concentrations of H_2O_2 (17). In some animal and cell culture models, CAT is induced by hyperoxia, oxidants, and cytokines. Tiina et al. reported that CAT was the only AOE to increase in both activity and mRNA expression throughout prenatal development in the lung but not in the liver, which raises the question of the role of this enzyme in the pulmonary defense system (20). In the present study, CAT expression in the lung did not change during the perinatal period with or without DEX administration. This suggests that CAT is not involved in the reduction of H_2O_2 during fetal lung development before birth.

Preterm infants are sensitive to ROS because their antioxidant defense systems are poorly developed. During the first days of life, preterm infants requiring ventilator therapy often have concurrent pulmonary inflammatory or infectious processes (21). Increases in Mn SOD and GSH-Px expression may be related to those inflammatory or infectious processes. Gradual increases in Mn SOD and GSH-Px levels may also be required in preparation for delivery. This suggests that the function of the antioxidant system varies as the result of different needs.

In the present study, DEX significantly increased i-NOS protein and mRNA expression in fetal rats on day 19 of gestation compared with control groups. In addition, e-NOS protein and mRNA expression gradually increased with increasing gestational and postnatal age in the lungs of control groups. DEX administration significantly increased e-NOS protein and mRNA expression on day 19 of gestation compared with the control group fetuses, but e-NOS protein did not increase significantly compared with that in control group neonates on postnatal days 1 and 3. The activities of e-NOS are developmentally regulated. Lin et al. reported that elevation of e-NOS in fetal lungs during late gestation suggests that the lungs are being prepared for the increase in pulmonary blood flow at birth (22). Furthermore, estrogen upregulates e-NOS enzyme activities in lysates of the fetal pulmonary artery endothelium (23). Asoh et al. reported that antenatal DEX administration significantly increased Ca^{2+}-sensitive NOS activity in rat fetal and neonatal lungs (24). Therefore, it was suggested that elevation of e-NOS during gestation induces the development of fetal lungs after birth, and antenatal glucocorticoid treatment prior to preterm delivery might lead to early development of the pulmonary i-NOS and e-NOS systems (25). Alveoli in the lungs in the DEX group increased in number in the present study.

On the other hand, NO generates cytotoxic compounds (e.g., nitrogen dioxide and peroxynitrite) (26) that may enhance inflammatory lung injury (27). Banks et al. (28) suggested that peroxynitrite-mediated oxidative stress contributes to the development of bronchopulmonary dysplasia (BPD) in premature infants. However, the effects of antenatal glucocorticoid therapy on peroxidants are unclear.

Recently, glucocorticoids have been administered to pregnant women at risk for premature delivery. It is possible that glucocorticoid treatment not only prevents

a deficiency of lung surfactants but also accelerates the maturation of fetal lungs (11 – 14). Andersson et al. reported that oxidation of natural surfactants may result in reduced function and contribute to chronic lung disease (29). The precise mechanisms by which antenatal glucocorticoid treatment improves pulmonary function are not clear. However, the effects of antenatal glucocorticoid treatment on i-NOS and e-NOS in the lungs of immature fetuses may reduce ROS-mediated injury and oxidative inactivation of surfactants and accelerate the development of the lungs. Further detailed studies are needed to clarify the mechanisms of these effects of DEX in the immature lung.

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