Chronic pulmonary hypertension increases fetal lung cGMP phosphodiesterase activity

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1Departments of Pediatrics, Anesthesiology, and Pharmacology, University of Washington School of Medicine, Seattle, Washington 98105-0037; 2Pediatric Heart-Lung Center, Department of Pediatrics, University of Colorado School of Medicine, Denver, Colorado 80218; and 3Department of Pediatrics, Brown University School of Medicine, Providence, Rhode Island 02903

Hanson, Kimberly A., James W. Ziegler, Sergei D. Rybalkin, Jim W. Miller, Steven H. Abman, and William R. Clarke. Chronic pulmonary hypertension increases fetal lung cGMP phosphodiesterase activity. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L931–L941, 1998.—An experimental ovine fetal model for perinatal pulmonary hypertension of the neonate (PPHN) was characterized by altered pulmonary vasoreactivity and structure. Because past studies had suggested impaired nitric oxide-cGMP cascade in this experimental model, we hypothesized that elevated phosphodiesterase (PDE) activity may contribute to altered vascular reactivity and structure in experimental PPHN. Therefore, we studied the effects of the PDE inhibitors zaprinast and dipyridamole on fetal pulmonary vascular resistance and PDE5 activity, protein, mRNA, and localization in normal and pulmonary hypertensive fetal lambs. Infusion of dipyridamole and zaprinast lowered pulmonary vascular resistance by 55 and 35%, respectively, in hypertensive animals. In comparison with control animals, lung cGMP PDE activity was elevated in hypertensive fetal lambs (150%). Increased PDE5 activity was not associated with either an increased PDE5 protein or mRNA level. Immunocytochemistry demonstrated that PDE5 was localized to vascular smooth muscle. We concluded that PDE5 activity was increased in experimental PPHN, possibly by posttranslational phosphorylation. We speculated that these increases in cGMP PDE activity contributed to altered pulmonary vasoreactivity in experimental perinatal pulmonary hypertension.

Persistent pulmonary hypertension of the newborn (PPHN) is the failure of postnatal adaptation of the pulmonary circulation at birth. PPHN is characterized by sustained elevations of pulmonary arterial pressure (PAP) and abnormal pulmonary vasoreactivity, leading to right-to-left shunting of blood across the ductus arteriosus (DA) and foramen ovale, causing severe hypoxemia (21). Clinical observations have led to the hypothesis that intrauterine stimuli may alter the pulmonary circulation before birth, leading to an inability to achieve or sustain the normal decrease in PVR at delivery (33). On the basis of observations that fetal hypertension can alter pulmonary vascular structure in lambs, an animal model of chronic fetal pulmonary hypertension involving compression or ligation of the DA in utero was developed to better understand the etiology and pathophysiology of PPHN (4, 29). This model was characterized by progressive pulmonary hypertension and altered vasoreactivity, characterized by the loss of vasodilation to some pharmacological (ACh) and physiological stimuli (such as increased oxygen and shear stress) (13, 36). In addition, these animals had right ventricular hypertrophy and sustained pulmonary hypertension after delivery, requiring ventilation with increasing oxygen concentrations. Recent studies (26, 42) demonstrated a decrease in endogenous endothelial NO synthase (eNOS) mRNA, protein, and activity in this chronic pulmonary hypertensive model; thus it may have contributed to sus-

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tained pulmonary hypertension in this model. In addition to decreasing eNOS, a pharmacological study (37) also demonstrated decreased guanylate cyclase activity in this model. Thus multiple abnormalities in the NO-cGMP cascade contributed to increasing PVR in this perinatal model.

Because vasodilation at birth is partly dependent on NO release and sustained elevation of cGMP in smooth muscle, we hypothesized that persistent or increased PDE5 may have contributed to the persistently elevated PVR in PPHN. To test this hypothesis, we studied the physiological effects of the PDE5 antagonists zaprinast and dipyridamole in fetal lambs with intrauterine pulmonary hypertension and in age-matched control lambs. To determine whether the effects of PDE5 were localized to pulmonary vascular tissue, we determined PDE5 protein in fetal ovine pulmonary tissue by immunocytochemistry (ICC). To determine whether pulmonary hypertension altered lung PDE activity, we also measured PDE5 activity, protein, and mRNA levels in fetal lambs with pulmonary hypertension and in age-matched control lambs.

METHODS

Physiological Studies

Surgical preparation. All procedures and protocols were reviewed and approved by the Animal Care and Use Committee at the University of Colorado Health Sciences Center (Denver). Mixed-breed (Columbia-Rambouillet) pregnant ewes were fasted for 24 h before surgery at 125–130 days of gestation (term, 147 days). Ewes were sedated with pentobarbital sodium (2–4 g iv infusion) and anesthetized with 1% isoflurane. Cerebral sodium (2–4 g iv infusion) and anesthetized with 1% isoflurane. Cerebral sodium (2–4 g iv infusion) and anesthetized with 1% isoflurane. Cerebral sodium (2–4 g iv infusion) and anesthetized with 1% isoflurane. Cerebral sodium (2–4 g iv infusion) and anesthetized with 1% isoflurane. Cerebral sodium (2–4 g iv infusion) and anesthetized with 1% isoflurane. Cerebral sodium (2–4 g iv infusion) and anesthetized with 1% isoflurane. Cerebral sodium (2–4 g iv infusion) and anesthetized with 1% isoflurane. Cerebral sodium (2–4 g iv infusion) and anesthetized with 1% isoflurane. 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mM magnesium acetate, 0.2 mg/ml of BSA, 1 μM cold cGMP, and [3H]cGMP (10^5 counts/min). The PDE5 inhibitor zaprinast was added at 4 μM. PDE1 activity was suppressed in the presence of EGTA (10 μM). To ensure appropriate linear kinetics of cGMP PDE activity, we initially assayed samples to determine the volume required for 20–30% hydrolysis of cGMP. All samples were assayed in triplicate. Protein concentrations were determined by the method of Bradford (8). cGMP PDE activity is expressed as picomoles of cGMP hydrolyzed per minute per milligram of measured protein.

Immunoblot analysis. PDE5 ANTIBODY PRODUCTION. Rabbits were used for the production of all the PDE antibodies. Based on the published sequence of bovine PDE5 (24), a peptide of 16 amino acids from the COOH-terminal region with the sequence C-R-K-N-R-Q-K-W-Q-A-L-E-Q-O-E-K-OH was selected. The peptide was conjugated to keyhole limpet hemocyanin (KLH) by the method of Green et al. (15).

The peptide-KLH conjugate (600 µg) was injected subcutaneously in rabbits; 3 wk later, a booster injection of peptide-KLH (300 µg) conjugate was given. The antibody specificity was determined by immunoblot analysis with partially purified bovine lung PDE5 obtained by the method of Thomas et al. (38). As anticipated from the conservation of homology among PDEs in various species, the PDE5 antibody also recognized mouse and rat PDE5. An immunoblot with preimmune serum and the COOH-terminal peptide (100 µg) mixed with PDE5 antibody was performed. These studies showed no reactivity, confirming that the antibody is specific for sheep PDE5.

PHOSPHORYLATED PDE5 ANTIBODY PRODUCTION. With the use of a peptide of 18 amino acids from the cGMP binding region, the sequence H-R-D-F-E-S-A-S-I-K-R-P-D-T-G-C-OH was synthesized with phospho- or dephosphoserine at position 92 (Quality Controlled Biochemicals). Antibodies were produced that were selective for phosphorylated and unphosphorylated PDE5. With the use of the method of Thomas et al. (39), partially purified bovine PDE5 was phosphorylated with protein kinase A for 60 min. The phosphorylated PDE5-selective antibody showed increased affinity for phosphorylated PDE5, whereas the unphosphorylated PDE5-selective antibody showed decreased affinity for phosphorylated PDE5 as assessed by both 32P incorporation and immunoblot analysis.

ACTIN ANTIBODY. α-Actin vascular smooth muscle antibody was obtained from Sigma.

Immunoblot methodology. Protein samples were prepared in 2× sample buffer containing 0.12 M Tris·HCl (pH 6.8), 20% glycerol, 5% SDS, 10% mercaptoethanol, and 0.01% bromphenol blue at both 1 and 2 mg/ml for immunoblot analysis. The immunoblot samples were boiled for 5 min and stored at –20°C. To determine the concentration of PDE5 protein in supernatant from DA-ligated and control animals and to account for variation in enhanced chemiluminescence (ECL) exposure, we developed standard curves of bovine PDE5 (concentrations ranging from 0.0625 to 0.5 µg) at 1–10 s. These experiments demonstrated that ECL was linear at 3 and 5 s over the range of added bovine PDE5 protein. Thus Western blots were subsequently developed at 3 and 5 s. These immunoblots were scanned by DATASCAN II and analyzed by National Institutes of Health (NIH) Image. With the use of NIH Image, each lane containing bovine PDE or sheep lung protein was analyzed vertically with the background subtracted, and the number of pixels under the curves was determined. Every PDE5 immunoblot contained the same internal standards (bovine PDE at 0.25 µg). Therefore, because samples were assayed on the linear portion of the ECL exposure curves and the same internal standard was present on each immunoblot, quantitative comparison of ovine PDE5 could be made from the immunoblots. Prestained broad molecular-weight and kaleidoscope molecular-weight standards (Bio-Rad) were used and boiled for 1.5 min. The samples were loaded onto an 8% SDS resolving gel and 5% stacking gel in a 1-mm-thick slab with the products of Protogel. The proteins were blotted onto nitrocellulose paper and reacted with the respective polyclonal antibodies. The specific PDE isoform and actin signals were detected with the supersignal chemiluminescent-horseradish peroxidase substrate system (Amersham). The immunoblots of α-actin were scanned by DATASCAN II and analyzed by NIH Image and are reported as the number of pixels.

Measurement of PDE5 mRNA by RNase protection assay.

TOTAL RNA EXTRACTION AND mRNA EXTRACTION. Sheep RNA was extracted with a modification of the single-step method by Chromczynski and Sacchi (11). A purified mRNA preparation was made from total RNA with reagents and protocols supplied in the Fast Trak 2.0 mRNA Isolation Kit (Clontech). mRNA was then quantitated via ultraviolet spectrophotometry and used in the preparation of a cDNA library. Sheep lung cDNA was made with the Marathon kit from Clontech. This cDNA pool provided amplified sheep lung PDE5 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene fragments via PCR as described in GENERATION OF OVINE PDE5 PCR FRAGMENTS AND PRODUCTION OF GAPDH CLONES, respectively.

GENERATION OF OVINE PDE5 PCR FRAGMENTS. Ovine PDE5 gene fragments were made via PCR. PDE5-specific oligopeptides based on bovine PDE5 sequences (Genetics Computer Group accession no. L16545) were synthesized in the University of Washington Department of Pharmacology Core Molecular Biology Facility (Seattle). Two sets of PCR primers were developed that would produce nonoverlapping PCR products.

Table 1. Gestational age and baseline hemodynamic measurements in normal (control) and hypertensive (DA-ligated) fetal lambs before dipyridamole or zaprinast treatment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>DA ligation</th>
<th>Control</th>
<th>DA ligation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age, days</td>
<td>134 ± 2</td>
<td>133 ± 1</td>
<td>134 ± 2</td>
<td>134 ± 1</td>
</tr>
<tr>
<td>LPA blood flow, ml/min</td>
<td>74 ± 2</td>
<td>54 ± 9*</td>
<td>76 ± 1</td>
<td>51 ± 8*</td>
</tr>
<tr>
<td>Mean PAP, mmHg</td>
<td>42 ± 2</td>
<td>77 ± 5*</td>
<td>41 ± 2</td>
<td>79 ± 5*</td>
</tr>
<tr>
<td>Mean AoP, mmHg</td>
<td>41 ± 1</td>
<td>40 ± 1</td>
<td>41 ± 2</td>
<td>41 ± 2</td>
</tr>
<tr>
<td>TPR, mmHg·m-1·min-1</td>
<td>0.57 ± 0.03</td>
<td>1.59 ± 0.26*</td>
<td>0.54 ± 0.03</td>
<td>1.70 ± 0.24*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5 animals/group. LPA, left pulmonary artery; PAP, pulmonary arterial pressure; AoP, aortic pressure; TPR, total pulmonary resistance in left lung. *P < 0.05 for comparison of baseline parameters in control vs. hypertensive lambs before dipyridamole or zaprinast infusion.
of PDE5. The first set of primers was designed to produce a PCR product corresponding to base pairs 1687–2399 of bovine PDE5. The primers used to generate this product were 5′-TGCAGTCCTTAGCGGCTGCT-3′ (sense primer) and 5′-CCGTTGTTGAATAGGCCAGG-3′ (antisense primer). The product of this PCR was termed PDE5-1. A second set of primers was designed to cover base pairs 1–1046 of bovine PDE5. The primers used to generate this ovine PCR product were 5′-GGGAGGGTCTCGAGGCGAGTTC-3′ (sense primer) and 5′-CTCCAGCAGTGAAGTCTCATAG-3′ (antisense primer). The product of this PCR reaction was termed PDE5-2. Sense and antisense primers (20 pmol each) were mixed with 5 µl of the cDNA pool described above. PCR was done in the following sequence: 94°C for 30 s, 60°C for 30 s, and 68°C for 5 min. This PCR reaction was performed for a total of 40 cycles, and the purity of PCR products was determined by running 20 µl of each reaction on a 1% agarose-1× tricine-KOH gel. Fragments generated were purified out of low-molecular-weight protein agarose-gel, and poly(A) + tails were added to PCR fragments to increase ligation efficiency with Tailing Reaction Mix (Promega). Escherichia coli (Invitrogen INVαF ′) was transformed with the above ligation reactions with the Original TA Cloning Kit protocol and reagents (Invitrogen).

Production of probes for internal control of total RNA loading for analysis of mRNA levels. Production of GAPDH clones. Consensus sequence (human, mouse, and rat) primers for PCR amplification of GAPDH were purchased from Clontech. The 5′ primer had the sequence 5′-ACCACAGTCATGCCATCAC-3′. The 3′ primer had the sequence 5′-TCCACCCCTGTGTGCTGTA-3′. With the use of the method described above for the production of PDE5 clones, ovine GAPDH clones were obtained. These GAPDH clones were confirmed by sequencing.

Production of probes for RNase protection assay. Both PDE5-1 and PDE5-2 clones were used to make probes for RNase protection assay (RPA) analyses. Plasmids were cut with appropriate enzymes, and 32P-labeled transcripts were made with the MaxiScript system (Ambion). RPAs were begun by adding 15 µg of sample of sheep lung RNA. This was mixed with 1 × 106 counts/min of one or more of the probes described above. Nucleic acid was precipitated with 0.5 M ammonium acetate and 70% ethanol for 15 min at −70°C and centrifuged (16,000 g, 15 min at 4°C). Pellets were resuspended in 20 µl of hybridization buffer (80% deionized formamide, 100 mM sodium citrate (pH 6.4), and 1 mM EDTA) and denatured (3 min at 90°C and 10-s vortex) and incubated overnight at 45°C. The next day, selected samples were digested for 1 h at 37°C with a mixture of RNase A (0.9 U/ml) and RNase T1 (36 U/ml) in a total volume of 220 µl.

Fig. 1. Pulmonary hemodynamic effects of zaprinast (ZAP) in control (CTL) and ductus arteriosus-ligated (hypertensive (HTN)) fetal lambs. ZAP lowered total pulmonary resistance (TPR; mean pulmonary arterial pressure divided by flow) in both study groups (n = 5 animals/group) expressed as absolute change in TPR (A) and %change from baseline (BL; B). *P < 0.05 vs. BL.

Fig. 2. Pulmonary hemodynamic effects of dipyridamole (DIP) in CTL and HTN fetal lambs. DIP lowered TPR in both study groups (n = 5 animals/group) expressed as absolute changes in TPR (A) and %change from BL (B). *P < 0.05 vs. BL.
RNA was then precipitated, air-dried, and resuspended in 4 µl of gel loading buffer, then visualized by SDS-PAGE (5% acrylamide-8 M urea) and autoradiography.

ICC. Portions of left and right fetal lung were prepared in 1% paraformaldehyde-PBS (pH 7.4) as previously described (17). The frozen tissue sections were blocked with a solution containing 5% goat serum to prevent nonspecific binding. The sections were incubated with anti-PDE5 COOH-terminal antibody at 1:1,000 dilution overnight. As controls, other sections were incubated overnight with preimmune serum at a 1:1,000 dilution or anti-PDE5 COOH-terminal antibody at a 1:1,000 dilution and 100 µg/ml of COOH-terminal peptide. The sections were washed and incubated with a biotinylated horse anti-rabbit antibody. In the tissue sections, intrinsic peroxidase activity was quenched by hydrogen peroxide (0.3%). The slides were covered with a streptavidin solution stained by the avidin-biotin-peroxidase complex method with ABC Vecastain Kit from Vector Laboratories.

RESULTS

Physiological Studies

Table 1 summarizes the gestational ages and baseline hemodynamic measurements in control and DA-ligated study groups. Gestational age was similar in each group. The DA-ligated group developed increased mean PAP and decreased LPA flow without changing aortic pressure. TPR (mean PAP divided by flow) was increased nearly threefold 8 days after DA ligation. At autopsy, fetal weights in control (3,135 ± 310 g) and hypertensive lambs (3,175 ± 85 g) were not different. Right ventricular hypertrophy, expressed as the ratio of right ventricular to left ventricular + septal weight was markedly elevated in the DA-ligated group (control 0.53 ± 0.03 vs. hypertension 0.82 ± 0.05; P < 0.05).

Fig. 3. Effects of DIP and ZAP on TPR in CTL and chronic HTN lambs. Responsiveness to phosphodiesterase (PDE) inhibitors was similar in both study groups.

Fig. 4. Effects of DIP and ZAP on TPR in CTL and chronic HTN lambs. Responsiveness to phosphodiesterase (PDE) inhibitors was similar in both study groups.

RNA was then precipitated, air-dried, and resuspended in 4 µl of gel loading buffer, then visualized by SDS-PAGE (5% acrylamide-8 M urea) and autoradiography.

Fig. 4. Effects of chronic pulmonary hypertension on average cGMP PDE activity at 1 µM cGMP in presence and absence of ZAP in CTL (n = 6) and HTN (n = 6) animals. Total cGMP PDE activity is significantly elevated in HTN fetal lung compared with CTL. Presence of ZAP demonstrates that majority of cGMP PDE activity in presence of EGTA is PDE5. Significantly different from CTL: *P < 0.0001; **P < 0.001.

Fig. 5. A: representative immunoblot of α-actin vascular smooth muscle (VSM) protein in fetal HTN (n = 3) and CTL (n = 3) animals. Nos. at left, molecular-weight markers. B: means ± SE of α-actin VSM protein by immunoblot analysis in fetal HTN (n = 6) and CTL (n = 6) lambs. After 30-s exposure, immunoblots were analyzed by DATASCAN II and NIH Image. Pixels were determined and averaged. Data demonstrate no significant difference between the 2 groups in α-actin VSM protein content and also illustrate lack of difference between individual samples.
Brief infusions of zaprinast and dipyridamole decreased TPR in both control late-gestation fetal lambs and DA-ligated animals (hypertensive). Figures 1 and 2 demonstrate the pulmonary effects of zaprinast and dipyridamole, respectively, in hypertensive vs. control fetal sheep. As shown in Fig. 1, zaprinast lowered TPR in both study groups. As shown in Fig. 2, dipyridamole also lowered TPR in both study groups.

Figure 3 is a summary of the maximum reduction in TPR in control and DA-ligated animals receiving either zaprinast or dipyridamole. These results demonstrate that the maximal reduction in TPR in the DA-ligated group was 35%, whereas in the control group, the maximal reduction in TPR was 46%. With the administration of dipyridamole, the maximal reduction in TPR was similar in both the control (63%) and DA-ligated groups (63%). In both groups (control and DA ligated), dipyridamole caused a slightly greater reduction in TPR than zaprinast.

Biochemical Studies
cGMP-dependent PDE activity in hypertensive animals was significantly elevated compared with control animals ($P < 0.0001$) (Fig. 4). Inhibition of cGMP-dependent PDE activity with zaprinast demonstrated that the increase in cGMP PDE activity was secondary to PDE5-specific activity.

Figures 5–8 show the immunoblot data for analysis of $\alpha$-actin, PDE5, and phosphorylated PDE5 in hypertensive vs. control fetal sheep. Figure 5 shows no significant difference in vascular smooth muscle $\alpha$-actin levels in hypertensive vs. control animals. Therefore, increases in PDE5 activity were not attributed to changes in vascular smooth muscle content.

Although cGMP-dependent PDE5 activity was increased in hypertensive animals, immunoblot levels of PDE5 were not significantly different between hypertensive and control lungs (Fig. 6). These results suggest that the increase in PDE5 activity in the experimental PPHN model may involve a posttranslational modification of PDE5 without a change in total protein. A previous study by Burns et al. (10) indicated that PDE5 activity may be increased posttranslationally by phos-
phorylation. Therefore, antibodies were developed to both phosphorylated and unphosphorylated PDE5. As a control, partially purified bovine lung PDE5 was incubated with protein kinase A in a phosphorylation reaction for 60 min, with increasing $^{32}$P incorporation observed after 60 min. Figure 7 shows the selectivity of the dephosphorylated PDE5 antibody for PDE5 before phosphorylation and lower selectivity after phosphorylation. The selectivity of the phosphorylated antibody increased after phosphorylation of PDE5. The phosphorylated antibody demonstrated lower selectivity for unphosphorylated PDE5. The unphosphorylated and

Fig. 8. A: representative immunoblots of COOH-terminal (immunoblot a), unphosphorylated (Dephospho; immunoblot b) and Phospho (immunoblot c) PDE5 protein in fetal HTN ($n = 3$) and CTL ($n = 3$) animals. Protein added to each lane was 50 µg. Total protein was similar between HTN and CTL animals. COOH-terminal Ab (immunoblot a), which recognizes both Phospho and Dephospho, was not significantly different between HTN and CTL animals. PDE5 was higher in CTL than in HTN animals (immunoblot b), whereas Phospho PDE5 was elevated in HTN compared with CTL animals (immunoblot c). B: immunoblots d and e demonstrate specificity of Ab for Dephospho and Phospho PDE5, respectively. Each immunoblot has 3 separate sections: partially purified bovine PDE5 (Std), fetal HTN ovine lung tissue (HTN), and CTL ovine lung tissue extract (CTL). Protein added to each lane was 50 µg. Immunoblot d: left, Ab specific for Dephospho PDE5 at 1:2,000; middle, preimmune serum; right, Ab for COOH-terminal region with Dephospho peptide (100 ng) to which Ab was made. Ab was specific for Dephospho PDE5, and Dephospho PDE5 was elevated in CTL compared with HTN animals. Immunoblot e left, Ab specific for Phospho PDE5 at 1:2,000; middle, preimmune serum; right, Ab for COOH-terminal region mixed with Phospho peptide (100 ng) to which Ab was made. Ab was specific for Phospho PDE5, and Phospho PDE5 was elevated in HTN compared with CTL animals. C: averages of Dephospho and Phospho PDE5 protein by immunoblot analysis in fetal HTN ($n = 6$) and CTL ($n = 6$) lambs. After 3-min exposure, immunoblots were analyzed by DATASCAN II and NIH Image as discussed in METHODS. Dephospho PDE5 was significantly elevated in CTL animals compared with HTN animals, and Phospho PDE5 was significantly elevated in HTN animals compared with CTL animals. *P < 0.05.
phosphorylated antibodies recognized both forms of PDE5 but with greater selectivity for unphosphorylated and phosphorylated PDE5, respectively.

Figure 8A shows representative immunoblots of three individual hypertensive and three age-matched control animals with the COOH-terminal antibody (immunoblot a; antibody that recognizes both phosphorylated and unphosphorylated forms of PDE5), dephosphorylated antibody (immunoblot b), and the phosphorylated antibody (immunoblot c). Although the COOH-terminal antibody demonstrated no difference in total PDE5 protein between hypertensive and control animals, the dephosphorylated PDE5 was increased in control animals and the phosphorylated PDE5 was increased in hypertensive animals. Figure 8B demonstrates the specificity of the dephosphorylated antibody (immunoblot d) and the phosphorylated antibody (immunoblot e). Figure 8C demonstrates the levels of the unphosphorylated and phosphorylated PDE5 in six hypertensive and six age-matched control animals. The protein levels of phosphorylated PDE5 were significantly elevated (P < 0.05) in the hypertensive animals compared with control sheep. With the use of these same animals, the unphosphorylated PDE5 protein levels were lower in the hypertensive animals and elevated in the control sheep.

Figure 9 shows the RPA analysis of PDE5 message and control GAPDH in hypertensive and control fetal sheep lungs. As with PDE5 COOH-terminal protein levels, there was no significant difference in the quantitative level of PDE5 mRNA in either hypertensive or control animals.

The localization of PDE5 in control fetal ovine sheep in pulmonary tissue is shown in Fig. 10. PDE5 protein was localized to vascular smooth muscle in both control fetal lung and hypertensive fetal lung. ICC also demonstrated a small amount of staining in bronchial cilia in both control and hypertensive lungs.

DISCUSSION

cGMP plays a crucial regulatory role in vascular tone. An elevation in cGMP induces vasorelaxation. In addition to regulating smooth muscle tone, cGMP was also able to inhibit smooth muscle cell proliferation (5, 7). Intracellular cGMP concentrations are regulated by a balance between synthesis by smooth muscle guanylate cyclases and degradation by cyclic nucleotide PDEs. Seven PDE gene families have been described based on different kinetic properties, cyclic nucleotide preferences, regulatory mechanisms, and sensitivities to pharmacological inhibitors (6). Recently, it was determined that within each major gene family, there were multiple PDE isoforms and splice variants, each exhibiting different characteristics and tissue expressions (6). PDE1 (calcium/calmodulin-dependent PDE), PDE2 (cGMP-stimulated PDE), PDE3 (cGMP-inhibited PDE), PDE4 (cAMP-specific PDE), and PDE5 (cGMP-specific PDE) were identified in vascular smooth muscle (23, 27, 35). PDE5 accounts for the majority of cGMP hydrolysis in vascular smooth muscle. Dipyridamole and zaprinast are potent inhibitors of PDE5 and PDE1C, with IC50 values in the 1–4 µM and 4–6 µM ranges, respectively (12, 32). A previous study (44) demonstrated that dipyridamole- and zaprinast-induced vasodilation was dependent on NO-cGMP cascade and not related to other nonspecific effects (such as increases in adenosine activity). Therefore, the effects of zaprinast and dipyridamole were studied in a chronic hypertensive fetal model and normotensive fetal control model to determine whether these inhibitors were effective
vasodilators in a pathological model of pulmonary hypertension.

We reported that dipyridamole and zaprinast caused pulmonary vasodilation during the development of intrauterine pulmonary hypertension by chronic compression of the DA in late-gestation fetal lambs. The percent decrease in TPR to either dipyridamole or zaprinast was similar in both normal fetal lambs and hypertensive fetuses. These data demonstrated the persistence of the potent pulmonary vasodilator response to PDE5 antagonists in the hypertensive lambs. A previous study (44) in normal fetal lambs demonstrated that inhibition of eNOS activity blocks the vasodilator effects of PDE5 antagonists. Therefore, dipyridamole- and zaprinast-induced vasodilation was more dependent on endogenous eNOS activity in the normal fetal lung. However, chronic pulmonary hypertension decreased endogenous eNOS activity, protein, and mRNA in perinatal lambs (26, 42). Despite this reduction in eNOS activity, we reported that the PDE5 antagonists still induce marked pulmonary vasodilation. These findings suggested that PDE5 activity regulated basal PVR in the normal fetus and remained critical in the hypertensive fetal lung circulation.

Fig. 10. Immunocytochemistry analysis of fetal CTL lung with PDE5 Ab (A), fetal HTN lung with PDE5 Ab (B), fetal CTL sheep lung cilia with PDE5 Ab (C), fetal CTL lung with PDE5 Ab and COOH-terminal peptide (100 µg/ml; D), and fetal HTN lung with PDE5 Ab and COOH-terminal peptide (100 µg/ml) (E). B, bronchiode; A, alveola. PDE5 is localized to pulmonary VSM and cilia. Similar results were obtained with HTN lung tissue.
Several other studies have reported the efficacy of dipyridamole and zaprinast as vasodilators in animal models of pulmonary hypertension. Rosenkrantz et al. (31) showed that dipyridamole attenuated the pressor response to acute hypoxia in pigs, an effect that was attributed to antiplatelet activity of dipyridamole. Mlczoch et al. (28) subsequently showed in a canine model of hypoxic pulmonary hypertension that dipyri-
damole administration inhibited hypoxic pulmonary vasoconstriction, even in dogs rendered thrombocytope-
nic by platelet antiserum. As noted in fetal lambs (44), these findings suggested a direct effect of dipyridamole in causing vasodilation in the adult pulmonary circulation. Similar findings were observed in normal new-
born lambs in response to zaprinast (9).

Our results demonstrated that cGMP PDE activity was elevated in the hypertensive model compared with normal control lungs, suggesting that higher PDE activity may have contributed to an elevated PVR after DA ligation. Although multiple cGMP PDE activities are present in lung tissue, PDE5 is present in high concentrations in the lung and is the only PDE known that is zaprinast sensitive in the presence of EGTA (which will inhibit all PDEs in the PDE1 family, including PDE1C). Previous studies demonstrated the IC50 for zaprinast for guinea pig and bovine PDE5 to be 0.4–8 µM. Therefore, our data suggested that the majority of the cGMP PDE activity in the presence of EGTA, 1 µM cGMP, and 4 µM zaprinast was PDE5. However, PDE5 protein levels and message levels did not differ between hypertensive and normal control lungs, suggesting that the increase in PDE5 activity without a concurrent rise in PDE5 protein in the hypertensive fetal lungs was secondary to posttransla-
tional control of PDE5. Previous work suggested that PDE5 activity increased after phosphorylation and that PDE5 activity decreased with dephosphorylation (10). Our results with an antibody that recognized only the phosphorylated state of PDE5 indicated that hyper-
tensive animals had an increase in phosphorylated PDE5 compared with control animals. Therefore, in the experimental model of PPHN, increases in PDE5 activity may be due to an increase in phosphorylation of PDE5. It was also reported that PDE5 activity post-
translationally may be altered by the presence of factors, referred to as a γ- and δ-factor (22). These factors may inhibit PDE5 activity similar to γ- and δ-factor mechanisms with PDE6 (34).

Our results localized PDE5 to fetal pulmonary vasculature by ICC (Fig. 10). We also observed a small amount of staining in the ciliary bodies of bronchioles in fetal lung tissue. The alterations in PDE5 in the hypertensive animals most likely reflected changes to vascular smooth muscle content.

In summary, physiological studies demonstrated the effectiveness of the PDE inhibitors zaprinast and dipyri-
damole in reducing a pathological elevation in PVR in a model of PPHN. Biochemical studies demonstrated that PDE5 activity is significantly elevated in PPHN, and this activity is not associated with an increase in PDE5 protein or mRNA. However, our results strongly implied that posttranslational modification of PDE5 by phosphorylation accounts for the increase in PDE5 activity in this model of PPHN. Therefore, a pathologi-
cal elevation in PDE5 in vascular smooth muscle, suggested by ICC localization, that resulted in an even lower cGMP level may have contributed to the elevated PVR by vasoconstriction and possibly smooth muscle vascular proliferation. We speculate that the elevation in cGMP PDEs may also be important in downregulat-
ing the activity of endogenous NO in the pulmonary hypertensive model. We further speculate that isozyme-
specific PDE inhibitors, alone or in combination with other cGMP-dependent dilators (inhaled NO), may prove to be potential treatments of severe pulmonary hypertension.

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