Sildenafil Stimulates and Dexamethasone Inhibits Pulmonary Vascular Development in Congenital Diaphragmatic Hernia Rat Lungs

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Key Words

Congenital diaphragmatic hernia · Pulmonary hypertension · Pulmonary vascular development · Sildenafil · Dexamethasone · Nitrofen

Abstract

Background: A predictor of neonatal mortality in infants with congenital diaphragmatic hernia (CDH) is disrupted pulmonary vascular development, clinically expressed as pulmonary hypertension. Objective: To determine if prenatal corticosteroids and phosphodiesterase-5 (PDE-5) inhibitors have a beneficial effect on pulmonary vascular development in CDH lungs. Methods: We induced CDH in fetal rats by giving nitrofen. We then exposed them to dexamethasone or to sildenafil. We separated them into three groups: (1) DEX, 4 pregnant rats received dexamethasone at days E16, E18 and E20; (2) SILD, 4 pregnant rats received sildenafil and L-arginine between E14 and E22, and (3) placebo. We then analyzed the lung of each fetus with CDH at E22. We examined the number of arterioles and arteries, and their percent of medial wall thickness (%MWT). Results: We obtained 30 CDH-positive fetuses. We analyzed 3,560 arterioles and 211 arteries. SILD showed a significant increase in the number of arterioles, but no significant increase in the number of arteries. No change was noted in the arteriolar %MWT. In contrast, DEX showed significant decreases in the number of arterioles and arteries and a significant increase in %MWT.

Conclusions: PDE-5 inhibitors may improve pulmonary arteriolar development in fetuses with CDH. In contrast, prenatal corticosteroids could have deleterious effects on arteriolar and arterial development in CDH lungs.

Introduction

Congenital diaphragmatic hernia (CDH) is the most common malformation in newborns causing severe respiratory distress with an incidence of approximately 1 in 2,500 live births. The mortality rate is still very high, between 40 and 80% depending on risk factors, despite new therapies in neonatal care \cite{1}. CDH remains a serious cause of neonatal mortality and long-term morbidity, mainly as a result of lung hypoplasia and pulmonary hypertension (PPHN), associated with abnormal pulmonary vascular development \cite{2, 3}. In 1971, Kitagawa et al. \cite{4} showed a decrease in lung arterial sprouting and an increase in arterial medial wall thickness (MWT). More recently, Roubliova et al. \cite{3} reported a failure of the normal decrease in arteriolar medial thickness in late gestation. Oue et al. \cite{5} showed that some signals related to angiogenesis, such as bFGF, TGF-β₁, and PDGF, are diminished in CDH lungs induced by nitrofen.
Because of the strong association between PPHN and pulmonary hypoplasia, nitric oxide (NO), endothelin-1 (ET-1), and its mediators have been analyzed. Studies have shown a decrease in eNOS and K+ channels [6]. At the same time, ET-1 and its ET-A receptor increases in animal models and patients with CDH [7]. Because ET-1 induces smooth muscle cell proliferation in the vessel wall, and NO, eNOS and cGMP play a role in vascular remodeling, changes in its expression could remodel lung vascular hyperplasia, and in this manner revert PPHN [2, 8].

Few antenatal interventions have demonstrated an impact on pulmonary vessels, with some contradictory results [9]. Prenatal steroids can increase some growth factors diminished with CDH, such as bFGF, TGF-β1 and PDGF [5, 10], and eNOS and K+ channels [11]. Therefore, prenatal steroids could remodel the pulmonary vasculature in CDH fetuses. NO works by increasing cGMP levels in the muscular vessel wall, which activates the K+ channels, decreasing calcium and producing vasodilatation. cGMP has a short half-life due to rapid degradation by phosphodiesterase-5 (PDE-5) in the lung. The combination of inhaled NO and PDE-5 inhibitors, such as sildenafil, has shown synergism in PPHN treatment in newborns with CDH [12]. Sildenafil is the most potent PDE-5 inhibitor and crosses the placenta easily [13]. Preliminary results showed that by increasing cGMP, sildenafil enhances pulmonary growth, promotes pulmonary angiogenesis, and decreases PPHN in a rat model of bronchopulmonary dysplasia [14]. The authors describe a synergism at increasing intracellular cGMP, which increases VEGF expression, a factor related to pulmonary angiogenesis [14, 15].

The recent study by Luong et al. [16] demonstrated that prenatally administered sildenafil in the nitrofen CDH model increased lung capillary density. However, the investigators did not separately analyze arterioles and arterial vessels in terms of number and MWT, both important markers of pulmonary hypertension [16]. As Luong et al. mention, the use of prenatal corticosteroids and its positive impact on lung vasculature in CDH has not been evaluated, thereby adding importance to our hypothesis regarding prenatal steroids [16]. Moreover, if a positive result is seen in the lung vasculature using separate prenatal administration of sildenafil and corticosteroids, a combined therapy could possibly be studied in the future.

We hypothesize that the signals responsible for inadequate vascular lung development in the CDH fetus constitute a susceptible point to be modified through agonists of this vascular development. Prenatal steroids and PDE-5 inhibitors enhance angiogenesis and vascular remodeling, increasing growth factors (FGF, VEGF) and intracellular signals (NO-cGMP).

**Methods**

**CDH Model in Rats Induced by Nitrofen**

The rat model of CDH was used in pregnant Sprague-Dawley dams. All procedures were in accordance with international standards for the use of laboratory animals [17]. The study was approved by the local institutional review board. CDH was induced in embryos by gavage of 45 mg/kg, twice a day of the herbicide nitrofen (Chem Service, West Chester, Pa., USA), dissolved in 2 ml of olive oil to rats at 9.5 days of pregnancy (term day 22) [18]. Control rats were administered 2 ml of pure olive oil.

**Prenatal Intervention Using Potential Agonists of Pulmonary Vascular Development in CDH**

Two potential agonists were used: (1) dexamethasone (Ora-dexon, 5 mg/ml; NV Organon, Oss, The Netherlands), a long-acting corticosteroid used in the prenatal period, and (2) sildenafil (Pfizer Inc., New York, N.Y., USA), a powerful inhibitor of PDE-5 [13]. Sildenafil was co-administered with L-arginine, a substrate of eNOS, to ensure optimal levels of cGMP in the fetal lung, since at early stages of pregnancy endogenous NOS inhibition can be found [19].

Three groups of dams in gestation, previously treated with nitrofen, were intervened: (1) dexamethasone group (DEX), 4 dams received intraperitoneal (IP) dexamethasone 0.25 mg/kg daily E16, E18 and E20 of gestation; (2) sildenafil group (SILD), 4 dams received sildenafil orally 45 mg/kg every 12 h and IP L-arginine 0.5 g/kg/day, both between E14 and E22 of gestation [20], and (3) placebo group, 4 pregnant rats received IP normal saline and/or sterile water orally.

**Post-Intervention Assessment**

At E22, pregnant dams were anesthetized and the fetuses were analyzed under a Nikon dissecting microscope to confirm the presence of CDH. Each newborn rat was weighed and visible malformations were evaluated. Lung and heart were removed. In addition, samples of brain, kidney and heart tissue were stored to evaluate possible secondary adverse effects.

**Immunohistochemistry**

Antibodies were used in order to detect constituent cells of the pulmonary vasculature: (1) monoclonal anti-PECAM-1 (platelet endothelial cell adhesion molecule-1; BD Pharmingen), a member of the immunoglobulin superfamily and major constituent of the intercellular junction of endothelial cells [21], and (2) monoclonal anti-α-SMA (α-smooth muscle actin; Sigma), a marker of smooth muscle cells [22].

**Morphological and Statistical Analysis**

Standard full lung coronal sections for each fetus were analyzed by light microscopy at a magnification of 20x and 400x in order to count all the arterial vessels visualized on a complete section and to make measurements of each arterial vessel individually. Precise

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lung area and vessel analysis were measured and analyzed by computer using Adobe Photoshop and SimplePCI C-Imaging Systems (Hamamatsu Co., Pa., USA). We did not perfuse the lung with constant pressure because our protocol was focused on lung arterial and arteriolar vasculature. Standardized methods of lung harvest, fixation, section and visualization were used in all experiments.

At E22 the pulmonary vascular development was compared by two independent observers. The number of major vessels and arterioles ≤70 μm, vessel diameter, percent of endothelium, and percent of MWT (%MWT) were analyzed in CDH+, CDH− coronal sections. %MWT, a way to assess pulmonary artery remodeling, was calculated as (2 × wall thickness/external diameter) × 100% [3, 14].

The statistical analysis comparing groups was performed through analysis of variance or Fisher’s protected least significant difference. Statistical analysis was performed using StatView (Abacus Concepts, Inc., Berkeley, Calif., USA). p ≤ 0.05 was considered statistically significant.

Results

Incidence of CDH in the Nitrofen Model and Lung Hypoplasia

We obtained a total of 30 left CDH-positive fetuses, 34% incidence of CDH from the nitrofen model: 12 in the DEX group, 9 in the SILD group, and 9 in the placebo group (fig. 1). Rats with nitrofen-induced CDH had a reduced body weight at term. The SILD group had the same body weight at term gestation as the placebo group, however, the DEX group had a lower body weight than the placebo and SILD groups (data not shown). The lung area was lower in nitrofen-induced CDH fetuses compared to that of the control group, demonstrating lung hypoplasia (data not shown).

Antenatal Sildenafil Increases Arteriole Number in CDH Lungs

We analyzed a total of 3,560 arterioles and 211 arteries. Compared to the placebo group, the SILD group showed a significant increase in the number of arterioles in both lungs (fig. 2, 3a), without change in %MWT (fig. 3b). The SILD group did not show an increase in the number of pulmonary arteries, however, there was an increase in the arterial %MWT compared to that of the placebo group (fig. 3c, d). Figure 3 shows a detailed quantitative comparison between left and right lungs. Figure 3a shows in the SILD-treated CDH group a significant and proportional increase in the number of arterioles in both left and right lungs compared to the placebo group. In contrast, the DEX-treated group shows a proportional decrease in the number of arterioles in both left and right lungs compared to the placebo group.

Antenatal Dexamethasone Decreases Arteriole and Arterial Number in CDH Lungs

Compared to the placebo and SILD groups, the DEX group showed significant decreases in the number of arterioles and arteries on right and left lungs (fig. 3a, c) and a significant increase in %MWT (fig. 3b, d). Figure 3b shows in the DEX-treated CDH group an increase in the
arteriolar %MWT, being more significant on the left lung than in the right lung compared to the placebo and SILD groups. Figure 3c shows in the DEX-treated CDH group a significant and equal decrease in arterial number in both the left and right lung compared to the placebo group, and figure 3d shows in the DEX group a significant and equal increase in the arterial %MWT in both left and right lungs compared to the placebo group. This significant vessel disruption in the DEX group was associated with diminished lung area (fig. 4).

**Number of Pulmonary Arterioles and Arteries Adjusted by Lung Area**

When we adjusted by lung area, the number of pulmonary arterioles was still significantly higher in the SILD group compared to the placebo group (fig. 4b). However, the significant decrease of arterioles in the DEX group disappears when we adjusted by lung area (fig. 4b). In terms of arteries, when we adjusted by area, the number of arteries in the DEX group was significantly lower than that of the placebo group, and no difference in the SILD group was visualized (fig. 4c).

**Discussion**

We have shown that PDE-5 inhibitors, such as sildenafil, can improve angiogenesis during pulmonary vascular development in CDH fetuses. In contrast, and contrary to original hypothesis, potent prenatal corticosteroids, such as dexamethasone, can have deleterious effects on vascular development in CDH lungs.

This study is consistent with the findings recently published by Luong et al. [16], where they showed that antenatal subcutaneous sildenafil improves pulmonary vessel density with no effect on arteriolar %MWT. However, we showed an increase in the arterial %MWT, the mechanism for which remains unclear. Probably by increasing...
cGMP, sildenafil enhances pulmonary angiogenesis via VEGF, finally decreasing PPHN [14].

Our protocol was designed to administer oral sildenafil because sildenafil has very good intestinal absorption, which could facilitate its prenatal clinical use in the future compared to intravenous or subcutaneous administration.

Sildenafil was co-administered with L-arginine, a NO precursor, to optimize levels of cGMP in the fetal lung, given that in the fetal circulation there is endogenous inhibition of NOS [19]. Without L-arginine, sildenafil may not maintain high levels of cGMP prenatally, due to inhibition of PDE-5. It is also possible that L-arginine may increase NO production with an effect by itself. It is more likely, however, that L-arginine in addition to sildenafil may only facilitate a positive effect on lung vasculature development.

Dexamethasone decreases not only the number of lung arterioles and arteries, but also increases %MWT in all the lung vessels, which could adversely affect PPHN at delivery. It is possible that the disruption of pulmonary vascular development associated with dexamethasone is the consequence of lower global lung development induced by potent steroids in this CDH model. On the other hand, vascular arrest could explain poor lung development based on the evidence that suggests vascular development is crucial for normal lung development [23].

This contrary observation with dexamethasone differs from the findings of previous authors who showed that prenatal steroids increase some growth factors that diminish with CDH (bFGF, TGF-β, and PDGF) [10], and enhance eNOS and K+ channels [11], all signals that would predict more developed pulmonary vasculature in the CDH fetuses.

The SILD group did not show an increase in the number of pulmonary arteries compared to the placebo group. In contrast, the DEX group showed a marked decrease in the number of pulmonary arteries and an increase in %MWT, which has been associated with increased PPHN and mortality [24]. The mechanisms of this disrupted vascular development are unclear, but a potent steroid like dexamethasone and the repeated doses of steroids could explain this finding. The inhibitory effect of corticosteroids on VEGF expression could explain the hypoplastic lung vasculature, as opposed to the positive effect of sildenafil on VEGF with increased angiogenesis in CDH lung arterioles, but not on arteries [25]. This supports the recommendation that the value of using antenatal steroids with newborns with CDH requires more evidence from clinical trials [26].

Fetal CDH is the most common prenatal diagnosis of severe PPHN at delivery. Sildenafil opens the possibility of treating CDH early in the gestation of this devastating disease and thus decreasing postnatal mortality. It is also promising that a low-cost prenatal therapy could be used postnataally over an extended period [27].

Our animal study using prenatal sildenafil has several limitations for clinical practice. It will be very important to study short- and long-term adverse effects, dosages, and timing on the fetus and pregnant women before starting sildenafil clinically, including pulmonary, cardiac, vascular, visual, and neurologic evaluations [28]. Recently, Luong et al. [16] showed that antenatal sildenafil had no adverse effects on retinal and brain development.

Fig. 4. Number of pulmonary arterioles and arteries adjusted by lung area in rat CDH induced by nitrofen. a Lung area in placebo, DEX and SILD groups (CDH+ n = 30, CDH− n = 31): * DEX vs. placebo CDH+ p < 0.0001; ** DEX vs. SILD p < 0.0001; SILD vs. placebo CDH+ p > 0.05. b Number of pulmonary arterioles adjusted by lung area (n = 30 CDH+): * SILD vs. placebo p < 0.04; DEX vs. placebo p > 0.05. c Number of pulmonary arteries adjusted by lung area (n = 30 CDH+): * DEX vs. placebo p < 0.02; SILD vs. placebo p > 0.05.
A major limitation of our study was that it did not include a control group of animals without nitrofen. Due to the huge number of vessels counted and measured (3,771), we chose to limit our study to a placebo control group. Also, another limitation of the model was that it did not include a control group with only L-arginine to differentiate the effect of sildenafil and that of L-arginine.

In conclusion, PDE-5 inhibitors may improve angiogenesis during pulmonary vascular development in fetuses with CDH. Potent prenatal corticosteroids could have deleterious effects on vascular development in CDH lungs. Further studies are necessary to determine the molecular signals involved and possible adverse effects before starting clinical studies.

We speculate that a better comprehension of the vascular development in pulmonary hypoplasia and the signals involved open the possibility to study the combination of other antenatal therapies with PDE-5 inhibitors, such as vitamin A, growth factors, endothelin inhibitors, and prenatal interventions such as tracheal occlusion, thus trying to reverse the arrest in lung development in fetuses which often ends in high mortality and morbidity rates.

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Disclosure Statement

The authors have no conflicts of interest to disclose.