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Antenatal BAY 41-2272 reduces pulmonary hypertension in the rabbit model of congenital diaphragmatic hernia

Vuckovic A, Herber-Jonat S, Flemmer AW, Strizek B, Engels AC, Jani JC. Antenatal BAY 41-2272 reduces pulmonary hypertension in the rabbit model of congenital diaphragmatic hernia. Am J Physiol Lung Cell Mol Physiol 310: L658–L669, 2016. First published February 12, 2016; doi:10.1152/ajplung.00178.2015.—Infants with congenital diaphragmatic hernia (CDH) fail to adapt at birth because of persistent pulmonary hypertension (PH), a condition characterized by excessive muscularization and abnormal vasoreactivity of pulmonary vessels. Activation of soluble guanylate cyclase by BAY 41-2272 prevents pulmonary vascular remodeling in neonatal rats with hypoxia-induced PH. By analogy, we hypothesized that prenatal administration of BAY 41-2272 would improve features of PH in the rabbit CDH model. Rabbit fetuses with surgically induced CDH at day 23 of gestation were randomized at day 28 for an intratracheal injection of BAY 41-2272 or vehicle. After term delivery (day 31), lung mechanics, right ventricular pressure, and serum NH2-terminal-pro-brain natriuretic peptide (NT-proBNP) levels were measured. After euthanasia, lungs were processed for biological or histological analyses. Compared with untouched fetuses, the surgical creation of CDH reduced the lung-to-body weight ratio, increased mean terminal bronchial density, and impaired lung mechanics. Typical characteristics of PH were found in the hypoplastic lungs, including increased right ventricular pressure, higher serum NT-proBNP levels, thickened adventitial and medial layers of pulmonary arteries, reduced capillary density, and lower levels of endothelial nitric oxide synthase. A single antenatal instillation of BAY 41-2272 reduced mean right ventricular pressure and medial thickness of small resistive arteries in CDH fetuses. Capillary density, endothelial cell proliferation, and transcripts of endothelial nitric oxide synthase increased, whereas airway morphometry, lung growth, and mechanics remained unchanged. These results suggest that pharmacological activation of soluble guanylate cyclase may provide a new approach to the prenatal treatment of PH associated with CDH.

Congenital diaphragmatic hernia; pulmonary hypertension of the newborn; BAY 41-2272; rabbit model

CONGENITAL DIAPHRAGMATIC HERNIA (CDH) is a life-threatening anomaly that occurs in 1 of 3,000 live births (64). It is characterized by protrusion of abdominal viscera into the thorax through a diaphragmatic defect and impaired growth and maturation of fetal lungs resulting in pulmonary hypoplasia. Besides airway and alveolar underdevelopment (4), CDH consists of a reduced number of pulmonary vessels per lung volume unit, excessive medial and adventitial thickening of pulmonary arteries, extension of muscularization into small intra-acinar arterioles, and reduced vascularization of the alveolar surface (27, 41, 56). Additionally, hypermuscularized pulmonary arteries display abnormal vasoreactivity, which originates from the combination of inappropriate synthesis of vasoactive mediators by endothelial cells and altered responsiveness of vascular smooth muscle cells (48). Consequently, structural and functional vascular abnormalities contribute to the failure of pulmonary vascular resistance to fall after birth, leading to persistent pulmonary hypertension (PH) of the newborn (48). Significant PH is a common clinical finding in CDH neonates, resulting in right-to-left shunting, hypoxemia, and acute right heart failure in the most severely affected patients (48). Despite additive postnatal therapies, PH associated with CDH lacks a causal therapy and remains a major determinant of outcome in CDH infants (69).

The nitric oxide (NO)-guanosine 3’,5’-cyclic monophosphate (cGMP) signaling pathway is a key regulator of pulmonary angiogenesis (32, 34) and perinatal vascular tone (25). Vascular endothelial cells produce NO under the action of endothelial NO synthase (eNOS), which converts L-arginine to L-citrulline (25). Upon activation by NO, soluble guanylate cyclase (sGC) synthesizes the second messenger cGMP, which modulates downstream effectors such as cGMP-dependent protein kinases and phosphodiesterases (PDEs) for the control of smooth muscle relaxation, cell growth and differentiation, or platelet aggregation (25). PDEs may in turn hydrolyze cGMP to regulate the signaling (25). The pathophysiology of PH related to CDH involves various disorders of NO/cGMP signaling in endothelial and vascular smooth muscle cells, including decreased eNOS expression (2, 9, 46, 55, 58, 66), lower activity or content of sGC (15, 57, 63), and increased PDE5 activity (15, 42). These alterations participate in the refractory response of CDH lungs to inhaled NO. Therefore, stimulating cGMP production in CDH fetuses might be effective in restoring the normal architecture and reactivity of the pulmonary vasculature. The pulmonary and systemic vasodilator BAY 41-2272 is a direct pharmacological activator of sGC that increases the production of cGMP in an NO-independent manner (59). Insofar as BAY 41-2272 reduces vascular smooth muscle proliferation (37) and stimulates neovessel formation (49), we hypothesized that antenatal BAY 41-2272 would stimulate capillary formation, decrease pulmonary arterial remodeling, and reduce functional signs of PH in the fetal rabbit model of CDH.

METHODS

Animals. Forty-nine New Zealand White female rabbits were obtained at 15 days of gestation from an authorized farm. They were...
housed as previously reported (66). The Institutional Ethics Committee of the “Université Libre de Bruxelles” (Brussels, Belgium) approved the protocol. All experiments respected U.S. National Institutes of Health guidelines for laboratory animal care and use.

Experimental protocol and tissue sampling. Anesthesia and general surgical procedures have been described elsewhere (65, 66). The feasibility of tracheal injection of medication has already been shown in the fetal rabbit (17, 33). Briefly, at 23 days of gestation (pseudoglandular stage of lung development), two ovarian-end fetuses per doc were subjected to the surgical creation of diaphragmatic hernia (DH) through a left thoracotomy (23). A stock solution of BAY 41-2272 (Sigma-Aldrich, Diegem, Belgium) was prepared at 20 mg/ml in DMSO and further diluted in sterile water to obtain doses of 2 mg/kg body wt in a 300 µl injection volume (<1% DMSO in final concentration). Solutions were then sterilized through filters. At 28 days (early sacral stage), DH fetuses were randomly assigned to a single tracheal injection of BAY 41-2272 (DH+BAY) or vehicle (DH+vehicle) with a 29-gauge needle, followed by sealing of the small tracheal hole with sterile fibrin glue (Tissucol Duo 0.5 ml, Baxter, Braine-l’Alleud, Belgium). In pilot experiments, tracheal injection of BAY 41-2272 using doses up to 16 mg/kg body wt did not affect fetal survival, body weight, or organ weights (data not shown). Moreover, the safety of fibrin sealant has already been reported in rabbit fetuses (33, 45). At term (31 days), living fetuses were delivered by cesarean section and weighed. Nonoperated fetuses of similar size were considered as controls. Operated and control fetuses were randomly allocated to postnatal investigations (Fig. 1). A first set of fetuses was euthanized before the first breath, to avoid pulmonary changes occurring at and after birth. Their lungs were immediately snap frozen in liquid nitrogen and stored at −80°C for biological studies. A second set of rabbit pups was anesthetized at birth and intubated for the assessment of postnatal lung mechanics followed by intratracheal instillation of BAY 41-2272. Rabbit fetuses underwent a partial excision of the diaphragm at day 23. They were randomized for intratracheal instillation of BAY 41-2272 (DH+BAY group) or vehicle (DH+vehicle group) at day 28. Nonmanipulated littersmates were used as controls. After term birth (day 31), live fetuses were randomly allocated to functional analyses (respiratory mechanics and hemodynamics) and lung histology or biological analyses performed on whole lung tissue [quantitative PCR (qPCR) and immunoblotting].

**Fig. 1.** Experimental design for the creation of diaphragmatic hernia (DH) in fetal rabbits and intrapulmonary administration of BAY 41-2272. Rabbit fetuses underwent a partial excision of the diaphragm at day 23. They were randomized for intratracheal instillation of BAY 41-2272 (DH+BAY group) or vehicle (DH+vehicle group) at day 28. Nonmanipulated littersmates were used as controls. After term birth (day 31), live fetuses were randomly allocated to functional analyses (respiratory mechanics and hemodynamics) and lung histology or biological analyses performed on whole lung tissue [quantitative PCR (qPCR) and immunoblotting].
in arteries with a complete muscular coat and intact elastic lamina.

Moreover, avidin-biotin-peroxidase immunohistochemistry using 3,3′-diaminobenzidine (DAB) staining was performed as previously described (65) by using mouse monoclonal anti-CD31 (1:50; M0823, Dako, Heverlee, Belgium) and anti-Ki67 (1:50; clone MIB-1, Dako) antibodies, which were used to detect endothelial and proliferating cells, respectively. Sections were lightly counterstained with hematoxylin. Negative controls were performed by primary antibody omission. The pixel area of positive CD31 staining (i.e., DAB-positive area) was determined with the ImageJ program by using the color deviation plug-in in a minimum of 10 random nonoverlapping fields per study subject at a magnification of ×40. Results were expressed as a ratio of pixel area of air-exchanging parenchymal lung tissue. The same method was applied to measure Ki67 area (i.e., DAB-positive nuclei) and the area of total nuclei (i.e., hematoxylin-stained area). Results were expressed as a percentage of Ki67-positive nuclei. In all cases, the peripheral parenchyma excluding blood vessels and bronchi was considered for measurements.

**Real-time quantitative PCR.** Total RNA from whole frozen lung specimens was isolated by using TRIzol reagent (Life Technologies, Ghent, Belgium). RNA concentration and integrity were assessed by spectrophotometry and 1.5% agarose gel electrophoresis (66). Before reverse transcription (66), the absence of genomic DNA was verified by real-time quantitative PCR (qPCR) on total RNA. As previously detailed (66), SYBR Green qPCR analysis was carried out in triplicate by using previously published primer sequences (66) for eNOS, vascular endothelial growth factor (VEGF), and corresponding receptors (VEGFR1 and VEGFR2). Newly designed primers for rabbit endothelin-1 gene (ET-1; accession no. NM_001101696) were CAAGCAGGAACGGAACTCA (forward) and TTGGAGCAGTGCTTTGCGT (reverse). To ensure the quality of measurements, negative and positive controls were included. Expression levels of target genes were expressed according to the efficiency-corrected model and normalized by the geometric mean of SDHA and ATP5B, which were identified as the most suitable normalizers among 10 candidate housekeeping genes tested (65, 66).

**Western blotting.** Protein extraction, immunoblotting, and densitometric analysis were performed as previously described (65). Briefly, 50 μg of total lung protein was separated by electrophoresis on 4–12% gradient SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were incubated with mouse monoclonal anti-CD31 antibody (1:1,000; M0823, Dako) followed by stripping and reprotein with GAPDH (1:5,000; Sigma-Aldrich). By using the NIH ImageJ gel analysis tool, signal intensities for CD31 protein were quantified by densitometry considering the integral of the entire optical density profile. Values were then normalized to those of GAPDH (26). Because samples were run on different gels, a standard sample of lysates from three intact rabbit lungs was run in each gel to correct for technical variations. Western blot analysis was done twice to ensure reproducibility of the results.

**Statistical analysis.** Values are reported as means ± SE. After ensuring normality and equality of variance, multiple comparisons were performed with one-way ANOVA or the Kruskal-Wallis test followed by the Bonferroni correction. Correlations were made with Pearson’s coefficient (r). A P value < 0.05 for two-tailed tests was considered significant (SPSS software, IBM, Armonk, NY).

**RESULTS**

**Surgical results and gross anatomical findings.** All does survived the two operations. The fetal survival rate after DH creation was 64% (63/99). Fetal survival rates after tracheal instillation of vehicle or BAY 41-2272 were not different (79 and 63%, respectively, P = 0.283). Moreover, the fetal body weight of DH+vehicle and DH+BAY fetuses did not differ from that of control fetuses, indicating good tolerance of fetal surgery and instilled substances (Fig. 2A). As an index of lung hypoplasia (5), the lung-to-body weight ratio, which was decreased by 45% in DH+vehicle fetuses compared with controls, did not improve after antenatal BAY 41-2272 therapy (Fig. 2B). Since heart hypoplasia has been described in experimental CDH (38), heart-to-body weight ratio was calculated as a marker of global heart hypoplasia. Values were, however, similar between groups (Fig. 2C).

**Effects of antenatal BAY 41-2272 on neonatal pulmonary hypertension.** To determine whether antenatal BAY 41-2272 improved neonatal PH, functional and morphological hallmarks of PH were evaluated in the rabbit model, such as right ventricular pressure, serum NT-proBNP as a marker of right ventricular overload, right ventricular hypertrophy index, and wall remodeling of pulmonary arterioles. First, direct measurements of right ventricular pressure were performed at room air pressure in open chest rabbit pups that were ventilated and sedated. Heart rate was not different between the three study groups (Fig. 3A). Mean right ventricular pressure, which increased by 38% in DH+vehicle fetuses, dropped to the level of controls in DH+BAY fetuses (Fig. 3B). Second, serum levels of NT-proBNP, which increased in the DH+vehicle group compared with controls (Fig. 3C), were not significantly reduced after treatment. Mean values in the DH+BAY group tended to be higher than in controls, but the difference was not
significant after post hoc correction \((P = 0.06)\). Mean right ventricular pressure values correlated with serum NT-proBNP levels (Fig. 3D), indicating the utility of this biomarker in reflecting PH immediately after birth in the rabbit model. Third, the right ventricular hypertrophy index, which rises after prolonged right ventricle pressure afterload, was not changed after DH creation or modified after antenatal BAY 41-2272 therapy (Fig. 3E). Finally, the effects of prenatal BAY 41-2272 injection on pulmonary vascular morphometry were assessed. Intra-acinar arteries with an external diameter less than 30 μm (Fig. 4A, bottom row) showed a rise in proportionate medial thickness after DH creation (Fig. 4B). The adventitial thickness, which was indistinguishable from surrounding connective tissue, could not be measured. In intra-acinar arteries with a diameter between 30 and 60 μm (Fig. 4A, middle row), medial and adventitial layers were thicker in the DH+vehicle group than in controls (Fig. 4, B and D). In larger arteries (Fig. 4A, top row), only the proportionate adventitial thickness increased after surgical DH (Fig. 4D) despite apparent, but nonsignificant, hypermuscularization (Fig. 4B). Antenatal BAY 41-2272 significantly attenuated the muscularization of the smallest pulmonary arteries (Fig. 4B) but had no effect on adventitial thickness (Fig. 4D). The proportionate medial thickness of small intra-acinar arteries was positively correlated with mean right ventricular pressure, which was consistent with the fact that small arterioles are the primary site of vascular resistance (Fig. 4C). Overall, these changes indicate an attenuation of PH in neonatal DH rabbits that received prenatal BAY 41-2272.

**Pulmonary effects of antenatal BAY 41-2272 on endothelial homeostasis.** Since BAY 41-2272 promotes angiogenesis in vitro (49), endothelial cell function, proliferation, and density were investigated. First, gene expression analyses in whole lungs were conducted at the term of gestation, just before the first breath. The following genes were considered: VEGF pathway components, as VEGF is the prototype mitogen for endothelial cells; eNOS, as NO is a crucial endothelium-derived relaxing factor and a downstream mediator of VEGF-induced angiogenesis; and ET-1, the potent vasoconstrictor synthesized by the vascular endothelium (25). Transcripts of eNOS were downregulated in DH+vehicle lungs but returned to control levels after prenatal BAY 41-2272 (Fig. 5A). Transcripts of the other genes were not changed by surgically induced DH, nor were they modulated by fetal therapy (Fig. 5B). Second, the effects of prenatal BAY 41-2272 on capillary bed formation were evaluated by using the endothelial marker CD31. Protein abundance of CD31 was similar in control and DH+vehicle fetuses, but increased after antenatal BAY 41-2272 (Fig. 5; semiquantified in Fig. 5D). Yet, whole lung analyses encompassed the expression of CD31 in endothelial cells from alveolar capillaries and blood vessels. Therefore, immunoblotting was completed by measurement of capillary density in the distal lung, except for blood vessels. The CD31 immunostained area relative to the surface area of parenchyma,
which reflects the capillary network, decreased by 57% in DH+vehicle lungs compared with controls, and increased to control levels after antenatal BAY 41-2272 (Fig. 5E; semi-quantified in Fig. 5F). Third, the proliferation marker Ki67 was immunolocalized to determine whether increased capillary bed was related to a rise in cell proliferation. Ki67 is a nuclear antigen expressed during all active stages of cell division, but not detected in quiescent cells (10). In all study groups, Ki67 was similarly evidenced in the nuclei of bronchial epithelial cells (Fig. 5G, left photomicrographs). Regarding pulmonary blood vessels, Ki67 was detected in the capillary bed (Fig. 5G, right photomicrographs) but was absent from endothelial and mural cells of arteries (Fig. 5G, left photomicrographs). This was in accordance with the highly proliferative potential of capillary endothelial cells, whereas cells from mature pulmonary arteries replicate poorly (34). In the DH+BAY group, there was an apparent increase in Ki67 expression within alveolar capillaries (Fig. 5G, right photomicrographs). This was verified by semiquantitative evaluation of nuclear staining, considering the alveolar parenchyma while excluding blood vessels and bronchi. Consistent with the physiological decline in cell proliferation toward apoptosis with advancing gestation (16, 54), Ki67 was faintly detected in alveolar epithelial cells or interstitial fibroblasts. Consequently, the whole lung periphery could be reasonably taken into account to reflect Ki67 expression in alveolar capillaries. The percentage of nuclei immunostained for Ki67 was approximately eight times higher in DH fetuses receiving BAY 41-
2272 than in fetuses exposed to the vehicle (Fig. 5H). Apparent differences with respect to the control group were not meaningful after post hoc correction. Together, these changes might suggest enhanced angiogenesis after antenatal BAY 41-2272 treatment.

**Effects of antenatal BAY 41-2272 on pulmonary hypoplasia.** Because adequate development of the peripheral lung requires a well-developed capillary network (35), respiratory mechanics and airway morphometry were evaluated to determine whether enhanced angiogenesis after BAY 41-2272 treatment promoted
the development of hypoplastic airway structures. Consistent with lung-to-body weight ratio values (Fig. 2), total lung capacity, a functional marker of lung size (23), was reduced after DH creation and unchanged by BAY 41-2272 therapy (Fig. 6A). Likewise, static compliance of the whole respiratory system was consistently reduced in DH fetuses receiving the vehicle or BAY 41-2272 (Fig. 6B). Besides, the forced oscillation technique was applied to evaluate mechanical properties of central airways (i.e., Newtonian resistance) and viscoelastic properties of the lung parenchyma (i.e., tissue damping and tissue elastance). The ratio of tissue damping, a parameter of energy dissipation, to tissue elastance, an indicator of lung stiffness, is known as hysteresivity and reflects heterogeneity owing to changes in connective tissue and/or surface-acting forces (23). DH fetuses receiving the vehicle or BAY 41-2272 had higher Newtonian resistance (Fig. 6C), tissue damping (Fig. 6D), and tissue elastance (Fig. 6E) compared with controls. Hysteresivity was significantly lower in the DH/H11001 BAY 41-2272 group compared with controls.

Fig. 5. Endothelial homeostasis following prenatal administration of BAY 41-2272 or vehicle in rabbit neonatal lungs with diaphragmatic hernia (DH). A and B: lung expression of genes controlling vasoreactivity and/or angiogenesis (eNOS, endothelial nitric oxide synthase; ET-1, endothelin-1; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor). Data normalized against the geometric mean of validated housekeeping genes were expressed as a percentage of controls, which was set to 1. **P < 0.01 vs. controls; †P < 0.05 vs. DH+vehicle (1-way ANOVA and Bonferroni correction; n = 7–10 per group). C and D: whole lung immunoblotting and corresponding densitometric analysis for the endothelial marker CD31. Data normalized against GAPDH values were expressed as a percentage of controls, which was set to 1. *P < 0.05 vs. controls (1-way ANOVA and Bonferroni test; n = 6–8 per group, distributed in two different gels processed in parallel). E: representative lung sections from the 3 study groups showing lung peripheral tissue immunostained for CD31 (brown stain). High-magnification views of gas-exchanging region correspond to the magnified area demarcated by hatched lines in left photomicrographs. Scale bars = 50 μm. F: measurements of percentage of Ki67-stained nuclei in the peripheral parenchyma. †P < 0.05 vs. DH+vehicle (1-way ANOVA and Bonferroni test; n = 6–9 per group). A.U., arbitrary units; N.S., not significant.

Fig. 6. Functional features of pulmonary hypoplasia in rabbit pups with diaphragmatic hernia after prenatal BAY 41-2272 therapy (DH+BAY) or vehicle (DH+vehicle). A and B: lung mechanics of the whole respiratory system (TLC, total lung capacity; Cst, static compliance). C–F: respiratory input impedance partitioned into airway (Rw, Newtonian resistance) and tissue components (Gt, tissue damping; Ht, tissue elastance; ηt, Gt/Ht). *P < 0.05 and ***P < 0.001 vs. controls (1-way ANOVA and Bonferroni correction; n = 10–17 per group). A.U., arbitrary units; N.S., not significant.
group compared with controls (Fig. 6F). Apparent lower values in the DH+/vehicle group compared with controls failed to reach significance after post hoc correction ($P = 0.065$). However, treated and untreated DH lungs showed comparable values for hysteresivity.

Morphometric measurements of airways were in accordance with unimproved lung tissue mechanics after BAY 41-2272 therapy. As such, mean terminal bronchial density, a parameter inversely proportional to the number of alveoli surrounding each bronchiole, was higher in DH+/vehicle lungs than in controls (Fig. 7B). The apparent decrease after antenatal BAY 41-2272 did not reach significance. Differences between groups in percentage of space occupied by lung tissue were not meaningful (Fig. 7C). Finally, mean wall transection length, which reflects the thickness of alveolar septa, was similar between the study groups (Fig. 7D). These findings suggest that prenatal activation of sGC by BAY 41-2272 did not significantly improve functional and morphological features of pulmonary hypoplasia in DH rabbits.

DISCUSSION

The limited success of pulmonary vasodilator therapies in PH associated with CDH has led to the development of experimental strategies to improve fetal pulmonary vascular growth and adaptation to extrauterine life. Considering the role of the disturbed NO/cGMP pathway in CDH pathogenesis and the benefit of BAY 41-2272 therapy in neonatal rats with hypoxia-induced PH (19), we presumed that antenatal BAY 41-2272 would counteract lung vascular impairment and attenuate the characteristics of PH in the rabbit model of CDH. Because pulmonary vasodilation and release of transpulmonary cGMP have been reported after inhalation of BAY 41-2272 (21), intratracheal instillation was herein considered as an attractive means of selective intrapulmonary drug delivery (11). This route of administration, which appears to be more efficient in targeting the fetal lung than intra-amniotic delivery (11), was previously used in fetal rabbits to modulate lung growth and maturation (17, 33). BAY 41-2272 was administered at the early saccular stage, when development of intracinar blood vessels and capillary expansion normally take place (34). Considering the short duration of the late developmental stages in rabbit lungs, brief exposure of the pulmonary epithelium to BAY 41-2272 may actually represent weeks on the scale of human gestation (51). We demonstrated that antenatal BAY 41-2272 reduced right ventricular pressure and hypermuscularization of pulmonary resistive arteries. Moreover, antenatal BAY 41-2272 increased eNOS transcript levels, capillary bed formation, and endothelial cell proliferation, which play a role in improved angiogenesis. However, BAY 41-2272 did not ameliorate lung growth and respiratory mechanics. These findings offer novel opportunities for the prenatal therapy of CDH, with PH as a specific target.

Failure of the transition from fetal to neonatal life leads to persistent PH of the newborn, which is defined as an abnormal state of sustained elevation of pulmonary artery pressure with right-to-left shunting through the ductus arteriosus and the foramen ovale resulting in profound hypoxemia (60). Apart from CDH, in which PH develops as a primary consequence of the underdeveloped pulmonary vasculature, failure of pulmonary vascular resistance to decrease at birth can arise from the functional maladaptation of an otherwise anatomically normal pulmonary circulation secondary to parenchymal lung disease.
of the newborn or perinatal distress (60). This frequent and reversible type of PH can be mimicked in neonatal animals exposed to acute or chronic hypoxia, intratracheal instillation of meconium, or infection. Moreover, neonatal PH can result from excessive muscularization and reduced vascular density without primary parenchymal lung disease (60). This form, referred to as idiopathic PH, has been related to antenatal constriction of the ductus arteriosus, which can be surgically induced in the fetal lamb. In this model, ductal ligation during late gestation generates severe pulmonary vascular remodeling and decreases angiogenesis (1). Interestingly, sustained intrauterine PH further impairs alveolarization and lung weight in utero (29).

In the absence of medical interventions targeting neonatal PH, a vicious circle of hypoxemia-vasoconstriction leads to right heart failure with fatal outcome. Therefore, cardiac evaluation is crucial for diagnosing PH and its severity and evaluating response to vasodilator therapies (48). Besides right ventricle dilatation at early neonatal echocardiography (43), rabbit CDH pups showed increased right ventricular pressure and serum NT-proBNP levels, which are good indicators of PH (48, 61). In accordance with fetal CDH lambs (38), but in contrast to fetal lambs with chronic intrauterine PH due to ductus arteriosus ligation (1), right ventricular hypertrophy index did not rise in rabbit CDH pups at birth, suggesting that PH and right ventricular hypertrophy did not develop prenatally. Indeed, increased right ventricular afterload following CDH-induced pulmonary vascular remodeling in utero would not greatly affect right heart function, because of flow redistribution through fetal right-to-left shunting. Preserved right heart contractility in human fetuses with severe CDH (18) and stable right ventricular overload markers in fetal rats with nitrofen-induced CDH at the term of gestation (8) support this assumption. A single antenatal instillation of BAY 41-2272 reduced right ventricular pressure but did not significantly decrease NT-proBNP levels, indicating attenuation, but not complete reversal, of PH. Because hemodynamic evaluation was performed 3 days after drug administration, functional effects were unlikely to be due to the persistence of direct pulmonary vasodilation. Consequently, we hypothesized that decreased medial remodeling of pulmonary arteries would participate in reduced right ventricular pressure after BAY 41-2272 therapy. According to the preferential localization of sGC in smooth muscle cells from the distal arterial tree of fetal lungs (13), antenatal BAY 41-2272 lessened medial hypertrophy of the smallest intra-acinar arteries, which represented the main site of vascular resistance. In agreement with the absence of sGC expression in adventitial cells of fetal lungs (13), antenatal BAY 41-2272 did not meaningfully change the adventitial thickness of pulmonary arteries. This may partially explain why PH did not fully reverse after prenatal treatment.

During late lung development, the pulmonary capillary network extends by sprouting from the preexisting vessels by angiogenesis, a process that requires NO/cGMP synthesis in response to VEGF (34). Defective angiogenesis in human and experimental CDH has been ascribed to low eNOS expression (2, 9, 46, 57). As confirmatory findings, rabbit CDH lungs displayed reduced eNOS transcripts and blood capillary density. In addition, downregulated eNOS cooccurred with VEGF overexpression in human CDH fetuses (9) and in rats with nitrofen-induced CDH (47), which was confirmed in vitro in endothelial cells harvested from fetal lambs with surgically induced CDH (2). This was interpreted as an inefficient attempt to overcome vascular hypoplasia (9). Nevertheless, decreased VEGF levels were associated with pulmonary artery endothelial cell dysfunction in the fetal lamb model of chronic intrauterine PH (28, 31). The reason for discrepancies in VEGF expression between animal models is not clear, but one putative mechanism might be related to the function of alveolar epithelial type II cells, which produce VEGF at late stages of lung development. On the one hand, decreased basal lung expansion associated with experimental CDH promotes an alveolar epithelial type II cell phenotype and delays the physiological differentiation into type I cells (14, 62). On the other hand, the acute rise in pulmonary blood flow after ductus arteriosus ligation followed by chronic remodeling of the vascular bed generates a lack of surfactant-associated protein C, which is the specific marker of alveolar epithelial type II cells (29). With regard to prenatal therapies targeting PH, VEGF restored eNOS expression and reduced pulmonary vascular remodeling in fetal lambs with intrauterine PH (30), whereas, in the present study, antenatal BAY 41-2272 increased eNOS transcripts, CD31 protein abundance, blood capillary density, and endothelial cell proliferation in rabbit CDH lungs, without changes in VEGF pathway components. This suggests that BAY 41-2272 might promote angiogenesis even in the absence of increased VEGF levels.

Since disruption of angiogenesis causes alveolar hypoplasia (35), improved vascular bed formation after antenatal BAY 41-2272 would theoretically enhance airway development. Besides, alveolization is impaired in mice deficient for the sGCα1 subunit (6). However, BAY 41-2272 did not ameliorate lung hypoplasia, as hinted at by decreased total lung capacity and lung-to-body weight ratio. Likewise, the remodeling of small airways appeared to be maintained in hypoplastic lungs receiving BAY 41-2272, as suggested by increased Newtonian resistance (23). Finally, impaired lung hysteresivity was in agreement with the persistence of thick septal walls, which suggests that BAY 41-2272 did not modify the parenchymal structure. These findings were consistent with unchanged airway morphometry after BAY 41-2272 treatment in hypoxia-induced PH in postnatal rats (19). Because alveolization involves complex and numerous molecular pathways (44), restoring eNOS levels and improving angiogenesis after BAY 41-2272 therapy may not be enough to rescue alveolar development in hypoplastic lungs.

In addition to promoting angiogenesis, the NO/cGMP pathway participates in the adaptation of pulmonary circulation to extrauterine life. Hence, dysfunctional or misexpressed eNOS contributes to the maintenance of high vasoreactivity not solely in the underdeveloped CDH vasculature (48) but also in other causes of neonatal PH (60). This was a rationale to administer inhaled NO to improve oxygenation as a replacement therapy for neonatal PH. However, decreased response and inability to sustain a response to inhaled NO are common features associated with neonatal PH (60). In CDH infants with left ventricular dysfunction, inhaled NO can worsen pulmonary edema and thus aggravate PH (60). Among the possible mechanisms explaining poor responsiveness to inhaled NO, alterations of downstream smooth muscle enzymes have also been experimentally described in experimental CDH and ovine fetuses with chronic intrauterine PH, including lower activity of sGC.
(12, 15, 63). Nevertheless, prenatal activation of sGC using BAY 41-2272 could still improve pulmonary arterial remodeling and angiogenesis in the rabbit model of CDH. Considering the previous literature, hypothetical mechanisms may be proposed to reconcile the positive effects of BAY 41-2272 in utero and lower sGC activity in CDH. NO activates sGC by heme binding, but oxidization of the heme moiety renders the enzyme insensitive to NO (59). Since alterations of the redox state have been reported in rat and sheep CDH lungs (2, 22), one possible hypothesis might be related to the ability of BAY 41-2272 to enhance sGC even in the context of oxidative stress (59). This is in keeping with potent vasodilation after sGC activation in rabbit fetuses with CDH (50) and ovine fetuses with chronic intrauterine PH (12, 20). Hence, one can hypothesize that antenatal BAY 41-2272 induces a sudden drop in fetal pulmonary pressure, a surge in pulmonary blood flow, and a subsequent rise in shear stress. These events may upregulate eNOS transcription (7) as a result of increased c-Jun activity or reduced protein kinase C inhibitory activity (25) and thus improve angiogenesis by forcing NO/cGMP synthesis. However, because the eNOS is dysfunctional in CDH, this first hypothetical mechanism is probably very limited. As a second and more probable mechanism, BAY 41-2272 might promote angiogenesis directly even in the absence of VEGF overexpression. Indeed, BAY 41-2272 induces the formation of capillary-like structures in vitro independently of VEGF (49). Apart from angiogenesis, NO downregulates transcripts of ET-1 in vitro via the action of sGC (40). Furthermore, blockade of ET receptor type A reduces medial wall thickness in neonatal rats with hypoxia-induced PH (3). Yet decreased ET-1 mRNA levels did not accompany increased eNOS expression after fetal treatment in the present study. As an alternative hypothesis, decreased pulmonary arterial remodeling might be explained directly by antiproliferative actions of BAY 41-2272 on vascular smooth muscle cells (37) or indirectly by modulation of growth factor activity such as TGF-β1 (67).

The rabbit model represents a valuable option in evaluation of antenatal therapies for CDH, compared with surgically induced CDH lambs, which are difficult to handle and have low fetal survival rates (57), and nitrogen-exposed CDH rats, in which physiological measurements are more challenging to obtain (42). However, some limitations should be acknowledged. Technical obstacles related to rabbit pup size prevented pharmacokinetic evaluation following intratracheal administration of BAY 41-2272. Moreover, nonmanipulated littersmates instead of sham-operated fetuses receiving the vehicle were used as external controls. Theoretically, sham surgery would have taken into consideration possible impairment in lung development owing to oligohydramnios and chest scarring after DH creation (65), pulmonary stretch induced by liquid instillation (17), and surgically induced inflammation (65). However, previous reports have shown that sham-operated rabbit fetuses did not display real features of lung hypoplasia (53, 68). Thus presumed differences between sham-operated and untouched littersmates were probably minor. In the present study, DH fetuses were randomized for the same injection volume of BAY 41-2272 or vehicle, similarly manipulated during the two surgical procedures, and mechanically ventilated at birth under the same conditions. Therefore, stretch-induced mechanisms due to the injected volume and/or mechanical ventilation as well as fetal stress after surgery could not reasonably account for the observed differences between treatment groups. Another limitation of the present work is reliance on the sample size based on previous studies (33, 65), which appears to be insufficient for a few comparisons with low statistical power (e.g., in Fig. 5H, 6F, 7B, and 7C). Finally, priority was given to functional and morphological analyses of the pulmonary circulation, as the main study outcomes. Fetal losses after surgery and postnatal invasive measurements plus the small amounts of tissue available from hypoplastic lungs limited the possibility of laboratory investigations. Hence, whole lung experiments were performed only at the time of term delivery. The key questions of biological changes and modulation of molecular signaling pathways at different time points and in different cell types therefore remained unanswered and warrant further studies.

Fetal safety is a major concern of prenatal interventions. Intratracheal administration of BAY 41-2272 was chosen to improve local availability and avoid putative pharmacological side effects in does and littersmates. Compared with vehicle, instillation of BAY 41-2272 into fetal rabbit airways did not impact fetal body weight, organ weights, or survival, suggesting good fetal tolerance. Because BAY 41-2272 was given late in gestation, the risk of gross malformations due to possible systemic absorption was likely reduced. In the perspective of clinical use, BAY 41-2272 might be administered by percutaneous ultrasound-guided tracheal puncture or minimally invasive endoscopic access (36). In this context, the potential benefit of prenatal therapy should be balanced against the risk of premature rupture of the membranes (36).

In summary, a single intratracheal instillation of BAY 41-2272 at the early saccular stage of lung development attenuates functional, morphological, and biological features of PH in newborn rabbits with surgically induced CDH. Additional evidence about the mechanisms of action, effectiveness, and fetal safety is warranted in other animal models of CDH before antenatal BAY 41-2272 is considered as a therapeutic option to ameliorate fetal lung vasculature and reduce persistent PH in infants with CDH.

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AUTHOR CONTRIBUTIONS

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