Cinaciguat, a soluble guanylate cyclase activator, augments cGMP after oxidative stress and causes pulmonary vasodilation in neonatal pulmonary hypertension

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Am J Physiol Lung Cell Mol Physiol 301: L755–L764, 2011. First published August 19, 2011; doi:10.1152/ajplung.00138.2010.—Although inhaled NO (iNO) therapy is often effective in treating infants with persistent pulmonary hypertension of the newborn (PPHN), up to 40% of patients fail to respond, which may be partly due to abnormal expression and function of soluble guanylate cyclase (sGC). To determine whether altered sGC expression or activity due to oxidized sGC contributes to high pulmonary vascular resistance (PVR) and poor NO responsiveness, we studied the effects of cinaciguat (BAY 58-2667), an sGC activator, on pulmonary artery smooth muscle cells (PASMC) from normal fetal sheep and sheep exposed to chronic intrauterine pulmonary hypertension (i.e., PPHN). We found increased sGC α1- and β1-subunit protein expression but lower basal cGMP levels in PPHN PASMC compared with normal PASMC. To determine the effects of cinaciguat and NO after sGC oxidation in vitro, we measured cGMP production by normal and PPHN PASMC treated with cinaciguat and the NO donor, sodium nitroprusside (SNP), before and after exposure to 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, an sGC oxidizer), hyperoxia (fraction of inspired oxygen 0.50), or hydrogen peroxide (H2O2). After treatment with ODQ, SNP–induced cGMP generation was markedly reduced but the effects of cinaciguat were increased by 14- and 64-fold in PPHN fetal PASMC, respectively (P < 0.01 vs. controls). Hyperoxia or H2O2 enhanced cGMP production by cinaciguat but not SNP in PASMC. To determine the hemodynamic effects of cinaciguat in vivo, we compared serial responses to cinaciguat and ACh in fetal lambs after ductus arteriosus ligation. In contrast with the impaired vasodilator response to ACh, cinaciguat–induced pulmonary vasodilation was significantly increased. After birth, cinaciguat caused a significantly greater fall in PVR than either 100% oxygen, iNO, or ACh. We conclude that cinaciguat causes more potent pulmonary vasodilation than iNO in experimental PPHN. We speculate that increased NO-insensitive sGC may contribute to the pathogenesis of PPHN, and cinaciguat may provide a novel treatment of severe pulmonary hypertension.

Bay 58-2667; cyclic GMP; nitric oxide; SGC stimulators; persistent pulmonary hypertension of the newborn; pulmonary hypertension therapy

PERSISTENT PULMONARY HYPERTENSION of the newborn (PPHN) is a clinical syndrome that is characterized by failure to achieve or sustain the decline in pulmonary vascular resistance (PVR) at birth, leading to extrapulmonary right-to-left shunting of blood across the ductus arteriosus (DA) or foramen ovale and severe hypoxemia (28). Mechanisms that increase PVR in PPHN include high pulmonary vascular tone, abnormal vaso-reactivity, hypertensive vascular wall structure, and reduced vascular growth (21, 31). Previous studies have shown that increased NO production causes fetal pulmonary vasodilatation at birth (2) and that impaired NO-cGMP signaling contributes to the pathogenesis of PPHN (2, 4, 10, 42, 43, 46). NO causes vasodilatation through stimulation of soluble guanylate cyclase (sGC) in pulmonary vascular smooth muscle cells (18, 19, 26, 30). sGC is a heterodimer consisting of a larger α-subunit and a smaller heme-binding β-subunit. NO binds to the reduced heme moiety of the β-subunit, stimulating sGC conversion of GTP to cGMP (18, 20, 26, 38–40). Increased cGMP production stimulates cGMP-dependent protein kinases, leading to reduction in cytoplasmic Ca2+ levels and decreased vasoconstriction (9, 30). Although sGC is expressed in the developing lung, little is known about its role in pulmonary vasoregulation in utero and at birth, but past studies suggest that sGC expression is decreased in experimental PPHN (3, 22, 44, 45).

Inhaled NO (iNO) is a potent and selective pulmonary vasodilator in neonates with PPHN, and, on the basis of several multicenter randomized trials, iNO has been approved by the Food and Drug Administration for the treatment of near-term and term infants with PPHN (1, 7, 23, 34, 35). Despite clear success in reducing the need for extracorporeal membrane oxygenation (ECMO), however, each of these multicenter trials suggested that up to 40% of patients do not respond to iNO therapy and require ECMO due to refractory pulmonary hypertension (1, 7, 34). Mechanisms underlying poor responsiveness to iNO therapy are incompletely understood, and novel therapies are needed to further improve outcomes of neonates with severe PPHN.

Since NO-mediated vasodilatation requires stimulation of sGC to generate cGMP in vascular smooth muscle, impaired sGC activity may contribute to poor responsiveness to endogenous or exogenous NO in the perinatal lung. Recent work from Stasch and colleagues (39–41) on sGC structure and function has led to the development of novel pharmacological agents that are capable of directly activating or stimulating sGC in an NO-independent fashion. sGC stimulators (BAY 41-2272, BAY 63-2521) have been shown to increase sGC activity in isolated cells and animal models of pulmonary hypertension independent of NO (6, 10–12). As observed with NO, these effects are dependent on the presence of a reduced heme.
moiety within sGC, and removal or oxidation of the heme group abolishes NO-mediated sGC stimulation (39, 41). In contrast, sGC activators (such as BAY 58-2667 or cinaciguat) remain uniquely capable of restoring sGC activity or reactivation of dysfunctional sGC. These agents provide pharmacological tools to assess the potential contribution of oxidized sGC in disease states and offer novel therapeutic approaches to diseases that are poorly responsive to NO, as observed in neonates with refractory PPHN.

Recent studies have suggested that generation of reactive oxygen and nitrosative species, such as superoxide, hydrogen peroxide (H₂O₂), and peroxynitrite, may impair vasoreactivity in adult and neonatal models of pulmonary hypertension (5, 13–15, 23, 25, 48, 49). Superoxide production is increased in experimental PPHN and treatment with recombinant human superoxide dismutase (rSOD) enhances vasodilation after birth (25). Importantly, hyperoxia impairs iNO-induced pulmonary vasodilation in normal lambs after birth (27). Mechanisms underlying these effects include increased cGMP-specific type 5 phosphodiesterase (PDE5) activity, which rapidly degrades cGMP in smooth muscle cells (SMC), which would potentially limit NO-induced vasodilation (14). However, whether oxidative stress further impairs pulmonary vascular tone and reactivity due to alterations of the redox state of sGC in perinatal pulmonary artery smooth muscle cells (PASMCs) is unknown. Oxidative stress can increase concentrations of oxidized or NO-insensitive sGC, rendering the enzyme unresponsive to NO stimulation (41).

We recently reported that cinaciguat (BAY 58-2667) causes potent and sustained pulmonary vasodilation in normal fetal sheep (6), but whether oxidized sGC contributes to high PVR or impaired vasodilation during treatment with iNO in experimental PPHN has not been studied. Therefore, we hypothesized that cinaciguat would cause greater pulmonary vasodilation than NO or NO-dependent agents in experimental PPHN and augment cGMP production by fetal SMC after exposure to oxidative stress. To determine the contribution of oxidized sGC in PPHN and to determine the potential role for sGC activators in the treatment of severe pulmonary hypertension, we performed a series of studies to examine the in vitro effects of cinaciguat on isolated PASMCs from fetal sheep with pulmonary hypertension and its physiological effects in animals with experimental PPHN. We found that oxidative stress augments cinaciguat-induced generation of cGMP in vitro and that cinaciguat causes more potent pulmonary vasodilation than iNO in lambs with experimental PPHN. These findings support the hypothesis that sGC activators may be effective agents for the treatment of refractory PPHN.

**METHODS**

Time-dated pregnant, mixed-breed (Colombia-Rambouillet) ewes were used in this study. All procedures and protocols were reviewed and approved by the Animal Care and Use Committee of the University of Colorado Health Sciences Center and followed the Guide for the Care and Use of Laboratory Animals established by the National Research Council.

**Drug Preparation**

A solution of cinaciguat (BAY 58-2667; kindly provided by Bayer, 0.05 mg/ml) was freshly diluted to study doses in normal saline immediately before each study. The infusion rate for in vivo studies was 0.3 ml/min over 10 min. Acetylcholine (ACH, Sigma, St. Louis, MO) was diluted in normal saline and the infusion rate was 0.3 ml/min in the in vivo studies. 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, Tocris Bioscience, Ellisville, MO, catalog no. D2650) was dissolved to a concentration of 10 mg/ml in DMSO (Sigma, catalog no. H2120) and was subsequently diluted with normal saline to achieve study doses. Sodium nitroprusside (SNP, MP Biomedicals, Solon, OH, catalog no. 152061) was diluted to study doses in normal saline immediately before each study. H₂O₂ (Fisher Scientific, Fair Lawn, NJ, catalog no. H325-500) was diluted in DMEM prior to each study. Gaseous NO or iNO (Ikaria, Clinton, NJ) was used at 20 and 40 ppm for the in vivo studies.

**SMC Culture Methods**

Primary cultures of fetal PASMC were prepared from intrapulmonary arteries isolated from late-gestation fetal lambs (126 ± 2 days gestation; term = 147 days). The adventitia was gently removed under sterile conditions and the vessel was washed in sterile phosphate-buffered saline (PBS). The vessel was sliced longitudinally and placed with the intima side down in a petri dish containing 0.5% collagenase for 10 min at 37°C. The intimal surface was then gently removed with a cell scraper. The remaining portion of the vessel was cut into small (1–2 mm) pieces and washed with HBSS without Mg or Ca²⁺ and with NaHCO₃ (20 mM) and HEPES (10 mM) for 30 min in the 37°C hybridization oven. Fragments were placed in a SMC digest that consisted of 7.5 ml HBSS with 40 μM Ca²⁺, 4.0 mg elastase (Roche, catalog no. 109907), 4.07 mg type 2 collagenase (Worthington, catalog no. LS4174 CLS-2), 15 mg albumin (Sigma, catalog no. A9647), and 147 μl soybean trypsin inhibitor (10 mg/ml) (Worthington, catalog no. LSO 3570) for 2 h in a 34°C hybridization oven. The digest was filtered through a 100-μm cell strainer, washed with 5 ml of 10% fetal bovine serum (FBS)/DMEM with 1% l-glutamine and 1% antibiotic and spun at 900 rpm for 6 min. The supernatant was removed and the pellet resuspended in 8 ml of 10% FBS/DMEM with 1% l-glutamine and 1% antibiotic and transferred to the culture flask. Cells were maintained in culture containing 10% FBS, 1% penicillin/streptomycin, and 1% l-glutamine, until they reached confluence. PASMC identity was confirmed by morphology and immunostaining. These studies demonstrated an absence of contamination with fibroblasts or endothelial cells.

**sGC Western Blot Analysis**

At 90% confluence, cultured PASMC from normal and PPHN animals were washed twice with ice-cold PBS and scraped off the culture dishes. PASMC lysates were sonicated and centrifuged at 10,000 g for 20 min at 4°C to remove cell debris. The supernatant was removed and protein content in the supernatant was determined by the bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL). Twenty-five microliters of protein sample per lane were resolved by SDS-PAGE, and proteins from the gel were transferred to nitrocellulose membrane by semidry transfer technique. sGC α₁ and β₁ blots were blocked for 30 min in 5% nonfat dry milk in Tris-HCl, 150 mM NaCl, and 0.05% Tween 20 (pH 8.0). sGC α₁ blots were incubated overnight at 4°C in a 1:500 dilution of monoclonal antibody to the sGC α₁ subunit (Alexis Biochemicals, Lausen, Switzerland, catalog no. ALX-804-648) in nonfat dry milk dissolved in buffer 1. sGC β₁ blots were incubated overnight at 4°C in a 1:150 dilution of monoclonal antibody to the sGC β₁ subunit (Cayman Chemical, Ann Arbor, MI, catalog no. 160897) in nonfat dry milk dissolved in buffer 1. The blots were washed and incubated for 1 h at room temperature with goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Chemicon, Billerica, MA; 1:2000 dilution). After incubation with the secondary antibody, blots were washed and the bands of interest were visualized by enhanced chemiluminescence (ECL kit, Amersham Pharmacia Biotech, Buckinghamshire).
shire, UK) and identified by molecular weight as designated by the manufacturer for the protein of interest. Both blots were stripped and reprobed with an antibody to β-actin (catalog no. A5316, Sigma). Densitometry was performed by use of NIH Image (version 1.61). Changes in protein expression were analyzed after normalization for β-actin expression.

cGMP Assay

cGMP content was determined through measuring generation of cGMP in PASMC with a standard cGMP ELISA (Cayman Chemical, catalog no. 581021). Samples were run in triplicate, relative to a cGMP standard curve by use of a Bio-Rad 680 XR microplate reader (Bio-Rad, Hercules, CA). Total protein concentration in the samples was quantified using a BCA protein assay kit (Thermo Scientific, Rockford, IL). Results are expressed as picomoles of cGMP per milligram of total protein. PASMC samples were incubated for 20 min at 37°C in a serum-free media reaction mixture consisting of 1 mM MgCl₂, 0.5 μM isobutylmethylxanthine (IBMX), 10 μM sildenafil, and the sGC stimulant. PASMC were stimulated with SNP (10 μM) or cinaciguat (10 μM). Both basal and stimulated cGMP contents were measured in the presence of ODQ (20 μM) and in the presence and absence of hyperoxia [fraction of inspired oxygen (FiO₂) 0.5], and H₂O₂ (75 μM).

Surgical Preparation

Surgery was performed in time-dated ewes at 126 ± 2 days gestation after 24 h of fasting. Animals were given intramuscular penicillin G (600,000 units) and gentamicin (80 mg) immediately before surgery. Ewes were sedated with intravenous ketamine (8 ml) and diazepam (2 ml) and intubated and ventilated with 1–2% isoflurane for the duration of the surgery. Under sterile conditions, a midline abdominal incision was made, and the uterus was externalized. A hysterotomy was performed, and the left fetal forelimb was fully freed from the maternal flank. After infusion of pancuronium bromide (0.1 mg/kg, inferior vena cava) to prevent fetal breathing, a hysterotomy was performed, and the fetus was intubated. The fetus was extracted from the uterus, dried, and warmed. The animal was placed on a heating pad and ventilated with a time-cycled, pressure-limited mechanical ventilator (Infant Star 950; Infrasonics, San Diego, CA) with room air. Initial ventilator settings were a rate of 30 breaths/min, peak inspiratory pressure (PIP) 35 cmH₂O, positive end-expiratory pressure 6 cmH₂O, and inspiratory time (IT) 1.0 s. Target blood gas parameters included achieving blood pH between 7.35 and 7.45, and arterial PCO₂ values between 35 and 45 Torr. If the PaCO₂ was below 35 Torr, PIP was decreased to a minimum of 22 cmH₂O. Ventilator rate and IT were gradually decreased if PaCO₂ remained below the target range. If PaCO₂ was above 45 Torr, ventilator rate was increased by 5 breaths/min and IT was shortened. The FiO₂ was maintained at 0.21 throughout the entire study, except for brief exposure to 1.00 for 10 min to test vasoreactivity. Hypotension was treated with volume infusions of normal (0.9%) saline (10 ml/kg over 5 min) as needed. Lambs were kept on a heating pad throughout the study.

Experimental Design

Protocol 1: sGC protein expression and cGMP content in fetal PASMC isolated from normal and PPHN sheep. To determine whether sGC protein expression is altered in SMC from PPHN lambs, we measured sGC α₁- and β₁-subunits from normal and experimental PPHN PASMC lysates. Cell lysates were collected from 90% confluent cells between passages 1–3 from normal and experimental PPHN PASMC. Protein expression for sGC α₁- and β₁-subunits was measured by Western blot analysis and values were normalized for β-actin expression. Initial analysis of sGC α₁ was performed, after which blots were stripped and reprobed for β₁-subunit and β-actin expression. cGMP was measured from normal and PPHN SMC lysates by ELISA.

Protocol 2: effects of ODQ on NO and cinaciguat stimulation of cGMP production in PPHN PASMC in vitro. The purpose of this protocol was to determine the effects of ODQ, an sGC oxidizer, on the ability of cinaciguat and SNP, a NO donor to activate sGC in experimental PPHN PASMC. PASMC were incubated for 20 min in 5 ml of culture-free medium containing 1 μM MgCl₂, 0.5 μM IBMX, 10 μM sildenafil, and cinaciguat (10 μM) or SNP (10 μM) in the presence and absence of ODQ (20 μM). After 20 min of incubation, the reaction mixture was removed, and 0.1 N HCl (150 l) was added to lyse the cells. Culture plates were scraped, and extracts were vortexed for 5 min and then centrifuged at 1,000 g for 10 min before the supernatants were removed. cGMP content was determined by ELISA.
Protocol 3: effects of cinaciguat and SNP on cGMP generation in the presence and absence of hyperoxia in normal and experimental PPHN PASMC. The purpose of this protocol was to determine whether moderate hyperoxia altered the ability of cinaciguat to activate sGC in normal and experimental PPHN PASMC. These effects were compared with the effects of SNP. PASMC were exposed to either room air or hyperoxia (FiO2, 0.50) for 24 h prior to cinaciguat or SNP treatment. After 24 h, culture media were removed and cells were incubated with either cinaciguat (10 μM) or SNP (10 μM) for 20 min in 5 ml of culture-free medium containing 1 mM M6Cl2, 0.5 μM IBMX, and 10 μM sildenafil. After 20 min of incubation, the reaction mixture was removed, the cells were lysed and cGMP content was determined by ELISA, as described above.

Protocol 4: effects of cinaciguat and SNP on cGMP production in the presence and absence of H2O2 in normal and PPHN PASMC. The purpose of this protocol was to determine the effects of H2O2 on cinaciguat and SNP stimulation of sGC in normal and experimental PPHN PASMC. PASMC were treated with H2O2 (75 μM) dissolved in 10% DMEM overnight at 37°C or 10% DMEM alone (control). Cell lysates were collected for assay of cGMP content, as described above. Selection of the H2O2 dose used for these studies was based on preliminary data that showed apoptosis with 100 μM but no apoptosis at 75 μM in these conditions.

Protocol 5: acute pulmonary hemodynamic effects of cinaciguat and ACh during the development of chronic pulmonary hypertension in utero. The purpose of this protocol was to compare the effects of acute pulmonary administration of ACh, an endothelium-dependent vasodilator, and cinaciguat during the progressive development of chronic pulmonary hypertension in chronically prepared fetal sheep. Studies were performed on days 1 and 5 after partial DA ligation. One day after surgery, saline (0.3 ml/min) was first infused into the LPA for ≥30 min to establish baseline measurements for QLPA, MPAP, AoP, LAP, and HR. After baseline measurements were stable for a 30-min period, ACh (15 μg) was infused over 10 min into the LPA catheter. After a minimum of 2 h recovery, cinaciguat (5 μg) was infused over 10 min into the LPA catheter. The dose of ACh used in this study was based on past studies that demonstrate a doubling of blood flow without systemic effects. The dose for cinaciguat was selected from our previous studies that demonstrated a twofold increase in pulmonary blood flow in normal fetal sheep (8). After each infusion, the catheter was subsequently flushed with saline (0.3 ml/min) for 10 min. Hemodynamic measurements were recorded every 10 min starting at the beginning of the infusion and continued for ≥30 min after the return to baseline for each drug infused. Arterial blood gas tensions were obtained at baseline before each drug infusion and at the point of maximal response of each drug infusion. Studies were repeated 5 days after surgery for comparison of changes in pulmonary vasoreactivity.

Protocol 6: acute effects of 100% O2, ACh, iNO, and cinaciguat after cesarean section delivery of newborn sheep with PPHN. To compare the pulmonary hemodynamic effects of iNO and cinaciguat in experimental PPHN, we studied the effects of these agents and other vasodilator stimuli (hyperoxia and ACh) after cesarean section delivery of sheep that had undergone DA ligation 10 days earlier. Saline (0.3 ml/min) was initially infused into the LPA catheter for at least 30 min after delivery, and baseline hemodynamic measurements were recorded at 10-min intervals (baseline period 1). Hemodynamic measurements included QLPA, MPAP, AoP, LAP, and HR. After baseline measurements were stable for at least 30 min, FiO2 was briefly increased to 100% for 10 min and hemodynamic measurements were recorded. After a 30-min recovery period (baseline period 2), the hemodynamic effects of iNO (20 and 40 ppm) was studied for 10 min at each dose. After another 30-min baseline period (baseline period 3), ACh (15.0 μg) was infused into the LPA over 10 min. After the ACh infusion was stopped, hemodynamic measurements were recorded for 30 min (baseline period 4). Cinaciguat (5.0 μg) was then infused into the LPA over 10 min. Arterial blood gas tensions were obtained during each baseline and infusion period. At the end of the study, sheep were killed with a large dose of pentobarbital sodium to confirm the location of catheters.

Statistical Analysis

Data are presented as means ± SE. Statistical analysis was performed with the Prism 5.0 software package (GraphPad, San Diego, CA). Statistical comparisons were performed by repeated-measures analysis of variance and paired t-tests. The significance level was set at P < 0.05.
RESULTS

Protocol 1: sGC Protein Expression and cGMP Content in Fetal PASMC Isolated from Normal and PPHN Sheep

In comparison with normal fetal PASMC, sGC α1- and β1-protein expression were increased in PPHN PASMC. sGC α1 and sGC β1 protein expression were 22 and 61% greater in PPHN PASMCs (Fig. 1, top). Despite the increase in sGC protein in PPHN SMCs, basal cGMP content was reduced from values measured in normal SMCs by 50% (P < 0.01; Fig. 1, bottom).

Protocol 2: Effects of ODQ on NO and Cinaciguat Stimulation of cGMP Production in PPHN PASMC In Vitro

Under basal conditions, treatment of fetal PPHN PASMC with cinaciguat (10 μM for 20 min) increased cGMP from 0.14 ± 0.01 (baseline) to 0.91 ± 0.12 pmol/mg protein (Fig. 2). SNP (10 μM) also increased cGMP (from 0.14 ± 0.01 to 27.60 ± 7.11 pmol/mg, P < 0.05; Fig. 2). In normal and PPHN PASMC stimulated with cinaciguat after ODQ treatment, cGMP generation markedly increased when compared with control levels (from 0.91 ± 0.12 to 63.90 ± 6.54 pmol/mg, P < 0.001; Fig. 2). In contrast, ODQ significantly reduced the effects of SNP on cGMP production (from 27.60 ± 7.11 to 30.0 ± 0.05 pmol/mg, P < 0.01).

Protocol 3: Effects of Cinaciguat and SNP on cGMP Generation in the Presence and Absence of Hyperoxia Exposure of Normal and PPHN PASMC

Under hyperoxic (FiO₂ 50%) conditions, cinaciguat increased cGMP production in normal and PPHN PASMC by nearly fourfold compared with its effects on cGMP levels in room air (Fig. 3). In contrast, the response to SNP was not different after hyperoxia compared with room air, but there was a trend for a reduction in cGMP levels.

Protocol 4: Effects of Cinaciguat and SNP on cGMP Generation in the Presence and Absence of H₂O₂ in Normal and PPHN Fetal PASMC

After treatment with H₂O₂, the effect of SNP on cGMP production was reduced by 90% in normal PASMC (12.3 ± 2.5 to 3.1 ± 0.9 pmol/mg, P < 0.01). H₂O₂ tended to decrease the effects of SNP in PPHN PASMC as well, but this did not reach statistical significance (Fig. 4). In contrast with the effect of H₂O₂ on the effects of SNP, the response to cinaciguat was not attenuated following H₂O₂ treatment and trended toward enhanced cGMP production in both normal and PPHN PASMC (Fig. 4). H₂O₂ treatment did not induce SMC apoptosis at the doses used in this study.

Protocol 5: Acute Pulmonary Hemodynamic Effects of Cinaciguat and ACh During the Development of Chronic Pulmonary Hypertension

As previously observed in normal fetal sheep, brief infusion of cinaciguat increased QLPA and decreased PVR in chronically prepared fetal sheep with pulmonary hypertension induced by DA ligation (Figs. 5 and 6). At day 1, cinaciguat (5 μg) increased Q_LPA by 69% (from 78.1 ± 5.7 to 131.7 ± 9.2 ml/min, P < 0.001; Fig. 6 and Table 1), reduced MPAP (from 74.3 ± 7.4 to 67.9 ± 6.8 mmHg; P < 0.01), and reduced PVR.
by 42% (from 0.93 ± 0.10 to 0.54 ± 0.08 mmHg·ml⁻¹·min⁻¹, \( P < 0.05 \)). After 5 days of pulmonary hypertension, the pulmonary vasodilator response to ACh was abolished (Figs. 5 and 6; Table 1), but the response to cinaciguat remained intact. At day 5, cinaciguat increased \( Q_{LPA} \) by 75% (from 110.0 ± 14.0 to 193.0 ± 20.0 ml/min, \( P < 0.001 \)), decreased MPAP by 16% (from 66.5 ± 10.1 to 55.7 ± 7.6 mmHg, \( P < 0.05 \)), and decreased PVR by 53% (from 0.68 ± 0.17 to 0.32 ± 0.07 mmHg·ml⁻¹·min⁻¹, \( P < 0.05 \)). At the doses used in this study, cinaciguat infusion decreased in mean AoP by 12 and 14% on days 1 and 5, respectively.

Protocol 6: Physiological Effects of 100% O₂, ACh, iNO, and Cinaciguat in PPHN Sheep After Cesarean Section Delivery

To further determine the pulmonary vascular effects of cinaciguat in experimental PPHN and to directly compare the hemodynamic effects of cinaciguat with iNO, we studied the hemodynamic effects of cinaciguat in neonatal sheep that were delivered by cesarean section and mechanically ventilated after birth. After establishing stable baseline hemodynamic and arterial blood gas values (as outlined in METHODS), newborn sheep were sequentially treated with 100% O₂, iNO (20 and 40 ppm), ACh (15 \( \mu \)g), and cinaciguat (5 \( \mu \)g) (Fig. 7; Table 2). As shown, acute hyperoxia caused a small but statistically significant rise in \( Q_{LPA} \) from 192 ± 36 to 216 ± 31 ml/min (\( P < 0.05 \)) but did not lower calculated PVR. Similarly, iNO at 40 ppm increased \( Q_{LPA} \) from 179 ± 20 to 233 ± 25 ml/min (\( P < 0.05 \)), but this effect was small and not associated with a significant fall in PVR (0.23 ± 0.05 vs. 0.20 ± 0.05; \( P = \) not significant). ACh did not cause pulmonary vasodilation, as reflected by the lack of change in \( Q_{LPA} \) or PVR. In contrast, cinaciguat increased \( Q_{LPA} \) by 42% and lowered PVR by 36%
Fig. 7. Hemodynamic response to 100% O₂, inhaled NO (iNO, 20 and 40 ppm), ACh (15.0 μg) infusion, cinaciguat (5.0 μg) infusion, and cinaciguat (5.0 μg) infusion in combination with iNO (40 ppm) during delivery study in lambs after partial ligation of the ductus arteriosus, as shown for PVR. Values represent means ± SE (n = 4 animals).

DISCUSSION

INO is well established as an effective therapy for newborns with PPHN, as demonstrated by improved oxygenation and a decreased need for ECMO therapy; however, up to 40% of neonates with severe PPHN are poor responders to iNO (1, 7, 34). To better understand mechanisms that contribute to decreased NO responsiveness in PPHN, we studied the potential role of oxidized sGC and the effects of iNO, a heme-independent sGC activator, in an experimental model of PPHN in fetal sheep. We found that sGC protein expression is increased in PASMC from experimental PPHN sheep, but basal cGMP levels were lower in PPHN than control SMC. Brief treatment with three different sources of oxidative stress, including the pharmacological SGC inhibitor ODQ, H₂O₂, and moderate hyperoxia, augmented the ability of cinaciguat to elevate cGMP production while attenuating or not enhancing the effects of the NO donor, SNP. We further report that cinaciguat caused more potent pulmonary vasodilation than iNO, brief hyperoxia, and ACh in the chronically prepared fetal lamb in utero and after birth. Overall, these findings support the hypothesis that despite increased sGC protein expression, impaired sGC activity may contribute to the pathogenesis of PPHN, and that cinaciguat effectively enhances cGMP production and pulmonary vasodilation in this experimental model. These results support our hypothesis that direct activation of sGC by the NO- and heme-independent sGC activator cinaciguat increases cGMP production in normal and PPHN PASMC and causes potent pulmonary vasodilation in this experimental model of PPHN.

This study supports the growing hypothesis that oxidative stress contributes to impaired pulmonary vascular tone and reactivity in the perinatal lung and plays an important role in the pathogenesis of PPHN. Increases in reactive oxygen species (ROS) such as superoxide and H₂O₂ in pulmonary arterial smooth muscle and adventitia have been previously demonstrated in this model of ovine PPHN (5, 48, 49). There are likely multiple sources for elevated ROS production, including mitochondria, increased NADPH oxidase or xanthine oxidase expression and activity, and uncoupled NO synthase (NOS) activity (5, 24, 29, 48, 49). ROS promote vasoconstriction directly and through multiple mechanisms including increased endothelin levels and oxidization of free fatty acids to create vasoconstrictor metabolites such as isoprostanes. Superoxide anions rapidly combine with NO, reducing its bioavailability and in the process forming peroxynitrite, a potent oxidant with the potential to produce vasoconstriction and cytotoxicity. Increased ROS in the pulmonary vasculature of the ductal ligation model of PPHN promotes dysfunction of NO-cGMP signaling at multiple steps in the pathway, including blunted NOS expression, uncoupled endothelial NOS (eNOS) activity, and increased activity and expression of cGMP-specific phosphodiesterases (13, 14, 16, 17, 24).

Superoxide dismutases (SOD) catalyze the conversion of superoxide anions to H₂O₂ and O₂. Because of the efficiency of the reaction between NO and superoxide, the local concentration of SOD is a key determinant of the biological half-life of endogenous NO. Decreased SOD activity has been observed in this model of PPHN (13). Further evidence for the critical role of ROS in altered vasoreactivity in PPHN is the recent observation that rhSOD administration in neonatal lambs with PPHN produced a sustained increase in oxygenation over a 24-h period, reduced production of isoprostanes and peroxynitrite, and restored normal eNOS expression and function (25).
Recent studies have convincingly shown that hyperoxia up-regulates PDE5 expression and activity in fetal pulmonary vascular smooth muscle, limiting cGMP production in response to NO (14).

This present study extends past work and suggests that, in addition to high PDE5 activity, impaired sGC activity further limits cGMP production upon exposure to NO. We found that sGC expression is increased in PPHN PASMC lysates, as reported by others in human SMC from patients with idiopathic pulmonary artery hypertension and rat models of pulmonary hypertension due to chronic hypoxia and monocrotaline (36). Despite the increase in sGC protein, sGC activity is reduced in this model of ovine PPHN. However, PPHN lambs and pulmonary artery endothelial cells remain markedly responsive to the sGC activator, cinaciguat, and the effects of cinaciguat are further enhanced in the setting of additional oxidative stress despite the loss of NO responsiveness in these conditions. Interpretation of these data is limited, however, because of the current lack of a direct tissue assay for oxidized sGC.

Along with the in vitro studies, our data further demonstrate that cinaciguat causes striking pulmonary vasodilation in experimental PPHN in vivo and that this response was far greater than the small vasodilator effects of iNO at 20 and 40 ppm. These findings of differential effects of NO and an sGC activator further suggest that oxidized sGC may contribute to poor NO responsiveness in PPHN. The physiological studies were performed during administration of room air and not hyperoxia; we speculate that even greater differences between these agents would be observed if these lambs were treated with marked elevations of inspired oxygen (27). Furthermore, low microgram doses of cinaciguat caused potent pulmonary vasodilation in PPHN sheep. Previous studies using the sGC stimulator BAY 41-2272 required 100 times the dose (500 μg) that was used in our study of cinaciguat (5 μg) to achieve similar degrees of pulmonary vasodilation (10). This difference in dose and response in vivo parallels past studies comparing the relative effects of sGC stimulators and activators in vitro. In addition, cinaciguat, at the doses used in this study, caused minimal (10%) but statistically significant decreases in systemic arterial pressure. Whether lower doses of cinaciguat would have been as effective as the doses used in this study without affecting systemic arterial pressure is unknown, and little is known about the pharmacokinetics of cinaciguat in the setting of PPHN. Thus further work is needed to define dose responses and the pharmacokinetics of cinaciguat before clinical studies in human infants with PPHN should be performed.

The results of the in vitro studies on the effects of cinaciguat during oxidative stress with fetal PASMCs are consistent with the pioneering work by Stasch et al. (39–41) that showed that purified sGC stimulation with cinaciguat is enhanced in the presence of ODQ. Previous studies have shown that cinaciguat acts in completely different fashion from other known sGC stimulators, including YC-1, BAY 41-2272, and BAY 41-8543 (12). Cinaciguat directly activates sGC by binding the unoccupied heme-binding pocket or by replacing the weakly bound oxidized heme moiety, producing selective sGC activation and subsequent vasodilation. This binding also inhibits ubiquitination-dependent sGC protein degradation. We found that cGMP generation due to in vitro stimulation of PPHN PASMCs with SNP was abolished in the presence of ODQ, again consistent with our prior work in normal PASMCs treated with ODQ prior to stimulation with cinaciguat (6). Brief hyperoxia treatment of normal and PPHN PASMCs caused a similar augmentation in cGMP production caused by cinaciguat stimulation and a similar inhibition of cGMP production caused by the NO donor SNP. Normal SMCs showed the same pattern after H2O2 treatment; this effect did not reach statistical significance in PPHN SMCs, but the trend was similar (Fig. 4). Prior studies have shown that removal or oxidation of the heme moiety led to the formation of an NO-insensitive form of sGC (19, 20, 32, 37–41). These concepts may explain NO refractory states in diverse vascular diseases, including pulmonary hypertension.

Previous studies demonstrated that cinaciguat caused potent vasorelaxation of rabbit saphenous artery rings with an IC50 160-fold more potent than BAY 41-2272 and >1,000-fold more potent than SNP or 3-morpholinosydnonimine (NO donors). Cinaciguat stimulated purified sGC in an additive manner when combined with NO, compared with the synergistic manner of older sGC stimulators, confirming a unique mechanism of sGC activation. As observed in this study, ODQ is known to potentiate the effects of cinaciguat on purified sGC enzyme activation (30). Importantly, inhalation of microparticles with cinaciguat produced dose-dependent pulmonary vasodilation, increased transpulmonary cGMP release, and increased systemic arterial oxygenation in juvenile lambs with acute pulmonary hypertension caused by U-46619; these effects were greatly increased after treatment with ODQ (11). Potential limitations in these data include the concern that part of the impaired response to iNO therapy may be related to decreased NO bioavailability due to superoxide-mediated catalysis of NO. In addition, although in vitro studies were performed with high concentrations of the PDE5 antagonist.
sildenafil, comparisons of the responses to iNO vs. cinaciguat in the presence or absence of sildenafil were not performed. Such experiments would not only clarify mechanisms of vasodilation in this experimental setting, but these studies may also provide insights into separate and combined therapies of refractory PPHN in human infants. Past studies from our laboratory have highlighted the persistent pulmonary vasodilation and augmentation of responsiveness to iNO in this model of PPHN (10). Future studies are planned to address these issues. We further acknowledge that the cGMP assays in our report were not performed with excess substrate, such as GTP, creatinine phosphate, and creatinine phosphokinase, which may differ within PPHN and control SMCs. Similarly, because of the importance of high PDE5 activity in this model of PPHN, high doses of sildenafil were applied in the in vitro studies, which may have affected the cGMP responses to the sGC agonists used in this study.

In conclusion, cinaciguat causes potent and sustained pulmonary vasodilation in experimental PPHN in vivo, which is more potent than iNO. In addition, in contrast with exogenous NO, the effects of cinaciguat on cGMP production were enhanced in fetal PASMC in the setting of diverse oxidative stresses caused by pharmacology (ODQ), hyperoxia, and $H_2O_2$. These observations suggest that impaired sGC activity contributes to altered vascular tone and reactivity in PPHN and that cinaciguat may have therapeutic potential as an alternate or adjuvant therapy for severe PPHN that is refractory to iNO and oxygen therapy.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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