0031-3998/06/6003-0252
PEDIATRIC RESEARCH

Vol. 60, No. 3, 2006
Printed in U.S.A.

Interaction of K_{ATP} Channels and Endothelin-1 in Lambs With Persistent Pulmonary Hypertension of the Newborn

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ABSTRACT: Persistent pulmonary hypertension of the newborn is a life-threatening condition in which half of infants fail to respond to inhaled nitric oxide. Development of new therapeutic pathways is crucial. The adenosine triphosphate (ATP)–sensitive potassium channels (K_{ATP}) may be important in this condition. Concentration-response curves to the K_{ATP} channel opener (SR47063) were performed in isolated pulmonary arterial rings from normal newborn lambs ($n = 8$) and pulmonary hypertensive lambs ($n = 7$) induced by intrauterine ductus arteriosus ligation. The effect of endothelin (ET) receptor antagonists was analyzed. Expression in the lung of the subunit Kir 6.1 of the K_{ATP} channel and of ET were analyzed using Western blot and immunohistochemistry. Relaxation to SR47063 was increased in ligated animals compared with the control group. Endothelium removal enhanced this response in ligated animals ($p < 0.01$). The inhibitory effect of the endothelium was reversed by the Endothelin-A receptor (ET-A) antagonist BQ 123 ($p < 0.01$). Kir 6.1 expression was not different between groups and that of endothelin-1 (ET-1) was increased threefold in ligated animals ($p = 0.007$). In pulmonary hypertensive lambs, vasodilation to K_{ATP} channel openers was enhanced compared with controls and further potentiated by ET-A blockade. These data might lead to new therapeutic strategies in infants with pulmonary hypertension. **(*Pediatr Res* 60: 252–257, 2006)**

Persistent pulmonary hypertension of the newborn (PPHN) is a severe disease characterized by sustained elevation of pulmonary vascular resistance after birth, resulting in significant morbidity and mortality in term and near-term infants (1,2). The pathophysiology of PPHN remains poorly understood. Endothelial dysfunction has been suggested to be partly responsible for this syndrome through an imbalance between the release of vasorelaxant substances and vasoconstrictive ones with an excess of ET-1 production (3–5).

ET-1 is a potent vasoactive agent that is produced by a number of cell types including pulmonary vascular endothelial cells. It produces its vasoactive effects through its interactions with at least two receptor subtypes, ET-A and ET-B, located on the vascular smooth muscle and endothelium. ET receptors are also present in other structures within the lung, such as fibroblasts, bronchial smooth muscle, and epithelial cells, but their respective distribution remains unknown (6). The pulmonary vasoconstrictor effect of ET-1 results mainly from activation of the ET-A receptor, whereas activation of the endothelial ET-B receptor has been shown to induce relaxation (5,7). Plasma levels and expression of ET-1 are increased in models of pulmonary hypertension as well as in children with PPHN (8–10).

The nitric oxide (NO) pathway, involved in endothelium-dependent pulmonary vasodilation has been shown to be deficient in PPHN (4,11–13). Although inhaled NO (iNO) has modified the poor prognosis of PPHN, half of the affected children are either refractory to iNO or remain dependent on it thereafter (14). There is a need for continued investigation of adjuncts, or even alternatives, to iNO for the treatment of PPHN.

Potassium channel activation is involved in the regulation of perinatal pulmonary vascular tone by inducing membrane hyperpolarization and vasodilation (15). In experimental PPHN in sheep, the expression of potassium channels (voltage-gated channels (K_V) and Ca^{2+}-sensitive K^+ (K_{Ca}) channels) have been shown to be decreased (16,17).

We have previously demonstrated an important vasorelaxant effect of ATP-sensitive potassium (K_{ATP}) channel openers at birth in piglets that failed to relax to acetylcholine, which exerts its vasodilator effect *via* NO (18). In addition, we have shown that K_{ATP} channels are strongly expressed in the perinatal human lung as assessed by the presence of the K_{ATP} subunit Kir 6.1 (19).

Furthermore, we have demonstrated that, at birth, the vasodilator effect of the K_{ATP} opener was reduced by the endothelium through the action of ET-1 (20). ET-1 has been shown to inhibit K_{ATP} channels in smooth muscle cells from rabbit

Received February 9, 2006; accepted April 19, 2006.
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This work was supported by a grant from the Fondation de l'Avenir pour la Recherche Médicale Appliquée.

DOI: 10.1203/01.pdr.0000233075.48306.57

Abbreviations: EC_{50}, concentration required to obtain half-maximal relaxation; **eNOS,** endothelial nitric oxide synthase; **ET-1,** endothelin-1; **ET-A,** endothelin A receptor; **ET-B,** endothelin B receptor; **iNO,** inhaled nitric oxide; **K_{ATP},** ATP sensitive-potassium channel; **L-NAME,** *N*-nitro-L-arginine methyl ester; **PPHN,** persistent pulmonary hypertension of the newborn

coronary and pulmonary arteries and in whole rat lungs *via* the ET-A activation (20–22). The excessive production of ET-1 in PPHN may in part inhibit K_{ATP} channel induced vasodilation. We hypothesized that in PPHN K_{ATP} channel openers could be an alternative therapy and that ET antagonists may potentiate their effect as vasodilators.

We have studied the pulmonary arterial responses to a K_{ATP} channel opener in a sheep experimental model of PPHN induced by intrauterine ductus arteriosus ligation (23). The inhibitory effect of ET on the K_{ATP} pathway was assessed.

METHODS

This study was approved by the local institutional ethics committee for animal research, and all animals received humane care in compliance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (National Institutes of Health publication no. 80-23, revised in 1996). We used an ovine experimental model of PPHN, produced by ligation of the ductus arteriosus 12 days before delivery (23).

Surgery was performed at 134–135 d of gestation (term = 147 d) under sterile conditions. Ewes were anesthetized with i.v. pentobarbital sodium (30 mg/kg) and were given immediately before surgery i.m. penicillin G (600 000 UI) and gentamicin (100 mg) and daily thereafter until delivery. The uterus was externalized, and the left fetal forelimb was exposed. The ductus arteriosus was ligated by hemoclip (Pilling-Weck, section 7 or 8) (ligated group $n = 7$). Ewes were then left to deliver naturally and lambs were allowed to breathe spontaneously for 2 h. All animals were born at 146–147 d and therefore had similar duration of ductus ligation. Eight lambs born spontaneously served as controls. In half of them, a thoracotomy had been performed without ductus arteriosus ligation at 134–135 d of gestation. No difference was observed between these sham animals and the nonoperated lambs, and the results were therefore pooled (control group, $n = 8$). All animals (2 h of age) were anesthetized with ketamine hydrochloride (0.3 mg/kg/min), diazepam (0.002 mg/kg/min) and fentanyl citrate (1 μg/kg/h) and killed by rapid exsanguination through a cardiac puncture. The heart and lungs were removed immediately after death and placed in a Krebs solution (in mmol/L: 119 NaCl, 5.4 KCl, 2.5 $CaCl_2$, 1.2 H_2PO_4, 25 $NaHCO_3$, 11.7 glucose) bubbled with a gas mixture (95% O_2, 5% CO_2).

To determine the presence of pulmonary hypertension, the fixed hearts of the lambs were dissected and weighed. The ratio of right ventricular weight to left ventricular + septum weights [RV/(LV + S)] was calculated (24). On histologic sections from the lungs, the medial wall thickness and external diameter of at least 20 arteries in a range of sizes were measured in each animal. The percentage wall thickness of each artery was assessed by the formula (2 × medial wall thickness/external diameter) × 100.

Pharmacological studies. Fourth-generation intralobar pulmonary arteries with a lumen diameter of 1.5–2 mm were isolated, cleaned of surrounding connective tissue, and cut into rings of approximately 2 mm length. From each animal, rings with (E+) or without (E−) endothelium were studied. The endothelium was removed by gently rubbing the luminal surface with watchmaker's forceps (18). Endothelium removal was confirmed during the course of experiment by lack of relaxation to acetylcholine (10^{-6} mol/L) during a U46619 contraction and at the end of the experiments, rings were fixed to confirm microscopically the presence or absence of endothelium. Vessel rings were mounted on two parallel stainless steel wire hooks in 5-mL glass organ baths filled with Krebs solution, maintained at 37°C and continuously bubbled with 95% O_2, 5% CO_2. Tissues were connected to a Statham force-displacement transducer. Isometric tension changes were recorded on a polygraph (Linseis 6512). After an initial stabilization period (60 min), each ring was stretched to its optimal point determined by the maximal force developed in response to 20 mmol/L of KCl. After resting tension was achieved, rings were equilibrated for 30 min. The cyclooxygenase inhibitor indomethacin (10^{-5} mol/L) was then added 30 min before the study and remained present throughout.

Rings were precontracted with the thromboxane analogue U46619 (10^{-6} mol/L, the concentration required to obtain half maximal relaxation (EC_{50}) determined in preliminary studies). In each animal, cumulative concentration-response curves to the K_{ATP} channel opener SR47063 (10^{-9}–10^{-5} mol/L) were obtained by adding the drug in log increments. This experiment was repeated in parallel in rings incubated for 30 min after precontraction with U46619 with either the NOS inhibitor *N*-nitro-L-arginine methyl ester (L-NAME) (10^{-5} mol/L), the potassium channel blocker glibenclamide (10^{-5} mol/L), the ET-A antagonist BQ 123 (10^{-5} mol/L) or the ET-B antagonist, BQ 788 (10^{-5} mol/L).

To further analyze the potential interaction between ET-1 and K_{ATP} channels, we analyzed the relaxant effect of SR47063 in rings precontracted with ET-1 (10^{-10} mol/L) in three ligated and three control animals.

At the end of the experiments, all rings were exposed to sodium nitroprusside (10^{-5} mol/L) to test the capacity of the smooth muscle to relax and then weighed.

Drugs. All drugs were obtained from Sigma Chemical Co. Aldrich (Saint Quentin Fallavier, France) except for SR47063 (a kind gift from Sanofi Aventis, Montpellier, France). Drugs were dissolved in distilled water, except indomethacin and SR47063, which were dissolved in ethanol. Drugs were freshly made every day. All concentrations are expressed as the final molar concentration in the organ chamber.

Immunohistochemistry. Serial sections (4 μm) of lung tissue blocks taken from the same animals used for pharmacological studies were immunostained with antiserum to ET-A, ET-B (Alexis Corporation, San Diego, CA) or to the Kir 6.1 subunit of K_{ATP} channels (Santa Cruz Biotechnology, Santa Cruz, CA). Tissue sections were rehydrated and heated for 40 min in buffered citrate at pH 6, incubated in hydrogen peroxide to block endogenous peroxidase activity, washed in Tris-buffered saline (TBS), and incubated for 1 h with either anti-Kir 6.1 (dilution 1/150), anti-ET-A (dilution 1/200), anti-ET-B (dilution 1/150) primary antibodies, or with normal serum used as a negative control. Sections were incubated for 15 min with a biotinylated secondary antibody and stained with streptavidin-labeled with peroxidase (Dako Corp., Carpenteria, CA). Diaminobenzidine tetrahydrochloride was used to visualize immunoreactive cells. Slides were counterstained using Harris hematoxylin.

Western blot analysis. Lung tissue from four controls and four ligated animals was homogenized in Tris-HCl containing protease inhibitors (0.1 mm) and was centrifuged at 10,000 × *g* for 10 min. Supernatant fractions were collected for total protein assay (Bradford assay kit, Bio-Rad, Hercules, CA) and Western blot analysis. Fifty micrograms of protein was separated by electrophoresis on a 12% polyacrylamide gel using Tris-glycine-sodium dodecyl (SDS) sulfate buffer. The separated proteins were transferred to nitrocellulose membranes at 1.2 mA/cm^2 for 1 h and 45 min in transfer buffer (25 mmol/L Tris-HCl, 192 mmol/L glycine, 20% methanol). The membranes were blocked with 5% nonfat dried milk in TBS 10 mmol/L Tris pH 7.4, 100 mmol/L NaCl, and then incubated overnight at 4°C with a rabbit polyclonal Kir 6.1 antibody (1:1000) (Santa Cruz Biotechnology). The membranes were then rinsed three times for 10 min each in TBS-0.1% Tween and incubated for 1 h at room temperature with an anti-rabbit immunoglobulin G–horseradish peroxidase conjugate (1:500). After three 10-min rinses, the reaction was developed using the Western Lightning Chemiluminescence Reagent *Plus* (Perkin Elmer Life Science, Boston, MA) on Kodak BioMax Light-1 films (Eastman Kodak, Rochester, NY). Membranes were washed with stripping buffer (β-mercaptoethanol 100 mmol/L, SDS 2%, Tris pH 6.8 20 mmol/L at 50°C for 15 min) and reprobed with a β-actin MAb used as housekeeping protein (Cytoskeleton Inc., Denver, CO) at a dilution of 1:1000 for 1 h. Band densitometry was performed using National Institutes of Health image analysis software.

Enzyme-linked immunosorbent assay (ELISA). The Biotrak ET-1 ELISA system (Amersham Pharmacia Biotech., Buckinghamshire, UK) was used to detect ET-1 production by the lungs of controls ($n = 5$) and ligated lambs ($n = 5$). Supernatant fractions from homogenized lung tissues were processed. ET-1 in the samples tested was captured by microtiter plates precoated with ET-1 antibody and detected by a peroxidase-labeled Fab′ fragment of ET-1 antibody conjugate.

Statistical analysis. Response to SR47063 was expressed as a percentage of relaxation from U46619 or ET-1–induced tone. EC_{50} was calculated by nonlinear regression analysis (Prism version 4.0), the maximal relaxation being that obtained at the highest concentration of SR47063 (10^{-5} mol/L). All values are expressed as mean ± SEM. Statistical analysis was performed on end point relaxation (10^{-5} mol/L) and EC_{50}. The effect of each vasoactive agent was compared by the paired *t* test using the Bonferroni correction when necessary. Comparisons between ligated and control lambs were performed using the Mann-Whitney *U* test. Protein expression was expressed as the percentage of control values and comparisons were performed using a *t* test. Differences were considered statistically significant when $p < 0.05$.

RESULTS

Morphologic suggestion of pulmonary hypertension. In the ligated animals, the ratio of right ventricular weights to left ventricular + septum weights was greater than in the control group (0.79 ± 0.05 and 0.62 ± 0.07; $p < 0.05$). These

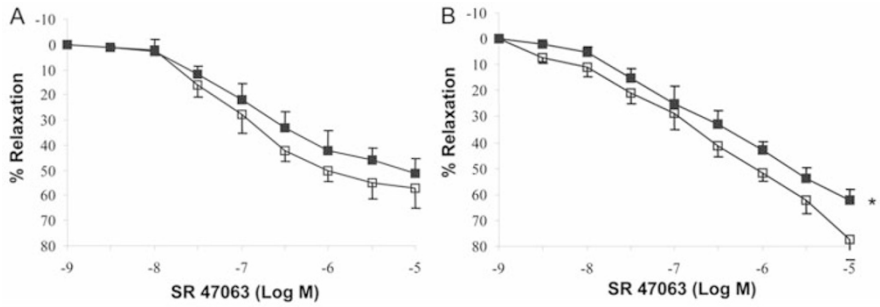

Figure 1. Cumulative concentration-response curves to SR47063 on rings precontracted with U46619 with (E+: ■) or without (E−: □) endothelium from control lambs ($n = 8$) (*A*) and ligated lambs ($n = 7$) (*B*).*$p < 0.05$ compared with ligated E− and controls.

findings show hypertrophy of the right ventricle that is associated with pulmonary hypertension (24). In addition, the percentage of wall thickness of intrapulmonary arteries calculated was greater in ligated than in control animals (59 ± 2% *versus* 42 ± 3%; $p < 0.05$), suggesting muscle cell hypertrophy or hyperplasia.

Organ chamber studies. Pulmonary artery ring weights were similar in ligated and control groups. The resting tension of the pulmonary artery rings was similar: 0.82 ± 0.03 g and 0.74 ± 0.04 g in ligated and control groups, respectively (not significant [NS]). The additional tone induced by U46619 (10^{-6} mol/L) in lamb pulmonary vessels was similar in both groups (0.69 ± 0.37 g and 0.61 ± 0.28 g in ligated and control groups, respectively; NS).

Pharmacological response to SR47063. SR47063 (10^{-5} mol/L) induced a greater arterial relaxation in ligated than in control groups (62.2 ± 4.3% and 51.2 ± 5.6%, respectively; $p < 0.05$) (Fig. 1) with a shift to the left of the concentration-response curve in the ligated group (−log EC_{50}: 6.83 ± 0.1 and 6.35 ± 0.2; $p < 0.05$) (Table 1). This relaxation was present in the presence or absence of endothelium (Fig. 1). However, in the ligated group, the removal of endothelium increased the vasodilation to SR47063 (10^{-5} mol/L) (62.2 ± 4.3% *versus* 77.6 ± 7.7% in E+ and E−, respectively; $p < 0.05$) (Fig. 1) and led to a significant shift to the left of concentration-response curve (Table 1).

Effect of L-NAME on SR47063-induced relaxation. The endothelial nitric oxide synthase (eNOS) inhibitor L-NAME had no inhibitory effect on SR47063 relaxation in ligated arteries (Fig. 2). However, it increased the relaxant effect of SR47063 from 51.2 ± 5.6% to 58 ± 10% in the control group ($p < 0.05$).

Effect of glibenclamide on SR47063-induced relaxation. The relaxation induced by SR47063 was abolished by the K_{ATP} channel blocker glibenclamide in both groups, confirming the specificity of SR47063 on K_{ATP} channels (Fig. 2).

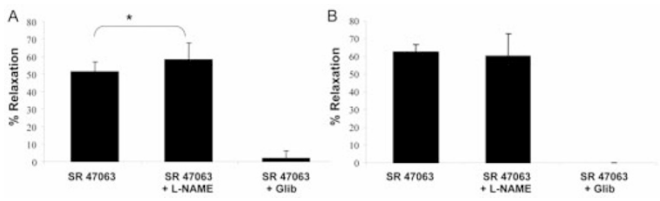

Figure 2. Histograms showing the effect of L-NAME (10^{-5} mol/L) and glibenclamide (10^{-5} mol/L) on relaxation to SR47063 (10^{-5} mol/L) on E+ rings precontracted with U46619 from control ($n = 8$) (*A*) and ligated lambs ($n = 7$) (*B*). *$p < 0.05$.

Effect of BQ 123 and BQ 788 on SR47063-induced relaxation. Blockade of ET-A receptors by BQ 123 potentiated the relaxant effect of SR47063 in both groups but more so in the ligated group (75.8 ± 7.1% *versus* 62.2 ± 4.3% with and without BQ 123, respectively, $p < 0.05$) (Fig. 3), with a significant increase in –log EC_{50} (Table 1). In E− rings, the relaxation to in E+ rings SR47063 was not further increased by BQ 123. In controls, BQ 123 increased the –log EC_{50} ($p < 0.05$) (Table 1), but not the maximum relaxation to in E+ rings SR47063 (57 ± 3.2% *versus* 51.2 ± 5.6% with and without BQ 123, respectively; NS). Preincubation with BQ 123 had no significant effect on baseline tone.

Preincubation with the ET-B antagonist BQ 788 induced an increased baseline tone in E+ rings in ligated and control rings, but had no effect on the SR47063 response (Fig. 3).

Effect of ET-1 precontraction. To confirm the inhibitory effect of ET-1 on K_{ATP} channels, we repeated concentration-response curves to SR47063 in rings precontracted with ET-1 (10^{-10} mol/L). This dose of ET-1 gave a precontraction comparable to that obtained with U46619 (10^{-6} mol/L) (0.8 ±

Table 1. *Effect of the removal of the endothelium or the ET-A (BQ 123) or the ET-B (BQ 788) receptor antagonists on SR 47063-induced relaxation in rings precontracted with U46619*

	E+	E+/BQ 123	E+/BQ 788	E−	E−/BQ 123	E−/BQ 788
Control						
−Log EC_{50}	6.35 ± 0.2*	6.58 ± 0.2†	6.38 ± 0.3	6.46 ± 0.2	7 ± 0.3‡	6.43 ± 0.2
E_{max}	51.2 ± 5.6%	57 ± 3.2%	52 ± 7.2%	57 ± 7.9%	59 ± 3.2%	55 ± 8.3%
Ligated						
−Log EC_{50}	6.83 ± 0.1	7.12 ± 0.2†	6.93 ± 0.2	7.2 ± 0.1†	7.3 ± 0.2†	7 ± 0.2
E_{max}	62.2 ± 4.3%	75.8 ± 7.1%	64 ± 5.6	77.6 ± 7.7%	78.3 ± 4.7%	73.2 ± 5.6%

* $p < 0.05$ compared to E+ rings in ligated group.
† $p < 0.05$ compared to E+ rings in each group.
‡ $p < 0.05$ compared to E+ and E− rings in controls.

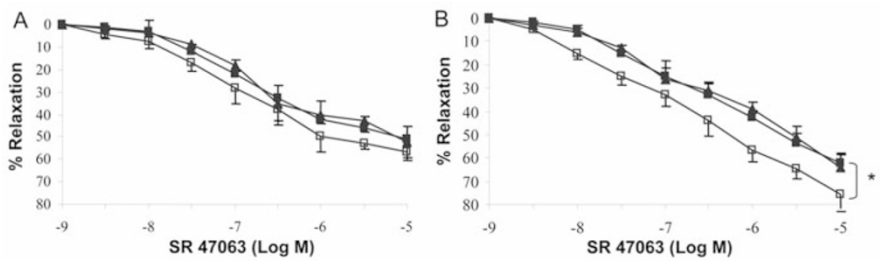

Figure 3. Cumulative concentration-response curves to SR47063 on E+ rings precontracted with U46619 from control ($n = 8$) (*A*) and from ligated lambs ($n = 7$) (*B*) in the presence of the ET-A antagonist BQ 123 or the ET-B antagonist BQ 788. E+ (■), E+/BQ123 (□), E+/BQ788 (▲). *$p <$ 0.05.

0.3 g and 0.7 ± 0.25 g in ligated and controls, respectively). Maximal relaxation to SR47063 (10^{-5} mol/L) was less when rings were precontracted with ET-1 than with U46619 (21 ± 5% *versus* 62.2 ± 4.3% in ligated and 17 ± 6% *versus* 51.2 ± 5.6% in controls; $p < 0.001$ in both). BQ 123 enhanced significantly the relaxation to SR47063 in both groups in E+ and E− rings (Fig. 4, Table 2).

Immunolocalization of ET-A, ET-B, and Kir 6.1. ET-A were detected on arterial smooth muscle cells in proximal arteries used for pharmacological studies and on distal pulmonary arteries (Fig. 5*A*). ET-B were detected on arterial smooth muscle cells and endothelial cells (Fig. 5*B*). The K_{ATP} channel subunit Kir 6.1 was detected on arterial smooth muscle cells in both proximal (≈1 mm) and in peripheral arteries (Fig. 5*C*).

Western blotting analysis of Kir 6.1 protein. Western blot analysis detected a single protein band for Kir 6.1 at 46 kD in both groups. Measurement of density showed no significant difference between control and ligated animals (Fig. 6).

Quantification of ET-1 protein by ELISA. ET-1 peptide levels in lung homogenates detected by ELISA is increased in ligated animals compared with controls by threefold ($p = 0.007$) (Fig. 7).

DISCUSSION

PPHN is a potentially life-threatening condition in which therapeutic options are poor. Understanding the contribution of the different vasoactive factors produced by the endothelium to the pathophysiology of this condition and their potential as therapeutic targets is still incomplete. In this study, we used a sheep model of fetal ligation of the ductus arteriosus, which has previously been shown to be a good model of neonatal pulmonary hypertension (23). We found as previously described in this model morphologic evidence of pulmonary hypertension with right ventricular hypertrophy and increased arterial muscularity (24). At birth, SR47063 induced a marked vasodilator response that was greater in the pulmonary hypertensive group and was enhanced by blocking the ET-A in this group. This vasodilative effect was demonstrated on fourth-generation pulmonary arteries (1.5–2 mm), whereas pulmonary vascular resistance involves also more distal vessels. We therefore looked for K_{ATP} channel expression in distal and proximal intrapulmonary arteries. To our knowledge, we have for the first time shown expression of these channels on smooth muscle cells of newborn pulmonary arteries in both groups using immunohistochemistry and Western blot. However, the increased response to SR47063 in the hypertensive animals was not related to an increase in Kir 6.1 protein.

The relaxant response to the K_{ATP} channels openers was important in both normotensive and hypertensive groups and was totally abolished by the K_{ATP} inhibitor glibenclamide, confirming the specificity of SR47063 on K_{ATP} channels (25). Although conflicting results have been reported on the activity of K_{ATP} channels in the perinatal period (26,27), we found a potent pharmacological effect in newborn lambs pulmonary arteries from both hypertensive and controls. Surprisingly, the relaxant effect of SR47063 was greater in our ligated animals than in controls, although the protein expression was comparable in both groups. This enhanced vasodilator effect of K_{ATP} channel openers has also been reported in a porcine hypoxic model of pulmonary hypertension (28,29). This could suggest an increased pharmacological effect of a potassium channel opener when the NO pathway is defective as it is in PPHN

Table 2. *Effect of the removal of the endothelium or ET-A antagonist BQ 123 on SR47063-induced relaxation in rings precontracted with ET-1*

	E+	E+/BQ 123	E−	E−/BQ 123
Control				
−Log EC_{50}	6.4 ± 0.2	7.0 ± 0.1*	6.9 ± 0.1	7.0 ± 0.2
E_{max}	17 ± 6%	53 ± 6%*	20 ± 7%	35 ± 10%
Ligated				
−Log EC_{50}	6.5 ± 0.1	7.0 ± 0.1*	6.8 ± 0.2	7.1 ± 0.1
E_{max}	21 ± 6%	46 ± 5%*	28 ± 6%	50 ± 7%*

* $p < 0.05$ compared to E+ rings in each group.

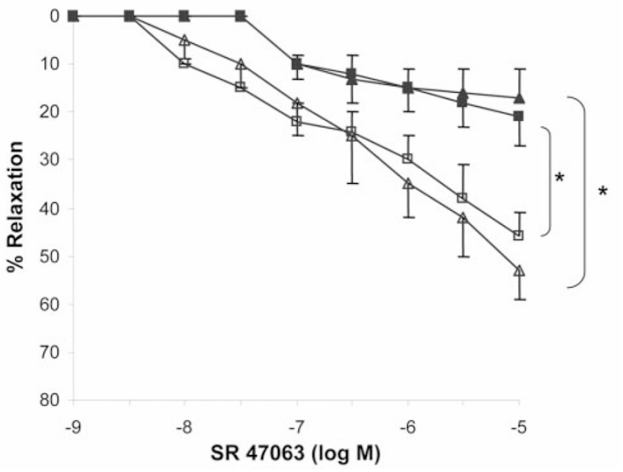

Figure 4. Cumulative concentration-response curves to SR47063 on E+ rings precontracted with ET-1 pulmonary arteries from control ($n = 3$) and ligated lambs ($n = 3$) in the absence or the presence of the ET-A antagonist BQ 123. Control (▲), control + BQ123 (△), ligated (■), ligated + BQ123 (□). *$p < 0.05$.

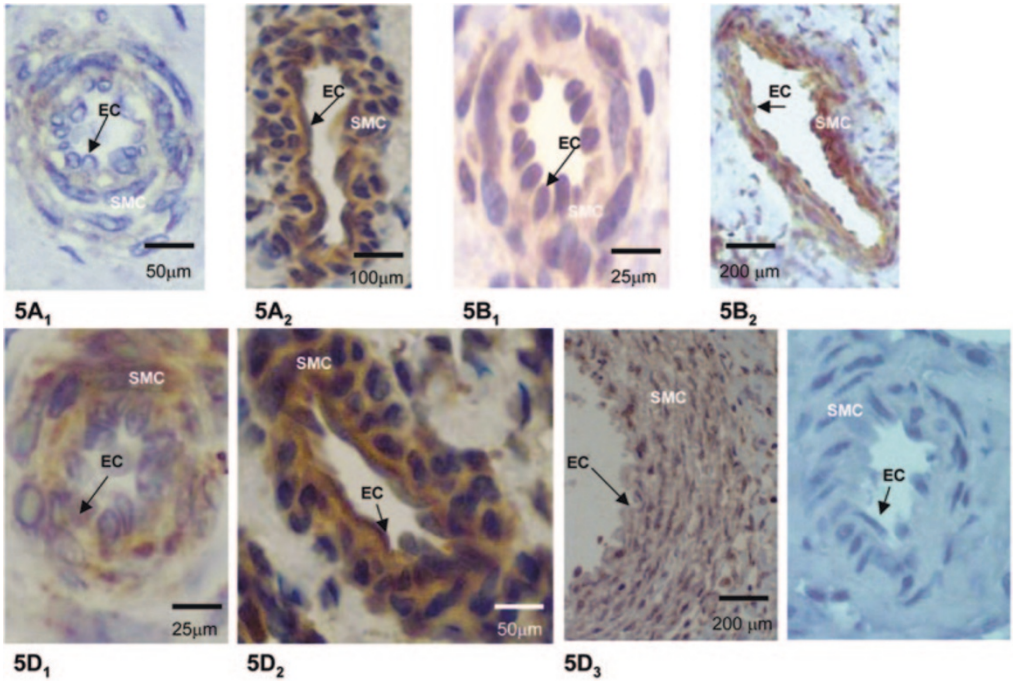

Figure 5. Immunohistochemical localization of ET-A, ET-B, and Kir 6.1 protein expression in the ligated group. Immunostaining (brown) for ET-A in smooth muscle cells (SMC) of intra-acinar pulmonary artery (A_1, distal pulmonary artery; A_2, proximal pulmonary artery). *B*, ET-B on endothelial cells and smooth muscle cells of distal (B_1) and proximal (B_2) pulmonary artery. Kir 6.1 immunostaining is shown on smooth muscle cells of distal (C^1) and proximal (C_2) intra-acinar arteries and intralobar artery (C_3) used for pharmacological studies. *D*, negative controls.

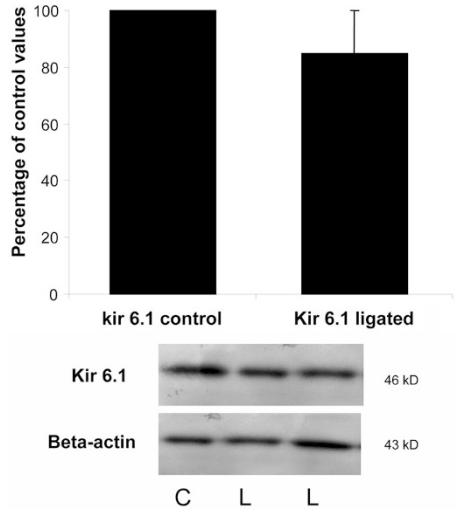

Figure 6. Western blot analysis for Kir 6.1 protein. Fifty micrograms of protein extracted from lung tissues from control ($n = 4$) and ligated lambs ($n = 4$) were analyzed on four blots. (*Upper panel*) The mean densitometric values expressed as a percentage of control data and found no statistical difference between control and ligated lambs. (*Lower panel*) Individual Western blot findings in one control (C) and two ligated (L) animals and β-actin bands probed on the same blot. Data were normalized to β-actin.

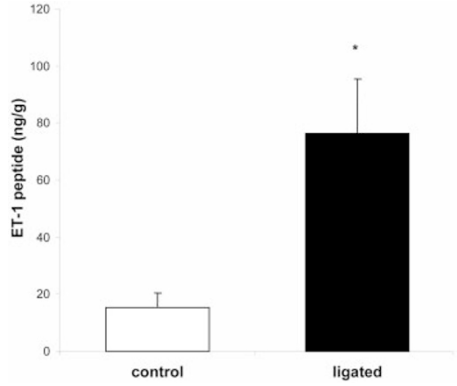

Figure 7. ELISA for ET-1 quantification. ELISA quantification of ET-1 in lung tissues from controls (□: $n = 5$) and ligated lambs (■: $n = 5$). There is a threefold increase in the ligated animals compared with control animals. *$p = 0.007$.

(4,11–13). In accordance with this hypothesis, the inhibition of the NO pathway by L-NAME enhanced the vasorelaxant effect of SR47063 in controls. NO is known to inhibit NOS activity through interaction with superoxide whose production is increased in PPHN (30,31). In addition, superoxide generation is involved in protein kinase C–mediated K_{ATP} blockade (32). The impaired NO pathway in PPHN could therefore partly explain the increased K_{ATP} activity.

In addition, SR47063-induced pulmonary vasorelaxation was greater in endothelium denuded rings than in intact rings. These results indicate that vasorelaxation to the K_{ATP} channel agonist involves vascular smooth muscle and that the endothelium produces inhibition of this relaxation. We have previously reported an inhibitory effect of the endothelium at birth in porcine pulmonary arteries where it was mediated by ET-1 (18).

Inhibition of K_{ATP} channels by ET-1 has been described in smooth muscle cells of the pulmonary and systemic arteries where ET-A receptors and protein kinase C are involved (22,33). Because ET-1 production by the endothelium is increased in PPHN and in our model, we hypothesized that it could be involved in inhibiting K_{ATP} channels. We tested both ET receptor antagonists on SR47063-induced relaxation. ET-B blockade increased basal tone, confirming a vasodilative effect of ET-1 at birth, as previously described (7), but did not affect relaxation to SR47063. By contrast, we found that the ET-A antagonist BQ 123 enhanced vasorelaxation to the K_{ATP} channel opener, suggesting an inhibitory effect of ET-1 through ET-A activation. This inhibitory effect depends on the release of ET-1 by the endothelial cells because BQ 123 had

no enhancing effect on vasodilation in endothelium denuded rings. In addition, the very poor vasodilation *via* K_{ATP} openers when rings were precontracted by ET-1 adds further to the argument for an inhibitory effect of ET-1 on K_{ATP} channels. This relaxation on rings precontracted with ET-1 was significantly enhanced by BQ 123, confirming that the inhibitory effect of ET-1 involves ET-A. This inhibitory effect of ET-1 on K_{ATP} channels, to our knowledge, has not been previously reported in newborn animals or those with PPHN. The nonspecific ET receptor antagonist bosentan has been shown to be effective in adults and children with pulmonary hypertension (34). Its efficacy on infants with PPHN is unknown, but caution is needed for the use of this drug in the perinatal period because of the potential vasodilative effect of ET-B.

In summary, the K_{ATP} opener SR47063 is a highly effective pulmonary vasodilator in this model of PPHN where the NO pathway is deficient (4,11–13). This rather is further potentiated by ET-A antagonists in our model. These data suggest an alternative and adjunctive therapeutic approach in PPHN for those who fail to respond to classic treatment.

Acknowledgments. The authors thank Dr. Nicholas Borenstein and Dr. Luc Behr (IMM Recherche, Institut Mutualiste Montsouris) for expert technical assistance.

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