Neonatal dexamethasone treatment increases the risk for pulmonary hypertension in adult rats

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Le Cras, Timothy D., Neil E. Markham, Kenneth G. Morris, Charles R. Ahrens, Ivan F. McMurry, and Steven H. Abman. Neonatal dexamethasone treatment increases the risk for pulmonary hypertension in adult rats. Am J Physiol Lung Cell Mol Physiol 278: L822–L829, 2000.—Dexamethasone (Dex) treatment during a critical period of lung development causes lung hypoplasia in infant rats. However, the effects of Dex on the pulmonary circulation are unknown. To determine whether Dex increases the risk for development of pulmonary hypertension, we treated newborn Sprague-Dawley rats with Dex (0.25 µg/day, days 3–13). Litters were divided equally between Dex-treated and vehicle control (ethanol) rats. Rats were raised in either room air or until 10 wk of age (normoxic groups) or room air until 7 wk of age and then in a hypoxia chamber (inspired O2 fraction = 0.10; hypoxic groups) for 3 wk to induce pulmonary hypertension. Compared with control vehicle rats, Dex treatment of neonatal rats reduced alveolarization (by 42%; P < 0.05) and barium-filled pulmonary artery counts (by 37%; P < 0.05) in 10-wk-old adults. Pulmonary arterial pressure and the ratio of right ventricle to left ventricle plus septum weights (RV/LV+S) were higher in 10-wk-old Dex-treated normoxic rats compared with those in normoxic control rats (by 16 and 16%, respectively; P < 0.05). Small pulmonary arteries of adult normoxic Dex-treated rats showed increased vessel wall thickness compared with that in control rats (by 15%; P < 0.05). After 3 wk of hypoxia, RV/LV+S values were 36% higher in rats treated with Dex in the neonatal period compared with those in hypoxic control rats (P < 0.05). RV/LV+S was 42% higher in hypoxic control rats compared with those in normoxic control rats (P < 0.05). We conclude that Dex treatment of neonatal rats caused sustained lung hypoplasia and increased pulmonary arterial pressures and augmented the severity of hypoxia-induced pulmonary hypertension in adult rats.

Lung development; glucocorticoids; alveolarization; congenital diaphragmatic hernia; bronchopulmonary dysplasia; hypoxia

NORMAL LUNG GROWTH IS DIVIDED into five distinct stages: embryonic, pseudoglandular, canalicular, saccular, and alveolar (7). In humans, the major phase of alveolarization occurs postnatally and lasts over at least the first 18–24 mo of life (7). In rats, alveolarization also occurs postnatally in the first 1–3 wk of life (4, 7, 27, 28, 32). Treatment of newborn rats with the synthetic glucocorticoid dexamethasone (Dex) impairs the process of alveolarization by reducing the formation of secondary septa and enhancing thinning of the alveolar wall (6, 26, 29, 46). High doses of antenatal and postnatal Dex are frequently used clinically to prevent respiratory distress syndrome (48) and to improve lung function in ventilator-dependent preterm infants with chronic lung disease (9). Although Dex can impair alveolarization in newborn rats, the long-term effects of steroids on pulmonary structure and function are not known.

Lung hypoplasia and a pattern of alveolar simplification occur in several neonatal lung diseases including bronchopulmonary dysplasia, congenital diaphragmatic hernia, and Down’s syndrome (7, 10, 12, 25, 39, 42). These diseases are also associated with an increased risk for development of pulmonary hypertension (1, 2, 45). Recently, Le Cras et al. (23) have observed that the Fawn-Hooded rat (FHR), a genetic strain that develops accelerated pulmonary hypertension after mild reductions in alveolar P O2 (at Denver’s altitude), also exhibits an abnormal pattern of lung development, resulting in reduced alveolarization. Treatment of FHR s with perinatal supplemental oxygen improved alveolarization as well as reduced the development of pulmonary hypertension, suggesting a link between these two phenomena (23). Although clinical diseases and experimental studies suggest important associations between lung hypoplasia and an increased risk of developing pulmonary hypertension, mechanisms that link abnormal lung growth and pulmonary hypertension are unclear.

Because the FHR is complicated by unknown genetic factors, it is difficult to determine the association between the lung hypoplasia, altered pulmonary vascular development, and the risk for pulmonary hypertension in this strain. The present study was designed to test the hypothesis that in normal rats Dex-induced lung hypoplasia would alter pulmonary vascular development and increase the risk for pulmonary hypertension. To test this hypothesis, we treated newborn Sprague-Dawley rats (SDRs) with Dex during a critical period of lung growth and assessed the long-term effects of Dex on lung growth, vascular growth, and the risk for pulmonary hypertension. We report that alveo-
lar simplification persists in 10-wk-old adult rats after neonatal Dex treatment. The Dex-induced lung hypoplasia was also associated with reduced vessel density, increased pulmonary arterial pressures, and an augmented severity of pulmonary hypertension when adult Dex-treated rats were exposed to 3 wk of hypoxia.

### METHODS

**Animals.** All procedures and protocols were approved by the Animal Care and Use Committee at the University of Colorado (Denver) Health Sciences Center. Treatment protocols are summarized in Fig. 1. Newborn SDRs were injected with Dex (0.25 µg/day, 20 µl/day subcutaneously, dissolved in 100% ethanol) from day 3 until day 13 (11 days of injections, ~50 µg/kg on day 3 to 8 µg/kg on day 13) to induce lung hypoplasia as previously described (29). Control SDRs were treated with 100% ethanol (20 µl/day) and served as vehicle controls. Each litter was divided equally between Dex-treated and vehicle control rats and kept with the same mother. Rats were raised either at Denver’s altitude until 10 wk of age (normoxic groups) or at Denver’s altitude until 7 wk of age and then in a hypobaric hypoxia chamber (inspired O2 fraction = 0.10; hypoxic groups) for 3 wk to induce pulmonary hypertension as previously described (35). Rats were euthanized for study by lethal injection of pentobarbital sodium at 2 and 10 wk of age. Lung and body weights and hematocrit were measured.

Lung histology and radial alveolar counts. Rat lungs were fixed for histology by tracheal installation of 10% buffered Formalin under constant pressure (10 cmH2O) as previously described (23). Alveolarization was assessed by the radial alveolar count method of Emery and Mithal (15) and Cooney and Thurlbeck (11). Briefly, radial counts were performed by identifying respiratory bronchioles as described by Randell et al. (37). From the center of the bronchiole, a perpendicular line was taken to the edge of the acinus (connective tissue septum or pleura), and the number of septa that intersected the line was counted. At least 10 counts were performed for each lung section.

Barium-gelatin arteriograms and arterial density counts. Barium-gelatin arteriograms and vessel density counts were performed with slight modifications of the technique reported by deMello et al. (13). Briefly, blood was flushed from the lungs by infusions of heparinized saline through a catheter in the main pulmonary artery. A heated barium-gelatin mixture was infused at 73 mmHg pressure until surface filling of the vessels with barium was seen uniformly over the surface of the lung. The main pulmonary artery was ligated under the infusion pressure, and the lungs were fixed by tracheal instillation of Formalin at constant pressure (10 cmH2O). The lungs were submersed in Formalin under pressure for at least 4 days. The left lungs were excised after fixation and imaged with X-ray radiography. After radiography, the left lungs were paraffin embedded, and sections were cut and stained with hematoxylin and eosin. Barium-filled pulmonary arteries per high-power field (×100 magnification) were counted. At least five high-power fields (×100 magnification) of distal lung next to the pleural surface per animal were counted. Fields containing large airways or major vessels were avoided to ensure consistency of counts between sections.

**Pulmonary arterial and systemic pressure measurements.** Pulmonary arterial and carotid arterial pressures were measured by placing catheters in the main pulmonary artery and right carotid artery as described in Stevens et al. (44).

**Right ventricular hypertrophy measurement.** At death, the hearts were removed and dissected to isolate the free wall of the right ventricle from the left ventricle and septum. The ratio of right ventricle (RV) weight to left ventricle plus septum (LV+S) weight (RV/LV+S) was used as an index of right ventricular hypertrophy (RVH).

**Vessel morphometry.** Morphometry was on slides from lungs fixed by tracheal installation of Formalin without arterial barium infusions. Vessel wall thickness measurements were performed by an observer blinded to the identity of the histology slides. Wall thickness measurements were performed on small pulmonary arteries (20–60 µm) associated with terminal bronchioles and distal air spaces with a Zeiss interactive digital-analysis system (ZIDAS). Wall thickness and external diameter were measured directly; the percent wall thickness was calculated as [(2 × wall thickness)/vessel diameter] × 100 to assess medial hypertrophy.

### RESULTS

Lung and body weights and hematocrit. Dex treatment reduced body weight by 23% in 2-wk-old rats compared with that in control rats (P < 0.05; Table 1). Body weight was not different between Dex-treated and control rat groups at 10 wk [normoxic groups; P = not significant (NS)]. Lung-to-body weight ratio was 12.5% lower in 2-wk-old Dex-treated rats compared with that

### Table 1. Body weight and lung-to-body weight ratio

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<tr>
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<th>Body Weight, g</th>
<th>Lung-to-Body Weight Ratio</th>
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<tr>
<td>2 wk</td>
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<tr>
<td>Control</td>
<td>27.6 ± 0.7</td>
<td>0.0016 ± 0.0005</td>
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<tr>
<td>Dex</td>
<td>21.3 ± 0.5*</td>
<td>0.0014 ± 0.0006*</td>
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Values are means ± SE. Dex, dexamethasone. Data for 10-wk-old rats are for normoxic groups. *P < 0.05 compared with control group.
in control rats (P < 0.05; Table 1). Lung-to-body weight ratios at 10 wk were not different between Dex-treated and control rat groups (normoxic groups; P = NS). Hematocrit was slightly lower in 10-wk-old Dex-treated rats compared with that in control rats (48.9 ± 1.3 vs. 52.5 ± 0.9; P < 0.05).

Lung histology and radial alveolar counts. Histology revealed a pattern of enlarged distal air spaces with reduced alveolar number (alveolar simplification) at 2 wk of age in Dex-treated rats (Fig. 2). This pattern was still evident in 10-wk-old adults (normoxic). Radial alveolar counts were reduced by 39% in 2-wk-old Dex-treated rats and by 42% in 10-wk-old adult Dex-treated rats compared with those in control rats (P < 0.05; Fig. 3).

Barium-gelatin arteriograms and arterial density counts. To examine pulmonary arterial structure and to determine arterial density, we infused heated barium-gelatin mixtures into the main pulmonary artery of 10-wk-old normoxic control and Dex-treated rats. Arteriograms of left lungs showed reduced barium filling of small pulmonary arteries in Dex-treated rats compared with that in control rats (Fig. 4). Barium-filled pulmonary artery counts were reduced by 37% in Dex-treated rats (P < 0.05; Fig. 5).

Pulmonary arterial and systemic blood pressures and small pulmonary artery morphology. Pulmonary arterial pressures were 16% higher in normoxic Dex-treated rats at 10 wk of age compared with normoxic control rats (P < 0.05; Fig. 6A). Systemic (carotid) arterial blood pressures were not different between Dex-treated and control rats (P = NS; Fig. 6B). Small pulmonary arteries of adult normoxic Dex-treated rats associated with terminal bronchioles and distal air spaces showed evidence of medial hypertrophy compared with that in control rats (Fig. 7A). Wall thickness of small pulmonary arteries (with external diameters ranging between 20 and 60 µm) in adult normoxic Dex-treated rats was 15% higher compared with that in normoxic control rats (P < 0.05; Fig. 7B). Three weeks of hypoxia increased small pulmonary artery wall thickness by 23% (hypoxic control vs. normoxic control rats; P < 0.05). Hypoxic Dex-treated rats did not show a further increase in small pulmonary artery wall thickness compared with hypoxic control rats (P = NS).

RVH. To assess the effects of Dex treatment on RVH, we determined the RV/LV:S values as a measure of RVH at 10 wk of age in normoxic and hypoxic Dex-treated and control groups. RV/LV:S was increased 16% in normoxic Dex-treated rats compared with that in normoxic control rats (P < 0.05; Fig. 8). The LV:S-to-body weight ratio was not different between control and Dex-treated rats (0.0023 ± 0.0002 and 0.0021 ± 0.0001, respectively).
respectively; \( P = \text{NS} \). RV/LV+S was 42% higher in hypoxic control rats compared with that in normoxic control rats \( (P < 0.05) \). In hypoxic Dex-treated rats, RV/LV+S was 36% higher compared with that in hypoxic control rats \( (P < 0.05; \text{Fig. 8}) \).

**DISCUSSION**

In this study, we report that Dex treatment of neonatal rats reduced alveolarization in 2-wk-old infant and 10-wk-old adult rats and decreased pulmonary arterial density. In addition, adult rats that were treated with Dex as neonates had higher pulmonary arterial pressures, increased small pulmonary artery wall thickness, and increased RVH compared with those in age-matched control rats. Systemic pressure and the ratio of LV+S to body weights were not different between control and Dex-treated rats at 10 wk of age, suggesting the lack of direct systemic vascular or cardiac effects of Dex at this time point. When adult Dex-treated and vehicle control rats were exposed to severe hypoxia for 3 wk, the Dex-treated rats developed significantly more RVH than the hypoxic control rats.

Collectively, Dex treatment during the neonatal period caused persistent decreases in alveolarization and pulmonary arterial density, increased pulmonary arterial pressure and small pulmonary artery hypertrophy, and increased the severity of hypoxia-induced pulmonary hypertension in adulthood. This study suggests that alterations of lung growth in the neonatal period can have long-term effects on cardiopulmonary structure and function later in life.

Although past studies have demonstrated that Dex treatment of neonatal rats impairs alveolarization and causes lung hypoplasia, effects on the pulmonary circulation were not studied. Massaro and co-workers \( (27, 29) \) reported that treatment of neonatal rats with low doses of Dex impaired alveolarization and caused lung hypoplasia at 2 wk of age. They suggested that the effects of Dex on alveolarization were due to inhibition of secondary septal formation. Further studies \( (6, 26, 40) \) have confirmed and extended these findings to show that Dex accelerates postnatal alveolar wall thinning and alters wall composition. The persistence of lung hypoplasia after Dex treatment has previously been

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**Fig. 4.** Barium-gelatin arteriograms of left lungs of Dex-treated Sprague-Dawley rats and vehicle control rats. Barium filling of pulmonary arteries was reduced in Dex-treated rats compared with that in vehicle control rats.

**Fig. 5.** Pulmonary arterial density is reduced in Dex-treated adult rats. Pulmonary artery vessel counts were performed on barium-filled fixed lung sections from 10-wk-old control and Dex-treated Sprague-Dawley rats (normoxic; \( \times100 \) magnification; \( A \)). Barium-filled pulmonary arteries were counted per high-power field (\( \times100 \) magnification; \( B \)). *\( P < 0.05 \) between Dex-treated and control rat lungs.
described in rats at 5 wk of age after Dex treatment from days 2 to 15 after birth (46). This study suggested that Dex may impair formation of secondary septa by accelerating parenchymal maturation and by inducing precocious microvascular maturation. This could potentially cause premature fusion of the double-capillary network that is a prerequisite for alveolar formation. In our study, Dex treatment of neonatal rats with the protocol developed by Massaro and Massaro (29) caused a reduction in alveolarization, which persisted for at least 8 wk after Dex treatment.

Several mechanisms may be responsible for an increased risk for pulmonary hypertension with lung hypoplasia. First, decreased alveolarization suggests that lung surface area is reduced and may lead to hypoxemia. Because hematocrits were not elevated in the Dex-treated adults, they were probably not hypoxic. The reduced arterial density that we observed suggests that the Dex-treated rats may have had a reduced cross-sectional area of the pulmonary vascular bed, leading to a higher pulmonary vascular resistance. Adult Dex-treated rats also showed increased small pulmonary artery wall thickness that may have contributed to increased pulmonary arterial pressure. In addition to structural factors, there may also be alterations in vasoreactivity and vascular tone over time; increased expression of the potent vasoconstrictor endothelin-1 has been described in models of pulmonary hypertension (14, 18, 41). Reduced production of vasodilators such as nitric oxide or prostacyclin may also be contributing factors (47).

The FHR is a genetic strain that develops severe pulmonary hypertension with modest decreases in alveolar PO$_2$ (at Denver’s altitude) (41). Le Cras et al. (23) recently reported abnormal lung development in the FHR at Denver’s altitude, resulting in enlarged distal

Fig. 6. Pulmonary arterial blood pressure but not systemic arterial blood pressure is increased in Dex-treated adult rats. A: pulmonary arterial blood pressure for 10-wk-old normoxic Dex-treated and control rats. *P < 0.05 between Dex-treated and control rats. B: systemic (carotid arterial) blood pressure for 10-wk-old normoxic Dex-treated and control rats. P = not significant (NS).

Fig. 7. Increased vessel wall thickness in small pulmonary arteries of adult Dex-treated rats. A: histology of pulmonary arteries in 10-wk-old normoxic Dex-treated and control Sprague-Dawley rats. Lungs were fixed by tracheal instillation of Formalin (without barium infusion of pulmonary artery). Arrows, small pulmonary arteries associated with terminal bronchioles (×200 magnification). B: small pulmonary artery (20–60 µm) wall thickness was increased in normoxic adult Dex-treated Sprague-Dawley rats compared with that in normoxic control rats. *P < 0.05 between Dex-treated and control rats.
air spaces and reduced arterial density in the adults. Perinatal treatment of FHRs with supplemental oxygen to mimic sea-level PO$_2$ improved alveolarization and reduced development of pulmonary hypertension (23). This suggested that FHRs have an altered oxygen sensitivity and that lung hypoplasia might be linked with development of pulmonary hypertension. Because the FHR is complicated by unknown genetic factors, it is difficult to determine the association between lung hypoplasia and the risk for pulmonary hypertension in this strain. The purpose of the present study was to test the hypothesis that Dex-induced lung hypoplasia in normal rats would increase the risk for pulmonary hypertension. Dex-treated adult SDRs had reductions in radial alveolar counts that were similar to adult FHRs (7.0 and 5.1 in Dex-treated SDRs and FHRs, respectively, vs. 12.1 in control SDRs) and similar increases in RV/LV+S (0.40 and 0.43 in Dex-treated SDRs and FHRs, respectively, vs. 0.34 in control SDRs). Hence, in both models, lung hypoplasia was associated with an increased risk for pulmonary hypertension.

Perinatal exposure of rats to hypoxia and hyperoxia has also been shown to reduce alveolarization (5, 30, 37). Whether these effects also increase the risk for pulmonary hypertension later in life has not been studied but is suggested because increased pulmonary reactivity has been reported in adult rats exposed to perinatal hypoxia (16).

A potential limitation of our study is that we were unable to differentiate whether the effects of Dex on the pulmonary circulation are due to indirect effects of Dex on airway development, which, in turn, affects vascular development, or due to direct effects of Dex on the pulmonary circulation itself. Dex has many effects on lung gene expression, including the production of surfactant proteins (34) and growth factors and cytokines such as tumor necrosis factor-α and transforming growth factor-β2 and -β3, which may mediate some of the effects of glucocorticoids on morphology and differentiation of the developing lung (19, 20, 31). Dex also increases expression of keratinocyte growth factor and hepatocyte growth factor receptor (36), growth factors produced by the mesenchyme. These and other factors are involved in epithelial-mesenchymal interactions, which are thought to be important in development of the pulmonary circulation (43). Dex may also have direct effects on the pulmonary circulation: glucocorticoids upregulate endothelin-1 expression in vascular smooth muscle cells (38) and downregulate cyclooxygenase-1 gene expression and prostacyclin synthesis in cultured fetal pulmonary artery endothelial cells (21).

Glucocorticoids have also been shown to suppress induction of vascular endothelial growth factor in cultured pulmonary vascular smooth muscle cells by platelet-activating factor, platelet-derived growth factor, and hypoxia (22, 33). Another limitation of our study is that we did not measure pulmonary blood flow, and, therefore, we cannot be sure whether increased pulmonary arterial pressure in the Dex-treated rats was due to increased pulmonary arterial resistance or to a change in flow. However, increased pulmonary arterial pressure was associated with increased RVH and small pulmonary artery hypertrophy. In this study, we did not measure vasoreactivity so we were unable to evaluate the contribution of increased vascular tone to pulmonary hypertension in the Dex-treated rats. Small pulmonary artery wall thickness was not different between hypoxic Dex-treated rats and hypoxic control rats. This suggests that increased RVH in the hypoxic Dex-treated rats relative to that in hypoxic control rats may be due to increased pulmonary vascular tone and reduced arterial density, which would contribute to higher pulmonary vascular resistance after Dex treatment.

Although the findings of this study may have implications regarding long-term outcomes of human neonates treated with high doses of steroids, care should be taken in extrapolating our findings. The treatment protocol followed in this study was not an attempt to reproduce clinically relevant doses and regimens; Dex was given over an 11-day period, which in human neonates is equivalent to a period of several months to a couple of years, the period of alveolar development for humans (7). However, it should be noted that the treatment dose that we used (8–50 µg/kg) was ~10- to 100-fold less than that used clinically (24). Although the short-term beneficial effects of antenatal and postnatal steroids on lung function and in the prevention of respiratory distress syndrome and treatment of chronic lung disease are clear, the long-term effects of steroids on lung growth and development are uncertain.

Several neonatal lung diseases in humans, including bronchopulmonary dysplasia, congenital diaphragmatic hernia, primary lung hypoplasia, lung hypoplasia associated with oligohydramnios or renal dysfunction, and Down's syndrome, are characterized by lung hypoplasia with decreased alveolarization and a high risk for pulmonary hypertension (1, 8, 10, 12, 17, 25, 39,
42, 45). Although uncertainty exists about the links between perinatal events and adult diseases, our experimental studies (23) and these clinical diseases suggest that lung hypoplasia may increase the risk for pulmonary hypertension.

In recent years, epidemiologic studies have suggested that factors affecting fetal and neonatal development may have long-term effects on adult diseases such as ischemic heart disease, hypertension, stroke, chronic bronchitis, and emphysema (3). This concept, the so-called “Barker hypothesis,” suggests that stress in the fetal or neonatal period can cause long-term alterations in the incidence of adult disease. In our study, we found that treatment of neonatal rats with Dex increased the risk for development of pulmonary hypertension in adult rats. Hence alterations of pulmonary structure induced in this early period by Dex treatment had long-term effects and increased the risk and severity for adult pulmonary sequelae. In our study, we found that lung hypoplasia may increase the risk for pulmonary hypertension. In vivo and in vitro studies (23) and these clinical diseases suggest that lung hypoplasia may increase the risk for pulmonary hypertension.

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REFERENCES


