Prevention of pulmonary hypoplasia and pulmonary vascular remodeling by antenatal simvastatin treatment in nitrofen-induced congenital diaphragmatic hernia

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Prevention of pulmonary hypoplasia and pulmonary vascular remodeling by antenatal simvastatin treatment in nitrofen-induced congenital diaphragmatic hernia. Am J Physiol Lung Cell Mol Physiol 308: L672–L682, 2015. First published January 23, 2015; doi:10.1152/ajplung.00345.2014.—Congenital diaphragmatic hernia (CDH) has a high mortality rate mainly due to lung hypoplasia and persistent pulmonary hypertension of the newborn (PPHN). Simvastatin has been shown to prevent the development of pulmonary hypertension (PH) in experimental models of PH. We, therefore, hypothesized that antenatal simvastatin would attenuate PPHN in nitrofen-induced CDH in rats. The efficacy of antenatal simvastatin was compared with antenatal sildenafil, which has already been shown to improve pathological features of PPHN in nitrofen-induced CDH. On embryonic day (E) 9.5, nitrofen or vehicle was administered to pregnant Sprague-Dawley rats. On E11, nitrofen-treated rats were randomly assigned to antenatal simvastatin (20 mg·kg⁻¹·day⁻¹ orally), antenatal sildenafil (100 mg·kg⁻¹·day⁻¹ orally), or placebo administration from E11 to E21. On E21, fetuses were delivered by cesarean section, killed, and checked for left-sided CDH. Lung tissue was then harvested for further pathobiological evaluation. In nitrofen-induced CDH, simvastatin failed to reduce the incidence of nitrofen-induced CDH in the offspring and to increase the body weight, but improved the lung-to-body weight ratio and lung parenchyma structure. Antenatal simvastatin restored the pulmonary vessel density and external diameter, and reduced the pulmonary arteriolar remodeling compared with nitrofen-induced CDH. This was associated with decreased lung expression of endothelin precursor, endothelin type A and B receptors, endothelial and inducible nitric oxide synthase, together with restored lung activation of apoptotic processes mainly in the epithelium. Antenatal simvastatin presented similar effects as antenatal therapy with sildenafil on nitrofen-induced CDH. Antenatal simvastatin improves pathological features of lung hypoplasia and PPHN in experimental nitrofen-induced CDH.

lung hypoplasia; persistent pulmonary hypertension of the newborn; sildenafil

DESPITE RECENT ADVANCES IN antenatal and neonatal intensive care, congenital diaphragmatic hernia (CDH) remains a life-threatening cause of severe respiratory failure in the newborn (12), mainly because of combined ventilatory deficiency and persistent pulmonary hypertension of the newborn (PPHN) (50). CDH results in lung hypoplasia and pulmonary vascular abnormalities, including pulmonary artery remodeling (42), whose severity mainly determines the outcome in CDH (1, 55). However, the pathogenesis of pulmonary epithelial and vascular anomalies in CDH remains largely unknown and the drugs available for the clinicians insufficiently efficacious.

Statins have been shown to exert beneficial effects in various experimental models of pulmonary hypertension (15, 36, 37). Simvastatin reversed established monocrotaline-induced pulmonary hypertension in rats by inducing apoptosis of neonatal smooth muscle cells (37) and chronic hypoxia-induced pulmonary hypertension by inhibiting the Rho signaling pathway (16). Simvastatin also improved pulmonary hypertension induced by a combination of vascular endothelial growth factor (VEGF) receptor inhibition and chronic hypoxia exposure by inducing endothelial apoptosis (53). Statins exert, indeed, direct anti-inflammatory (3), antiproliferative, and proapoptotic effects on vascular wall cells (11, 36, 37, 53) and improve endothelium-dependent relaxation by increasing endothelial nitric oxide (NO) synthase (34) and by reducing endothelin expression (18, 19).

We therefore hypothesized that antenatal maternal administration of simvastatin might show benefits in experimental nitrofen-induced CDH in rats. The efficacy of antenatal simvastatin therapy was compared with antenatal sildenafil administration, which has already shown beneficial effects on experimental (29) and human (38) CDH.

METHODS

All procedures and protocols were approved by the Institutional Committee on Animal Welfare of the Faculty of Medicine at the Université libre de Bruxelles (Brussels, Belgium).

Animal model. Pregnant Sprague-Dawley rats (Janvier, Saint-Barthevin, France) were gavage fed with 100 mg herbicide nitrofen (2,4-dichloro-4-nitrodiphenil ether; Fluka, Deisenhofen, Germany) dissolved in 1.5 ml olive oil as vehicle at embryonic day (E) 9.5 (predicted term: E22). Control rats received olive oil vehicle only. Animals were randomized into four groups as follows: control rats, nitrofen-treated rats, nitrofen + simvastatin (20 mg·kg⁻¹·day⁻¹)-treated rats, and nitrofen + sildenafil (100 mg·kg⁻¹·day⁻¹)-treated rats. The simvastatin dose was chosen on the basis of previous studies evaluating the pharmacokinetics of simvastatin (8), its safety in experimental (29) and human (38) CDH.

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lungs (n = 5 in each group, from two different litters) were embedded in toto in paraffin for histopathological evaluation. Because not all fetuses develop CDH, only those who developed CDH in nitrofen-, nitrofen + simvastatin-, and nitrofen + sildenafil-treated rats were further analyzed.

**Lung architecture and pulmonary artery morphometry.** Five-micrometer serial sections were taken along the longitudinal axis of the lung lobes and stained with hematoxylin-eosin for overall morphology assessment. Morphometric analysis of the overall lung architecture was performed estimating the mean wall thickness (wLun) and the radial alveolar count (RAC), as previously described by Roubliova et al. (45) and Cooney et al. (6), respectively. The total surface of each lung section was virtually divided in up to 20 random nonoverlapping fields for morphometric lung parenchyma analysis. Pulmonary artery morphometry was evaluated as previously described (44). Only small pulmonary arteries with an external diameter (ED) <100 μm and a complete muscular coat were measured. Medial thickness (MT) was related to arterial size by the formula %MT = (MT/ED) × 100 and was measured by counting at least 50 pulmonary arteries per lung lobe from each fetus. Pulmonary vascular density (number of pulmonary arteries with ED <100 μm/mm²) was evaluated using at least 50 microscopic fields (in ×100 total magnification) of pulmonary sections randomly selected. All analyses were performed with a LEICA ICC 50 HC Plan (Switzerland) in ×100 total magnification by two independent investigators in a blinded manner. The mean value was used for analysis.

**Real-time polymerase chain reaction.** Total RNA was extracted from snap-frozen lung tissue using TRIzol reagent (Life Technologies, Carlsbad, CA) followed by a chloroform/ethanol extraction and a final purification using the QIAGEN RNeasy Mini kit (QIAGEN, Hilden, Germany), according to the manufacturer’s instructions. RNA concentration was determined by standard spectrophotometric techniques, and RNA integrity was assessed by visual inspection of GelRed (Biotium, Hayward, CA)-stained agarose gels. Reverse transcription was performed using random hexamer primers and SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions.

For real-time polymerase chain reaction, sense and antisense primers were designed, using the Primer3 program, for *Rattus norvegicus* preproendothelin-1 (PPET-1), endothelin-converting enzyme 1 (ECE1), endothelin receptor type A (ETα) and B (ETβ), endothelial (eNOS) and inducible (iNOS) nitric oxide synthase, bone morphogenetic protein type 2 receptor (BMPR2), gremlin1, inhibitor of DNA binding 1 (Id1), Bax, Bcl-2, and hypoxanthine phosphoribosyltransferase 1 (HPRT1; used as housekeeping gene) mRNA sequences (Table 1). To avoid inappropriate amplification of residual genomic DNA, intron-spanning primers were selected when exon sequences were known. For each sample, amplification reaction was performed in triplicate using SYBRGreen PCR Master Mix (Quanta Biosciences, Gaithersburg, MD), specific primers, and diluted template cDNA. Result analysis was performed using an iCycler System (Bio-Rad Laboratories). Relative quantification was achieved with the comparative 2−ΔΔCt method by normalization with the housekeeping gene (HPRT1). Results were expressed as relative fold increase over the mean value of relative mRNA expression of the control group arbitrarily fixed to one.

**Protein extraction and dosage.** Proteins were extracted from snap-frozen pulmonary tissue samples by homogenization in an appropriate amount of ice-cold homogenizing lysis buffer [Complete Mini, protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) in phosphate-buffered saline and 0.1% Triton X-100]. The homogenates were centrifuged at 4°C, and the supernatants were collected. The protein content of total lung extract homogenates was measured by the method of Lowry et al. (28), using 5–25 μg homogenate and bovine serum albumin as standard (2–80 μg/assay).

Table 1. Sense and antisense primers used for RTQ-PCR analysis in rat tissue samples

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<tr>
<th>Genes</th>
<th>Primer Sequences</th>
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<td>Preproendothelin-1</td>
<td>Sense: 5'-CAGACCAAGGGAACAGATGC-3'</td>
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<td>Antisense: 5'-AGGCTTTTCTGATGATG-3'</td>
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<td>Sense: 5'-GCCACACAAACAGAAGATT-3'</td>
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<td></td>
<td>Antisense: 5'-GACCCCGATCAACGAAAT-3'</td>
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<td></td>
<td>Antisense: 5'-GCTGCCATTTCTCCTGG-3'</td>
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<tr>
<td>Endothelin receptor type B</td>
<td>Sense: 5'-CGATGGATGCTGCTGGTG-3'</td>
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<td></td>
<td>Antisense: 5'-GGAGCATTCTTCTAGC-3'</td>
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<td>Sense: 5'-GTTTCCCACGTTCTCCA-3'</td>
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<td></td>
<td>Antisense: 5'-CTGTCATTTGCGGCGAC-3'</td>
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<td>Antisense: 5'-GTTAAGCAACGGTCTGGTC-3'</td>
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<tr>
<td></td>
<td>Antisense: 5'-TTGGTCCCCCGAATCTC-3'</td>
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<tr>
<td>Inhibitor of DNA binding 1</td>
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<td>Bax</td>
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<td>Antisense: 5'-TCACGGAGAAATGTCCAG-3'</td>
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<tr>
<td>Hypoxanthine</td>
<td>phosphoribosyltransferase 1</td>
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<td></td>
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<tr>
<td></td>
<td>Antisense: 5'-TCCACATTTTCTGGTACGACC-3'</td>
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Enzyme-linked immunosorbent assay for lung content evaluation in endothelin-1. Endothelin-1 protein levels were determined with an Endothelin-1 QuantiKine enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN) in lung protein extracts (200 μg), according to the manufacturer’s instructions. Endothelin-1 concentrations were obtained by referring to a standard curve realized in parallel. Results represented the mean values of two separate measurements preformed in duplicate.

**Quantification of pulmonary total nitrate/nitrite levels.** Because levels of more stable NO metabolites (nitrates and nitrates) have been used to measure indirectly the levels of NO in biological samples, total nitrate/nitrite (NOx) levels were determined using the Total Nitric Oxide Assay (R&D Systems) according to the manufacturer’s instructions. Briefly, lung protein extracts (200 μg) were ultrafiltered through a 10,000 molecular weight cutoff filter to eliminate proteins. After reduction of nitrate to nitrite by nitrate reductase, total nitrite level was measured spectrophotometrically at 540 nm after incubation with Griess reagent at room temperature for 10 min. Pulmonary NOx levels were obtained by referring to a standard curve realized in parallel and expressed as micromoles per gram total protein. Results are represented as the mean values of two separate measurements preformed in duplicate.

**Terminal deoxynucleotidyl transferase dUTP nick-end labeling staining.** Apoptotic cells were detected by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (catalog no. L673 SIMVASTATIN IN EXPERIMENTAL CONGENITAL DIAPHRAGMATIC HERNIA L673 doi:10.1152/ajplung.00345.2014 • www.ajplung.org

Downloaded from http://ajplung.physiology.org/ by 10.220.0.47 on October 20, 2017
S7101: Chemicon, Temecula, CA) according to the manufacturer’s instructions. Negative control run without TdT enzyme and positive control pretreated with DNase I were tested. For each lung specimen, 10 different randomly chosen epithelial fields were examined. The epithelial apoptotic rate was calculated as the ratio of apoptotic nuclei (TUNEL-positive or brown nuclei) to total nuclei (brown + blue nuclei) ($\times 100$ to be expressed in percentage). The same procedure was used to evaluate the vascular apoptotic rate. All counts were performed by two independent investigators in a blinded manner. The mean value was used for analysis.

**Statistical analysis.** All data were expressed as means ± SE. Statistical analyses were performed using StatView 5.0 Software. Comparisons of parameters between two groups were performed using one-way ANOVA. When the $F$ ratio of this analysis reached a critical $P$ value <0.05, comparisons were made with a modified two-tailed Student’s $t$-test. $P$ values < 0.05 were considered to be statistically significant.

**RESULTS**

**Effects of antenatal simvastatin on the incidence of CDH, body weight, and lung hypoplasia.** In nitrofen-exposed litters, CDH was found in 19 of the 26 fetuses, thus 73% of the fetuses developed left-sided CDH. Neither antenatal simvastatin nor antenatal sildenafil treatment influenced the incidence of nitrofen-CDH (Fig. 1A).

Fetuses with nitrofen-induced CDH had a reduced body weight (by 22%) compared with controls. Antenatal simvastatin further reduced the body weight of nitrofen-CDH fetuses (by 37% compared with controls), whereas antenatal sildenafil did not (Fig. 1B). The lung-to-body weight ratio, a major indicator of lung hypoplasia, was lower (by 17%) in nitrofen-CDH compared with control fetuses (Fig. 1C). Antenatal simvastatin increased lung-to-body weight ratio in the nitrofen-CDH fetuses (by 12% compared with nitrofen-CDH fetuses), whereas antenatal sildenafil had no effect on the lung-to-body weight ratio (Fig. 1C). Lung weight did not change between nitrofen-CDH fetuses treated or not with simvastatin ($111 \pm 4$ vs. $115 \pm 4$ mg), suggesting that simvastatin-induced improvement in lung-to-body weight ratio could be (at least partly) the result of the decreased body weight.

**Antenatal simvastatin improves lung architecture in fetuses with nitrofen-induced CDH.** In fetuses with nitrofen-CDH, the thickness of the alveolar septa (assessed by $L_{mv}$) was higher when compared with controls (Fig. 2, A and C). Antenatal simvastatin reduced $L_{mv}$ in fetuses with nitrofen-CDH to similar levels as those observed in controls, whereas antenatal sildenafil failed to reduce significantly the $L_{mv}$ (Fig. 2A).

**RAC,** an indicator of lung maturity, was lower in nitrofen-CDH fetuses. Antenatal simvastatin restored the RAC in nitrofen-CDH fetuses, as well as antenatal sildenafil. The RAC levels observed after antenatal simvastatin and sildenafil administration were similar to those of controls (Fig. 2B).

**Antenatal simvastatin increases pulmonary vascular density and reduces pulmonary vascular remodeling in nitrofen-induced CDH.** In fetuses with nitrofen-CDH, pulmonary arteries were less abundant and smaller (assessed by decreased ED; Fig. 3, A and B). Antenatal simvastatin, as well as antenatal sildenafil, restored the pulmonary vascular density and the pulmonary artery ED in fetuses with nitrofen-CDH (Fig. 3, A and B).

As illustrated in Fig. 3, C and D, the medial wall thickness increased in the smallest pulmonary arteries (ED <25 µm) from fetuses with nitrofen-CDH. Antenatal simvastatin, as well as antenatal sildenafil, decreased the medial wall thickness in these small pulmonary arteries from fetuses with nitrofen-CDH (Fig. 3, C and D). When the pulmonary artery MT was assessed in all pulmonary arteries (from all types of size), the resulting data did not show any differences between analyzed groups (data not shown). This suggests major remodeling of the smallest pulmonary arteries in fetuses with nitrofen-CDH.

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**Fig. 1.** Effects of antenatal simvastatin and sildenafil on the incidence of congenital diaphragmatic hernia (CDH), body weight, and the lung-to-body weight ratio. The proportion of CDH (A), fetal body weight (in mg; $B$), and the lung weight-to-body weight ratio (C) was evaluated in each litter from control (white bars), nitrofen-induced CDH (CDH; black bars), nitrofen-induced CDH treated with simvastatin (light gray bars), and sildenafil (dark gray bars) groups. eNOS, endothelial nitric oxide synthase; HPRT1, hypoxanthine phosphoribosyltransferase 1; iNOS, inducible nitric oxide synthase; NOx, nitrates plus nitrites. Values are expressed as means ± SE. *0.01 < $P$ < 0.05 and ###$P$ < 0.001 vs. control fetuses; #0.01 < $P$ < 0.05 and ####$P$ < 0.001 vs. nitrofen-CDH fetuses; $SSSP$ < 0.001 vs. nitrofen-CDH treated with simvastatin fetuses.
Antenatal simvastatin restores the expression of molecules regulating the pulmonary vasomotion in nitrofen-induced CDH. Lung gene expression of the precursor of endothelin-1, PPET-1, and ET\textsubscript{A} increased in fetuses with nitrofen-CDH, whereas lung expression of the ECE1 and ET\textsubscript{B} did not change (Fig. 4, A–D). This was confirmed at the protein level, with increased lung protein expression of endothelin-1 in the lungs from fetuses with nitrofen-CDH (Fig. 5). This strongly suggests an imbalance of vasoconstrictor/vasodilator expression in nitrofen-CDH, in favor of an increased vasoconstriction.

In nitrofen-CDH, antenatal simvastatin restored lung gene expression of PPET-1 and ET\textsubscript{A} to control lung expression (or even lower for PPET-1) (Fig. 4, A and C). This was also observed for antenatal sildenafil in nitrofen-CDH (Fig. 4, A and C). In nitrofen-CDH, lung protein expression of endothelin-1 decreased after antenatal simvastatin and sildenafil treatments but remained higher than lung endothelin-1 expression in controls (Fig. 4E). Antenatal simvastatin, as well as antenatal sildenafil, restored lung gene expression of eNOS and iNOS and lung NO\textsubscript{x} levels in nitrofen-CDH to similar levels as those observed in controls (Fig. 5, A–C). This suggests restoration of the altered vasoactive balance after antenatal simvastatin and sildenafil administration in nitrofen-CDH.

Antenatal simvastatin restores BMPR2 signaling in nitrofen-induced CDH. Lung gene expression of BMPR2 and Id1, a target gene of the BMPR2 signaling, decreased in fetuses with nitrofen-CDH, whereas expression of gremlin1, a BMPR2 antagonist, increased (Fig. 6, A–C). Antenatal simvastatin restored BMPR2 and Id1 expression in the lungs of fetuses with nitrofen-CDH (Fig. 6, A and C), whereas lung expression of gremlin1 remained high after simvastatin administration (Fig. 6B). Antenatal sildenafil induced similar effects as antenatal simvastatin on lung expression of BMPR2, Id1, and gremlin1 (Fig. 6, A–C).

Antenatal simvastatin acts on the epithelial and the pulmonary vascular apoptosis. Nitrofen-CDH was associated with decreased lung expression of proapoptotic Bax mitochondrial member, whereas lung expression of antiapoptotic Bcl-2 did not change (Fig. 7, A and B). The resulting proapoptotic Bax/Bcl-2 ratio was decreased in the lungs of fetuses with nitrofen-CDH (Fig. 7C). Antenatal simvastatin, as well as antenatal sildenafil, restored lung expression of proapoptotic Bax and of the proapoptotic Bax/Bcl-2 ratio to similar levels as those of the control fetuses (Figs. 7A and 6C).

To assess whether the upregulation in upstream apoptotic mitochondrial pathways was accompanied by completion of apoptotic processes and to localize the areas where the apoptotic processes were mostly activated, TUNEL staining was...
performed (Fig. 7, D and E). As illustrated in Fig. 7, D and E, nitrofen-CDH was associated with decreased apoptosis within the epithelium and the pulmonary vascular wall. Antenatal simvastatin restored the epithelial apoptotic rate in the lungs from fetuses with nitrofen-CDH to similar levels as those observed in controls, whereas it failed to restore it in the remodeled pulmonary vessels from nitrofen-CDH (Fig. 7, D and E). The epithelial and pulmonary vascular apoptotic rates remained low in the lungs of nitrofen-CDH fetuses after antenatal sildenafil administration (Fig. 7, D and E).

**DISCUSSION**

The present results show that antenatal maternal administration of simvastatin improves characteristic features of lung hypoplasia and PPHN in experimental nitrofen-CDH. This includes improvement of lung parenchyma architecture and decreased pulmonary arteriolar remodeling that appear to be related to the restoration of endothelin and NO systems, BMPR2 signaling, and activation of apoptotic processes (mainly in the epithelium).

CDH remains one of the greatest challenges in perinatal medicine (50). Malformations observed in CDH include failure of both pulmonary alveolar and vascular development (13, 23, 35), responsible for severe respiratory failure and PPHN in survivors. Here, we used nitrofen-induced CDH, which is a well-established experimental model of CDH recapitulating the major pulmonary abnormalities observed in human CDH, including lung hypoplasia and pulmonary vascular remodeling (33). In this experimental model, we showed that antenatal simvastatin significantly attenuated lung hypoplasia and improved lung parenchyma maturation in fetuses with nitrofen-CDH. The alveoli in the hypoplastic lungs presented an immature form with reduced alveolarization and thickened intra-alveolar septa, responsible for a reduced capillary-air interface, which is essential for gas exchange. Simvastatin significantly improved these features, which are essential for the fetal outcome in CDH.

The endothelial dysfunction characterized by the imbalance in the production of vasoconstrictors (e.g., endothelin-1) and vasodilators (e.g., NO) together with the remodeling of the
pulmonary arteries contributes to high pulmonary vascular resistance and to the development of pulmonary hypertension in fetuses with CDH (32, 47). Here, we showed pulmonary arteriolar remodeling with muscularization of preacinar arteries in fetuses with nitrofen-CDH. Antenatal simvastatin reduced pulmonary vascular remodeling by decreasing medial wall thickness in the smallest pulmonary arteries. This is consistent with previous studies showing that simvastatin prevented and reversed pulmonary hypertension and pulmonary vascular remodeling in different experimental models of pulmonary hypertension (14, 36, 37, 53).

Interactions between pulmonary airways and blood vessels have been shown to play critical roles in normal lung development (51). Inhibition of VEGF in rat fetuses led to arrested alveolar development (21, 54). Moreover, the NO signaling played a critical role in vascular growth during septation and in modulation of perinatal pulmonary vascular tone (40). Consequently, promoting vascular growth before birth may improve lung maturation in CDH. Here, we showed decreased pulmonary vascular density and reduced pulmonary artery ED in fetuses with nitrofen-CDH, suggesting pulmonary vascular instability and defective lung angiogenesis in these pups. This was associated with reduced pulmonary expression of eNOS and iNOS, as previously described in the lungs from human (2) and experimental (39, 56) CDH. All of these altered features were largely improved by antenatal simvastatin treatment, which is consistent with previous studies showing that simvastatin promoted angiogenesis in vitro and in vivo (4, 25) and improved endothelium-dependent relaxation through the up-regulation of eNOS expression and NO production (25, 26). Moreover, eNOS has been shown to play a pivotal role in the transition of the pulmonary circulation from fetal to neonatal life (48). Therefore, the restoration of eNOS and iNOS expression by antenatal simvastatin treatment in nitrofen-CDH may correlate with improved pulmonary endothelial function and therefore with the potential positive outcome of these pups.

Endothelin is an endothelium-derived potent vasoconstrictor (32) that has been shown to play important roles in modulating...
eNOS (57) and BMPR2 signaling (31) in pulmonary vascular cells, contributing therefore to the pulmonary artery remodeling. It acts via two different receptors, the type A and B receptors (ET\textsubscript{A} and ET\textsubscript{B}). Present on vascular smooth muscle cells, ET\textsubscript{A} receptor mediates vasoconstriction, whereas ET\textsubscript{B} receptor, expressed on both endothelial and vascular smooth muscle cells, mainly mediates vasodilatation. Here, we showed increased gene expressions of endothelin precursor and ET\textsubscript{A} receptor, as well as increased endothelin-1 protein content in the lungs from nitrofen-CDH while ET\textsubscript{B} expression did not change. This is consistent with previous studies showing up-regulated endothelin signaling in the lungs from experimental (9) and human (7) CDH and beneficial effects of dual ET\textsubscript{A}-ET\textsubscript{B} antagonism on the regulation of pulmonary vascular tone in experimental CDH (49). As described above, nitrofen-CDH was associated with decreased lung expression of eNOS and iNOS, both responsible for decreased NO\textsubscript{x} production and consequently for vasodilator effects within the pulmonary vasculature. Antenatal simvastatin restored lung gene expressions of endothelin precursor, ET\textsubscript{A} receptor, eNOS and iNOS,

Fig. 5. Antenatal simvastatin and sildenafil attenuate imbalance between pulmonary vasoconstrictor and vasodilator-nitric oxide signaling. Relative gene expressions of eNOS (A), iNOS (B), and levels of NO\textsubscript{x} (C) in the lungs from control (white bars; \( n = 12 \)), nitrofen-induced CDH (black bars; \( n = 11 \)), nitrofen-induced CDH treated with simvastatin (light gray bars; \( n = 10 \)), and sildenafil (dark gray bars; \( n = 10 \)) groups. Data are expressed as means ± SE. *0.01 < \( P < 0.05 \) and **0.001 < \( P < 0.01 \) vs. control fetuses.

Fig. 6. Bone morphogenetic protein (BMP) signaling pathway. Relative gene expression of bone morphogenetic protein type 2 receptor (BMPR2; A), bone morphogenetic protein antagonist gremlin1 (B), and bone morphogenetic protein target gene, the inhibitor of DNA-binding 1 (Id1; C), in the lungs from control (white bars; \( n = 12 \)), nitrofen-induced CDH (black bars; \( n = 11 \)), nitrofen-induced CDH treated with simvastatin (light gray bars; \( n = 10 \)), and sildenafil (dark gray bars; \( n = 10 \)) groups. Data are expressed as means ± SE. *0.01 < \( P < 0.05 \) vs. control fetuses; #0.01 < \( P < 0.05 \) vs. nitrofen-CDH fetuses.
as well as lung endothelin-1 protein content and NO level (assessed by nitrate plus nitrite levels) in fetuses with nitrofen-CDH, which suggests restoration of pulmonary endothelial function in these pups.

The exact mechanisms by which simvastatin improved PPHN and lung hypoplasia in CDH remain to be clarified. However, simvastatin has been shown to enhance the expression of BMPR2 in vascular endothelial cells (20), contributing therefore to the endothelial function and the regression of vascular disease. Fluvastatin treatment of microvascular endothelial cells induced Id1 expression (41), suggesting upregulated BMPR2 signaling induced by fluvastatin treatment. Similarly, sildenafil has been shown to potentiate BMP signaling in pulmonary artery smooth muscle cells preventing the development of pulmonary vascular remodeling in experimental pulmonary hypertension (59). We previously reported that BMP/BMPR2 signaling was downregulated in the lungs from fetuses with experimental nitrofen-CDH (30). Here, we showed that antenatal simvastatin and sildenafil treatment both restored BMP/BMPR2 signaling in the lungs of fetuses with nitrofen-CDH. Because BMP/BMPR2 is largely involved in regulating the balance between cell proliferation and apoptosis, antenatal simvastatin and sildenafil probably act on the epithelial and vascular cell proliferation/apoptosis balance through the restoration of the BMPR2 signaling.

Disturbed balance between cell proliferation and apoptosis contributes to the development of lung hypoplasia (22) and pulmonary vascular remodeling (5) associated with CDH induced by nitrofen in rats. Here, we showed decreased activation of apoptotic processes in the epithelial and vascular layers in the lungs of nitrofen-CDH fetuses. Antenatal simvastatin partly reversed this decreased activation of apoptotic processes.
in the epithelium, but not in the vessels, while antenatal sildenafil did not affect epithelial and vascular apoptosis. Our data are consistent with simvastatin effects reported in the epithelium in an experimental model of lung ischemia-reperfusion injury (58). However, we did not see any change in the percentage of vascular TUNEL-positive cells, even though MT was reduced after antenatal simvastatin treatment. Similarly, antenatal sildenafil did not alter epithelial and vascular apoptotic rates, while $L_{nw}$ and MT were decreased. A possible explanation for these apparent discrepancies could be differences in proliferation rates and/or elimination of vascular cells through apoptotic processes already accomplished on E21.

Based on animal and human data, maternal administration of simvastatin during pregnancy seems to be safe for the fetuses (43, 46). The simvastatin dose tested in the present study was chosen on the basis of these previous studies performed in pregnant rats. Here, we found reduced fetal body weight induced by antenatal simvastatin, suggesting intrauterine growth retardation. This is consistent with previous studies showing reduced body weight, but no reduced offspring survival at birth and during the neonatal period after antenatal administration of simvastatin (10, 17). We did not evaluate the infant morbidity, which could be related to this fetal growth retardation. This should be clearly addressed in the further clinical trials. Our results on antenatal sildenafil administration in nitrofen-CDH were consistent with those obtained by Luong et al. in terms of improved characteristic features of lung hypoplasia and PPHN in nitrofen-CDH (29). Antenatal simvastatin was as effective as antenatal sildenafil in terms of lung hypoplasia and PPHN improvement in experimental nitrofen-CDH. However, even if pulmonary morphological and biological data obtained after antenatal simvastatin treatment in nitrofen-CDH were encouraging, one major limitation of the present study remains the lack of evaluation of physiological parameters, including pulmonary artery pressure and lung compliance. Experiments testing the efficacy of simvastatin in larger experimental models of CDH should be performed.

Because CDH can be diagnosed early (at 22 wk of gestation) by usual ultrasound examination in humans, antenatal therapies are therefore feasible. By analogy to the current use of glucocorticoid treatment used to induce the maturation of fetal lungs and prevent postnatal complications in the newborns (27), we reasoned that antenatal simvastatin, an already used medication in pregnant women for other complications (such as pre-eclampsia), could improve lung epithelial and vascular development in CDH. However, further clinical studies testing the timing, dosing, and toxicity of maternal simvastatin administration in fetuses with CDH are clearly needed to evaluate efficacy and potential side effects (including intrauterine growth retardation) of antenatal simvastatin.

In conclusion, the present study shows that antenatal simvastatin treatment improves lung structure and decreases pulmonary arteriolar remodeling in nitrofen-CDH as efficaciously as antenatal sildenafil treatment.

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DISCLOSURES

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS


REFERENCES


AJP-Lung Cell Mol Physiol • doi:10.1152/ajplung.00345.2014 • www.ajplung.org
hypoxic pulmonary hypertension: effect of simvastatin treatment (Ab-

16. Girgis RE, Mozammel S, Champion HS, Li D, Peng X, Shimoda L, 

17. Henck JW, Craft WR, Black A, Colgin J, Anderson JA. Pre- 

3-methylglutaryl-CoA reductase inhibitors, atorvastatin and simvastatin, on the expression of endothelin-1 and endothelial nitric oxide synthe- 

expression by simvastatin in vascular endothelial cells. Circ Res 87: 

20. Hu H, Sung A, Zhao G, Shi L, Qiu D, Nishimura T, Kao PN. Simvastatin enhances bone morphogenetic protein receptor type II expres-

21. Jakkula M, Le Cras TD, Gebb S, Hirth KP, Tuder RM, Voelkel NF, 
Abman SH. Inhibition of angiogenesis decreases alveolarization in the 

explains pulmonary hypoplasia in the nitrogen model of congenital dia-

23. Kitagawa M, Hislop A, Boyden EA, Reid L. Lung hypoplasia in 
congenital diaphragmatic hernia. A quantitative study of airway, artery, 

24. Kumawat K, Ikawa M, Kidoya H, Hasuwa H, Saito-Fujita T, Mo-
rioka Y, Takakura N, Kinuma T, Okabe M. Pravastatin induces plas-
cental growth factor (PGF) and ameliorates preeclampsia in a mouse 

WC, Walsh K. The HMG-CoA reductase inhibitor simvastatin activates 
the protein kinase Akt and promotes angiogenesis in normocholester-

26. Laufs U, Fata VL, Liao JK. Inhibition of 3-hydroxy-3-methylglutaryl 
(HMG-CoA) reductase blocks hyoxia-mediated down-regulation of en-

27. Langlais GC, Matsuoka TN. A controlled trial of antepartum glucocorticoid 
treatment for prevention of the respiratory distress syndrome in premature 

28. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measure-

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Acute effect of a dual 
ETA-ETB receptor antagonist on pulmonary arterial vasculature in pre-
term lamb fetuses with surgically induced hypoxic hernia. Pediatr 

30. Luquet S, Fenton A, Jalabert A. Arteriogenesis in experimental congeni-

31. Makanga M, Dewachter C, Kerbaul F, Kung F, Feder P, Brimioulle S, 
Abman SH. Prolonged overcirculation-induced pulmonary arterial 
hypertension as a cause of right ventricular failure. Eur Heart J 33: 

32. Nishimura T, Vaszar LT, Faul JL, Zhao G, Berry GJ, Shi L, Qiu D, 
Benson G, Pearl RG, Kao PN. Simvastatin rescues rats from fatal pulmonary hypertension by inducing apoptosis of neonatal smooth 

of simdifenil in neonates and infants with congenital diaphragmatic hernia 

34. North AJ, Moya FR, Mysore MR, Thomas VL, Wells LB, Wu LC, 
Shaul PW. Pulmonary endothelial nitric oxide synthase gene expression 
is decreased in a rat model of congenital diaphragmatic hernia. Am J Res-

35. North AJ, Star RA, Brannon TS, Ujije K, Wells LB, Lowenstein CJ, 
Snyder SH, Shaul PW. Nitric oxide synthase type I and type III gene 
expression are developmentally regulated in rat lung. Am J Physiol Lung 

36. Nishimura T, Faul JL, Berry GJ, Vaszar LT, Qiu D, Pearl RG, Kao PN. 

37. Nishimura T, Vaszar LT, Faul JL, Zhao G, Berry GJ, Shi L, Qiu D, 

38. Sood BG, Wykes S, Landa M, De Jesus L, Rabah R. Expression of 

WC, Walsh K. The HMG-CoA reductase inhibitor simvastatin activates 
the protein kinase Akt and promotes angiogenesis in normocholester-

40. Laufs U, Fata VL, Liao JK. Inhibition of 3-hydroxy-3-methylglutaryl 
(HMG-CoA) reductase blocks hyoxia-mediated down-regulation of en-

41. Langlais GC, Matsuoka TN. A controlled trial of antepartum glucocorticoid 
treatment for prevention of the respiratory distress syndrome in premature 

42. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measure-

43. Luong C, Rey-Perra J, Vadivel A, Gilmour G, Sauve Y, Koonen D, 
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Acute effect of a dual 
ETA-ETB receptor antagonist on pulmonary arterial vasculature in pre-
term lamb fetuses with surgically induced hypoxic hernia. Pediatr 

44. Shepard TH. Catalog of Teratogenic Agents. Bethesda, MD: National 

45. Shinkai T, Shima H, Solari V, Puri P. Expression of vasoactive medi-
ators during mechanical ventilation in nitrofen-induced congenital dia-

46. Sood BG, Wykes S, Landa M, De Jesus L, Rabah R. Expression of 

47. Statopoulos E, Rolland PH, Hery G, de Magnee C, Paut O, Couchot 
WC, Walsh K. The HMG-CoA reductase inhibitor simvastatin activates 
the protein kinase Akt and promotes angiogenesis in normocholester-

48. Laufs U, Fata VL, Liao JK. Inhibition of 3-hydroxy-3-methylglutaryl 
(HMG-CoA) reductase blocks hyoxia-mediated down-regulation of en-

49. Langlais GC, Matsuoka TN. A controlled trial of antepartum glucocorticoid 
treatment for prevention of the respiratory distress syndrome in premature 

50. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measure-
