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Antenatal Glucocorticoid Corrects Pulmonary Immaturity in Experimentally Induced Congenital Diaphragmatic Hernia in Rats

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ABSTRACT. Congenital diaphragmatic hernia, a highly lethal condition, displays at term the pulmonary biochemical and morphologic immaturity characteristic of premature delivery. We hypothesized that antenatal glucocorticoid, now the standard treatment to prevent hyaline membrane disease in premature human beings, might correct the parameters of the pulmonary biochemical and morphologic immaturity in severe congenital diaphragmatic hernia. A total of 112 fetal rats with or without nitrofen-induced congenital diaphragmatic hernias from 34 pregnancies were treated antenatally with either saline or dexamethasone. Antenatal dexamethasone increased the lung disaturated phosphatidylcholine content, reduced the lung glycogen concentration, reduced the saccular septal thickness, and increased the mean saccular size and volume fraction of saccules in the lungs of rats with large congenital diaphragmatic hernia in comparison with similar rats not so treated. All differences were statistically significant. Antenatal glucocorticoid therapy was efficacious in treating rats with nitrofen-induced congenital diaphragmatic hernia. This encouraging finding warrants further investigation in a large animal model with surgically created congenital diaphragmatic hernia. (*Pediatr Res* 35: 523–529, 1994)

Abbreviations

CDH, congenital diaphragmatic hernia
non-CDH, noncongenital diaphragmatic hernia
DSPC, disaturated phosphatidylcholine
i.p., intraperitoneally
RNase, ribonuclease
ET1, endothelin 1

CDH occurs when the pleuroperitoneal septum fails to separate the thoracic and abdominal cavities; it results in death for many affected infants despite the introduction of novel interventional therapies in conjunction with improved neonatal intensive care (1, 2). Death of infants with CDH is associated with profound pulmonary hypoplasia (3–5) and persistent pulmonary hypertension (6). Convincing evidence has accumulated that the neonatal lung in the presence of lethal CDH is both biochemically and morphologically immature. Blackburn *et al.* (7) in 1977 and Wigglesworth *et al.* (8) in 1981 showed that CDH lungs had significantly reduced lecithin (phosphatidylcholine) content and a smaller number of lamellar granules in type II pneumocytes; furthermore, the lecithin/sphingomyelin ratio was reduced and phosphatidylglycerol was absent in the amniotic fluid surrounding these otherwise normal full-term fetuses (9, 10), although others have observed more normal ratios (11, 12). George *et al.* (13) documented that lungs from 10 neonates dying of CDH (seven of them full term) were morphologically immature for their gestational age.

Antenatal maternal administration of glucocorticoids is an established therapy well known for diminishing the severity of hyaline membrane disease in premature babies (14, 15). Its observed propitious effects on the lung include the accelerated appearance, accumulation, and secretion of surfactant (16–18); a reduction of mesenchymal volume and narrowing of alveolar septal thickness (19); an increase in maximal lung volume (20) and compliance (21); and an increase of antioxidant enzyme activities in the fetal lung (22), leading to decreased incidence, severity, and deaths resulting from neonatal respiratory distress syndrome associated with premature delivery (14, 15).

We recently reported that neonatal rat lungs in the presence of large CDH created experimentally by maternal feeding of nitrofen (2,4-dichloro-4′-nitrodiphenyl ether) were biochemically and morphologically immature, as manifested by smaller, atelectatic lungs with diminished DSPC and a higher glycogen content (23). We used this relatively inexpensive, short-gestation, small-animal model to study whether treatment of these animals with antenatal dexamethasone could significantly improve important parameters of pulmonary maturity. The experimental findings of this study indicate that the lungs in full-term human neonates with severe CDH, which are known to display similar biochemical and morphologic characteristics of prematurity, might respond to prenatal glucocorticoid therapy and thus improve their dismal outcome. Further studies in large non-nitrofen-treated animals will be needed to confirm these findings.

MATERIALS AND METHODS

The experimental design is outlined in Figure 1. Timed pregnant Sprague-Dawley rat mothers (Holtzman Laboratories, Madison, WI; vaginal smear positive, d 0; term, d 22), briefly anesthetized with ether on d 9.5 of gestation, were tube fed 100 mg of nitrofen (technical grade, 92% purity, Zhejiang Chemicals Import and Export Corporation, China) dissolved in 2 mL of olive oil to create CDH in a proportion of neonatal rats. This treatment and subsequent animal care were carried out in accordance with institutional guidelines (MGH Accession 92-4124). Some mothers (treated) were injected with dexamethasone before delivery, and others (untreated) were injected with saline or not injected at all. The dams were killed on d 21.5 of gestation, and fetuses were delivered by cesarean section. Pups with diaphragmatic hernias occupying more than 50% of the thoracic

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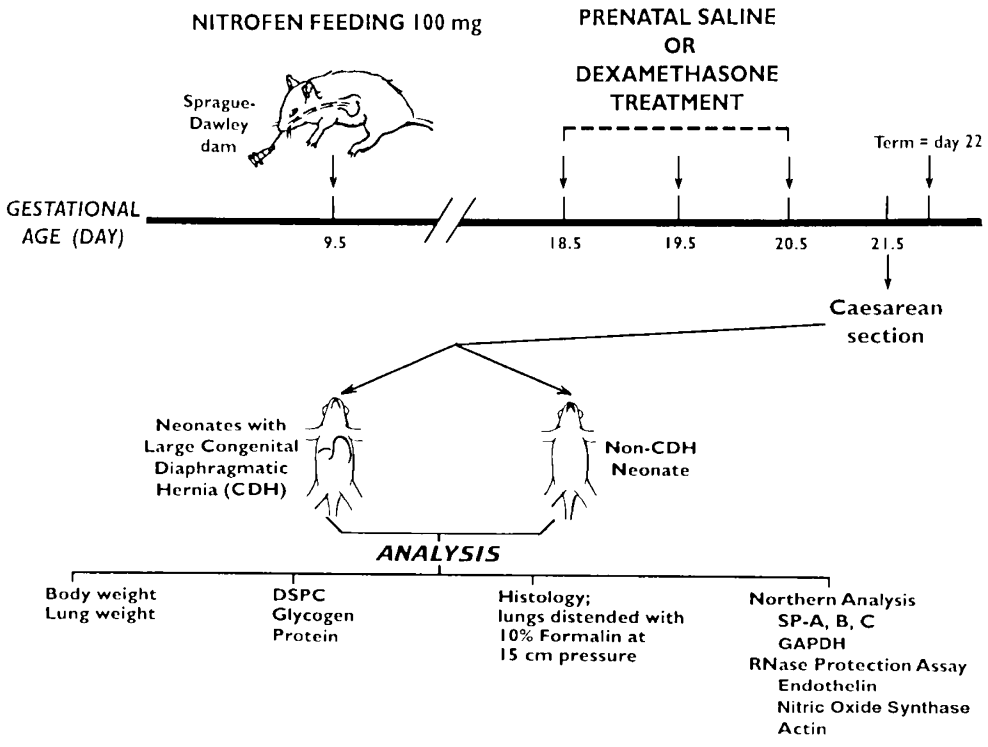

Fig. 1. Experimental design.

cavity were designated CDH rats, and pups without diaphragmatic hernias were designated non-CDH rats. All CDH rats were studied. Because non-CDH rats always outnumbered CDH rats, only a similar number of non-CDH rats from each pregnancy were randomly chosen for subsequent analysis. Pups with diaphragmatic hernias occupying less than 50% of the thoracic cavity were not analyzed so that a uniform degree of pulmonary hypoplasia among the analyzed CDH rats could be maintained. Comparisons between treated and untreated rats were performed with biochemical, morphologic, and molecular methods. Three dosages of dexamethasone were tested in the biochemical analyses. Because dexamethasone produced greater reduction of birth weight and lung weight in both the CDH and non-CDH rats, the lowest of the three dosages of dexamethasone that resulted in significant biochemical improvement but the least inhibition of somatic and pulmonary growth was used in the morphometrical and molecular experiments.

Biochemical analyses. To study biochemical differences, we gave animals three different dosages of dexamethasone (Gensia, Irvine, CA): 0.25 mg/kg i.p. on d 18.5 and 19.5 (n = 2 dams); 0.25 mg/kg ip on d 18.5, 19.5, and 20.5 (n = 3 dams); and 1 mg/kg i.p. on d 18.5, 19.5, and 20.5 (n = 3 dams). The dose schedule of 0.25 mg/kg for 3 d was chosen because it approximates doses currently used in human pregnancy to hasten maturation of fetal lungs (24). The lower and higher doses were chosen empirically to study the dose-related response. The pups of five pregnant dams served as controls, with three dams uninjected and two injected with 0.15 mL (the volume of dexamethasone used in the treated groups) of normal saline i.p. on d 18.5, 19.5, and 20.5. On d 21.5, 132 neonatal rats were delivered and their birth weights recorded. There were 30 CDH rats (24 left-sided and six right-sided); all were subsequently analyzed. Thirty-five non-CDH rats were randomly selected from a pool of 92 for subsequent analysis. Ten other rats with only small CDH were excluded. Lungs from nine neonatal rats whose mother was fed olive oil only without nitrofen were also assayed for comparison to understand the effect of nitrofen on lung development. The number of rats used in each treatment group is shown in Figure 2*A*.

Each heart-lung block was removed from the chest, and the lungs were cleared of surrounding parenchyma, frozen in liquid nitrogen, and stored at −80°C. Lungs at the time of assay were weighed and homogenized (Polytron, Brinkmann Instruments, Westbury, NY) on ice after adding 19 volumes of distilled water to create a 20-fold dilution (assuming lung density of 1 g/mL). Six smaller lungs (three CDH and three non-CDH lungs) were diluted 30-fold. Lung homogenates were assayed for DSPC, protein, and glycogen content as previously reported (23). Briefly, to assay for DSPC, we extracted lipid on 0.15 mL of the homogenate with a chloroform-methanol 2:1 (vol/vol) mixture. After centrifugation, the supernatant was washed with 0.1 M potassium chloride solution according to Folch *et al.* (25). After the upper phase was removed, the lower phase was evaporated to dryness with dry nitrogen gas. The lipid extract was reacted with osmium tetroxide, evaporated again, and the residue dissolved in chloroform-methanol 20:1 (vol/vol). DSPC was isolated through an activated neutral alumina column after elution by chloroform-methanol-7M ammonium hydroxide 70:30:2 (vol/vol/vol) (26). The amount of DSPC was determined by assaying the phosphorus content in the eluent by the method of Bartlett (27).

Glycogen was measured by the method of Lo *et al.* (28); 0.5 mL of the lung homogenate was digested with 30% potassium hydroxide saturated with sodium sulfate and precipitated with 95% ethanol. The glycogen precipitate was redissolved in water and the amount of glycogen determined by the phenol-sulfuric acid color reaction. Protein was determined by the method of Bradford (29).

Morphometrical analysis. For morphometrical analysis, pup lungs from six dexamethasone-treated (0.25 mg/kg i.p., d 18.5 and 19.5) and five untreated (saline-injected on the same days) dams were collected on d 21.5 by cesarean section. Before delivery from the uterus, each pup was given an i.p. injection of 6.5 mg pentobarbital to prevent subsequent insufflation by breathing air. After delivery, the trachea was cannulated and the lungs distended *in situ* with the chest open at 15-cm pressure with 10% formalin. Each trachea was then ligated to maintain the lungs in standardized inflation during fixation. Midcoronal paraffin sections (8 μm) stained with hematoxylin and eosin were studied stereologically with a Nikon Diaphot inverted microscope (Nikon, Garden City, NY) equipped with a 10× objective

with lung maturation (38). Among the CDH rats, the mean lung glycogen concentrations were significantly decreased by antenatal dexamethasone treatment at doses of 0.25 mg/kg i.p. for 3 d (0.108 ± 0.023 mg/mg protein, $p = 0.042$) and at doses of 1 mg/kg i.p. for 3 d (0.115 ± 0.008 mg/mg protein, $p = 0.01$) but not at doses of 0.025 mg/kg i.p. for 2 d (0.142 ± 0.014 mg/mg protein, $p = 0.494$) when compared with the CDH rats not treated with dexamethasone (0.153 ± 0.008 mg/mg protein) (Fig. 2*B*). The effects of dexamethasone on the nitrofen non-CDH rats were also apparent. Without dexamethasone treatment, no statistical significant difference was observed in the lung glycogen mean concentrations among the olive oil (0.163 ± 0.01 mg/mg protein), nitrofen non-CDH (0.139 ± 0.012 mg/mg protein), and nitrofen CDH (0.153 ± 0.008 mg/mg protein) groups. Nitrofen administration decreased birth weight and lung weight, and dexamethasone further reduced these two parameters in a dose-related manner in both the CDH and non-CDH rats (Fig. 3).

Morphologically, lungs in the untreated CDH rats have smaller saccules and thicker saccular septal walls compared with untreated non-CDH rats, whereas antenatal dexamethasone enlarged the pulmonary saccules and decreased the saccular septal wall thickness in the lungs of rats with large CDH (Fig. 4). The mean saccular volume of the untreated CDH rats (93 200 ± 18 400 μm^3), which was much smaller than that of the untreated non-CDH rats (275 000 ± 17 000 μm^3, $p < 0.001$), was significantly increased by dexamethasone treatment (148 000 ± 17 300 μm^3, $p = 0.03$, Fig. 5*A* and *B*). The volume fraction of saccules in the lung of the untreated CDH rats (0.24 ± 0.01), which was much smaller than that of the untreated non-CDH rats (0.4 ± 0.001), was also increased by dexamethasone treatment (0.3 ± 0.02, $p = 0.007$, Fig. 5*A* and *C*).

No significant differences were seen in the pulmonary SP-A, SP-B, SP-C (Fig. 6), ET1 (Fig. 7), and endothelial nitric oxide synthase (Fig. 8) mRNA concentrations measured for whole-lung extracts between the untreated CDH and untreated non-CDH rats fed nitrofen. After antenatal dexamethasone treatment at the dose of 0.25 mg/kg on d 18.5 and 19.5, only ET1 gene expression (Fig. 7) showed a 1.8-fold increase in the non-CDH rats ($p < 0.001$) and 2-fold increase in the CDH rats ($p < 0.001$). The other differences after antenatal dexamethasone treatment were all less than 30% (Figs. 6 and 8). We observed no up-regulation of SP-B mRNA in control animals fed olive oil (no nitrofen) and later treated with dexamethasone.

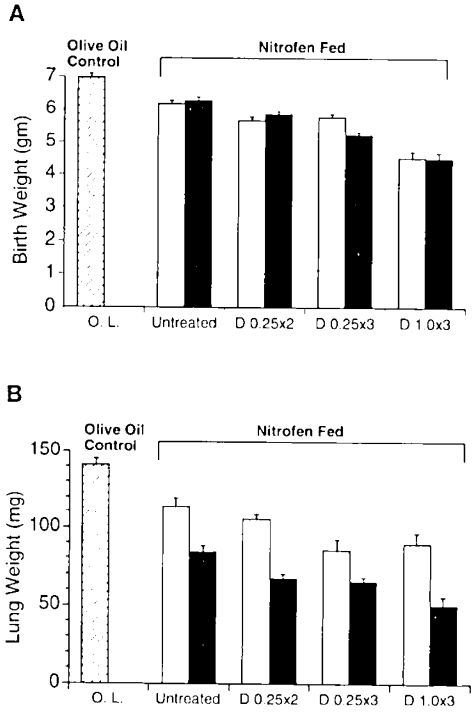

Fig. 3. Effects of antenatal dexamethasone on birth weight and lung weight. Antenatal dexamethasone decreased birth weight (*A*) and lung weight (*B*) in a dose-dependent manner in both the CDH (■) and non-CDH rats (□). See Figure 2*A* for number of animals represented by each bar.

DISCUSSION

The observation that antenatal dexamethasone corrects the pulmonary immaturity in rats with large CDH indicates that antenatal glucocorticoid therapy might be efficacious in the treatment of human babies with severe CDH. The incidence of CDH in the human population is approximately 1 in 3000 live births (39), leading, in the United States with 4 million live births, to approximately 1333 cases. The 60% mortality rate of CDH (40) would result in approximately 800 deaths a year, which is 25% of deaths caused by congenital heart disease in the 1st y, or 1.3 times the deaths caused by leukemia from 0 to 14 y (41, 42).

Pulmonary hypoplasia, persistent pulmonary hypertension, and surfactant deficiency are pathognomonic of severe and lethal CDH lungs. A similar pathophysiologic condition is found in the lungs of premature infants with hyaline membrane disease. Because antenatal glucocorticoid in premature babies has been proved to accelerate pulmonary maturity, thus reducing the incidence and severity of hyaline membrane disease and leading to improved survival, it is logical to consider the use of prenatal glucocorticoid therapy to accelerate pulmonary maturity in CDH. We confirmed that nitrofen alone can suppress DSPC content in lung and reduces the birth weight and lung weight. However, we have shown that antenatal glucocorticoid treatment elevated the DSPC content and reduced the glycogen concentration compared with nitrofen-treated animals who received no dexamethasone and that DSPC improvement was more dramatic in the CDH lungs. Antenatal glucocorticoid treatment also reduced the saccular septal thickness and increased the saccular size and volume fraction of saccules in the lungs of rats with nitrofen-induced large CDH. Our observation that antenatal dexamethasone did not increase surfactant-associated protein mRNA levels in either nitrofen- or nonnitrofen-treated controls differs from results observed by others who reported increases of SP-A, SP-B, and SP-C mRNA levels in immature non-CDH lungs of rats treated with dexamethasone (33, 34). These differences may be the result of variations in dexamethasone dosage or timing of treatment. Further investigations by *in situ* hybridization and immunohistochemical localization may yield important cellular differences not appreciable by whole-lung extraction.

Antenatal glucocorticoid, when used to treat premature babies, is less effective when given after 34 wk gestation or if the baby is delivered more than 7 d after therapy (14, 15). Although most babies with CDH are full term, we predict that their lungs will respond to glucocorticoid because their lungs reflect the biochemical and morphologic patterns characteristic of prematurity (9, 10, 13) and fall within the well-known window of responsiveness to glucocorticoid therapy.

Although glucocorticoid therapy in this study inhibited both somatic and pulmonary growth, long-term follow-up in other studies of both animals (43) and human beings (44, 45) treated for prematurity alone detected no subsequent deleterious effects on somatic, pulmonary, psychologic, or intellectual development. Other salutory effects included a decreased incidence of patent ductus arteriosus (46), necrotizing enterocolitis (47), and intraventricular hemorrhage (48, 49), and stimulation of lung antioxidant enzymes (22).

Because antenatal glucocorticoid and postnatal surfactant replacement therapy have additive beneficial effects in the respiratory distress syndrome of prematurity (50), the present study suggests that they may be similarly additive in neonates with

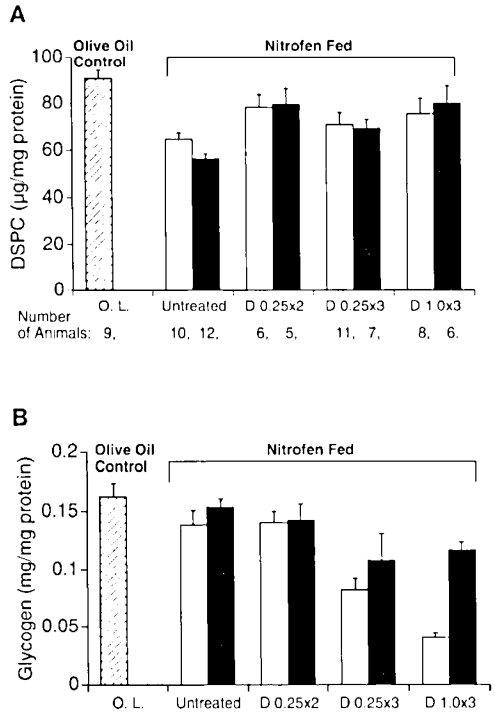

Fig. 2. Effects of antenatal dexamethasone on lung DSPC and glycogen. ■, CDH rats and □, non-CDH rats; *O.L.*, rats whose mothers were fed olive oil only, without nitrofen; *Untreated*, not antenatally treated with dexamethasone; *D 0.25 × 2*, 0.25 mg/kg dexamethasone i.p. on d 18.5 and 19.5; *D 0.25 × 3*, 0.25 mg/kg dexamethasone i.p. on d 18.5, 19.5, and 20.5; and *D 1.0 × 3*, 1 mg/kg dexamethasone i.p. on d 18.5, 19.5, and 20.5. *A*, Nitrofen treatment reduced the DSPC content in non-CDH lungs (*lane 2* compared with *lane 1*). The presence of CDH (*lane 3*) further decreased DSPC content. Antenatal dexamethasone at the three doses tested corrected the DSPC levels toward normal. *B*, Lung glycogen concentrations were significantly reduced by higher doses of antenatal dexamethasone in both the nitrofen-treated CDH and non-CDH rats. Without dexamethasone treatment (*lanes 1, 2,* and *3*), lung glycogen concentrations were similar in olive oil controls and in the nitrofen CDH and non-CDH lungs. The number of animals denoted in *A* applies to *B*.

and a Dage-MTI model 65DX video camera (Michigan City, IN). The image was projected onto a Sony video monitor (Sony Corp., Tokyo, Japan). The randomized midcoronal sections of the right and left lungs were studied separately without knowledge of previous treatment. With right and left lungs counted separately, 18 lung sections from nine treated CDH rats were compared with 18 lung sections from nine untreated CDH rats and 16 lung sections from eight untreated non-CDH rats. Whenever possible, four fields on each lung section (randomly chosen from the superior, inferior, lateral, and medial aspects) were studied. In 11 of the 52 lung sections, only one to three fields were available for study because of pulmonary hypoplasia. A computer-generated grid containing 70 regularly spaced points, which had been previously calibrated with a 0.01-mm micrometer, was superimposed on the lung image on the video monitor. With the help of a cursor, the length of all intercepts (l_0) through test points overlying saccules were measured in a random direction, and the value of $\pi/3 \cdot (\overline{l_0^3})$ was used as an unbiased estimate of the mean saccular volume sampled (30, 31). An average of 82 l_0 measurements were made for each lung section. The ratio of points intercepting saccules to the points overlying the lung parenchyma is in turn an objective estimate of the volume fraction of saccules in the lung (31).

Molecular analysis. Gene expression of surfactant-associated proteins SP-A, SP-B, SP-C, ET1, and endothelial nitric oxide synthase was measured with RNA blot hybridization or an RNase protection assay. Three dams were treated with 0.25 mg/kg dexamethasone i.p. on d 18.5 and 19.5, and two dams were saline injected on the same days. From these pregnancies, six dexamethasone-treated and six untreated CDH rats were identified, and six treated and six untreated non-CDH rats were randomly chosen for analyses. Lungs harvested from these pups on d 21.5 of gestation were immediately frozen in liquid nitrogen. RNA was extracted by the guanidine isothiocyanate/cesium chloride method (32). For RNA blot hybridization, 10-μg RNA samples were fractionated on a formaldehyde-agarose gel and transferred to a nitrocellulose membrane. The membrane was sequentially hybridized with ^{32}P-labeled cDNA probes for SP-A, SP-B, and SP-C (33, 34). The membrane was hybridized with ^{32}P-labeled 1-kb *Pst*1 restriction fragment of a cDNA encoding human glyceraldehyde-3-phosphate dehydrogenase to standardize the amount of RNA in each sample (35). The quantity of hybridizing probe bound to the membrane was measured with a Betascope 603 blot analyzer (Betagen Corp., Waltham, MA). The SD of loading as determined by the glyceraldehyde-3-phosphate dehydrogenase mRNA level in individual samples of specimen was 21%. SP-B mRNA was similarly studied in nonnitrofen (olive oil)-treated controls with (n = 6) and without (n = 6) glucocorticoid treatment for comparison.

For RNase protection assay, RNA samples were incubated with radiolabeled antisense cRNA probes derived from restriction fragments of the rat ET1 gene (Bloch KD, unpublished observations) and of a partial rat endothelial nitric oxide synthase cDNA (Bloch KD, unpublished observation). For preparation of a uniformly labeled antisense ET1 cRNA probe, pGEM4-ET1 (containing exon 2) was linearized with *Eco*RI and incubated with T7 RNA polymerase in the presence of ^{32}P-uridine triphosphate. A partial endothelial nitric oxide synthase cDNA was amplified from rat lung RNA with degenerate oligonucleotides corresponding to amino acids 1012 to 1018 and 1114 to 1120 of human endothelial nitric oxide synthase (36). An approximately 250 bp *Bam*HI/*Eco*RI restriction fragment was ligated into pGEM7zf. For preparation of a cRNA probe for rat endothelial nitric oxide synthase, the plasmid containing the partial cDNA was linearized with *Bam*HI and incubated with T7 RNA polymerase. A radiolabeled antisense cRNA probe for human γ-actin (37) was added to each hybridization to confirm that equal concentrations of RNA were hybridized. RNA fragments protected from RNase were fractionated on a 6% urea-polyacrylamide gel. Hybridization of ET1 RNA probe with ET1 mRNA protected a predominantly 141-nucleotide radiolabeled fragment. Rat γ-actin mRNA protected an approximately 80-nucleotide fragment of human γ-actin probe. The quantity of protected probe was measured with the Betascope 603 blot analyzer. The SD of loading as determined by the γ-actin mRNA levels for the endothelin and the endothelial nitric oxide synthase experiments were 43% and 24%, respectively.

Results were expressed as mean ± SEM. Comparisons were analyzed statistically by analysis of variance, independent t test, and *post hoc* testing; $p < 0.05$ was statistically significant.

RESULTS

The DSPC content, which normally rises with maturity, was reduced significantly in the nitrofen non-CDH rats (64.6 ± 2.7 μg/mg protein) compared with the olive oil rats (90.8 ± 3.5 μg/mg protein, $p < 0.001$). The presence of CDH further decreases the DSPC content (56.3 ± 2.2 μg/mg protein, $p = 0.026$ compared with the nitrofen non-CDH rats). Among the CDH rats, the DSPC content was increased or partially corrected by dexamethasone at doses of 0.25 mg/kg i.p. for 2 d (79.5 ± 6.8 μg/mg protein, $p = 0.001$) and for 3 d (69.1 ± 3.8 μg/mg protein, $p = 0.037$) and at doses of 1.0 mg/kg i.p. for 3 d (80.0 ± 7.4 μg/mg protein, $p < 0.001$) (Fig. 2*A*). A similar dexamethasone response was observed among the nitrofen non-CDH rats.

Lung glycogen mean concentrations normally fall coincident

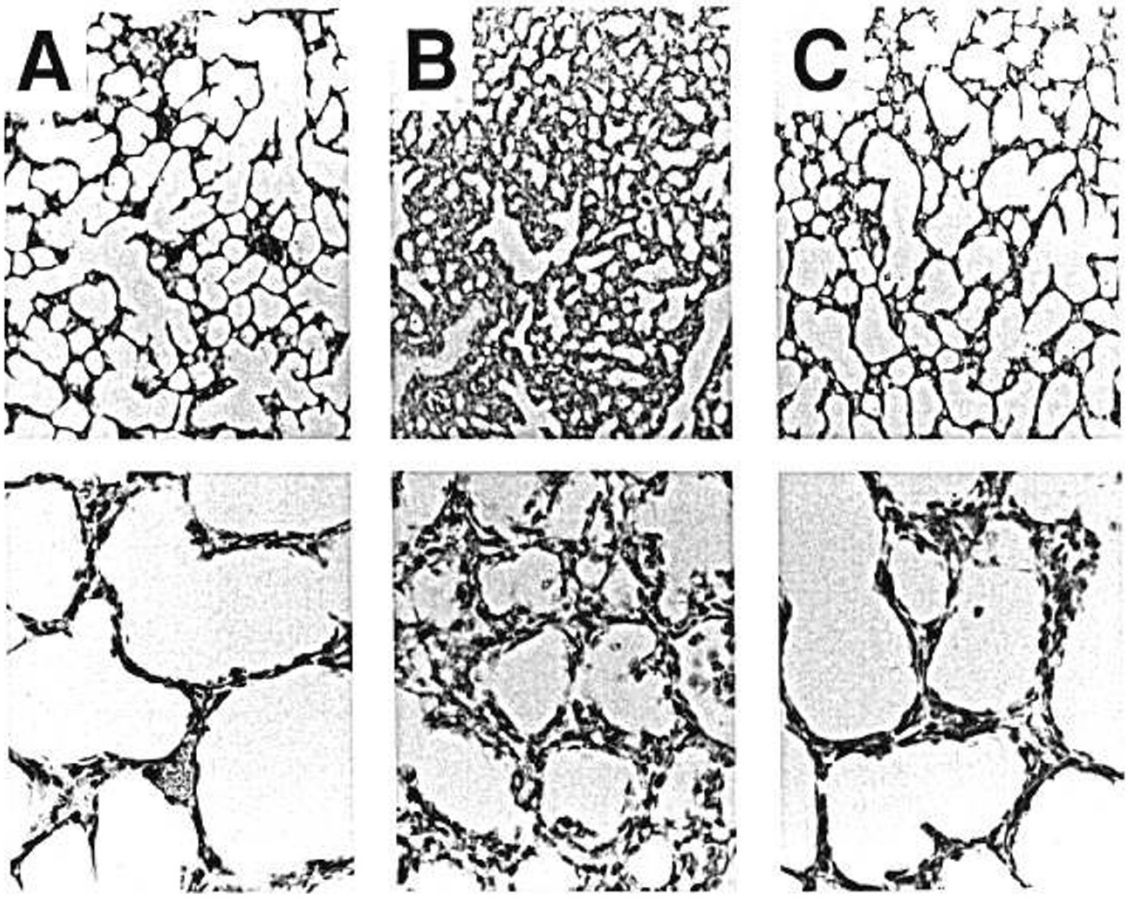

Fig. 4. Effects of antenatal dexamethasone on lung structure. (*Top*, magnification ×79; *bottom*, ×313). The untreated CDH lungs (*B*) had small saccules and thick saccular septae compared with the treated CDH lungs (*C*) in which the histologic tissue, corrected by antenatal dexamethasone, resembled the untreated non-CDH lungs (*A*).

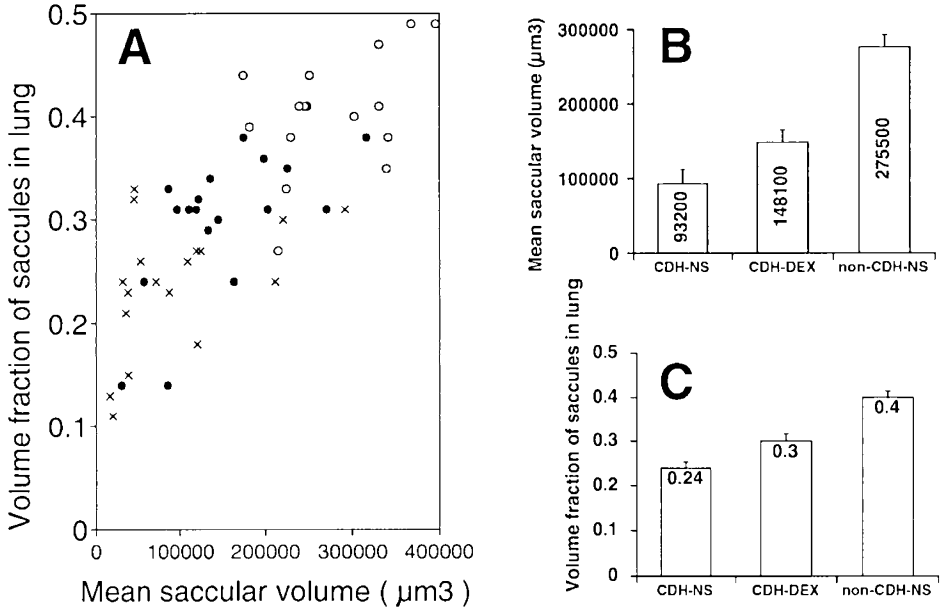

Fig. 5. Effects of antenatal dexamethasone on lung morphometry. *A*, All the individual data points from the stereologic study were shown. Despite scattering within each group, it can be appreciated that most of the dexamethasone-treated CDH values (●) fall between the untreated CDH values (*x*) and the untreated non-CDH values (○). *B*, The mean saccular volume of the untreated CDH rats (*CDH-NS*), which was much smaller than that of the untreated non-CDH rats (*non-CDH-NS*), was improved in the treated CDH rats (*CDH-DEX*). *C*, The volume fraction of saccules in the lung of the untreated CDH rats (*CDH-NS*), which was much smaller than that of the untreated non-CDH rats (*non-CDH-NS*), was significantly improved in the treated CDH rats (*CDH-DEX*).

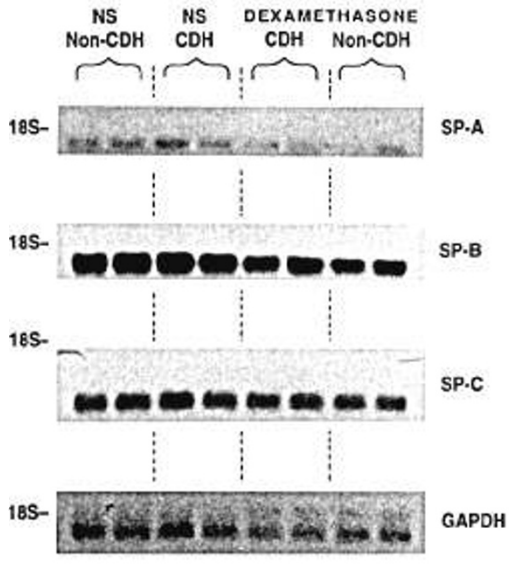

Fig. 6. Results of RNA blot hybridization showing pulmonary SP-A, SP-B, and SP-C mRNA levels in CDH and non-CDH rats with either antenatal saline (*NS*) or dexamethasone treatment. Glyceraldehyde-3-phosphate dehydrogenase mRNA level was used to standardize any differences in loading between samples. Position of 18S rRNA is indicated.

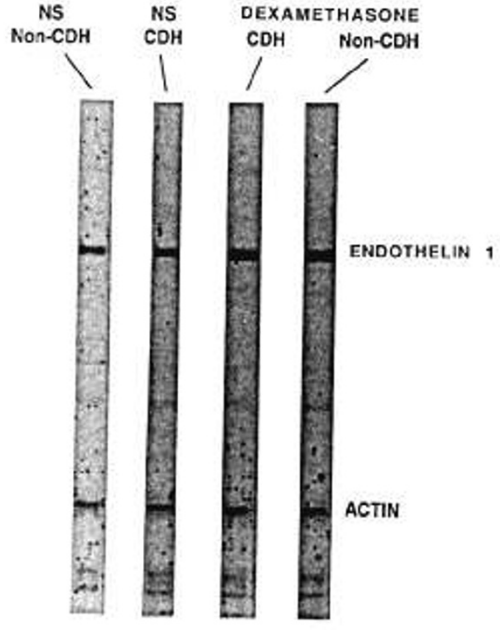

Fig. 7. Representative results of RNase protection assay showing no change in pulmonary endothelin 1 mRNA level between untreated CDH and non-CDH rats. There was, however, a 1.8-fold increase in the non-CDH rats ($p < 0.001$) and 2-fold increase in the CDH rats ($p < 0.001$) after antenatal dexamethasone treatment. *NS*, normal saline.

prenatally diagnosed CDH. Antenatal ultrasonography can visualize these defects, and cases exhibiting criteria indicative of a near 100% mortality rate can be predicted with accuracy (1, 2); therefore, treatment of these babies with an established agent such as antenatal glucocorticoid should be considered as a therapeutic option after confirmation of the present observations in larger animal models with surgically created CDH without the use of nitrofen. When appropriate, antenatal glucocorticoid treatment should be compared or combined with surfactant replacement therapy (51), extracorporeal membrane oxygenation (52), or nitric oxide inhalation therapy (53) in the treatment of babies with severe CDH.

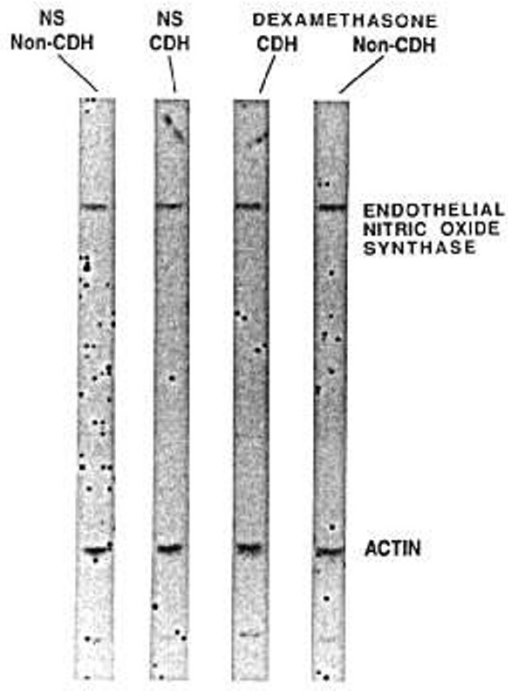

Fig. 8. Representative results of RNase protection assay showing no change in pulmonary endothelial nitric oxide synthase mRNA level between untreated CDH and non-CDH rats or between untreated and dexamethasone treated CDH and non-CDH rats. *NS*, normal saline.

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