Effects of chronic estrogen-receptor blockade on ovine perinatal pulmonary circulation

THOMAS A. PARKER,1 SAM AFSHAR,2 JOHN P. KINSELLA,1 THERESA R. GROVER,1 SARAH GEBB,1 MARK GERACI,2 PHILIP W. SHAUL,3 CHAD M. T. CRYER,1 AND STEVEN H. ABMAN1

The Pediatric Heart Lung Center, Departments of 1Pediatrics and 2Internal Medicine, University of Colorado School of Medicine, Denver, Colorado 80262; and 3Department of Pediatrics, University of Texas Southwestern Medical Center, Dallas, Texas 75235

Received 16 June 2000; accepted in final form 24 April 2001

Parker, Thomas A., Sam Afshar, John P. Kinsella, Theresa R. Grover, Sarah Gebb, Mark Geraci, Philip W. Shaull, Chad M. T. Cryer, and Steven H. Abman. Effects of chronic estrogen-receptor blockade on ovine perinatal pulmonary circulation. Am J Physiol Heart Circ Physiol 281: H1005–H1014, 2001.—Prolonged infusions of 17β-estradiol reduce fetal pulmonary vascular resistance (PVR), but the effects of endogenous estrogens in the fetal pulmonary circulation are unknown. To test the hypothesis that endogenous estrogen promotes pulmonary vasodilation at birth, we studied the hemodynamic effects of prolonged estrogen-receptor blockade during late gestation and at birth in fetal lambs. We treated chronically prepared fetal lambs with ICI-182,780 (ICI, a specific estrogen-receptor blocker, n = 5) or 1% DMSO (CTRL, n = 5) for 7 days and then measured pulmonary hemodynamic responses to ventilation with low- and high-fraction inspired oxygen (FiO2). Treatment with ICI did not change basal fetal PVR or arterial blood gas tensions. However, treatment with ICI abolished the vasodilator response to ventilation with low FiO2 [change in PVR −30 ± 6% (CTRL) vs. +10 ± 13%, (ICI), P < 0.05] without reducing the vasodilator response to ventilation with high FiO2 [change in PVR, −73 ± 3% (CTRL) vs. −77 ± 4%, (ICI); P = not significant]. ICI treatment reduced prostacyclin synthase (PGIS) expression by 33% (P < 0.05) without altering expression of endothelial nitric oxide synthase or cyclooxygenase-1 and -2. In situ hybridization and immunohistochemistry revealed that PGIS is predominantly expressed in the airway epithelium of late gestation fetal lambs. We conclude that prolonged estrogen-receptor blockade inhibits the pulmonary vasodilator response at birth and that this effect may be mediated by downregulation of PGIS. We speculate that estrogen exposure during late gestation prepares the pulmonary circulation for postnatal adaptation.

Parker, Thomas A., Sam Afshar, John P. Kinsella, Theresa R. Grover, Sarah Gebb, Mark Geraci, Philip W. Shaull, Chad M. T. Cryer, and Steven H. Abman. Effects of chronic estrogen-receptor blockade on ovine perinatal pulmonary circulation. Am J Physiol Heart Circ Physiol 281: H1005–H1014, 2001.—Prolonged infusions of 17β-estradiol reduce fetal pulmonary vascular resistance (PVR), but the effects of endogenous estrogens in the fetal pulmonary circulation are unknown. To test the hypothesis that endogenous estrogen promotes pulmonary vasodilation at birth, we studied the hemodynamic effects of prolonged estrogen-receptor blockade during late gestation and at birth in fetal lambs. We treated chronically prepared fetal lambs with ICI-182,780 (ICI, a specific estrogen-receptor blocker, n = 5) or 1% DMSO (CTRL, n = 5) for 7 days and then measured pulmonary hemodynamic responses to ventilation with low- and high-fraction inspired oxygen (FiO2). Treatment with ICI did not change basal fetal PVR or arterial blood gas tensions. However, treatment with ICI abolished the vasodilator response to ventilation with low FiO2 [change in PVR −30 ± 6% (CTRL) vs. +10 ± 13%, (ICI), P < 0.05] without reducing the vasodilator response to ventilation with high FiO2 [change in PVR, −73 ± 3% (CTRL) vs. −77 ± 4%, (ICI); P = not significant]. ICI treatment reduced prostacyclin synthase (PGIS) expression by 33% (P < 0.05) without altering expression of endothelial nitric oxide synthase or cyclooxygenase-1 and -2. In situ hybridization and immunohistochemistry revealed that PGIS is predominantly expressed in the airway epithelium of late gestation fetal lambs. We conclude that prolonged estrogen-receptor blockade inhibits the pulmonary vasodilator response at birth and that this effect may be mediated by downregulation of PGIS. We speculate that estrogen exposure during late gestation prepares the pulmonary circulation for postnatal adaptation.

prostacyclin; prostacyclin synthase; cyclooxygenase; newborn
tal role in gas exchange. Although specific mechanisms that contribute to the perinatal pulmonary vasodilation are incompletely understood, past studies suggest that both nitric oxide (NO) and prostacyclin (PGI2) are released within the lung in response to specific birth-related stimuli (1, 16). However, factors that mature these vasodilator systems, and thereby prepare the fetal pulmonary circulation for transition, are unknown.

Exposure to endogenous estrogen may be among the factors that contribute to the release of NO and PGI2 at birth. Estrogen circulates at high levels in the late-gestation fetus, increases rapidly immediately before birth, and remains elevated for the first 24–48 h in the newborn (4, 7). Physiological studies demonstrate that estrogen causes relaxation of numerous adult vascular beds, often by releasing NO (28). In addition, we have previously reported that prolonged infusions of estradiol (E2) cause a marked and sustained increase in pulmonary blood flow in late gestation fetal lambs (22). In isolated ovine fetal pulmonary artery endothelial cells, estrogen increases expression of both endothelial NO synthase (eNOS) and cyclooxygenase (COX) and acutely stimulates the release of NO (14, 15, 18). Together, these studies suggest that endogenous estrogens may enhance the ability of the perinatal lung circulation to produce NO and PGI2 during the transition to extruterine life. However, the pulmonary vascular effects of endogenous estrogen during the transition have not been directly studied.

We hypothesized that exposure to high estrogen levels in late gestation enhances the ability of the pulmonary circulation to dilate in response to birth-related stimuli during the transition. To address this hypothesis, we measured the pulmonary hemodynamic response of chronically prepared fetal lambs to birth-related stimuli after prolonged treatment with the specific estrogen-receptor antagonist ICI-182,780 (ICI). We report that ICI selectively abolishes pulmonary vasodilation caused by rhythmic lung distension but

IN THE FETUS, pulmonary vascular resistance (PVR) is high, limiting pulmonary blood flow to <10% of the combined ventricular output (23). At birth, a rapid decrease in PVR leads to increased pulmonary blood flow and allows the lung to assume its normal postna-
does not affect the vasodilator response to ventilation with high oxygen. To address the potential mechanism by which estrogen may influence pulmonary blood flow, we performed Western blot analysis of lung tissue from ICI-treated and control lambs. We report that estrogen-receptor blockade reduces prostacyclin synthase (PGIS) expression, but does not change lung eNOS or COX protein expression.

METHODS

Pregnant, mixed-breed (Columbia-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 Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1996).

Fetal Surgical Preparation

The surgical preparation for this model has been described in detail previously (12). Surgery was performed at 128 ± 2 day gestation (term = 147 days) after ewes had fasted for 2 days. Animals were sedated with intravenous pentobarbital sodium and 1% intrathecal tetracaine hydrochloride (3 ml) and were given intramuscular penicillin G (600,000 units) and gentamycin (80 mg) immediately before 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 into the ascending aorta and superior vena cava, respectively. A left thoracotomy and pericardial incision were made, and the heart and great vessels were exposed. With the use of a 16-gauge intravenous placement unit (Angiocath, Travenol; Deerfield, IL), a 22-gauge catheter was placed through purse-string sutures into the left pulmonary artery (LPA) for infusion of selective drugs. Catheters (20 gauge) were placed in the main pulmonary artery (MPA) and the left atrium (LA) to measure pressure. A 6-mm ultrasonic flow transducer (Transonic Systems; Ithaca, NY) was placed around the LPA to measure blood flow. 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 infused into the amniotic cavity. The catheters 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 standing within 8 h. Fetuses were treated with ampicillin (500 mg) infused into the venous catheter for the first two postoperative days. All catheters were gently infused daily with 1–2 ml of normal (0.9%) saline with added heparin to maintain catheter patency. Animals were allowed to recover for at least 72 h before studies were initiated.

Physiological Studies

During physiological studies, MPA, LA, and aortic pressure measurements were determined by connecting the externalized catheters to computer-driven pressure transducers (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. Heart rate was determined from phasic pressure tracings. Arterial blood gas measurements included pH, PCO₂, PO₂ (ABL 500, Radiometer), and oxygen saturation, and hemoglobin (OSM3 Hemoximeter, Radiometer).

After physiological studies were completed, fetuses were killed with a large dose of pentobarbital. A midline thoracotomy was rapidly performed and the heart and lungs were removed en bloc. A tracheal catheter was placed, and the airway was simultaneously perfused with 10% formalin. The lungs were then immersed and stored in formalin for later processing.

Drug Preparation

A stock solution of ICI (Tocris; Ballwin, MO) was prepared in 100% DMSO. Infusate was made daily by diluting the stock solution with saline to a final concentration of 5 µg/ml. This dose was based on previously published studies in humans and rats demonstrating antiestrogenic effects at a similar per kilogram dose (6, 10). Acetylcholine (ACH; A-6625, Sigma) was dissolved in saline (15 µg/ml) immediately before use.

Statistical Analysis

Results are reported as means ± SE. Comparisons within groups were made by repeated measures analysis of variance (ANOVA). For normally distributed data, comparisons between groups at discrete time points were made by ANOVA. Where significant differences were detected, Student-Newman-Keuls post hoc testing was used. For nonnormally distributed data, Wilcoxon signed-rank test was used. Statistical significance was set at P < 0.05.

Protocols

Protocol 1. Effects of prolonged treatment with ICI on fetal pulmonary hemodynamics and on NO-dependent vasodilation. After an initial period of at least 30 min of stable baseline hemodynamics, ACh (1.5 µg/min × 10 min), an eNOS agonist, was infused into the LPA catheter. Pressure and flow measurements were recorded at baseline (before ACh), at the end of the infusion, and then every 10 min until values returned to baseline. Animals were then randomized to treatment with either ICI (5 µg/h, n = 5) or 1% DMSO (CTRL, 1 ml/h, n = 5). Either ICI or CTRL was infused continuously into the LPA catheter for 7 days. Hemodynamic measurements and the vasodilator response to ACh were repeated at the end of the 7-day treatment period. In addition, arterial blood gas measurements were recorded at the start and end of treatment.

Protocol 2. Effects of prolonged treatment with ICI on pulmonary hemodynamic response to birth-related stimuli. After 7 days of treatment, ICI and CTRL infusions were discontinued, and lambs were delivered by cesarean section as previously described with minor modifications. Before delivery, ewes were sedated with intravenous pentobarbital and intrathecal tetracaine hydrochloride (1%, 3 ml). A hysterotomy was performed and the fetal head and chest were externalized. Intravenous pancuronium bromide (0.3 mg) was administered to the fetus to prevent spontaneous breathing. Aortic, MPA, and LA catheters were connected to pressure transducers and pressures were measured continuously during the study. After sampling arterial blood and recording baseline hemodynamic measurements (“fetal baseline” time point), a tracheostomy was performed, and lambs were intubated with a 3.5-mm endotracheal tube. Lambs were treated with endotracheal surfactant (Infasurf, 6 ml, kindly provided by E. A. Eagan) and placed on a time-cycled, pressure-limited neonatal ventilator (Infant Star 950, Infrasonics; San Diego, CA). During the first 15 min of the study, the umbilical circulation was kept intact to ensure stability of the animal.
preparation. Lambs were initially ventilated with low oxygen fraction of inspired oxygen (FIO2) of <10% to maintain the Paco2 at fetal levels and to test the response to rhythmic lung distension (“ventilation” time point). After 15 min of ventilation with low FIO2, the FIO2 was increased to 1.0, the umbilical cord was ligated, and hemodynamic measurements were recorded for an additional 25 min (“100% O2” time point).

A treatment protocol for changes in ventilator settings was followed closely after delivery to ensure that lambs were treated consistently. Initial ventilator settings included rates, 30 breaths/min; an inspiratory time (IT), 1.0 s; peak inspiratory pressure, 35 cmH2O; and positive end-expiratory pressure, 6 cmH2O. Subsequent changes from these initial settings were determined by arterial blood gas values and chest wall excursions. Target blood gas parameters were pH, 7.35–7.45 units and arterial partial CO2 pressure (Paco2), 35–45 Torr. If Paco2 fell below 35 Torr, peak inspiratory pressure was progressively decreased to a minimum of 22 cmH2O. If Paco2 remained <35, ventilator rate and IT were gradually decreased. Alternatively, if the Paco2 was above target range, ventilator rates were increased and IT was decreased to maintain the inspiratory-to-expiratory ratio at 1:1. Treatment with sodium bicarbonate or cardiotoxic agents was not allowed during the study period.

Protocol 3. Effects of prolonged treatment with ICI on expression of eNOS, COX-1, COX-2, and PGIS in distal lung. Previous studies have demonstrated that generation of NO by eNOS contributes to the fall in PVR in response to birth-related stimuli. In addition, generation of PGI2 from arachidonic acid by COX-1 and -2 and PGIS contributes to the vasodilator response to rhythmic lung distension.

At the end of the above physiological studies, CTRL and ICI lambs were killed as described above, and left lung tissue was harvested and rapidly frozen. Immunoblot analysis for COX-1, COX-2, and PGIS was performed on peripheral lung homogenates using methods modified from those previously described (3). Lung tissue was thawed and homogenized on ice with a ground glass homogenizer in 50 mM Tris buffer (pH 7.5) containing 2 mg/ml peptatin, 20 mg/ml leupeptin, 20 mg/ml aprotenin, 1.2 mM Na-p-tosyl-l-lysine-chloromethyl ketone, 20 mM tetrahydrobiopterin, 3.0 mM dithiothreitol, 250 mM 2-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 12,000 g at 4°C for 10 min and the supernatant collected and ultrasonically disrupted (Branson Ultrasonics; Chicago, IL). The protein content was determined by the Bradford method (5). Proteins were separated on a Bis-Tris-HCl-buffered (pH 6.4) polyacrylamide gel (4–12%) (Novex; San Diego, CA) and electrophoretically transferred to nitrocellulose paper. Membranes were blocked in buffer containing TBS with 0.5% Tween and 5% dried milk. eNOS monoclonal antibody (Transduction Laboratories; Lexington, KY) diluted 1:200 was applied for 2 h at 4°C. The membranes were then washed and incubated in 1:1,000 dilution of anti-mouse immunoglobulin (Santa Cruz Biotechnology) for 2 h at room temperature. Proteins were visualized using chemiluminescence (ECL Western Blotting Analysis system, Amersham Pharmacia Biotech). Densitometry was performed with a scanner and National Institutes of Health Image software.

Protocol 4. Effects of prolonged treatment with exogenous estrogen on expression of COX-1, COX-2, and PGIS in distal lung. The purpose of this protocol was to complement the expression studies in protocol 3 by determining whether treatment with exogenous estrogen increases lung expression of COX-1, COX-2, and PGIS. Tissues used for this protocol were from fetal lambs whose hemodynamic response to prolonged infusions of 17β-E2 selectively into the LPA (E2, 250 µg/h; n = 4) has previously been reported (22). E2 infusion ended if lambs demonstrated a sustained doubling of LPA flow, an absolute LPA flow of >150 ml/min, or no response after 8 days. Duration of treatment ranged from 2 to 8 days, with six of the nine treated animals responding to E2. Control lung tissue was from fetal lambs treated with 0.025% ethanol at 1 ml/h (n = 2) or saline (n = 2). Lung tissue was rapidly frozen after completion of fetal infusions and lambs were neither delivered nor mechanically ventilated. Lung eNOS expression in this group of animals has been previously reported (22) and was not repeated in this study.

Protocol 5. Localization of PGIS expression in the lungs of newborn lambs. To determine the site of expression of PGIS in the lung of neonatal lambs, we performed both in situ hybridization and immunohistochemistry (IHC) on sections taken from lambs after delivery studies. At the conclusion of delivery studies, lungs were fixed by slow infusion of 10% buffered formalin into the trachea. Fixed tissue was stored in 70% ethanol until the time of localization studies. After paraffin embedding, 3- to 5-μm sections were cut and mounted on Super Frost Plus slides (Fisher Scientific; Pittsburgh, PA).

In situ hybridization was performed according to published methods with minor modifications (11). Briefly, sense and antisense riboprobes were generated using linearized templates from a 1.5 kbp rat PGIS cDNA (13). cRNA probes were labeled with [33P]UTP (2,000 Ci/mmol, New England Nuclear; Boston, MA). The PGIS transcripts were hydrolyzed in 80 mM NaHCO3 and 120 mM Na2CO3 to yield fragments of ~300–400 base pairs. After overnight hybridization and high-stringency washes, the slides were dipped in NTB-2 nuclear track emulsion (Eastman Kodak; Rochester, NY) and developed 22 days later with Kodak D19 developer at 16°C.
For IHC, slides were deparaffinized in Hemo-De and then rehydrated by serial immersion in graded ethanols. Antigen retrieval was accomplished by digestion with Proteinase K (50 µg/ml, Roche Diagnostics; Indianapolis, IN) for 15 min and endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxidase in methanol for 20 min. After being rinsed in PBS, nonspecific binding was blocked with DAKO serum-free protein block (DAKO; Carpinteria, CA) for 15 min and with 10% goat serum/2% sheep serum for 30 min. PGIS polyclonal primary antibody (9) or rabbit IgG (Vector Labs; Burlingame, CA) was applied at a 1:500 dilution (Research Genetic; Huntsville, AL) for 2 min.

### RESULTS

**Protocol 1. Effects of Prolonged Treatment with ICI on Fetal Pulmonary Hemodynamics and on NO-Dependent Vasodilation**

Two of five CTRL animals and three of five ICI animals were males. Fetal systemic and pulmonary hemodynamic and arterial blood gas variables were similar between groups at the start of the study. Arterial blood pressure increased in CTRL animals during the 7-day treatment period but was not different from ICI animals after treatment (Table 1). Pulmonary hemodynamic and arterial blood gas variables did not change in either group during the 7-day treatment period (Table 1). Ach decreased PVR to a similar degree in each group at the start of the study [−49 ± 9 vs. −48 ± 2%, CTRL vs. ICI, P = not significant (NS)]. After 7 days of treatment with ICI or CTRL, the vasodilator response to Ach did not change and was not different between groups (−52 ± 6 vs. −53 ± 4%, CTRL vs. ICI, P = NS).

### Protocol 2. Effects of Prolonged Treatment with ICI on Pulmonary Hemodynamic Response to Birth-Related Stimuli

Hemodynamic and blood gas variables at each of the delivery study time points are summarized for both study groups in Table 2. At the start of the delivery study ("fetal baseline" after surgery but before initiation of mechanical ventilation), aortic pressure and pulmonary artery pressure were higher in ICI animals than CTRLs (P < 0.05). Left pulmonary arterial flow (QLPA) and arterial blood gas tensions were similar between groups.

During ventilation with low FiO2 ("ventilation" time point), QLPA was higher and PVR was lower in the CTRL group compared with the fetal baseline (Fig. 1). In contrast, QLPA and PVR were not different from baseline fetal values in the ICI lambs. When expressed as fractional change from fetal baseline, PVR fell by 30 ± 6% in CTRL lambs in response to ventilation with low FiO2 (Fig. 2). In contrast, PVR rose by 10 ± 13% in ICI lambs in response to ventilation with low FiO2 (P < 0.05). ICI lambs had lower PaCO2 and higher pH compared with CTRL lambs (P < 0.05), but PaO2 and PAP were not different between groups.

### Table 1. Hemodynamic and arterial blood gas values in fetal lambs before and after 7 days of treatment with 1% DMSO or ICI-182,780

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ICI-182,780</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 7</td>
</tr>
<tr>
<td>QLPA, ml/min</td>
<td>85 ± 7</td>
<td>79 ± 6</td>
</tr>
<tr>
<td>PAP, mmHg</td>
<td>46 ± 2</td>
<td>49 ± 1</td>
</tr>
<tr>
<td>PVR, mmHg·ml⁻¹·min⁻¹</td>
<td>0.53 ± 0.04</td>
<td>0.62 ± 0.05</td>
</tr>
<tr>
<td>AoP, mmHg</td>
<td>44 ± 2</td>
<td>49 ± 1*</td>
</tr>
<tr>
<td>pH, units</td>
<td>7.39 ± 0.01</td>
<td>7.36 ± 0.01</td>
</tr>
<tr>
<td>PO2, Torr</td>
<td>46 ± 2</td>
<td>50 ± 1</td>
</tr>
<tr>
<td>PaO2, Torr</td>
<td>21 ± 1</td>
<td>21 ± 1</td>
</tr>
</tbody>
</table>

Values are means ± SE. CTRL, 1% DMSO; ICI, ICI-182,780. H1008

### Table 2. Hemodynamic and arterial blood gas values during delivery studies of control and ICI-182,780-treated lambs

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ICI-182,780</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Ventilation</td>
</tr>
<tr>
<td>QLPA, ml/min</td>
<td>79 ± 3</td>
<td>104 ± 7*a</td>
</tr>
<tr>
<td>AoP, mmHg</td>
<td>50 ± 2</td>
<td>49 ± 3</td>
</tr>
<tr>
<td>PAP, mmHg</td>
<td>51 ± 2</td>
<td>49 ± 4</td>
</tr>
<tr>
<td>LAP, mmHg</td>
<td>1 ± 1</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>PVR, mmHg·ml⁻¹·min⁻¹</td>
<td>0.64 ± 0.05</td>
<td>0.45 ± 0.04*</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>232 ± 17</td>
<td>203 ± 16</td>
</tr>
<tr>
<td>pH, units</td>
<td>7.29 ± 0.02</td>
<td>7.32 ± 0.01</td>
</tr>
<tr>
<td>PO2, Torr</td>
<td>49 ± 3</td>
<td>38 ± 3</td>
</tr>
<tr>
<td>PaO2, Torr</td>
<td>17 ± 1</td>
<td>17 ± 1</td>
</tr>
<tr>
<td>Saturation, %</td>
<td>39 ± 6</td>
<td>39 ± 4</td>
</tr>
</tbody>
</table>

Values are means ± SE. LAP, left atrial pressure; HR, heart rate. °P < 0.05 vs. baseline. †P < 0.05 vs. control.
arterial saturation were not different between groups and were unchanged from the “fetal baseline” time point.

During ventilation with 100% oxygen, both CTRL and ICI lambs had higher \( Q_{LPA} \) and lower PVR than fetal baseline, and there was no difference between groups in any hemodynamic parameter (Fig. 1, Table 2). The change in PVR in response to ventilation with 100% oxygen was similar between the two groups (\( P < 0.05 \), ICI vs. CTRL).

Protocol 3. Effects of Prolonged Treatment with ICI on Expression of eNOS, COX-1, COX-2, and PGIS in Distal Lung

eNOS, COX-1, and PGIS protein were detected by Western blot analysis in each sample from both groups. COX-2 protein was not detected in any sample. eNOS and COX-1 protein expression were similar between groups (Fig. 3). In contrast, PGIS expression was reduced by 33% in the ICI group compared with CTRL lambs (\( P < 0.05 \)) (Fig. 4).

Protocol 4. Effects of Prolonged Treatment with Exogenous Estrogen on Expression of COX-1, COX-2, and PGIS in Distal Lung

COX-1 and PGIS protein were detected by Western blot analysis in each sample from both groups. COX-2 protein was not detected in any sample from either group. COX-1 protein expression was similar between groups (Fig. 5). In contrast, PGIS protein expression was increased by twofold in the E2 group compared with CTRL (\( P < 0.05 \)) (Fig. 6).

DISCUSSION

In this study, we tested the hypothesis that endogenous estrogen modulates the pulmonary vasodilator response to birth-related stimuli in the late gestation fetal lamb. We report that chronic treatment with ICI, a selective estrogen-receptor antagonist, abolishes the vasodilator response to rhythmic lung distension with hypoxic gas. In contrast, the vasodilator response to increased oxygen tension remained intact. Furthermore, prolonged treatment with ICI reduced PGIS protein expression but had no effect on COX-1 or eNOS content. Complementary studies demonstrate that prolonged treatment with E2 increases PGIS protein expression but does not change COX-1 or eNOS content. From these results, we conclude that high endogenous estrogens in late gestation contribute to the normal perinatal pulmonary vasodilation and speculate that this effect is mediated by increasing the expression of PGIS. From these studies, we speculate that endogenous estrogen exposure during late gestation prepares...
the pulmonary circulation for postnatal adaptation at birth.

Previous studies have demonstrated that the fetal pulmonary circulation undergoes a developmental increase in the ability to respond to several pharmacological and physiological dilator stimuli during late gestation (17, 20). Maturational changes in the production of vasoactive substances, including NO and PGI2, may underlie these developmental changes (2, 24). Although regulation of these vasodilator systems in the near-term fetal lung are poorly understood, exposure to endogenous estrogen may be among the factors that modulate their activity. Estrogens circulate at high levels in the near-term fetus, increase rapidly immediately before birth, and remain elevated for 48–72 h postnatally (4, 7). The temporal association between rising fetal estrogen levels and the progressive increase in the ability of the fetal lung to respond to vasodilator stimuli has suggested the possibility that endogenous estrogen may enhance fetal pulmonary vascular responsiveness and the subsequent perinatal pulmonary vasodilation. The current study extends the findings of our previous report on the marked pulmonary vasodilator effects of exogenous estrogen in the late-gestation fetus (18). Although we previously reported that infusions of 17β-E2 caused nearly a threefold increase in pulmonary blood flow, application of that data to the pulmonary vascular effects of endogenous estrogen is limited by the possibility that the observed effects resulted from pharmacological local estrogen concentrations within the lung (22). The current study lends further support to the hypothesis that endogenous estrogens contribute to the vasodilator response to specific birth-related stimuli during the transition to extrauterine life.

The mechanism by which estrogen-receptor blockade alters pulmonary vasoreactivity at birth is uncertain. Previous studies demonstrate that release of NO and PGI2 from the pulmonary circulation both contribute to the fall in PVR at birth (1, 16). Pharmacological blockade of NOS decreases basal fetal pulmonary blood flow and blunts the normal vasodilator response to both rhythmic lung distension and ventilation with oxygen (1, 9, 19). In contrast, blockade of prostaglandin synthesis reduces the fall in PVR caused by rhythmic lung distension with nitrogen without changing basal PVR or the response to ventilation with oxygen (21, 27). From these previous studies, the alterations in vasoreactivity caused by ICI treatment suggest that estrogen-receptor blockade selectively impairs the ability of the perinatal lung to release PGI2 at birth. To determine whether changes in the ability of the lung to produce PGI2 might underlie our physiological observations, we studied the expression of COX-1, COX-2, PGIS, and eNOS in the lungs of CTRL and ICI-treated lambs. Although COX-1, COX-2, and eNOS were not affected, we found that PGIS expression was reduced by 33% after ICI treatment. These findings support the possibility that ICI treatment selectively impairs the release of PGI2 from the pulmonary circulation at

Fig. 3. Cyclooxygenase-1 (COX-1, A) and endothelial nitric oxide (eNOS, B) protein content in lungs of lambs treated with CTRL (n = 4) or ICI (n = 4).
birth. Although the change in PGIS protein expression is small, a previous study suggests that a similar increase in PGIS expression can be associated with measurable increases in production of PGI\textsubscript{2} (12). Further study is necessary to determine whether exposure to either endogenous or exogenous estrogen in fetal lambs produces measurable systemic changes in levels of PGI\textsubscript{2} or its stable metabolite, 6-keto-prostaglandin F\textsubscript{1\alpha}. Although PGI\textsubscript{2} is a potent vasodilator, regulation of its production in the fetal lung is poorly understood. PGI\textsubscript{2} is produced by the conversion of arachidonic acid by COX and PGIS via an intermediate prostanoid, prostaglandin H\textsubscript{2} (26). Expression of COX-1 rises during late gestation in fetal lambs and is increased by estrogen exposure in isolated fetal pulmonary artery endothelial cells (3, 14). These studies have suggested that increasing COX-1 expression is responsible for increasing PGI\textsubscript{2} activity in the late gestation fetal lung. We were unable to detect an estrogen-induced change in COX-1 expression in the current study. Because COX-1 is expressed in multiple cell types, our studies of whole lung homogenates may lack the sensitivity to detect small differences in endothelial cell COX-1 expression. Therefore, our data do not rule out a potential regulatory role for estrogen on COX-1 expression. However, they do suggest that estrogen may induce changes at other sites in the PGI\textsubscript{2} synthetic pathway that can result in substantial physiological changes.
In an extensive previous study using IHC, COX-1 was expressed in both the vascular endothelium and the airway epithelium in the lungs of fetal and newborn lambs (3). In the same study, COX-2 could not be detected by IHC. Ours is the first study to examine expression of PGIS in the newborn lung. We found by both IHC and in situ hybridization that the airway epithelium is the predominant site of PGIS expression in the newborn lung. Interpretation of these findings must be viewed with caution, as previous studies clearly demonstrate that the pulmonary vasculature has the capacity to produce PGI2 in substantial amounts. Nonetheless, our findings suggest the possibility that airway production of PGI2 may contribute to the vasodilation of adjacent small pulmonary arterioles. This hypothesis is particularly intriguing in light of previous reports that PGI2 selectively modulates the pulmonary vasodilator response to rhythmic distension of the airway (16, 27).

Regulation of PGIS has not previously been addressed in the fetal pulmonary circulation. In adult rats, Geraci et al. (13) have recently found that selective overexpression of PGIS in the pulmonary circulation protects against the development of hypoxic pulmonary hypertension. Both that study and our current report suggest the possibility that PGIS expression may directly alter the proportion of PGH2 that is converted to the dilator PGI2 compared with other constrictor prostanoids. In addition, our results are the first to suggest that PGIS expression may be regulated by estrogen and that estrogen-regulated expression of PGIS may have important hemodynamic effects in an intact lung. Support for a potential role of estrogen in the regulation of PGIS is strengthened by the demonstration that both endogenous and exogenous estrogen exposure alters PGIS expression in the fetal lamb. Additional work focusing on regulation of gene expression and maturational changes in PGIS in the developing fetal lung are needed.

The apparent lack of effect of endogenous estrogens on NOS expression and activity in the current study was unanticipated. Extensive in vitro studies (15, 18) demonstrate E2 increases expression of eNOS and release of NO in isolated fetal pulmonary artery endothelial cells. In addition, E2-induced vasodilation of the ovine fetal lung is dramatically reduced by Nω-nitro-L-arginine, a nonspecific NOS antagonist, suggesting that NOS activation contributes to the vasodilator effects of exogenous estrogen (22). Nonetheless, several lines of evidence strongly suggest that ER blockade did not diminish NO production in the current study. First, ICI treatment did not change in basal pulmonary blood flow.
flow or diminish the response to ACh. Previous studies demonstrate that NOS blockade decreases both basal and ACh-stimulated pulmonary blood flow in the fetus (1, 8, 25). Second, the vasodilator response to ventilation with oxygen, which is dramatically reduced by nonselective NOS inhibitors (9), remained completely intact after ICI treatment. Finally, Western blot analysis detected no difference in eNOS protein content after ICI treatment. Several explanations may account for the discrepancy between the current study and previous ones with regard to the effects of estrogen treatment on eNOS/NO axis in the fetal pulmonary circulation. First, the studies that demonstrate that estrogen increases eNOS expression and NO release have been performed in isolated cell culture systems, a technique that may introduce fundamental changes to the endothelial cell (15, 18). Second, previous studies (15, 18, 22), including in vivo ones, have examined the effects of exogenous estrogen, whereas the current study is intended to determine the effects of endogenous estrogen. Finally, differences in the degree of estrogen-receptor blockade may account for the differences between studies, particularly given the lack of useful endpoints to indicate the degree of receptor blockade.

In summary, we found that chronic estrogen-receptor blocker blockade abolishes the pulmonary vasodilator response to rhythmic lung distension and reduces expression of PGIS in the late gestation fetal lamb. We conclude that exposure to endogenous estrogens thereby contributes to the perinatal pulmonary vasodilation in response to birth-related stimuli. We speculate that the hemodynamic effects of endogenous estrogens are mediated, in part, by modulation of PGIS expression. These studies suggest that further investigation of the regulation of PGIS in the perinatal pulmonary circulation is warranted.

We deeply appreciate the technical assistance of Todd S. Sherman, Jie De Fan, and Yu-Ching Fan Chang.

This study was supported by a Professional Development Award from The Children’s Hospital Research Institute, American Heart Association (Desert/Mountain Affiliate) Grant 9960284Z, and National Heart, Lung, and Blood Institute Grants HL-04206, HL-01932, HL-41012, HL-46481, and HL-53546.

REFERENCES

24. Tiktinsky MH, Cummings JJ, and Morin FC III. Acetycholine increases pulmonary blood flow in intact fetuses via endo-