Antenatal Sildenafil Treatment Attenuates Pulmonary Hypertension in Experimental Congenital Diaphragmatic Hernia

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Background—Lung hypoplasia and persistent pulmonary hypertension of the newborn limit survival in congenital diaphragmatic hernia (CDH). Unlike other diseases resulting in persistent pulmonary hypertension of the newborn, infants with CDH are refractory to inhaled nitric oxide (NO). Nitric oxide mediates pulmonary vasodilatation at birth in part via cyclic GMP production. Phosphodiesterase type 5 (PDE5) limits the effects of NO by inactivation of cyclic GMP. Because of the limited success in postnatal management of CDH, we hypothesized that antenatal PDE5 inhibition would attenuate pulmonary artery remodeling in experimental nitrofen-induced CDH.

Methods and Results—Nitrofen administered at embryonic day 9.5 to pregnant rats resulted in a 60% incidence of CDH in the offspring and recapitulated features seen in human CDH, including structural abnormalities (lung hypoplasia, decreased pulmonary vascular density, pulmonary artery remodeling, right ventricular hypertrophy), and functional abnormalities (decreased pulmonary artery relaxation in response to the NO donor 2-(N,N-diethylamino)-diazenolate-2-oxide). Antenatal sildenafil administered to the pregnant rat from embryonic day 11.5 to embryonic day 20.5 crossed the placenta, increased fetal lung cyclic GMP and decreased active PDE5 expression. Antenatal sildenafil improved lung structure, increased pulmonary vessel density, reduced right ventricular hypertrophy, and improved postnatal NO donor 2-(N,N-diethylamino)-diazenolate-2-oxide–induced pulmonary artery relaxation. This was associated with increased lung endothelial NO synthase and vascular endothelial growth factor protein expression. Antenatal sildenafil had no adverse effect on retinal structure/function and brain development.

Conclusions—Antenatal sildenafil improves pathological features of persistent pulmonary hypertension of the newborn in experimental CDH and does not alter the development of other PDE5-expressing organs. Given the high mortality/morbidity of CDH, the potential benefit of prenatal PDE5 inhibition in improving the outcome for infants with CDH warrants further studies.

Key Words: pulmonary circulation ■ pulmonary disease ■ angiogenesis ■ congenital diaphragmatic hernia
(NO).\textsuperscript{13,14} Even among survivors, the prognosis is guarded and chronic pulmonary hypertension beyond the neonatal period is increasingly recognized in this patient population.\textsuperscript{15,16} Consequently, the ultimate therapeutic goal to improve survival of infants affected with this devastating malformation is to promote lung growth before birth and to develop more efficient strategies to treat PPHN.\textsuperscript{17}

The mechanisms underlying refractory PPHN in CDH remain unknown, but may relate to some combination of (1) altered vasoreactivity (lack of vasodilatation/increased vaso-constriction), (2) vascular remodeling (smooth muscle cell [SMC] proliferation), and (3) a hypoplastic pulmonary vascular bed. Although there are 11 cyclic GMP (cGMP)–specific phosphodiesterase (PDE) gene families expressed in mammalian SMCs, PDE type 5 (PDE5) is the most active cGMP-hydrolyzing PDE active under basal low-calcium conditions.\textsuperscript{18} PDE5 inhibition is used therapeutically in erectile dysfunction, and although it is expressed in all visceral and vascular SMCs, it has only a modest effect on systemic vasoreactivity.\textsuperscript{19} These properties have been harnessed for the treatment of pulmonary hypertension in adult patients.\textsuperscript{20,21} Consequently, we hypothesized that antenatal PDE5 inhibition with sildenafil would attenuate pulmonary vascular abnormalities in experimental CDH.

Methods

All procedures and protocols were approved by the animal care and use committee at the University of Alberta, Edmonton, Alberta, Canada.

Animal Model

Pregnant Sprague-Dawley rats were gavaged fed 100 mg of the herbicide nitrofen dissolved in 1 mL olive oil vehicle at embryonic day (E) 9.5, as previously described.\textsuperscript{22} Control animals received olive oil only. Nitrofen is not toxic to adult rodents, but induces CDH\textsuperscript{22} and vascular SMCs, it has only a modest effect on systemic vasoreactivity and although it is expressed in all visceral and vascular SMCs, it has only a modest effect on systemic blood pressure. In addition, the PDE5 inhibitor sildenafil dilates the pulmonary vasculature and has antiproliferative effects on human pulmonary artery (PA) SMC.\textsuperscript{19} These properties have been harnessed for the treatment of pulmonary hypertension in adult patients.\textsuperscript{20,21} Consequently, we hypothesized that antenatal PDE5 inhibition with sildenafil would attenuate pulmonary vascular abnormalities in experimental CDH.

Plasma Sildenafil Concentration

Maternal and fetal (E21.5) blood samples were collected at 1 hour, 2 hours, 6 hours, 12 hours, and 24 hours post injection. Plasma was isolated from these samples by centrifugation at 10 000 RPM for 10 minutes. Fetal plasma samples were pooled (at 1 hour [n=10], 2 hours [n=12], 6 hours [n=3], 12 hours [n=5], and 24 hours [n=10]), and sildenafil concentrations were determined by high-performance liquid chromatography with mass spectrometric detection.

Plasma samples were analyzed for sildenafil and its N-desmethyl metabolite by a modification of previously described methodology.\textsuperscript{23} Briefly, plasma samples (50 \textmu L) were mixed with internal standard (structurally related analog of sildenafil) and extracted by solid-phase extraction. Extracts were separated by high-performance liquid chromatography using a 5 \textmu m C18 column (50×4.6 mm, Hypersil-100, Thermo-Hypersil, Runcorn, UK) and quantified using triple quadrupole mass spectrometric detection in positive-ion magnetic resonance microscopy mode (API 4000, MDS, Sciex, Concord, Ontario, Canada). The quantitation range was 2 to 1000 ng/mL for both analytes.

Lung cGMP Levels

Lungs (E21.5) from control, nitrofen-CDH, nitrofen-CDH+sildenafil, and sildenafil control groups were fixed for histology by tracheal instillation of 10% buffered formalin under 20 cm H2O constant pressure. After ligation of the trachea, the lungs were immersed in fixative overnight. Lungs were processed and embedded in paraffin. Serial sections were taken throughout the medial right lung lobe and stained with hematoxylin and eosin (H&E). The stained sections were analyzed with OpenLab Imaging System (Quorum Technologies Inc., Guelph, Ontario, Canada). Alveolar structure was quantified by the mean linear intercept as previously described.\textsuperscript{23}

Western Blot Analysis

Snap frozen lungs were homogenized on ice in homogenization buffer (50 mmol/L Tris HCl, 150 mmol/L NaCl) containing protease inhibitor cocktail I and II (Sigma). Samples were sonicated and centrifuged at 10 000 g for 20 minutes at 4°C. Protein content in the supernatant was determined by the Bradford method using BSA as the standard. Thirty micrograms of protein sample per lane were subjected to SDS-PAGE, and proteins from the gel were transferred to nitrocellulose membranes by electroblotting. Immunodetection was performed with a mouse anti–endothelial nitric oxide synthase (eNOS) polyclonal antibody (610296, BD Sciences, Mississauga, Ontario, Canada) diluted 1:1000, a rabbit polyclonal to vascular endothelial growth factor (VEGF) antibody (ab46154; Abcam, Cambridge, MA) diluted 1:1000, and a phospho-specific PDE5A-antibody (PPD5–140AP, FabGennix Inc, Frisco, TX) diluted 1:500, and a phospho-specific PDE5A-antibody (PD5A–112AP, BD Sciences, Mississauga, Ontario, Canada) diluted 1:1000, a rabbit polyclonal to vascular endothelial growth factor (VEGF) antibody (ab46154; Abcam, Cambridge, MA) diluted 1:1000, cGMP-dependent PDE Type 5A-antibody (PD5A–112AP, FabGennix Inc, Frisco, TX) diluted 1:500 overnight at 4°C. After the blots were washed to remove unbound antibody, secondary antibodies, antimouse horseradish peroxidase antibody (1:1000, Santa Cruz Biotechnology) for eNOS detection, and an antirabbit horseradish peroxidase (1:3000, Santa Cruz Biotechnology) for VEGF, PD5A and phospho-specific PDE5A detection were applied for 2 hours at room temperature. After being washed, bands were visualized by enhanced chemiluminescence using ECL Plus detection (Amersham, Baie d’Urfe, Quebec, Canada). In addition, each gel was stripped and reprobed with actin as a housekeeping protein to normalize for protein loading.

Barium-Gelatin Arteriograms and Arterial Density Counts

The main PA of E21.5 rats was cannulated and injected with warmed barium gelatin. The lungs were then fixated by tracheal instillation of formalin at a constant pressure of 20 cm H2O, removed from the body cavity and submerged in formalin for at least 2 days. To assess gross vascular morphology, fixed lungs were imaged using a computed tomography imaging system (Gamma Medica). For quantitative assessment, lungs were paraffin embedded, sectioned and H&E stained. The number of barium-filled vessels was counted in 30 high-power fields (400×) per lung, in 5 animals per group.\textsuperscript{24} Fields containing large airways or major PAs were avoided to maintain consistency of counts between sections.
Medial Wall Thickness
To assess PA remodeling, the percentage medial wall thickness (MWT), a surrogate marker of pulmonary hypertension, was calculated as \( \frac{2 \times \text{wall thickness}}{\text{external diameter}} \times 100\% \).26 Medial wall thickness measurements were performed on small PAs (30 to 100 \( \mu \)m) on H&E-stained lung sections using OpenLab.

Right Ventricular Hypertrophy
The right ventricle (RV) and left ventricle (LV) plus septum (S) were weighed separately to determine the ratio (RV/LV + S) as an index of right ventricular hypertrophy (RVH).26

Organ Bath Studies of Newborn Rat Pulmonary Arteries
Intrapulmonary arteries (diameter <100 \( \mu \)m, length=2 mm) were mounted in a wire myograph (Myodaq; Danish Myo Technology, Denmark) and bathed in a Krebs-Henseleit buffer bubbled with 21% \( \text{O}_2 \)/5% \( \text{CO}_2 \)/balance \( \text{N}_2 \).27 Pulmonary arteries were preconstricted with the thromboxane A2 analog U-46619 (10\(^{-7}\)M), and isometric changes in response to NO donor 2-(\( \text{N},\text{N}-\text{diethylamino})\)-diazenolate-2-oxide (DEANO) (10\(^{-7}\)–10\(^{-5}\)M) were compared between PAs from the 4 age-matched groups. Pulmonary artery relaxation was assessed as a percentage of maximal constriction from U-46619. All drugs were purchased from Sigma-Aldrich (Saint Louis, MO).

Retina Studies
The functional integrity of the retina was assessed using a standard clinical test, the electroretinogram (ERG), which is a noninvasive measure of the electric changes of the retina in response to light flashes. The ERG was recorded from both eyes of 30-day old rats that had either received antenatal sildenafil (n=6 rats) or saline injections (n=4 rats). At this age, the retina’s functional maturity is reached.28 In brief, ERG responses (to increasing flash intensities) were recorded in xylazine-ketamine anesthetized rats, firstly, under dark adaptation (scotopic ERG). Two ERG waves were quantified, the a-wave (reflecting photoreceptor activity) and the b-wave (reflecting activation of the retina circuit downstream to photoreceptors). Electroretinogram recordings were then repeated under light adaptation (photopic ERG, under 30 cd/m\(^2\) luminance background). Finally, photopic ERG responses to flickering flashes (1.37 log cd/m\(^2\) luminance), presented at increasing frequencies, were recorded. Criterion amplitudes were set at 20 \( \mu \)V for a- and b-waves and at 5 \( \mu \)V for flicker amplitudes. The anatomic integrity of the retina was assessed using Nissl staining on retinal cross sections (to examine the retina’s laminar organization; n=4 control and n=4 sildenafil control), as well as with fluorescein angiography on retinal flatmounts (to examine the inner retinal vasculature; n=4 control and n=4 sildenafil). For Nissl staining, retina cryosections were stained in 0.1% cresyl violet solution, dehydrated, cleared in xylene, and mounted on glass slides.
For fluorescein angiography, rats were transcardially perfused with fluorescein isothiocyanate dextran 500 000-conjugate (Sigma). The eyes were removed and postfixed, and then retina flat mounts were dissected to be postfixed. Tissues were mounted on slides, visualized with a Zeiss LSM 510 confocal microscope, and processed with Photoshop 6.0 software (Adobe, San Jose, CA) to adjust contrast levels if required. Two variables were assessed using ImageJ (National Institutes of Health; http://rsbweb.nih.gov/ij/index.html): (1) branching points and (2) density of labeled blood vessels. Four windows were analyzed for the right eye of each animal; these corresponded to the midperiphery of the retina in the 4 respective quadrants (nasal, temporal, dorsal, and ventral).

Brain Studies
Brains collected at E21.5 were fixed in formalin and paraffin. Antibodies used for immunostaining were directed against calbindin protein (a marker of a subpopulation of GABAergic interneurons) (1/2000, rabbit polyclonal; SWant, Bellinzona, Switzerland), calretinin (a marker of a subpopulation of GABAergic interneurons) (1/2000, rabbit polyclonal; SWant), glial fibrillary acidic protein (GFAP, a marker of astrocytes) (1/500, rabbit polyclonal; Dako, Glostrup, Denmark), and tomatolectin (a marker of microglia-macrophages) (1/500, biotinylated lectin; Vector, Burlingame, CA). These antibodies were detected using an avidin-biotin-horseradish peroxidase kit (Vector), as instructed by the manufacturer. Immunolabeled cells were quantified at the level of the parietal cortex. Two fields were analyzed in each experimental group for each animal (5/group) and for each marker.

In a separate series of animals (n=4 to 6) fresh flash-frozen brains from postnatal day (P) 30.5 rats were mounted in freezing medium and the brains sectioned from the anterior pole to 5 mm posterior to bregma. Adjacent sections 20 μm thick were slide mounted and used to assess cytoarchitecture, cytogenesis, and dying cells. Antibodies recognizing the neuronal cell marker NeuN (1/1000, mouse monoclonal), the astrocyte marker glial fibrillary acidic protein (GFAP) (1/700, mouse monoclonal) and 5-bromo-2-deoxyuridine (BrdU), a marker of replicating/dividing cells (1/600, mouse monoclonal) were immunoreacted with the sections, then incubated with the species-relevant secondary immunoglobulin G antibody, amplified with avidin-biotin complex and visualized with 3,3-diaminobenzidine. Semiquantitative field count score was used to count BrdU-positive cells. To identify the presence of degenerating cells, the sections were incubated with Fluoro-jade-B according to standard protocols. Scores from abutting fields (3 to 6 fields depending on the size of the brain region) were obtained from the hippocampus, cortex, subventricular zone, basal ganglia (data not shown), and thalamus (data not shown).

Statistical Analysis
Statistical analysis was performed using software (SPSS for Windows, version 13; SPSS, Chicago, IL). Values were expressed as...
mean ± SEM. Comparisons of parameters between 2 groups were made by unpaired Student t test. One-way ANOVA determined significance of litter and rat where relevant. If litter was not significant, then comparisons of parameters were made by 1-way ANOVA and posthoc analysis with Student-Newman-Keuls (SNK) test. If a litter effect was significant, then statistical analysis was completed with a linear mixed-effects model to account for within-litter correlations. Repeated-measures ANOVA was used to assess differences in PA relaxation studies. Statistical significance was defined as \( P < 0.05 \).

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Maternal-Administered Sildenafil Crosses the Placenta and Inhibits PDE5 Activity in Fetal Rat Lungs

Maternal and fetal plasma concentrations of sildenafil were measured by high-performance liquid chromatography from samples obtained at 1, 2, 6, 12, and 24 hours postinjection (Figure 1A). Maternal and fetal plasma concentration profiles followed a similar trend, peaking at \( \approx 6 \) hours after maternal administration, indicating that sildenafil effectively crossed the placenta and entered the fetal circulation.

Biological activity of sildenafil in fetal lung was confirmed by measuring cGMP concentrations (Figure 1B), as well as PDE5A expression (Figure 1C) in fetal rat lungs. Nitrofen exposure at E9.5 was associated with a significant decrease in lung cGMP concentration and increased activated PDE5A expression. Sildenafil treatment from E11.5 to E20.5 produced a marked increase in lung cGMP in nitrofen-CDH fetuses and in controls and a significant attenuation in active PDE5A expression, indicating that sildenafil was biologically active in the fetal lung.

Effects of Antenatal Sildenafil on Body Weight, Incidence of CDH, and Lung Hypoplasia

Rats with nitrofen-induced CDH had significantly reduced body weights at E21.5 (Figure 2A). Antenatal sildenafil had no effect in pups with nitrofen-induced CDH. Sildenafil had no effect on the incidence of CDH (Figure 2B).

The lung weight–to–body weight ratio (LW/BW), a crude indicator of lung hypoplasia was lower in nitrofen-CDH fetuses compared to controls (Figure 2C). Antenatal sildenafil decreased LW/BW in normal fetuses. Sildenafil had no effect on LW/BW in the nitrofen-CDH sildenafil rats.

Antenatal Sildenafil Improves Lung Architecture in Congenital Diaphragmatic Hernia Rats

The mean linear intercept was significantly higher in animals with nitrofen-induced CDH compared with controls (Figure 3A and 3B). Fetal rats in the nitrofen-CDH sildenafil group had mean linear intercept values that were similar to those of controls (Figure 3B). Sildenafil had no effect on lung architecture in control pups.

Antenatal Sildenafil Increases Pulmonary Vessel Density in Congenital Diaphragmatic Hernia

Nitrofen-CDH animals had significantly fewer pulmonary vessels compared with controls (Figure 4A and 4B). Sildenafil significantly increased pulmonary vessel count in nitrofen-CDH animals, but decreased pulmonary vessel density in control animals. This was associated with increased lung eNOS and VEGF protein expression (Figure 4C).

Antenatal Sildenafil Attenuates Features of Pulmonary Hypertension in Congenital Diaphragmatic Hernia

Even though MWT was increased in rats with nitrofen-induced CDH compared with controls and antenatal sildenafil decreased MWT in animals with nitrofen-induced CDH (Figure 5A), using the appropriate linear mixed model taking both litter and rat effect into account these differences were not statistically significant.

RVH was significantly greater in nitrofen-CDH animals compared with control (Figure 5B). Antenatal sildenafil treatment attenuated RVH in animals with nitrofen-
induced CDH. Sildenafil had no effect on RVH in control rats. RV weight was significantly reduced in control sildenafil-treated animals. LV weight was significantly reduced in both nitrofen-CDH and nitrofen-CDH/sildenafil–treated animals.

Antenatal Sildenafil Enhances PA Responsiveness to the Nitric Oxide Donor DEANO
The contractile response of PAs in response to U46619 was similar between groups (Figure 5C). Pulmonary arteries from fetal rats with nitrofen-induced CDH relaxed less to DEANO compared with controls, paralleling the attenuated response to inhaled NO seen in infants with CDH (Figure 5D). In contrast, PAs from nitrofen-induced CDH animals treated with sildenafil showed an enhanced response to the DEANO. Relaxation of these vessels at the highest DEANO concentration approached preconstriction levels.

Retina Studies
Antenatal sildenafil had no side effects on retinal function (as assessed by ERG, Figure 6A) or anatomy (Nissl staining, Figure 6B, and fluorescein angiography, Figure 6C). All ERG components, including a- and b-waves as well as oscillatory potentials (not illustrated) had similar amplitudes and implicit times (not illustrated) between the 2 groups studied (control versus sildenafil, Figure 6A). Nissl staining shows that all layers were identical between both groups (Figure 6B). Likewise, retinas from both control and sildenafil-exposed rats had similar blood vessel patterns as observed on flat-mounts (Figure 6C). There was no difference between control and treated groups in arterial density (0.17±0.02. versus

Figure 4. Antenatal sildenafil improves lung capillary density and lung expression of eNOS and VEGF. A, Representative computed tomography scan of barium-injected pulmonary vessels. B, Representative H&E-stained sections of barium-injected pulmonary vessels. Quantification demonstrates decreased density of pulmonary vessels in nitrofen-CDH rats. Antenatal sildenafil restores vessel density (linear mixed model ANOVA P<0.0001; P values: *control versus CDH 0.004, control versus CDH+sildenafil 1.0, †control versus sildenafil control 0.02, **CDH versus CDH+sildenafil 0.002, CDH versus sildenafil control 0.007). C, Western blots for eNOS and VEGF expression in E21.5 fetal rat lungs. D, Mean data showing that eNOS and VEGF expression is decreased in nitrofen-CDH animals and is increased with antenatal sildenafil treatment (eNOS ANOVA P=0.019; SNK P values: *control versus CDH <0.05, control versus CDH+sildenafil >0.05, control versus sildenafil control >0.05, *CDH versus CDH+sildenafil <0.05, *CDH versus sildenafil control <0.05, and CDH+sildenafil versus sildenafil control >0.05; VEGF ANOVA P<0.0001; SNK P values: *control versus CDH <0.05, †control versus CDH+sildenafil <0.05, control versus sildenafil control >0.05, *CDH versus CDH+sildenafil <0.05, *CDH versus sildenafil control <0.05, and CDH+sildenafil versus sildenafil control >0.05). CDH indicates congenital diaphragmatic hernia; HPF, high-powered fields; Ctrl, control; VEGF, vascular endothelial growth factor; and eNOS, endothelial nitric oxide synthase.
Sildenafil Does Not Affect Brain Maturation

Parietal neocortexes of E21.5 pups were collected. Antenatal sildenafil had no detectable effect on the density of GFAP-positive astrocytes, tomatolectin-positive microglia-macrophages, and calretinin-positive and CaBP-positive interneurons (data not shown). Likewise, at P30 no differences were found in brain GFAP (Figure 7A), NeuN (Figure 7B), and BrDU (Figure 7C) immunoreactivity between groups. Fluoro-jade staining for dying/degenerating cells was negative in both groups (Figure 7D).

Figure 5. Antenatal sildenafil treatment attenuates features of pulmonary hypertension in the nitrofen-induced rat model of CDH. A, Medial wall thickness ratio. After using the appropriate linear mixed model taking both litter and rat effect into account, there were no significant differences between CDH and CDH+sildenafil-treated rats. B, Right ventricular hypertrophy in nitrofen-CDH rats was significantly reduced by sildenafil. (RV/LV+S linear mixed model ANOVA P = 0.005; P values: *control versus CDH 0.006, control versus CDH+sildenafil 1.0, control versus sildenafil control 1.0, **CDH versus CDH+sildenafil 0.03, CDH versus sildenafil control 0.055, and CDH+sildenafil versus sildenafil control 1.0; RV weights ANOVA P < 0.02; P values: control versus CDH 0.30, *control versus CDH+sildenafil 0.04, control versus sildenafil control 0.27, CDH versus CDH+sildenafil 0.35, *CDH versus sildenafil control 0.04, and **CDH+sildenafil versus sildenafil control 0.003; LV+S weights ANOVA P < 0.0001; P values: *control versus CDH < 0.0001, *control versus CDH+sildenafil < 0.0001, control versus sildenafil control 0.40, CDH versus sildenafil control 0.23, *CDH versus sildenafil control < 0.0001, and *CDH+sildenafil versus sildenafil control < 0.0001). C, The contractile response of PAs to U46619 was similar between groups (P values: control versus CDH 0.27, control versus CDH+sildenafil 0.39, control versus sildenafil control 0.67, CDH versus CDH+sildenafil 0.97, CDH versus sildenafil control 0.59, and CDH+sildenafil versus sildenafil control 0.67). D, Pulmonary arteries of nitrofen-CDH rats relaxed significantly less than controls in response to the NO donor DEANO. Antenatal treatment with sildenafil significantly enhanced relaxation of Nitrofen-CDH PAs (P values: *control versus CDH < 0.05 and **CDH+sildenafil versus CDH < 0.01). PA indicates pulmonary artery; MWT, medial wall thickness; CDH, congenital diaphragmatic hernia; RV, right ventricle; LV, left ventricle; S, septum; and DEANO, nitric oxide donor 2-((N (N-diethylamino)-diazenolate-2-oxide).

Discussion

We show that maternal sildenafil treatment enhances pulmonary vessel density, reverses RVH, and improves the pulmonary vasodilatory response to NO in the nitrofen-induced CDH rat model. This is achieved without significant effect on retinal structure and function (P30), and brain development assessed by histology (E21.5 and P30). Our data suggest the opportunity of further exploring the therapeutic potential of sildenafil as a prenatal medical therapy for CDH.

Congenital diaphragmatic hernia remains one of the greatest challenges in perinatal medicine.29 Many infants with CDH respond poorly to inhaled NO.13,14 Survivors with severe PPHN and hypoplastic lungs suffer significant mor-
bidity and show a new emerging pattern of severe late and chronic pulmonary hypertension. In humans, CDH can be accurately diagnosed at 22 weeks gestation during routine ultrasound examination and is thus amenable to antenatal therapies. Over the past decades, a number of surgical strategies have attempted to improve lung growth before birth. The idea of the herein-proposed antenatal use of sildenafil for regression of PPHN parallels the use of antenatal glucocorticoid treatment for women with threatened preterm labor to mature the fetal lung and prevent postnatal complications in premature infants. By analogy, we reasoned that antenatal sildenafil, an approved and safe medication in adults, could have similar beneficial effects on lung vascular development in CDH.

Experimental nitrofen-induced CDH in rats is a well established and reliable model that recapitulates the pulmonary abnormalities described in human CDH, including lung hypoplasia and pulmonary vascular remodeling. The mechanism by which nitrofen induces the diaphragmatic defect and lung hypoplasia is not fully understood, and the herbicide also affects overall fetal growth, suggesting potential systemic effects. Studies of nitrofen metabolism in pregnant rats suggest that its teratogenicity is not mediated via generation of mutagenic intermediates through nitro-reduction of the parent compound. Rather, the embryo is exposed to the parent compound alone, and appears to be a deep compartment for accumulation of nitrofen. This is further corroborated by lung explant studies that show that removing the lung leads to spontaneous recovery unless repeatedly treated with nitrofen.

Recent evidence suggest that interactions between airways and blood vessels are critical for normal lung development. In 1959, Liebow observed that the alveolar septa in centrilobular emphysema were remarkably thin, and almost avascular. He postulated that a reduction in the blood supply of the small precapillary blood vessels might induce the disappearance of alveolar septa. Pharmacological VEGF inhibition in neonatal and adult rats leads to arrested alveolar development and loss of alveoli, respectively. These data suggest that inhibition of vascular growth itself may directly impair postnatal lung development. We and others showed that VEGF-driven angiogenesis reverses postnatal hypoxia-induced alveolar hypoplasia. Consequently, enhancing vascular growth before birth may be a therapeutic strategy to promote lung growth in CDH. We previously showed that sildenafil promotes lung angiogenesis in vitro and in the developing lung postnatally.

There have been several studies from various laboratories using the nitrofen-induced CDH model, including several that...
have shown decreased lung eNOS and VEGF expression in CDH versus control lungs. More interestingly, NO and VEGF promote airway branching in normal and CDH lung explants, and in vivo, inhaled NO prolongs survival in CDH rat pups and this effect could be enhanced with prenatal glucocorticoids. Here, we investigated whether administration of antenatal sildenafil could have a beneficial effect on the hypoplastic nitrofen lung in vivo. Consistent with previous studies in this model, we show that antenatal sildenafil from E11.5 to E20.5 improved lung maturation and increased vessel density in rats with nitrofen-induced CDH. Accordingly, decreased lung eNOS and VEGF protein expression in nitrofen-induced CDH was restored by antenatal sildenafil. Hara et al had previously shown that antenatal tracheal occlusion (another therapeutic strategy to promote lung growth in CDH) increases VEGF-A protein expression and suggested that VEGF-A mediates previously described changes in lung vascular and parenchymal development caused by tracheal occlusion. Similar data were reported by Cloutier et al in mice. The mechanism by which sildenafil increases lung eNOS and VEGF expression remains speculative. It is suggested that sildenafil activates \( K_{\text{ATP}} \) channels and induces nitrate-like effects, which induces the production of interstitial adenosine. Adenosine is thought to increase VEGF protein and messenger RNA expression by adenosine receptors. Sildenafil also activates phosphorylation and activation of eNOS expression, and this might contribute to the upregulation of VEGF expression. Finally, sildenafil may increase VEGF expression through induction of thioredoxin-1 and hemeoxygenase-1, both known to upregulate VEGF expression.

Intriguingly, normal rats that received antenatal sildenafil had decreased lung-vessel density at term compared with controls. The mechanisms underlying this finding are unclear, and require further investigation. The few teratogenicity studies have suggested that sildenafil is safe during pregnancy in a variety of species.

In addition to lung hypoplasia, PH is a limiting factor of survival in CDH. Pulmonary hypertension in CDH is characterized by PA remodeling with excessive muscularization of preacinar arteries, reduced external diameter of pre- and intra-alveolar arteries with increased MWT, and poor response to inhaled NO. In addition to acting as a vasodilator, sildenafil also functions as a potent inhibitor...
of adult human PASMC proliferation as well as an inducer of apoptosis. Even though antenatal sildenafil attenuated pulmonary smooth media remodeling as assessed by MWT, the difference was not statistically different in the nitrofen model.

Another interesting finding was that the sildenafil-induced reduction in the RV/LV+S ratio was mostly due to a combined improvement in RV and LV+S. Indeed, nitrofen-induced CDH rats had significant LV hypoplasia, similar to what has been described in the sheep model and in humans with CDH. Another important finding of our study was the enhanced pulmonary vasorelaxation in response to DEA NO, suggesting that priming of the pulmonary vasculature before birth may enhance the response to postnatal therapies. Given that sildenafil was administered <24 hours before delivery, a certain degree of PDE5 inhibition may still be present in the pulmonary vascular tissue so that enhanced vasorelaxation can probably not solely be attributed to attenuation of PA remodeling. This hypothesis is further supported by the observation that sildenafil significantly reduces pulmonary vascular resistance in normal and ductus arteriosus–ligated fetal sheep in response to birth-related stimuli such as oxygen and shear stress.

The sildenafil dose chosen for the pregnant rats was 100 mg · kg⁻¹ · d⁻¹ on the basis of previous studies that examined the pharmacokinetics of sildenafil in rodents. In long-term in vivo studies in rats, this dose yielded mean free plasma concentrations comparable to levels obtained in humans at doses of 1 mg · kg⁻¹ · d⁻¹. This difference reflects the near 100-fold higher rate of metabolism of sildenafil in rats. At this dose, there were no adverse visual and neurological effects seen in the offspring. We did not investigate changes that may have occurred in pregnant dams that were treated with sildenafil. This is important to note, because there have been a small number of cases that have suggested a possible relationship between central serous chorioretinopathy and optic neuropathy with sildenafil use in humans. Furthermore, although there is no proven association between sildenafil use and central serous chorioretinopathy or optic neuropathy, it is prudent to consider cessation of sildenafil therapy in a patient that experiences sudden loss of vision. Further clinical challenges include the choice of the timing (eg, introducing maternal sildenafil treatment early enough during gestation to positively affect the outcome), dosing, and length of treatment. Other limitations of this study include the lack of survival data and, inherent to the small animal model, the lack of physiological data. Experiments in the fetal sheep model are underway to answer these questions. Finally, in this study, data from littermates were treated as independent observations. This is a common assumption in studies using the nitrofen model, because nitrofen does not affect all the offspring in a comparable manner relative to presence and severity of the diaphragmatic defect, degree of lung hypoplasia, or growth restriction, even within the same litter.

In conclusion, antenatal treatment with sildenafil improves lung structure, increases vessel density, decreases RVH, and enhances dilatation to NO in experimental, nitrofen-induced CDH in rats. We speculate that the sildenafil-induced pulmonary arterial tissue PDE5 inhibition resulted in enhanced DEA NO-dependent relaxation in the fetuses. Antenatal strategies may improve responsiveness to postnatal pulmonary vasodilator therapies, and ultimately the outcome of infants with CDH. The relative pulmonary vascular specificity of sildenafil, its low cost, and its postmarketing safety record makes it an attractive therapeutic option for infants with CDH.

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Disclosures
Dr Walker is an employee of and has stock options in Pfizer. The other authors report no conflicts.

References


CONGENITAL DIAPHRAGMATIC HERNIA REMAINS THE MOST LIFE-THREATENING CAUSE OF RESPIRATORY FAILURE IN NEWBORNS.

Lung hypoplasia and pulmonary hypertension refractory to inhaled nitric oxide are the main factors limiting survival. Currently, there is no specific treatment for CDH. Here, we show in a herbicide-induced CDH rat model that maternal treatment with sildenafil, a strategy reminiscent of antenatal steroid treatment given to women in preterm labor to mature the fetal lung, effectively crosses the placenta, increases fetal lung cyclic GMP, promotes lung growth, attenuates features of pulmonary hypertension and increases pulmonary artery relaxation in response to a nitric oxide donor in vitro. Our findings open new therapeutic avenues for pharmacological antenatal strategies to improve the outcome of infants with CDH. Given the high mortality/morbidity of CDH, the relative pulmonary vascular specificity of sildenafil, its low cost, and its postmarketing safety record make it an attractive therapeutic option that warrants further studies in infants with CDH.
Antenatal Sildenafil Treatment Attenuates Pulmonary Hypertension in Experimental Congenital Diaphragmatic Hernia

Christina Luong, Juliana Rey-Perra, Arul Vadivel, Greg Gilmour, Yves Sauve, Debby Koonen, Don Walker, Kathryn G. Todd, Pierre Gressens, Zamaneh Kassiri, Khurram Nadeemi, Beverly Morgan, Farah Eaton, Jason R. Dyck, Stephen L. Archer and Bernard Thébaud

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