Cinaciguat, a soluble guanylate cyclase activator, causes potent and sustained pulmonary vasodilation in the ovine fetus

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Cinaciguat, a soluble guanylate cyclase activator, causes potent and sustained pulmonary vasodilation in the ovine fetus. Am J Physiol Lung Cell Mol Physiol 297: L318–L325, 2009. First published May 22, 2009; doi:10.1152/ajplung.00062.2009.—Impaired nitric oxide-cGMP signaling contributes to severe pulmonary hypertension after birth, which may in part be due to decreased soluble guanylate cyclase (sGC) activity. Cinaciguat (BAY 58-2667) is a novel sGC activator that causes vasodilation, even in the presence of oxidized heme or heme-free sGC, but its hemodynamic effects have not been studied in the perinatal lung. We performed surgery on eight fetal lambs (full term = 147 days) and placed catheters in the main pulmonary artery, aorta, and left atrium to measure pressures. An ultrasonic flow transducer was placed on the left pulmonary artery to measure blood flow, and a catheter was placed in the left pulmonary artery for drug infusion. Cinaciguat (0.1–100 μg over 10 min) caused dose-related increases in pulmonary blood flow greater than fourfold above baseline and reduced pulmonary vascular resistance by 80%. Treatment with 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), an sGC-oxidizing inhibitor, enhanced cinaciguat-induced pulmonary vasodilation by >120%. The pulmonary vasodilator effect of cinaciguat was prolonged, decreasing pulmonary vascular resistance for >1.5 h after brief infusion. In vitro stimulation of ovine fetal pulmonary artery smooth muscle cells with cinaciguat after ODQ treatment resulted in a 14-fold increase in cGMP compared with non-ODQ-treated cells. We conclude that cinaciguat causes potent and sustained fetal pulmonary vasodilation that is augmented in the presence of oxidized sGC and speculate that cinaciguat may have therapeutic potential for severe neonatal pulmonary hypertension.

BAY 58-2667; cGMP; nitric oxide; persistent pulmonary hypertension of the newborn; pulmonary hypertension

THE FETAL PULMONARY CIRCULATION is characterized by high pulmonary vascular resistance (PVR), which results in low blood flow to the lungs. At birth, within the first few minutes of life, PVR decreases dramatically, increasing pulmonary blood flow up to eightfold. Mechanisms that mediate pulmonary vasodilation at birth are incompletely understood, but they include loss of fetal lung liquid, increased PaO2 (9, 44), breathing, or ventilation (14), and release of vasodilators, including nitric oxide (NO) (3, 12, 27). NO plays a crucial role in the regulation of vascular tone and reactivity in the fetal lung and contributes to the fall in PVR at birth (3, 4, 12). The effects of NO on pulmonary vascular tone are largely mediated through stimulation of soluble guanylate cyclase (sGC) in vascular smooth muscle cells (11, 22, 45, 46, 49). sGC is a heterodimer consisting of a larger α-subunit and a smaller heme-binding β-subunit (29). NO binds to the reduced heme moiety of the β-subunit, stimulating sGC conversion of GTP to cGMP (33, 36, 39, 47). Increased cGMP production stimulates cGMP-dependent protein kinases, leading to reduction in cytoplasmic Ca2+ levels and decreased vasoconstriction (13, 29). Although sGC is expressed in the developing lung (4, 27), not enough is known about its role in pulmonary vasoregulation in the fetus and during the transition of the pulmonary circulation at birth (3, 22).

Persistent pulmonary hypertension of the newborn (PPHN) is the clinical syndrome that is characterized by failure of the pulmonary circulation to achieve or sustain the normal fall in PVR at birth (20, 21). High PVR leads to extrapulmonary right-to-left shunting of blood across the ductus arteriosus and foramen ovale, which results in severe hypoxemia (26, 30, 34). Treatment of neonates with severe PPHN includes the use of inhaled NO (iNO) as a potent and selective pulmonary vasodilator (7, 10, 38). Three multicenter, randomized clinical trials have shown that iNO improves oxygenation and decreases the need for extracorporeal membrane oxygenation in PPHN (7, 10, 37). However, each of these studies suggests that ~40% of newborns with PPHN do not respond or sustain a response to iNO therapy and continue to require extracorporeal membrane oxygenation. Mechanisms underlying poor responsiveness to iNO therapy are incompletely understood, and studies are needed to develop new therapies to improve outcomes of sick neonates with PPHN.

Since NO-mediated vasodilation requires stimulation of sGC for generation of cGMP in vascular smooth muscle, impaired sGC activity may contribute to poor NO responsiveness in the perinatal lung. Recent insights regarding the structure and function of sGC have led to the development of novel pharmacological agents capable of directly stimulating or activating sGC (19). sGC stimulators (BAY 41-2272 and BAY 63-2521) increase sGC activity independent of NO, but their effects depend on the presence of the reduced heme moiety of sGC, similar to NO (19). Removal or oxidation of the heme moiety abolishes NO-mediated sGC stimulation; in contrast, sGC activators [e.g., BAY 58-2667 (cinaciguat)] remain capable of increasing sGC activity despite oxidation, suggesting a potentially unique approach for pulmonary hypertension therapy (41).
Cinaciguat may have more potent effects than other agents in pathophysiological settings, especially in PPHN. The hemodynamic effects of cinaciguat and its capability to activate oxidized sGC in the perinatal pulmonary circulation are unknown. For this reason, we hypothesize that activation of sGC with cinaciguat will cause potent and sustained pulmonary vasodilation in normal fetal sheep, even in the presence of oxidized sGC.

Past studies have shown that direct sGC activators, including BAY 41-2272, cause NO-independent pulmonary vasodilation, suggesting potential utility for the management of pulmonary hypertension (15–19). However, recent studies have suggested that generation of reactive oxygen species, such as superoxide and peroxynitrite, produced during conditions of oxidative stress, may impair vasoreactivity in models of pulmonary hypertension (8, 31). Changes in the redox state of sGC caused by oxidative stress (43) may lead to increased concentrations of oxidized or NO-insensitive sGC within the pulmonary artery smooth muscle cell (PASMC), which is insensitive to exogenous NO.

To begin to explore the potential utility of sGC activators in the treatment of newborns with refractory PPHN, we examined the pulmonary hemodynamic effects of cinaciguat, an sGC activator, on high PVR in the chronically prepared, late-gestation fetal lamb. We found that cinaciguat causes potent and sustained fetal pulmonary vasodilation that is augmented in the presence of oxidized sGC and speculate that cinaciguat may have therapeutic potential for severe neonatal pulmonary hypertension.

METHODS

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, 0.05 mg/ml; provided by Bayer) was diluted to study doses in normal saline immediately before each study and infused at 0.3 ml/min over 10 min. ACh (Sigma, St. Louis, MO) was diluted in normal saline and infused at 0.3 ml/min. 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; catalog no. 0880, Tocris Bioscience, Ellisville, MO) was dissolved to a concentration of 10 mg/ml with 500 ml of DMSO (catalog no. D2650, Sigma) and then diluted with normal saline to study doses. Sodium nitroprusside (SNP; catalog no. 152061, MP Biomedicals, Solon, OH) was diluted to study doses in normal saline immediately before each study.

Fetal Surgical Preparation

Surgery was performed at 126 ± 2 days gestation (full term = 147 days) after ewes had fasted for 24 h. Animals were given intramuscular penicillin G (600,000 U) 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 made, and the left fetal forelimb was exposed. Polyvinyl catheters (20 gauge) were placed in the left axillary artery and vein and advanced in the ascending aorta and superior vena cava, respectively. A left thoracotomy and pericardial incision were made, and the heart and great vessels were exposed. Using a 16-gauge intravenous placement unit (Angiocath, Travenol, Deerfield, IL), a 22-gauge catheter was placed through purse-string sutures in the left pulmonary artery (LPA) to allow for selective drug infusions. A 14-gauge intravenous placement unit (Angiocath) was used to place 20-gauge catheters in the main pulmonary artery (MPA) and left atrium. After gentle, blunt dissection of the bifurcation of the MPA, a flow transducer (Transonic Systems, Ithaca, NY) was placed around the LPA to measure blood flow to the left lung (QL_Pa). A catheter was placed in the amniotic cavity to serve as a pressure referent. The uterus was sutured, and a dose of ampicillin (500 mg) was given in the amniotic cavity. The catheters and flow transducer were externalized to a flank pouch on the ewe after the abdominal wall was closed. Postoperatively, ewes were allowed to eat and drink ad libitum and were generally standing within 1 h. All animals were treated with scheduled buprenorphine (0.6 mg) for 48 h postoperatively and then as indicated (based on veterinary assessment of pain). All catheters were gently flushed daily with 1–2 ml of heparinized normal saline (0.9%) saline to maintain catheter patency.

General Study Design

Ewes were allowed to recover from surgery for ≥24 h before the initiation of physiological studies. During each study, pulmonary arterial, aortic, and left atrial pressures were measured by connecting the externalized catheters to computer-driven pressure transducers (model MP100A, Biopac Systems, Santa Barbara, CA). Pressure transducers were calibrated using a mercury column manometer before each study. Pressure measurements were referenced to simultaneously recorded amniotic pressure. The flow transducer was connected to an internally calibrated flowmeter (Transonic Systems, Ithaca, NY) to measure QL_Pa. Before infusion of study drugs, a 30-min period of stable baseline hemodynamics was established during infusion of normal saline. Hemodynamic variables, including MPAP, aortic pressure (AoP), and QL_Pa, were measured continuously for the duration of each study protocol. Left atrial pressure (LAP) was measured in 5 animals (2.1 ± 0.7 mmHg) to ensure that values were constant throughout all protocols and consistent with previous studies in sheep. Left lung PVR was calculated as follows: (MPAP – LAP)/QL_Pa, where LAP = 2.1 mmHg. Heart rate (HR) was determined from phasic pressure traces. Arterial blood gas measurements included pH, Pco2, Po2, oxygen saturation, and Hb (model ABL 800, Radiometer, Copenhagen, Denmark).

Cell Culture Methods

Primary cultures of fetal PASMCs were prepared from intrapulmonary arteries isolated from late-gestation (126 ± 2 days) fetal lambs. Adventitia was gently removed under sterile conditions leaving only the medial layer, and the vessel was washed in sterile PBS. The vessel was cut open longitudinally and placed endothelial side down in a petri dish containing 0.5% collagenase for 10 min at 37°C. The endothelial surface was then removed with a cell scraper. The remaining portion of the vessel was cut into small (1–2 mm) pieces and washed with HBSS without Mg2+ or Ca2+ and with NaHCO3 (20 mM) and HEPES (10 mM) for 30 min in a 37°C hybridization oven. Fragments were placed in a smooth muscle cell digest that consisted of 7.5 ml of HBSS with 40 μM Ca2+, 4.0 mg of elastase (catalog no. 100907, Roche), 4.07 mg of type 2 collagenase (catalog no. LS04174 CLS-2, Worthington), 15 mg of albumin (catalog no. A9647, Sigma), and 147 μl of soybean trypsin inhibitor (10 mg/ml; catalog no. LSO 3570, Worthington) for 2 h in a 34°C hybridization oven. The digest was filtered through a 100-μm cell strainer and washed with 5 ml of 10% FBS-DMEM with 1% t-glutamine and 1% antibiotic. The digest was then spun at 900 rpm for 6 min. The supernatant was removed, and the pellet was resuspended in 8 ml of 10% FBS-DMEM with 1% t-glutamine and 1% antibiotic and transferred to the culture flask. Cells were maintained in culture containing 10% FBS, 1% penicillin-
sGC Enzyme Activity Assay

sGC activity was determined by measurement of cGMP generation in PASMCs with a cGMP ELISA (catalog no. 581021, Cayman Chemical, Ann Arbor, MI). Samples were run in triplicate, relative to a cGMP standard curve, using a microplate reader (model 680 XR, Bio-Rad, Hercules, CA). Total protein concentration in the samples was determined using a bichinchoninic acid 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 medium reaction mixture consisting of 1 μM MgCl2, 0.5 μM IBMX, 10 μM sildenafil, and the sGC stimulant. PASMCs were stimulated with SNP (10 μM) or cinaciguat (10 μM). Basal and stimulated sGC activities were measured in the presence and absence of ODQ (20 μM).

Experimental Design

Protocol 1: pulmonary hemodynamic effects of brief cinaciguat infusion. The purpose of protocol 1 was to determine the effects of brief intrapulmonary administration of cinaciguat on fetal pulmonary hemodynamics. After a 24-h recovery from surgery, saline (0.3 ml/min) was infused into the LPA catheter for ≥30 min, and baseline hemodynamic measurements were recorded every 10 min for QLPA, MPAP, AoP, LAP, and HR. After baseline measurements were stable for a 30-min period, cinaciguat was infused at one of several doses in random order (0.1, 1.0, 5.0, 10.0, and 100 μg) into the LPA over 10 min. Hemodynamic measurements were recorded for ≥30 min after the return to baseline values before the next drug infusion. Arterial blood gas tensions were obtained as part of baseline measurements and at the peak response of each infusion.

Protocol 2: pulmonary hemodynamic effects of cinaciguat and ACh during brief infusions. The purpose of protocol 2 was to determine whether the pulmonary vasodilator effect of cinaciguat was more sustained than that of ACh, a well-established fetal pulmonary vasodilator that acts through direct stimulation of endothelial NO synthase (1, 5). Saline (0.3 ml/min) was infused into the LPA for ≥30 min to establish baseline measurements. Then, cinaciguat (5.0 μg) was infused over 10 min into the LPA catheter. After hemodynamic measurements returned to baseline and were stable for a 30-min period, ACh (15 μg) was infused over 10 min into the LPA catheter. 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.

Protocol 3: pulmonary hemodynamic effects of cinaciguat and ACh after sGC oxidation by ODQ. The purpose of protocol 3 was to compare the hemodynamic response to cinaciguat with the hemodynamic response to ACh after pretreatment with ODQ, an sGC oxidizer. After 30 min of stable baseline measurements, ODQ (0.5 mg) was infused over 10 min into the LPA catheter. Dose selection for ODQ was based on preliminary studies that demonstrated minimal effects on basal pulmonary vascular tone or systemic effects. After the ODQ infusion, ACh (15 μg) was infused over 10 min into the LPA catheter. Immediately after the ACh infusion, cinaciguat (5.0 μg) was infused over 10 min into the LPA catheter. Hemodynamic measurements were recorded every 10 min starting at the beginning of the first infusion and continued every 10 min until measurements returned to baseline following the final infusion. Comparisons were made with infusions of each drug in the absence of ODQ in the same study animals on a separate day. Preliminary studies showed no difference in effect from changing the order of drug infusion. In some cases, the cinaciguat vasodilator effect was so prolonged after ODQ treatment that it was difficult to complete the ACh infusion within the same study day. ACh infusions were therefore studied before cinaciguat infusions after ODQ treatment. Arterial blood gas tensions were obtained at baseline before each infusion and after each infusion following return to baseline.

Protocol 4: in vitro effects of cinaciguat and SNP on cGMP generation in the presence or absence of ODQ in ovine fetal PASMCs. The purpose of protocol 4 was to determine the effects of ODQ, an sGC oxidizer, on the ability of cinaciguat to activate sGC in fetal PASMCs. These effects of cinaciguat were compared with the effects of SNP, an NO donor. PASMCs were isolated from late-gestation fetal sheep and grown to confluence in 150 × 25 mm culture dishes (see above). Once confluent, the culture medium was removed, and cells were incubated for 20 min in 10 ml of culture-free medium reaction mixture containing 1 μM MgCl2, 0.5 μM IBMX, 10 μM sildenafil, and cinaciguat (10 μM) or SNP (10 μM), in presence and absence of ODQ (20 μM). After 20 min of incubation, the reaction mixture was removed, and 0.1 N HCl (300 μl) was added to lyse the cells. Culture plates were scraped, and extracts were vortexed for 5 min and then spun at 1,000 g for 10 min before the supernatants were removed. cGMP content was determined by ELISA (see above).

Statistical Analysis

Statistical analysis was performed with the Prism 4.0 software package (GraphPad, San Diego, CA). Statistical comparisons were performed using repeated-measures analysis of variance and unplanned multiple comparisons. Significant differences were determined by Bonferroni’s post hoc test.

Fig. 1. Dose-response effects of cinaciguat on fetal pulmonary circulation. Top: infusion of cinaciguat (0.1–100 μg) caused a dose-related fall in pulmonary arterial pressure. Bottom: cinaciguat caused a dose-related fall in left pulmonary artery blood flow. Values are means ± SE (n = 8). *P < 0.05; **P < 0.01 vs. baseline.

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At higher doses, cinaciguat progressively increased QLPA and vasodilation (Fig. 3). In fetal lambs infused with cinaciguat and ACh, brief infusions of cinaciguat caused sustained pulmonary vasodilation in chronically prepared fetal sheep (Fig. 1). Brief infusions of cinaciguat increased Q_{LPA} from baseline values at a threshold dose of 1.0 μg (P < 0.05; Fig. 1) and decreased PVR from baseline values at a threshold dose of 0.1 μg (P < 0.01; Fig. 2). At higher doses, cinaciguat progressively increased Q_{LPA} and reduced PVR without altering blood gas tensions (P < 0.01). Infusions of cinaciguat at higher doses increased HR and reduced MPAP (Table 1). Brief infusion of cinaciguat did not alter arterial P_{O2}, P_{O2}, Hct, AoP, or LAP (Table 1).

**RESULTS**

**Protocol 1: Pulmonary Hemodynamic Effects of Brief Cinaciguat Infusion**

Cinaciguat caused dose-related pulmonary vasodilation in chronically prepared fetal sheep (Fig. 1). Brief infusions of cinaciguat increased Q_{LPA} from baseline values at a threshold dose of 1.0 μg (P < 0.05; Fig. 1) and decreased PVR from baseline values at a threshold dose of 0.1 μg (P < 0.01; Fig. 2). At higher doses, cinaciguat progressively increased Q_{LPA} and reduced PVR without altering blood gas tensions (P < 0.01). Infusions of cinaciguat at higher doses increased HR and reduced MPAP (Table 1, Fig. 1). Brief infusion of cinaciguat did not alter arterial P_{O2}, P_{O2}, Hct, AoP, or LAP (Table 1).

**Protocol 2: Pulmonary Hemodynamic Effects of Cinaciguat and ACh During Brief Infusions**

Brief infusions of cinaciguat caused sustained pulmonary vasodilation (Fig. 3). In fetal lambs infused with cinaciguat (5.0 μg over 10 min), Q_{LPA} initially doubled above baseline values at 10 min and continued to increase after termination of the infusion (from 68 ± 4 to 166 ± 14 ml/min, P < 0.001; Fig. 3), and PVR fell 60% (from 0.65 ± 0.04 to 0.25 ± 0.04 mmHg·ml⁻¹·min⁻¹, P < 0.001; Fig. 3). Q_{LPA} remained elevated for ~1 h after the infusion, and, on average, hemodynamics returned to baseline levels by 2 h after cinaciguat infusion. In contrast, brief infusion of ACh caused a similar rise in Q_{LPA} at 10 min (from 69 ± 4 to 100 ± 12 ml/min, P < 0.05; Fig. 3) and a fall in PVR (from 0.60 ± 0.03 to 0.40 ± 0.05 mmHg·ml⁻¹·min⁻¹, P < 0.01; Fig. 3), but the vasodilator effect rapidly ended after termination of the infusion. Brief infusions

<table>
<thead>
<tr>
<th>Table 1. Blood gases, AoP, LAP, HR, and Hct after cinaciguat infusion into the LPA catheter</th>
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<tr>
<td>Cinaciguat Infusion</td>
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<tr>
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</tr>
<tr>
<td>pH</td>
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<tr>
<td>P_{O2}, Torr</td>
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<tr>
<td>P_{O2}, Torr</td>
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<tr>
<td>O_{2} saturation, %</td>
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<tr>
<td>AOp, mmHg</td>
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<tr>
<td>LAP, mmHg</td>
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<tr>
<td>HR, beats/min</td>
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<td>Hct, %</td>
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</table>

Values are means ± SE. Cinaciguat was infused into the left pulmonary artery catheter for 10 min at 0.3 ml/min. AoP, aortic pressure; LAP, left atrial pressure; HR, heart rate. *P < 0.05; †P < 0.001 vs. baseline.
of cinaciguat and ACh did not change arterial blood gas tensions, pH, Hct, AoP, or LAP (Table 2).

Protocol 3: Pulmonary Hemodynamic Effects of Cinaciguat and ACh After sGC Oxidation by ODQ

To determine the effects of sGC oxidation on cinaciguat-induced pulmonary vasodilation, we studied the effects of cinaciguat before and after ODQ infusions. ODQ enhanced pulmonary vasodilation caused by cinaciguat (Fig. 4). ODQ reduced the vasodilator response to ACh, as reflected by pulmonary blood flow, by 71% (114 ± 2.1 to 39.8 ± 2.9 pmol/mg, P < 0.01; Fig. 4, Table 3). In striking contrast, ODQ enhanced the pulmonary vasodilator response to ACh but enhanced the vasodilator response to cinaciguat, as shown for left pulmonary artery blood flow (A) and PVR (B). Values are means ± SE (n = 8).

Fig. 4. Effects of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) on the fetal pulmonary vasodilator response to ACh and cinaciguat. ODQ reduced the vasodilator response to ACh but enhanced the vasodilator response to cinaciguat, as shown for left pulmonary artery blood flow (A) and PVR (B). Values are means ± SE (n = 8).

Table 2. Blood gases, MPAP, AoP, LAP, HR, and Hct at baseline and at maximal response after brief infusion of cinaciguat and ACh into the left pulmonary artery catheter

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Cinaciguat (5.0 μg)</th>
<th>ACh (15.0 μg)</th>
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<tbody>
<tr>
<td>pH</td>
<td>7.37 ± 0.01</td>
<td>7.32 ± 0.01</td>
<td>7.36 ± 0.01</td>
</tr>
<tr>
<td>Pco2, Torr</td>
<td>47.8 ± 2.1</td>
<td>49.5 ± 1.6</td>
<td>45.3 ± 1.4</td>
</tr>
<tr>
<td>Po2, Torr</td>
<td>19.6 ± 0.9</td>
<td>18.8 ± 1.3</td>
<td>20.9 ± 1.1</td>
</tr>
<tr>
<td>O2 saturation, %</td>
<td>84.5 ± 5.0</td>
<td>84.1 ± 4.5</td>
<td>82.1 ± 4.6</td>
</tr>
<tr>
<td>MPAP, mmHg</td>
<td>43.9 ± 3.6</td>
<td>39.8 ± 2.9</td>
<td>42.1 ± 2.1</td>
</tr>
<tr>
<td>AoP, mmHg</td>
<td>46.0 ± 5.0</td>
<td>41.2 ± 4.3</td>
<td>38.1 ± 1.6</td>
</tr>
<tr>
<td>LAP, mmHg</td>
<td>2.3 ± 0.2</td>
<td>2.0 ± 0.2</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>166.6 ± 8.0</td>
<td>182.1 ± 8.5</td>
<td>171.1 ± 5.1</td>
</tr>
<tr>
<td>Hct, %</td>
<td>33.0 ± 1.9</td>
<td>29.1 ± 1.6</td>
<td>30.4 ± 1.3</td>
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</table>

Values are means ± SE. MPAP, main pulmonary artery pressure. Cinaciguat and ACh were infused into the left pulmonary artery catheter at 0.3 ml/min for 10 min.

was significantly reduced after ODQ treatment compared with baseline values (P < 0.05; Table 3). There were no significant changes in arterial blood gas tensions, pH, AoP, or LAP after ODQ treatment for cinaciguat or ACh infusions.

Protocol 4: In Vitro Effects of Cinaciguat and SNP on cGMP Generation in the Presence or Absence of ODQ in Ovine Fetal PASMCs

Under basal conditions, treatment of fetal PASMCs with cinaciguat (10 μM) for 20 min increased cGMP [from 0.68 ± 0.08 (baseline) to 1.21 ± 0.07 pmol/mg, P < 0.01; Fig. 5]. SNP (10 μM) also increased cGMP (from 0.68 ± 0.08 to 2.94 ± 0.36 pmol/mg, P < 0.01; Fig. 5). In PASMCs treated with ODQ (20 μM) alone, cGMP generation decreased by 44% (from 0.68 ± 0.08 to 0.38 ± 0.01 pmol/mg, P < 0.05; Fig. 5). In PASMCs stimulated with cinaciguat after ODQ treatment, cGMP generation increased by >2,500% compared with controls (from 0.68 ± 0.08 to 18.00 ± 3.23 pmol/mg, P < 0.01; Fig. 5). In contrast, ODQ reduced the effects of SNP on cGMP generation by 63% (from 0.25 ± 0.02 to 0.68 ± 0.08 pmol/mg, P < 0.05; Fig. 5).

DISCUSSION

Even though iNO is an effective therapy for infants with PPHN, up to 40% of these babies are poor responders to iNO (7, 10, 37). To determine whether a novel NO- and heme-independent activator of sGC can provide an alternate therapy for PPHN, we studied the effects of cinaciguat (BAY 58-2667) in vivo in the fetal lamb and in vitro in fetal ovine PASMCs. In the fetal lamb, cinaciguat significantly increased pulmonary blood flow in a dose-dependent manner by over fourfold and decreased PVR by 80%, and the pulmonary vasodilator effect was enhanced by sGC oxidation. In addition, compared with
Table 3. Maximal pulmonary hemodynamic response at baseline, after ODQ infusion, and after ACh and cinaciguat infusions

<table>
<thead>
<tr>
<th></th>
<th>Pre-ODQ</th>
<th>Post-ODQ</th>
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<tr>
<td></td>
<td>Baseline before</td>
<td>ODQ (0.5 mg)</td>
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<tr>
<td></td>
<td>drug infusion</td>
<td>ACh (15.0 µg)</td>
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<tr>
<td></td>
<td></td>
<td>Cinaciguat (5.0 µg)</td>
</tr>
<tr>
<td>Ql, PA, ml/min</td>
<td>68.2 ± 4.1</td>
<td>139.1 ± 10.2*</td>
</tr>
<tr>
<td>MPAP, mmHg</td>
<td>43.5 ± 1.6</td>
<td>44.1 ± 1.8</td>
</tr>
<tr>
<td>PVR, mmHg·mL⁻¹·min</td>
<td>0.63 ± 0.1</td>
<td>0.32 ± 0.03*</td>
</tr>
<tr>
<td>AoP, mmHg</td>
<td>42.0 ± 1.4</td>
<td>41.0 ± 2.5</td>
</tr>
<tr>
<td>LAP, mmHg</td>
<td>2.1 ± 0.1</td>
<td>2.1 ± 0.1</td>
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</table>

Values are means ± SE. ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; Ql,PA, left pulmonary artery blood flow; PVR, pulmonary vascular resistance.

*P < 0.05 vs. baseline. †P < 0.05 vs. ODQ baseline.

ACh, an NO synthase stimulator, the pulmonary vasodilator response of cinaciguat was more potent and sustained. Results from our in vitro studies of PASMCs paralleled our studies in the whole animal. Generation of cGMP from PASMCs in response to cinaciguat stimulation was enhanced in the presence of oxidized sGC. These results support our hypothesis that direct activation of sGC by the NO- and heme-independent sGC activator cinaciguat causes potent and sustained vasodilation in the fetal sheep, and this response is augmented in the presence of oxidized sGC.

These results are important, because this is the first report describing the ability of cinaciguat to cause potent and sustained pulmonary vasodilation in the developing pulmonary circulation of the fetal lamb. In our studies, microgram doses of cinaciguat caused a potent pulmonary vasodilator response in the fetal lamb. Previous studies using the sGC stimulator BAY 41-2272 required 500 times the dose (500 µg) used in our study of cinaciguat (1 µg) to achieve a doubling of MPA blood flow and to reduce PVR by ∼50% from baseline (16).

Our studies also showed that cinaciguat caused a sustained vasodilation in the ovine fetal lung by directly activating sGC and increasing cGMP levels. This response is different from many endothelium-dependent agonists, including ACh, bradykinin, histamine, tolazoline, oxygen, and shear stress, which only cause a transient vasodilation (1, 2, 5, 6). Conversely, agents that directly increase smooth muscle cell cGMP, such as atrial natriuretic peptide, 8-bromo-GMP, and iNO, can cause a sustained vasodilation (2, 28). In our study, the cinaciguat vasodilator response was greater than that induced by these other agents and persisted long after termination of drug infusion.

Our studies also show that cinaciguat induced significant pulmonary vasodilation in the presence of oxidized sGC caused by ODQ treatment. In addition, pulmonary vasodilation was increased >120% above baseline in the presence of ODQ in the fetal lamb. In our in vitro studies, treatment of PASMCs with cinaciguat + ODQ led to a 2,500% increase in the amount of cGMP generated compared with controls. These results are consistent with in vitro work by Stasch et al. in 2002 (42), which showed that purified sGC stimulation with cinaciguat is enhanced in the presence of ODQ. cGMP generation due to in vitro stimulation of PASMCs with SNP, an NO donor, was abolished in the presence of ODQ. Prior research showed that removal or oxidation of the heme moiety led to the formation of an NO-insensitive form of sGC (24, 25, 32, 35, 40, 48). Clinically, these concepts are important, inasmuch as they may explain one possible mechanism for NO insensitivity in some babies with PPHN. Should there be a higher concentration of heme-deficient or oxidized sGC in these babies, then cinaciguat may provide a novel targeted therapeutic option for this population of PPHN babies.
 Previous studies have shown that cinaciguat behaved in a manner completely different from all other known direct-acting sGC stimulators, including YC-1, BAY 41-2272, and BAY 41-8543 (23, 42). Cinaciguat directly activates sGC by binding the unoccupied heme-binding pocket or by replacing the weakly bound oxidized heme moiety (39, 42). Previous studies demonstrated that cinaciguat caused potent vasorelaxation of rabbit saphenous artery rings with an IC_{50} 160-fold more potent than BAY 41-2272 and >1,000-fold more potent than SNP or 3-morpholininosydnonimine (NO donors) (42). Cinaciguat has potent antihypertensive effects in spontaneously hypertensive rats and anesthetized dogs (42). 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 fetal lambs in vivo, ODQ was found to potentiate sGC enzyme activation by cinaciguat in vitro (42). Importantly, inhalation of microparticles with cinaciguat produced dose-dependent pulmonary vasodilation, increased transpulmonary cGMP release, and increased systemic arterial oxygenation in lambs with acute pulmonary hypertension caused by U-46619; these effects were greatly increased after treatment with ODQ (18).

In this study, cinaciguat caused a significant increase in HR at higher doses. Cinaciguat did not decrease systemic arterial pressure; however, this agent was infused directly into the LPA. Administration into the systemic circulation may reduce systemic pressure, as would likely occur in the clinical setting. In addition, this study examined the hemodynamic effects of cinaciguat in the normal fetus. Whether cinaciguat can cause potent and sustained pulmonary vasodilation in sheep with experimental PPHN remains to be studied.

In conclusion, cinaciguat causes potent and sustained pulmonary vasodilation in fetal sheep and increases cGMP production in PASMCs. Both of these responses to treatment with cinaciguat are augmented in the presence of oxidized sGC. These observations suggest that cinaciguat may have therapeutic potential as an alternate or adjuvant therapy for severe neonatal pulmonary hypertension, leading to our speculation that cinaciguat could provide a novel treatment strategy for PPHN.

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