Impaired VEGF and nitric oxide signaling after nitrofen exposure in rat fetal lung explants

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CONGENITAL DIAPHRAGMATIC HERNA (CDH) is a complex disease that causes severe hypoxemic respiratory failure and high morbidity and mortality (8, 43). In the most severely affected newborns, CDH is characterized by lung hypoplasia, severe pulmonary hypertension, and poor cardiac function (33). Despite many advances in neonatal care, CDH often remains refractory to diverse medical interventions, such as inhaled nitric oxide (NO) therapy, and persists as a common indication for extracorporeal membrane oxygenation therapy (1). Although many factors contribute to survival of infants with CDH, the overall severity of CDH is largely due to an underlying disruption of normal lung growth and development, which leads to striking decreases in airway branches, alveolarization, and vascular growth and development (20, 40). Recent studies have suggested a potential genetic basis for CDH in some cases, but mechanisms that contribute to impaired lung growth in CDH remain poorly understood (4, 5).

Several animal models in mice, rats, rabbits, and sheep have been established to explore the pathogenesis of CDH (53). During the past two decades, a well-established model to study CDH in rodents has been developed through the use of the herbicide nitrofen (2,4-dichloro-p-nitrophenyl ether) (14, 28, 35, 50). When a maternal rat or mouse is fed nitrofen at precise time points of lung embryogenesis (E9.5 in rats, E8.5 in mice), the fetus often develops features of CDH that include bilateral lung hypoplasia, with more severe changes on the left lung, and in many cases, diaphragmatic defects (6, 14, 23, 25, 35, 50). Nitrofen exposure induces small lungs with reduced airway branching and septation, thickened interstitium, and reduced vascular density (10, 35). Interestingly, abnormal lung growth has been shown to precede the normal timing of diaphragm closure, suggesting that pulmonary hypoplasia is at least partly due to a direct teratogenic insult (14, 25). Thus, prenatal nitrofen treatment provides a useful model for studies of molecular and biochemical mechanisms that may cause abnormalities of lung structure in CDH (14, 42, 45, 46).

Previous studies of nitrofen-induced CDH have demonstrated early impairment of vascular growth in addition to abnormalities of the distal air space, as described in human infants with CDH (25). The precise relationship between alveolar and vascular growth is incompletely understood, but recent studies suggest that disruption of angiogenesis can inhibit alveolarization in the developing lung (2, 9, 17, 26, 29, 37, 51, 52). Although diverse mechanisms contribute to angiogenesis in the developing lung, VEGF has been shown to play a striking role during normal fetal and postnatal lung development. VEGF is an endothelial cell-specific mitogen and survival factor that promotes normal vessel growth and structure (15). Experimental studies have shown that vascular growth and alveolarization are closely related and that blood vessel growth in the lung promotes normal alveolar development (16, 21, 29) and contributes to the maintenance of alveolar structure throughout life (31). For example, disruption of VEGF signaling with the VEGF receptor (VEGFR) inhibitor SU-5416 or a VEGF aptamer decreases lung vascular and alveolar growth and can cause pulmonary hypertension in neonatal rats and fetal sheep (24, 29, 37). Past studies have suggested that VEGF and VEGFR expression is decreased in animal models of CDH,
including studies of nitrofen-induced lung hypoplasia (11, 25). Whether impaired VEGF signaling contributes to lung hypoplasia in experimental CDH and mechanisms through which disruption of VEGF signaling contributes to CDH remain unclear.

Recent studies have suggested that VEGF-induced lung angiogenesis is in part mediated by NO (30, 55). As a downstream effector and an upstream mediator of angiogenic factors, NO may play a critical direct and indirect role in lung vascular development (26, 30, 55). Abnormal lung vascular and alveolar growth has been described in endothelial nitric oxide synthase (eNOS)-deficient fetal mice (27). In addition, neonatal treatment with the VEGFR inhibitor, SU-5416, which causes lung hypoplasia, also downregulates eNOS protein and NO production, and treatment with inhaled NO improves vascular and alveolar growth in this model (7, 48). Importantly, past studies have shown that NO donors enhance lung branching in the normal fetal rat lung explant, further supporting a critical role for NO in normal lung growth (54). However, whether changes in VEGF signaling are modulated by altered eNOS expression or activity in experimental CDH remains unclear.

Therefore, we hypothesized that impaired fetal lung growth in experimental CDH after maternal nitrofen treatment may be due to disruption of VEGF signaling and that altered NO production may modulate the severity of lung structure in this model. To test this hypothesis, we performed a series of experiments to examine changes in lung structure and VEGF and eNOS expression and the effects of exogenous VEGF and NO on fetal lung growth after nitrofen treatment. To further determine the potential role of impaired VEGF signaling in this model, we also performed parallel studies to compare the effects of SU-5416, a VEGFR inhibitor, on fetal lung branching morphogenesis, as well as the effects of exogenous VEGF or NO treatment, with explants exposed to maternal nitrofen.

MATERIALS AND METHODS

Animals. All procedures and protocols were approved by the Animal Care and Use Committee at the University of Colorado Health Science Center. Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) and maintained in room air at Denver’s altitude (1600 m; barometric pressure, 630 mmHg; inspired oxygen tension, 122 mmHg) from gestational day 7 (E7) to E15. Animals were fed ad libitum and exposed to day-night cycles alternatively every 12 h.

Study design. Study animals included time-dated pregnant rats that were treated with nitrofen (100 mg in 1 ml of olive oil; Chem Service, West Chester, PA) by gavage feeding on E9.5. Maternal rats were killed by nitrogen inhalation on E15, and fetal lung explants were harvested by hysterectomy and placed on a 0.4-μm pore filter in a 12-well plate (Corning, Corning, NY) with DMEM (Sigma, St. Louis, MO). Lung explants were cultured for 3 days in DMEM, 1% L-glutamine, and 1% penicillin-streptomycin in humidified incubators (Forma Scientific, Marietta, OH) at 37°C in 3% O2, 5% CO2, and balance nitrogen. Studies were performed in 3% O2 to mimic fetal conditions. For studies on the effects of VEGF signaling on lung growth, fetal lung explants from control pregnant rats were harvested at E15 and exposed to SU-5416 (10 μM) in media and 3% O2 for 3 days. In some studies, exogenous VEGF and NO treatments were administered. Each study group consisted of six to eight lung explants that were treated with recombinant human VEGF (50 ng/ml), exogenous NO (10 ppm), the VEGFR inhibitor (SU-5416; 10 μM), or combinations of these agents. We conducted concurrent studies of control lung explants in parallel with each experimental group for comparison. Lung explants were harvested for morphometric analysis, immunohistochemistry, and Western blot studies, as described below.

Morphometry. Morphometric analyses were conducted using a skeletonization approach (Fig. 1) (36). Fetal lung explants were photographed daily with a dissecting microscope to document serial changes in growth. Images were digitized, and airways were marked using Adobe Photoshop and then skeletonized. Branching was assessed by automatic counting of the number of nodes. Changes in branching were expressed as the ratio of difference in branch points from 0 to 72 h divided by the number of branch points at 0 h. Results were expressed as the percentage increase in branching between day 0 (D0) and D3 of the experiment. We concurrently used a panel of standards approach as a second method for verification. In this model, photographs of the explants were randomly submitted to five independent, blinded observers and scored for lung complexity.

Immunohistochemistry. Explants were fixed in 4% paraformaldehyde for 1 h at 4°C, washed twice in PBS for 1 h at 4°C, and stained with Fast Red (1:10, Nuclear Fast Red H-3403; Vector, Burlingame, CA). Explants were washed in PBS and allowed to equilibrate overnight in PBS with sucrose (30%) at 4°C. The explants were then placed in embedding molds filled with 100% Optimal Cutting Temperature compound (Sakura Finetek, Torrance, CA), frozen, and stored in a –80°C freezer.

Fig. 1. Morphometric analysis of fetal lung growth in vitro. This figure illustrates the overall appearance of the fetal lung explant (A), marking of the airways (B), and skeletonization (C) for morphometric analysis.
Seven-micrometer-thick sections from the explants were obtained by cryosectioning, mounted on SuperFrost/Plus slides, and placed successively in 4% paraformaldehyde and methanol/acetone (1:1). Sections were rinsed with PBS and blocked with BSA (0.1%; Fisher, Fair Lawn, NJ) in PBS with horse serum (2%, Vector) for 1 h at room temperature. Immunohistochemistry for localization of eNOS protein and active caspase-3 protein was performed on sections that were incubated with either mouse anti-eNOS antibody (1:400, 610297; BD Sciences, San Jose, CA) or rabbit anti-active caspase-3 (1:200, AB 3623; Chemicon, Temecula, CA) at 4°C overnight. Sections were then washed, and secondary antibody for eNOS detection (goat anti-mouse Alexa Fluor 488, A11029, green, 1:1,000; Molecular Probes, Eugene, OR) or active caspase-3 (donkey anti-rabbit Alexa Fluor 504, A21207, red, 1:1,000; Molecular Probes) was applied for 2 h at room temperature. Sections were stained with 4',6-diamidino-2-phenylindole (DAPI, Vector) and visualized using an Olympus IX71 fluorescence microscope (Olympus America, Center Valley, PA). Three different filters were used to acquire images, DAPI (blue), eNOS (green), and active caspase-3 (red).

Western blot analysis. To obtain sufficient tissue for Western blot analysis, lung explants from each experiment were pooled for assay. Lung explants were collected and homogenized on ice in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS) containing a protease inhibitor cocktail (Sigma). Samples were sonicated and centrifuged at 10,000 g for 20 min at 4°C. Protein content in the supernatant was determined by the Bradford method (Pierce BCA assay, Rockford, IL) using BSA as the standard. Fifteen micrograms of protein sample per lane were subjected to SDS-PAGE, and proteins from the gel were transferred to nitrocellulose membranes by electroblotting. Blots were blocked overnight at 4°C in 5% nonfat dried milk or 2% ECL advance blocking agent (Amersham, Piscataway, NJ). Immunodetection was performed with a mouse anti-eNOS polyclonal antibody (610297, BD Sciences) diluted 1:1,000 and a rabbit anti-human polyclonal VEGF antibody (SC-152; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:500 overnight at 4°C. After the blots were washed to remove unbound antibody, secondary antibodies, anti-mouse horseradish peroxidase antibody (1:20,000, Chemicon) for eNOS detection, and anti-rabbit horseradish peroxidase (1:5,000, SC-2054; Santa Cruz Biotechnology) for VEGF detection were applied for 1 h at room temperature. After being washed, bands were visualized by enhanced chemiluminescence using ECL Plus detection (Amersham). In addition, each gel was stripped and reprobed with β-actin as a housekeeping protein to normalize for protein loading.

Statistical analysis. Data are presented as means ± SE. Statistical analysis was performed with the InStat 3.0 software package (GraphPad Software, San Diego, CA). Statistical comparisons were made using analysis of variance and Fisher’s protected least significant difference test. *P < 0.05 was considered significant.

RESULTS

Effects of VEGFR inhibition on branching morphogenesis and lung VEGF and eNOS expression in fetal lung explants. In contrast with the progressive increase in branching and lung complexity of control fetal rat lung explants, SU-5416-treated explants had decreased distal branching and increased distal air space size (Fig. 2A). To quantitate observed differences in lung structure, we used the skeletonization method, as originally described by Tschanz and Burri (51a) and verified with the use of a panel of standards approach, as described above. Compared with controls, SU-5416 treatment decreased branching after 3 days in culture by 25% (Fig. 2B). Immunostaining
for eNOS demonstrated strong signal within the lung mesenchyme but not distal airway epithelium of normal fetal lung explants (Fig. 3A). In contrast, eNOS immunoreactivity was markedly reduced after SU-5416 treatment (Fig. 3B).

To quantify changes in VEGF and eNOS expression after VEGFR inhibition, Western blot analysis was performed on pooled samples of lung homogenates from control and SU-5416-treated explants (n = 6 explants per group). As shown in Fig. 4, lung VEGF and eNOS protein level were markedly decreased after SU-5416 treatment.

Effects of NO and VEGF treatments on fetal lung branching morphogenesis after SU-5416 treatment. Since VEGFR inhibition altered lung structure and downregulated VEGF and eNOS protein level, we studied the effects of exogenous VEGF and NO after SU-5416 treatment to further define the roles of VEGF and NO on lung growth. As shown, treatment of lung explants with VEGF (50 ng/ml) did not alter lung structure (Fig. 5A, a and b) or increase the number of branches after SU-5416 treatment (Fig. 5B). In contrast, exogenous NO increased lung complexity (Fig. 5A, a and c) and branching (Fig. 5B). Morphometric analysis demonstrated a 1.5-fold increase in branching after NO treatment compared with SU-5416-treated lung explants. VEGF treatment did not affect VEGF or eNOS expression in pooled samples of lung explants (Fig. 6). In contrast, exposure to NO restored expression of VEGF and eNOS after VEGFR inhibition (Fig. 6).

Effects of maternal nitrofen exposure on lung structure and VEGF-NO signaling in fetal lung explants. Fetal lungs that were harvested after maternal nitrofen exposure showed a more simplified architecture with less branching than controls and were also smaller in size (Fig. 7A). Following 3 days in culture, however, this difference in lung structure did not persist, as the nitrofen-exposed lungs underwent progressive branching and growth (Fig. 7A, b and d). Morphometric analysis with skeletonization showed a greater increase in distal branching in the nitrofen group (1.5-fold) compared with control lungs (Fig. 7B). Although maternal nitrofen treatment markedly decreased VEGF expression in the explants, there was no difference in eNOS protein level when compared with control lungs (Fig. 8). Immunofluorescence staining for eNOS demonstrated persistent eNOS immunoreactivity within the mesenchyme after nitrofen exposure (Fig. 3C).

Effects of NO or VEGF treatment on fetal lung branching morphogenesis following maternal nitrofen exposure. Treatment with exogenous VEGF and NO further enhanced branching and distal structural complexity of lung explants after maternal nitrofen exposure (Fig. 9A). Morphometric analysis demonstrates a 1.4- and 1.7-fold increase in branching after VEGF and NO treatment, respectively, when compared with control-nitrofen-treated explants (Fig. 9B). NO treatment increased VEGF protein level by nearly 10-fold (Fig. 10). Lung eNOS protein level was not modified by treatment with either VEGF or NO (data not shown).

Mechanism of the NO-induced increases in fetal lung branching. NO preserved or enhanced lung growth in fetal explants after SU-5416 and nitrofen exposure, but whether endogenous NO contributes to lung structure in fetal lung explants and if the effects of NO were mediated by cGMP-dependent or -independent mechanisms were unknown. To investigate this, we treated fetal rat lung explants with the NOS inhibitor l-NA and found a significant decrease in branching (Fig. 11). We further found that cotreatment with 8-bromo-cGMP and NO preserved lung structure (Fig. 11).

To further explore mechanisms through which NO enhances lung structure, we determined the effects of SU-5416 and nitrofen on lung apoptosis as assessed by active caspase-3 immunofluorescence (Fig. 12). Compared with controls, both SU-5416 and nitrofen markedly increased active caspase-3 immunoreactivity, which was localized within the mesenchyme (Fig. 12). Treatment with exogenous NO decreased active caspase-3 staining after SU-5416 and nitrofen treatment (Fig. 11).

DISCUSSION

CDH is a complex clinical disorder characterized by decreased vascular and alveolar growth, but mechanisms that cause lung hypoplasia in CDH remain elusive. Based on recent studies of lung development, we hypothesized that impaired VEGF signaling contributes to abnormal lung
structure in CDH. To test this hypothesis, we studied the effects of nitrofen exposure on fetal rat lung structure and then compared these effects after treatment with SU-5416, a VEGFR inhibitor. We found that nitrofen exposure impaired fetal lung growth and distal air space branching, induced mesenchymal apoptosis, and reduced VEGF but not eNOS expression, a key downstream target of VEGF. In addition, treatment with VEGF and NO improved lung growth, and NO treatment increased VEGF expression. In comparison, SU-5416 treatment had many similar effects as nitrofen,
including decreased lung branching, increased mesenchymal apoptosis, and reduced VEGF expression. VEGF treatment failed to increase lung growth after VEGFR inhibition, but NO treatment increased lung growth and VEGF expression. In both models, NO reduced apoptosis within the developing lung mesenchyme. Overall, these findings suggest that in the nitrofen model of CDH, impaired VEGF signaling contributes to altered lung growth and structure, but it is unlikely that nitrofen-induced lung hypoplasia can be completely explained by decreased VEGF signaling alone. These findings further demonstrate the striking effects of VEGF and NO treatment on enhancing lung growth in experimental CDH.

These findings directly compared the effects of disruption of VEGF signaling with nitrofen in fetal rat lung explants. Although previous studies have measured changes in lung VEGF content in the nitrofen model of CDH, these data are conflicting (42). Oue et al. (42) showed that VEGF protein level is increased in rat lungs following exposure to nitrofen, whereas two other studies reported decreased VEGF expression (11, 12). Our findings that nitrofen exposure decreased lung VEGF expression are consistent with those published by Chang et al. (11) and Chinoy et al. (12). Moreover, we further explored the implications of these findings by comparing the effects of nitrofen treatment with VEGF and NO treatment on lung VEGF and eNOS protein level (pooled samples). SU-5416 markedly reduced lung VEGF protein content, which was not altered with VEGF treatment. In contrast, NO treatment increased VEGF expression by 7-fold (left). SU-5416 also reduced eNOS expression, which was increased by NO but not VEGF treatment (right).

Fig. 6. Effects of VEGFR inhibition on lung VEGF and eNOS protein level (pooled samples). SU-5416 markedly reduced lung VEGF protein content, which was not altered with VEGF treatment. In contrast, NO treatment increased VEGF expression by 7-fold (left). SU-5416 also reduced eNOS expression, which was increased by NO but not VEGF treatment (right).

Fig. 7. A: effects of maternal nitrofen administration on fetal lung explants. As shown, compared with control fetal lung explants on the day of harvesting (day 0; a), nitrofen-exposed lungs appeared simplified (day 0; c). However, after 3 days in culture (day 3), branching spontaneously increased in nitrofen-exposed lungs (d) and appeared similar to control explants (b). Magnification ×2. B: changes in fetal rat lung structure after maternal nitrofen exposure. As expressed by the percentage increase in distal branching between day 0 and day 3, nitrofen explants grew rapidly and demonstrated a significant increase in branching and complexity. *P < 0.01 vs. control, n = 6 explants in each group.
SU-5416, a VEGFR inhibitor. Overall, these findings strongly suggest that reduced VEGF signaling is associated with the lung developmental changes observed in the nitrofen model of CDH.

Previous studies have explored the role of NO in branching morphogenesis in the normal fetal rat lung explant (54) and in nitrofen-induced CDH (46). Shinkai et al. (46, 47) showed that NO treatment increases branching in fetal lung explants after nitrofen exposure, but reported no change in eNOS expression. In contrast, North et al. (41) reported decreased lung eNOS mRNA and protein content in this model. As observed by Shinkai et al., we found that eNOS...
protein level was unchanged after maternal nitrofen exposure. However, treatment with exogenous NO enhanced lung branching and growth in the nitrofen group, suggesting that perhaps NO production or bioavailability is reduced, despite sustained eNOS expression. Alternatively, lung eNOS expression may be reduced early in this model, and lung eNOS content returned to baseline values while maintaining the explant in culture.

Mann et al. (38) previously reported that inhaled NO improved survival of newborn rats after prenatal nitrofen exposure, but mechanisms leading to improved outcomes was not studied. We found that NO enhanced lung growth after nitrofen exposure and SU-5416 treatment, and this may be due to NO-mediated reduction of apoptosis within the fetal lung mesenchyme. Past work has shown increased apoptosis due to oxidant stress after nitrofen treatment (22, 34), suggesting that NO may reduce apoptosis through its antioxidant effects. Since SU-5416 treatment also increased lung apoptosis, we speculate that impaired VEGF signaling after nitrofen may increase apoptosis, which is protected by treatment with NO. Previous work from our laboratory demonstrated that exposure of neonatal rats with inhaled NO improved prenatal lung alveolar and vascular growth after SU-5416 treatment in vivo (48). As observed in our explant studies, SU-5416 treatment markedly increased lung apoptosis, primarily in vascular endothelium, and inhaled NO inhibited endothelial apoptosis in this model (49). Overall, these findings support our speculation that nitrofen may induce mesenchymal apoptosis due to impaired VEGF signaling and that NO enhances lung growth by preserving endothelial survival and function.

Previous studies have explored mechanisms of nitrofen-induced abnormalities of lung structure, but to our knowledge, this is the first study to specifically explore the effects of nitrofen on air space growth using fetal explants at a later period of gestation. Other studies have studied lung explants at an earlier stage of development, generally at E13.5 (11, 13, 14, 44–46). As observed in our study, these previous studies reported that maternal nitrofen exposure reduces lung size and structure by nearly 50% (13). Thus, in addition to its embryonic effects, nitrofen exposure can impair distal lung complexity during later stages of fetal lung development.

In addition, we found that nitrofen-exposed fetal explants initially had significantly fewer branches than control lungs at day 0, which rapidly increased to achieve a similar level of complexity at day 3. A previous study, using fetal rat explants, reported a significant decrease in the number of branches following maternal nitrofen exposure at E13 or E14, but whether this difference in branching persisted throughout gestation was not examined (25). Our study is the first to demonstrate spontaneous recovery in branching morphogenesis after removal of the nitrofen-induced lung. The rate of branching morphogenesis in vitro is greater in the nitrofen group, suggesting catch-up-like growth following withdrawal of the lung explants from the maternal environment. In comparison, Keijzer and colleagues (32) did not find differences in branching in maternal exposed fetal lung explants, which may be due to sustained in vitro treatment with nitrofen. Montedonico and colleagues (39) described a persistent difference in branching after 96 h in culture but did not take into consideration preexisting differences in branching at the day of harvesting. As opposed to past studies, our experiments were conducted under low oxygen (3%) conditions to reproduce the fetal oxygen environment, as fetal lung branching morphogenesis is enhanced in culture in low oxygen tension (3, 18, 19, 52).

Interestingly, we found that VEGF protein is downregulated in both nitrofen explants and SU-5416-treated explants, but eNOS expression is downregulated in SU-5416 explants but preserved following maternal nitrofen exposure. The latter could explain why nitrofen-treated explants spontaneously recovered despite dramatic decrease in VEGF expression as opposed to SU-5416-treated explants. NO signaling is a key regulator of branching morphogenesis, promoting lung branching as suggested by the restoration of both eNOS and VEGF protein level in SU-5416-treated explants after NO treatment.

Fig. 10. NO treatment increases VEGF protein level in nitrofen-exposed fetal lung explants (pooled samples).

Fig. 11. Effects of NO synthase inhibition on lung growth in fetal lung explants. As shown, treatment of lung explants with nitro-L-arginine (LNA), a NOS inhibitor, reduced lung branching by 40%, which was restored by treatment with NO and 8-bromo-GMP (†P < 0.01 vs. controls, *P < 0.05 vs. LNA treatment). 8Bromo, 8-bromo-guanosine monophosphate.
NO has previously been shown to enhance fetal lung development, and VEGF-induced pulmonary angiogenesis is partly mediated by NO (7, 51). Impaired NO production and loss of its regulation could explain some of the abnormalities observed in nitrofen fetal explants and following VEGFR inhibition.

A potential limitation of this study is the use of the nitrofen as an experimental model for CDH, which induces similar structural changes as observed in human CDH, but whether or not this model shares pathogenic mechanisms as observed in human CDH is unclear.

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Fig. 12. Effects of SU-5416 and nitrofen on apoptosis in fetal rat lung explants. Compared with control explants (A), SU-5416 (B) and nitrofen (C) increased active caspase-3 immunofluorescence throughout the lung mesenchyme.

Fig. 13. Effect of NO treatment on apoptosis in SU-5416 and nitrofen-treated fetal rat lung explants. Active caspase-3 immunofluorescence is decreased following NO treatment (B) compared with untreated SU-5416 explants (A) and after nitrofen exposure (C and D).
In summary, we found that maternal exposure to nitrofen decreases lung VEGF expression, increases lung apoptosis, and alters lung structure. As VEGFR inhibition induces many features observed in fetal lung explants after nitrofen exposure, we speculate that nitrofen-induced disruption of VEGF signaling may contribute to lung hypoplasia in this model. We further speculate that NO either restores or improves distal lung growth after VEGFR inhibition and nitrofen treatment via anti-apoptotic effects and that impaired NO activity may contribute to lung hypoplasia in this experimental model of CDH.

REFERENCES


