Ventilation and Oxygenation Induce Endothelial Nitric Oxide Synthase Gene Expression in the Lungs of Fetal Lambs

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Abstract

At birth, ventilation and oxygenation immediately decrease pulmonary vascular resistance (PVR) and increase pulmonary blood flow (PBF); more gradual changes occur over the next several hours. Nitric oxide, produced by endothelial nitric oxide synthase (eNOS), mediates these gradual changes. To determine how ventilation and oxygenation affect eNOS gene expression, 12 fetal lambs were ventilated for 8 h without changing fetal descending aortic blood gases or pH (rhythmic distension) or with 100% oxygen (O_2 ventilation). Vascular pressures and PBF were measured. Total RNA, protein, and tissue sections were prepared from lung tissue for RNase protection assays, Western blotting, and in situ hybridization. O_2 ventilation increased PBF and decreased PVR more than rhythmic distension ($P < 0.05$). Rhythmic distension increased eNOS mRNA expression; O_2 ventilation increased eNOS mRNA expression more and increased eNOS protein expression ($P < 0.05$). To define the mechanisms responsible for these changes, ovine fetal pulmonary arterial endothelial cells were exposed to 1, 21, or 95% O_2 or to shear stress. 95% O_2 increased eNOS mRNA and protein expression ($P < 0.05$). Shear stress increased eNOS mRNA and protein expression ($P < 0.05$). Increased oxygenation but more importantly increased PBF with increased shear stress induce eNOS gene expression and contribute to pulmonary vasodilation after birth. (*J. Clin. Invest.* 1997. 100: 1448–1458.) Key words: nitric oxide • endothelial nitric oxide synthase • endothelial cells • pulmonary circulation • development

Introduction

After birth, with initiation of ventilation by the lungs, and the subsequent increase in pulmonary and systemic arterial blood oxygen tensions, pulmonary vascular resistance decreases and pulmonary blood flow increases by 8–10-fold to match systemic blood flow (1–4). This process is regulated by a complex and incompletely understood interplay between mechanical and metabolic factors (5). For example, the replacement of fluid with gas in the alveoli changes alveolar surface tension, which unkinks the small pulmonary arteries, and causes an immediate decrease in pulmonary vascular resistance and increase in pulmonary blood flow (2, 5). There is also release of vasoactive substances, such as prostacyclin (PGI_2), which decrease pulmonary vascular resistance and increase pulmonary blood flow (6, 7). Both rhythmic distension of the fetal lamb lung and ventilation without oxygenation produce partial pulmonary vasodilatation (2).

Ventilation of the fetus with air or oxygen produces nearly complete pulmonary vasodilatation (5). The increase in alveolar or pulmonary and systemic arterial blood oxygen tensions decreases pulmonary vascular resistance either directly by dilating the small pulmonary arteries, or indirectly by stimulating the production of vasodilator substances such as nitric oxide (NO)[1] (8–10). Endothelial cells synthesize NO and L-citrulline from L-arginine by the action of the enzyme endothelial nitric oxide synthase (eNOS) (11, 12). NO diffuses from the endothelial cell into the smooth muscle cell, activates guanylate cyclase, increases cyclic guanosine-3′,5′-monophosphate production, and initiates a cascade that results in smooth muscle relaxation and vasodilation (13–15). Inhibition of eNOS attenuates the increase in pulmonary blood flow due to oxygenation of fetal lambs either by maternal hyperbaric oxygen exposure or by ventilation of the fetus with air or oxygen (8–10, 16, 17). There is also an increase in eNOS mRNA and protein expression in fetal rat lungs as they near term, which decreases 1 wk after birth (18, 19). These studies suggests an important role for NO in mediating pulmonary vascular tone in the perinatal period. The factors responsible for the changes in eNOS gene expression are not well understood.

An additional mechanism by which pulmonary vasodilation occurs relates to the increase in shear stress on the pulmonary vascular endothelium induced by the increase in pulmonary blood flow. Initially, NOS activity and NO production are increased when endothelial cells are exposed to increased shear stress (12, 14, 15, 20–23). If the shear is maintained there are then subsequent increases in eNOS mRNA and protein expression (24–26). The immediate increase in pulmonary blood flow at birth may increase shear forces on the pulmonary vascular endothelium inducing eNOS mRNA and protein expression. This would result in increased NO production and a continued, more gradual decrease in pulmonary vascular resistance and increase in pulmonary blood flow in the next hours and even days after birth.

This study had several purposes. First, to determine

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1. *Abbreviations used in this paper:* eNOS, endothelial nitric oxide synthase; NO, nitric oxide.

whether eNOS mRNA and protein expression are induced in the fetal lung with ventilation and oxygenation at birth, RNase protection assays and Western blot analyses were performed on lung tissue from near-term fetal lambs ventilated in utero with a gas mixture which did not change fetal descending aortic blood gases or pH, or with 100% oxygen. Second, to determine the cell type responsible for the increase in eNOS mRNA expression, in situ hybridization was performed on lung tissue sections from near-term fetal lambs ventilated with 100% oxygen. Breathing at birth produces increased oxygenation, increased pulmonary blood flow, and likely increased shear stress; therefore, whole animal studies cannot determine which of these effects are responsible for the increase in eNOS mRNA and protein expression. Therefore, pulmonary arterial endothelial cells were harvested from near-term fetal lambs and maintained in culture. These cells were exposed to different oxygen environments, or to shear stress, and changes in eNOS mRNA and protein levels were measured.

Our results show that the increases in descending aortic PO_2 and pulmonary blood flow are greater after ventilation of the near-term fetal lamb with 100% oxygen. Increases in eNOS mRNA and protein levels parallel these physiologic changes. The increase in eNOS expression is confined to the endothelium of small and large blood vessels. In cultured ovine fetal pulmonary arterial endothelial cells, shear stress induces eNOS mRNA and protein expression more than increased oxygenation does. Increased oxygenation but more importantly increased pulmonary blood flow with increased shear stress induce eNOS gene expression and contribute to pulmonary vasodilation after birth and the successful transition to extrauterine life.

Methods

Physiologic studies

Surgical preparation. All procedures and protocols were approved by the Committee on Animal Research of the University of California at San Francisco. 12 mixed-breed Western ewes (136–138 d of gestation, term = 145 days) were operated on under sterile conditions using local anesthesia (2% lidocaine hydrochloride), intramuscular sedation (20 mg valium, 600 mg ketamine hydrochloride, and 2 mg atropine), and intravenous anesthesia (1,000–2,000 mg ketamine hydrochloride). A venous catheter was placed in the maternal hind limb for the administration of intraoperative anesthesia and postoperative antibiotics.

A laparotomy was performed. Through a small uterine incision, a fetal hind limb was exposed. 1% lidocaine hydrochloride was used for local anesthesia. Polyvinyl catheters were inserted into the pedal artery and vein and advanced to the descending aorta and inferior vena cava, respectively. The fetus was then anesthetized with an intravenous injection of ketamine hydrochloride (25 mg), and spontaneous movement was prevented with an intravenous injection of succinylcholine chloride (5 mg). The fetal skin and uterine incisions were closed in layers.

Through a separate uterine incision, the fetal left hemithorax was exposed. A left lateral thoracotomy was performed in the fourth intercostal space to expose the heart and great vessels. Polyvinyl catheters were inserted into the main pulmonary trunk, left pulmonary artery, and left atrium. A 6-mm ultrasonic flow transducer (Transonic Systems, Inc., Ithaca, NY) was placed around the left pulmonary artery. Through a midline neck incision, the trachea was isolated and ligated proximally. Through an incision in the midtrachea, a 4.5-mm endotracheal tube was inserted. Polyvinyl catheters were inserted into the carotid artery and jugular vein, and advanced to the ascending aorta and right atrium, respectively. A polyvinyl catheter was inserted into the amniotic cavity.

The fetal, uterine, and maternal skin incisions were closed in layers. Amniotic fluid losses were replaced with warmed 0.9% saline. To ensure unobstructed drainage of the tracheal fluid into the amniotic cavity, the endotracheal tube was connected by tubing to the amniotic cavity catheter (3, 10). All catheters and the flow transducer were exteriorized to the left flank of the ewe and placed in a vinyl pouch. To maintain catheter patency, heparin sodium (1,000–2,000 U) was instilled into the catheters immediately after surgery, and then daily. Penicillin G potassium (1,000,000 IU) and gentamicin sulfate (100 mg) were injected intravenously into the ewe and into the amniotic fluid during surgery, and then daily. 3 d were allowed for recovery.

Measurements. Descending aortic, left and right atrial, and pulmonary arterial pressures, zero referenced to amniotic pressure, were measured using P23 Db pressure transducers (Statham Instruments, Oxnard, CA) and recorded continuously on a direct-writing polygraph (Gould Instruments, Valley View, OH). Mean pressures were obtained by electrical integration. Heart rate was measured by a cardiotachometer triggered from the phasic descending aortic pressure tracing. Left pulmonary arterial blood flow was measured with an ultrasonic flow transducer and flowmeter (Transonic Systems, Inc.). Left pulmonary vascular resistance was calculated as the difference between mean left pulmonary arterial pressure and mean left atrial pressure divided by left pulmonary blood flow per kilogram of body weight. Descending aortic blood gases and pH were measured on a blood gas analyzer (Corning Medical and Scientific, Medfield, MA). Hemoglobin concentration and oxygen saturation were measured by a hemoximeter (Radiometer, Copenhagen, Denmark).

Drug preparation. Exosurf™ (108 mg colfosceril palmitate, 12 mg cetyl alcohol, 8 mg tyloxapol, and 48 mg sodium chloride; Burroughs-Wellcome, Research Triangle Park, NC) was dissolved in 10 ml of normal saline.

Experimental protocol. During the study, the ewes stood quietly in a cage with free access to food and water. Baseline measurements of the hemodynamic variables (descending aortic, left and right atrial, and pulmonary arterial pressures, heart rate, and left pulmonary arterial blood flow), descending aortic blood gases, pH, hemoglobin, and oxygen saturation were made. The fetal lambs were then randomized (using a table of random numbers) to one of two groups: rhythmic distension or O_2 ventilation. In the rhythmic distension group ($n = 6$), the fetal lambs were ventilated with an $N_2/O_2/CO_2$ mixture which did not change fetal descending aortic blood gases or pH. In the O_2 ventilation group ($n = 6$), the fetal lambs were ventilated with 100% oxygen.

To ventilate the fetus, first, the endotracheal tubing was disconnected from the amniotic cavity catheter to drain the tracheal fluid (3, 10). Then, Exosurf™ (10 ml) was instilled into the trachea. After 10 min, in utero ventilation was begun using a standard animal ventilator (Harvard Apparatus, Inc., South Natick, MA); the ventilator settings were a rate of 20–25 breaths/min, an inspiratory time-to-expiratory time ratio of 0.45, and a tidal volume of 30–50 ml.

The hemodynamic variables were measured continuously, and descending aortic blood gases, pH, hemoglobin concentration, and oxygen saturation were measured every 30 min. After 8 h of in utero ventilation, the ewe and fetus were killed with an intravenous injection of pentobarbital sodium (Euthanasia CII; Central City Medical, Union City, CA) followed by bilateral thoracotomy. Catheter position was confirmed at autopsy.

Statistical analysis. The means±standard error were calculated for the hemodynamic variables, left pulmonary vascular resistance, and descending aortic blood gases, pH, hemoglobin concentration, and oxygen saturation during all experimental conditions (27). The maximum effects of rhythmic distension and O_2 ventilation on these variables were compared with their respective baselines by the paired t test or the Wilcoxon signed rank test. During rhythmic distension and O_2 ventilation, the maximum effects on these variables were compared by the unpaired t test or the Mann-Whitney U test. $P < 0.05$ was considered statistically significant (27).

Cellular and molecular studies

Tissue preparation. The fetal heart and lungs were removed en bloc. To prepare total RNA, lung tissue was removed, snap-frozen in liquid nitrogen, and stored at −70°C until used. Total RNA was prepared from snap-frozen lung tissue (after pulverization) or from cultured ovine fetal pulmonary arterial endothelial cells (see below) by brief homogenization in 4 M guanidinium isothiocyanate, followed by extraction with acid phenol, and precipitation in isopropanol (28). To prepare protein, lung tissue was rinsed in cold (4°C) normal saline to remove blood, then minced, and finally homogenized using a Tissuemizer (2 × 15 s at 80% power) at 4 vol/wet weight of Triton lysis buffer (20 mM Tris-HCl, pH 7.6, 0.5% Triton X-100, and 20% glycerol) supplemented with protease inhibitors; cultured ovine fetal pulmonary arterial endothelial cells were sonicated in 3 vol/wet weight. Extracts were then centrifuged at 15,000 *g* for 15 min. The supernatant was then removed for protein determination and Western blot analysis (29, 30).

For in situ hybridization, the pulmonary vascular tree was rinsed with cold PBS to remove blood and fixed by perfusion with cold 4% paraformaldehyde. The pulmonary artery was then clamped. The airways were fixed at 20 cm of H_2O pressure by filling the trachea with cold 4% paraformaldehyde. When the lungs were distended at this pressure, the trachea was clamped. The lungs were fixed for 24 h at 4°C by immersion in 4% paraformaldehyde. Representative slices from each lobe were removed, placed in 30% sucrose until they sank, placed in OCT, frozen on dry ice, and stored at −70°C until sectioned. 10–20-μm sections were cut using a cryostat, transferred to aminoalkylsilane-treated slides (Superfrost Plus; Fisher Scientific, Santa Clara, CA), and stored at −70°C (30–32).

Generation of ovine eNOS cDNA and antiserum. Sequence comparisons between the three isoforms of NOS (endothelial, inducible, and neuronal) were performed using dot-matrix homology plots to determine a region of minimal homology between them (15, 30). This region corresponded to an area within the heme binding domain. Oligonucleotides were synthesized (using the bovine eNOS as a template) to allow amplification of this region within the ovine eNOS sequence. The sequences of the oligonucleotides were 5′-CCTCCGGAGGGGCCCAAGTTCCCTCGC-3′ for oligonucleotide 1 and 5′-CACGTCGAAGCGCCGTTTCCGGGGGT-3′ for oligonucleotide 2. The region amplified corresponds to amino acids 62–288 of the eNOS protein (24, 33–35). Total RNA, prepared from ovine fetal lung, was used in RT-PCR reactions (kit from Perkin-Elmer, Foster City, CA). The cDNA fragment generated (681 bp) was then cloned directly into the pCR II vector (Invitrogen, San Diego, CA), sequenced (Sequenase kit; United States Biochemical Corp., Cleveland, OH), and then subcloned into pBluescript KS^+ (Stratagene, La Jolla, CA). The sequence in this region for ovine eNOS is 96.6% identical to bovine eNOS.

The eNOS cDNA fragment was subcloned in frame into the pET23a expression vector to overexpress the corresponding eNOS protein fragment (30) (Novagen, Madison, WI). After confirming the reading frame at both the 5′- and 3′-ends, the pET23a clone was transformed into the bacterial strain BL21(DE3)plys S, which contains a lysogen of T7 bacteriophage and a plasmid encoding lysozyme to reduce constitutive expression of the T7 RNA polymerase. Cultures (1 liter) were grown from a single colony under ampicillin selection until the OD_{600} reached ∼ 0.6; isopropyl β-D-thiogalactoside was then added (final concentration 0.4 mM). After 3 h, the cells were pelleted, resuspended in one-tenth volume of imidazole buffer, and sonicated to disrupt the cell membranes and shear chromosomal DNA. The lysate was cleared by centrifugation, passed over the Ni^{2+} column, washed, and eluted with 1 M imidazole. The eluted fraction was concentrated by passage through a concentrator with the addition of sterile distilled water to reduce the imidazole concentration. The resultant partially purified eNOS protein fragment was then injected into New Zealand White rabbits (Animal Pharm. Services, Healdsberg, CA) to produce a polyclonal eNOS antiserum. The specificity of the antiserum was assessed by Western blot analysis on protein extracts prepared from a variety of tissues.

RNA probe synthesis, RNase protection assay, and in situ hybridization. The plasmid containing the eNOS cDNA fragment was linearized with the appropriate restriction enzyme (GIBCO BRL, Gaithersburg, MD). Antisense and sense radiolabeled single-stranded RNA eNOS probes were synthesized by in vitro transcription using either T3 or T7 RNA polymerases (Boehringer-Mannheim, Indianapolis, IN) in the presence of cold rCTP, rGTP, and rATP. RNA probes labeled with ^{32}P-UTP (New England Nuclear, Boston, MA) were used for RNase protection assays (36); RNA probes labeled with ^{35}S-UTP were used for in situ hybridization (33–35).

RNase protection assays were performed as described previously (36). Antisense radiolabeled single-stranded RNA eNOS probes were hybridized overnight at 42°C with total RNA isolated from ovine fetal lung (50 μg) or from ovine fetal pulmonary arterial endothelial cells (20 μg) in 80% formamide, 50 mM Pipes (pH 6.4), 0.4 M NaCl, and 1 mM EDTA. Single-stranded RNA was digested for 1 h at 37°C with an RNase A/T1 mixture (Ambion, Austin, TX). After phenol/$CHCl_3$ extraction and ethanol precipitation, the protected fragments were analyzed by electrophoresis on a 6% denaturing polyacrylamide gel. Also included was a probe for 18S to serve as a control for the amount of input total RNA and the recovery of protected probe fragments.

In situ hybridization was performed as previously described (33–35). Studies were done on serial sections of ovine fetal lung, using the antisense radiolabeled cRNA eNOS probe and the corresponding nonhybridizing sense radiolabeled cRNA eNOS probe. Frozen tissue sections were allowed to come to room temperature. All sections were fixed in 4% paraformaldehyde in PBS, treated with proteinase K, and acetylated (0.25% acetic anhydride in 0.1 M triethanolamine-HCl, pH 7.5). After washing in 0.5× SSC, the sections were covered with hybridization solution (50% deionized formamide, 0.3 M NaCl, 20 mM Tris, pH 8, 5 mM EDTA, 1× Denhardt's, 10% dextran sulfate, and 10 mM DTT), and prehybridized for 1–3 h at 55°C. In the presence of tRNA, the radiolabeled RNA probe (600,000 cpm/slide) was applied to the hybridization solution, and the sections were then hybridized for 12–18 h at 55°C. After hybridization, the sections were washed for 20 min (2× SSC, 10 mM β-mercaptoethanol, and 1 mM EDTA), treated with RNase A (20 mg/ml), and washed for 20 min (2× SSC, 10 mM β-mercaptoethanol, and 1 mM EDTA). The sections were then washed in high stringency buffer (50% deionized formamide, 2× SSC, and 0.1 M DTT) for 30 min at 65°C. The final wash was in 0.5× SSC for 20 min. The sections were dehydrated, the slides dipped in photographic emulsion (Ilford, St. Louis, MO), stored at 4°C, developed after 2–10 wk of exposure, and counterstained with hematoxylin and eosin. For each experiment, four antisense and four sense slides (containing one to two sections) were studied. For each experiment, new radiolabeled probes were synthesized.

Western blot analysis. Western blot analysis was performed as described previously (30). Protein extracts isolated from ovine fetal lung (100 μg) or from cultured ovine fetal pulmonary arterial endothelial cells (25 μg) were separated on a 6% denaturing polyacrylamide gel and electrophoretically transferred to Hybond-PVDF membranes (Amersham, Arlington Heights, IL). The membranes were blocked with 5% nonfat dry milk in TBS containing 0.1% Tween. After blocking, the membranes were incubated at room temperature with 1:1,000 dilution of the eNOS antiserum, washed with TBS containing 0.1% Tween, and then incubated with a mouse anti–rabbit IgG horseradish peroxidase conjugate. After washing, chemiluminescence was used to detect the eNOS protein bands.

Cell culture techniques. The heart and lungs were obtained from fetal lambs (138–140 d of gestation) after killing. These fetal lambs had not undergone previous surgery or study. The main and branch pulmonary arteries were removed and dissected free; the adventitia was removed. The exterior of the vessel was rinsed with 70% ethanol. The vessel was then opened longitudinally and the interior rinsed with PBS to remove any blood. Using a cell scraper, the endothelium was lightly scraped away, placed in medium DME-H16 (with 10% FBS and antibiotics), and incubated at 37°C in 21% O_2/5% CO_2 bal-

anced with N_2. After 5 d, islands of endothelial cells were cloned to ensure purity. Basic fibroblast growth factor (10 ng/ml, a gift from Denis Gospodarowicz, Chiron Corporation, Emeryville, CA) was added to the media every other day. When confluent, the cells were passaged to maintain them in culture or frozen in liquid nitrogen. Endothelial cell identity was confirmed by their typical cobblestone appearance, contact inhibition, specific uptake of DiI-Ac-LDL (Molecular Probes, Eugene, OR), and positive staining for von Willebrand factor (Dako, Carpinteria, CA). Ovine fetal pulmonary arterial endothelial cells were studied between passages 3 and 10.

Oxygen studies. Because tissue culture plastic adsorbs oxygen, ovine fetal pulmonary arterial endothelial cells were grown on glass plates (37). These plates were washed and baked to remove organic residue before use. Confluent cultures were placed in an airtight Plexiglas chamber inside a standard CO_2 incubator. The chamber was gassed with 1% O_2/5% CO_2 balanced with N_2 (PO_2 of the culture medium 30–39 torr [1% O_2]), or with 95% O_2/5% CO_2 (PO_2 of the culture medium 549–644 torr [95% O_2]). Ovine fetal pulmonary arterial endothelial cells tolerate these treatments without change in morphology or cell death. Other confluent cultures were placed in 21% O_2/5% CO_2 balanced with N_2 (PO_2 of the culture medium 125–132 torr [21% O_2]). The cells were harvested in triplicate after 0, 8, or 24 h of exposure.

Shear stress studies. We have built a cone-plate viscometer to accept 15-cm tissue culture plates (38). This apparatus consists of a cone of shallow angle (α) rotating at angular velocity (ω) on top of a tissue culture plate, placed inside a standard CO_2 incubator (21% O_2/5% CO_2 balanced with N_2). The monolayer of confluent ovine fetal pulmonary arterial endothelial cells was subjected to a radially constant fluid shear stress as calculated using the formula $\tau = \mu\omega/\alpha$, where μ is the viscosity of the medium covering the ovine fetal pulmonary arterial endothelial cells (38, 39). A cone angle of 0.5° was used to achieve laminar flow rates representing levels of shear stress within the physiological range (5–25 dynes/cm^2). When rotated at 175 rpm, 20 dynes/cm^2 of shear stress was applied for 0, 2, 4, or 8 h.

Data analysis. Quantitation of autoradiographic results were performed by scanning (SCA Jet IICX; Hewlett Packard Inc., Palo Alto, CA) the bands of interest into an image editing software program (Adobe Photoshop, Adobe Systems, Mt. View, CA). Band intensities from RNase protection assays and Western blot analysis were analyzed densitometrically on a Macintosh computer (model 9500; Apple Computer, Inc., Cupertino, CA) using the public domain NIH Image program (developed at the National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image) (40). For RNAase protection assays, to control for the amount of input RNA and the recovery of protected probe fragments, the eNOS signal was normalized to the corresponding 18S signal for each lane. Results from unventilated fetal lamb lungs or from 0 h of exposure in cell culture experiments were assigned the value of 1 (relative eNOS mRNA). For Western blot analysis, to insure equal protein loading, duplicate polyacrylamide gels were run. One was stained with Coomassie blue. Results from unventilated fetal lamb lungs or from 0 h of exposure in cell culture experiments were assigned the value of 1 (relative eNOS protein). The means±standard error were calculated for relative eNOS mRNA and relative eNOS protein during all experimental conditions and were compared by the Kruskal-Wallis Test or ANOVA. Multiple comparison testing was performed. $P < 0.05$ was considered statistically significant.

Results

Physiologic studies. After 8 h of rhythmic distension of the fetal lung in utero by ventilation with a hypoxic gas mixture, there were no changes in fetal descending aortic PO_2 (before ventilation, 15.5±1.8 mmHg; during ventilation, 15.6±1.4 mmHg), PCO_2 (before ventilation, 51.3±1.0 mmHg; during ventilation, 49.0±1.7 mmHg), or pH (before ventilation, 7.35±0.01; during ventilation, 7.35±0.02). Rhythmic distension increased left pulmonary arterial blood flow ($P < 0.05$, Fig. 1, *top*) without changing mean left pulmonary arterial pressure (before ventilation, 61.3±3.9 mmHg; during ventilation, 64.0±3.0 mmHg) or mean descending aortic pressure (before ventilation, 61.3±3.9 mmHg; during ventilation, 60.0±3.0 mmHg). Left pulmonary vascular resistance decreased ($P < 0.05$, Fig. 1, *bottom*).

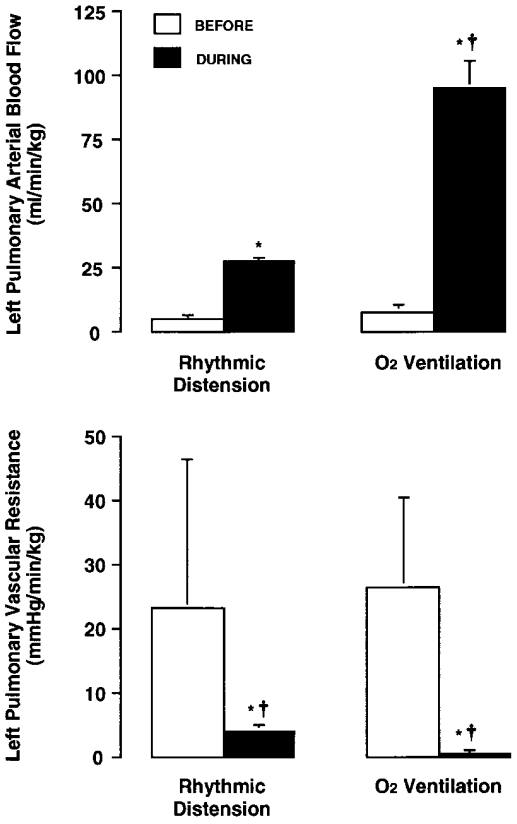

Figure 1. The effects of in utero ventilation on left pulmonary artery blood flow (*top*) and left pulmonary vascular resistance (*bottom*). 12 fetal lambs were ventilated in utero for 8 h with a gas mixture which did not change fetal descending aortic blood gases or pH (rhythm distension) or with 100% oxygen (O_2 ventilation). Rhythm distension increased left pulmonary blood flow and decreased left pulmonary vascular resistance. The effects were greater with O_2 ventilation. Values are mean±standard error. $n = 6$. $^*P < 0.05$ before ventilation vs. during ventilation (within treatments). $^\dagger P < 0.05$ during ventilation vs. during ventilation (between treatments).

After 8 h of in utero ventilation of the fetal lung with 100% oxygen, fetal descending aortic PO_2 (before ventilation, 18.8±1.9 mmHg; during ventilation, 327.2±70.4 mmHg, $P < 0.05$) and pH increased (before ventilation, 7.27±0.03; during ventilation, 7.42±0.02, $P < 0.05$); fetal descending aortic PCO_2 decreased (before ventilation, 52.0±4.0 mmHg; during ventilation, 35.8±3.0 mmHg, $P < 0.05$). O_2 ventilation increased left pulmonary arterial blood flow ($P < 0.05$, Fig. 1, *top*) and decreased left pulmonary vascular resistance ($P < 0.05$, Fig. 1, *bottom*) more than rhythmic distension. Mean left pulmonary arterial pressure decreased (before ventilation, 63.7±4.6 mmHg; during ventilation, 46.3±1.7 mmHg, $P < 0.05$), while mean descending aortic pressure was unchanged (before ventilation, 65.0±9.5 mmHg; during ventilation, 63.6±5.0 mmHg).

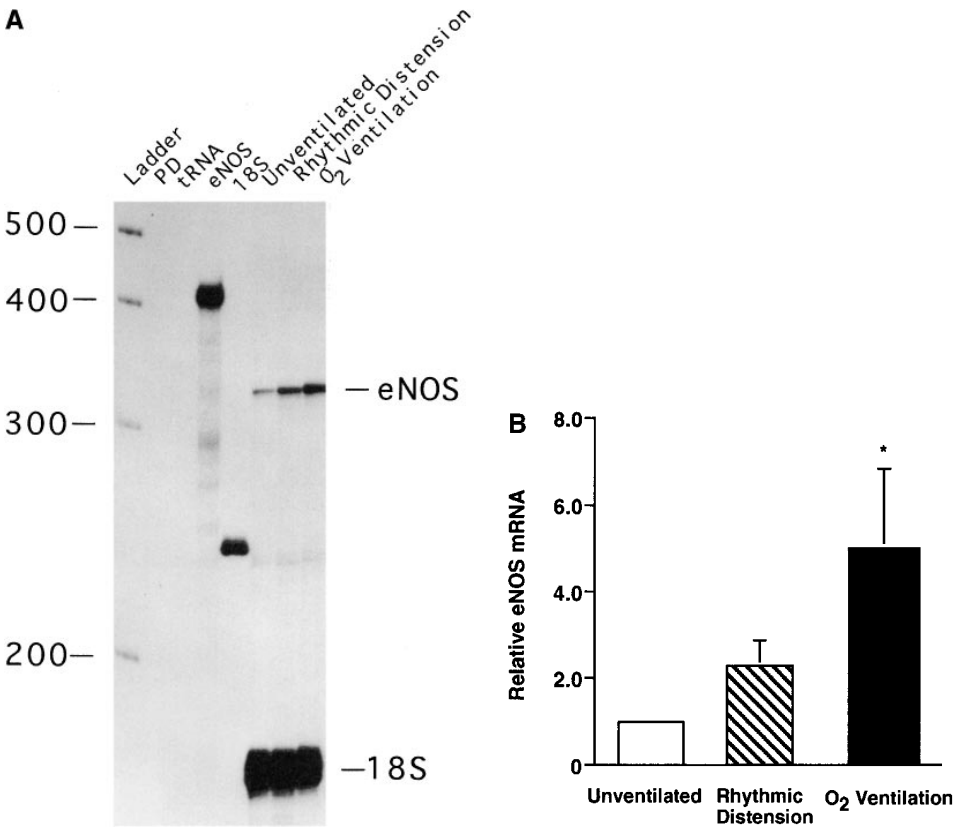

Figure 2. RNase protection assay for eNOS mRNA in lung tissue from near-term fetal lambs: unventilated, and after 8 h of in utero with a gas mixture which did not change fetal descending aortic blood gases or pH (rhythm distension) or with 100% oxygen (O_2 ventilation). (*A*) A cRNA probe for ovine eNOS was hybridized overnight to 50 μg of total lung RNA prepared from three near-term fetal lambs (unventilated, rhythmic distention, and O_2 ventilation). There is an increase in eNOS mRNA expression with rhythmic distension and a greater increase with O_2 ventilation. No protected fragments were detected in the lanes where the probe was hybridized without RNA (*PD*) or in the presence of tRNA. eNOS is undigested probe. A cRNA for ovine 18S was also hybridized to serve as a control for RNA loading. (*B*) The densitometric values for relative eNOS mRNA (normalized to 18S mRNA and to unventilated) from five different experiments. Rhythmic distension increased relative eNOS mRNA by 2.3-fold; O_2 ventilation increased relative eNOS mRNA by 5-fold ($P < 0.05$). Values are mean±standard error. $n = 5$. *$P < 0.05$ unventilated vs. O_2 ventilation.

Cellular and molecular studies. After 8 h of in utero ventilation, eNOS mRNA expression was increased in vivo in the fetal lung (Fig. 2). O_2 ventilation increased eNOS mRNA expression by 5-fold compared with the unventilated fetal lamb lung ($P < 0.05$); rhythmic distension increased eNOS mRNA expression by 2.3-fold. This increase in eNOS mRNA expression is confined to the endothelial cells of small and large arteries and veins (Fig. 3). After 8 h of rhythmic distension, there was no change in eNOS protein expression; O_2 ventilation increased eNOS protein expression by 1.9-fold compared with the unventilated fetal lamb lung ($P < 0.05$) (Fig. 4). When ovine fetal pulmonary arterial endothelial cells were exposed to 95% O_2 for 24 h, there was a 3.3-fold increase in eNOS mRNA expression and a 2.3-fold increase in eNOS protein expression ($P < 0.05$) (Fig. 5). Exposure to 1% O_2 did not change eNOS mRNA or protein expression. When ovine fetal pulmonary arterial endothelial cells were exposed to fluid shear stress to simulate the increase in pulmonary blood flow after birth, there were increases in eNOS mRNA expression of 2.5-, 3.4-, and 4.9-fold after 2, 4, and 8 h ($P < 0.05$), respectively (Fig. 6, *A* and *B*). eNOS protein expression was increased 1.2-fold after 8 h ($P < 0.05$) (Fig. 6, *C* and *D*).

Discussion

The purpose of this study was to evaluate whether birth-related events induce changes in eNOS gene expression. This study shows that ventilation of the near-term fetal lamb with oxygen to increase fetal descending aortic PO_2 decreases pulmonary vascular resistance and increases pulmonary blood flow more than rhythmic distension of the lung with a gas mixture which does not change fetal descending aortic blood gases or pH. Associated with these physiologic changes, eNOS mRNA and protein expression in the lung increases. This increase in eNOS mRNA expression is confined to the endothelium of small and large blood vessels. Both increased oxygenation and increased shear stress induce eNOS mRNA and protein expression in ovine fetal pulmonary arterial endothelial cells. The induction of eNOS mRNA by increased shear stress occurs more rapidly. Therefore, increased oxygenation, and more importantly, increased pulmonary blood flow with increased shear stress induce eNOS gene expression and contribute to pulmonary vasodilation after birth.

There is increasing evidence that NO mediates pulmonary vascular tone in the perinatal period (8–10, 16, 17). First, in fetal lambs, acetylcholine decreases pulmonary vascular resistance and increases pulmonary blood flow (41); these hemodynamic effects are blocked by N^{ω}-nitro-L-arginine or other L-arginine analogs which inhibit NO synthesis (41). Second, inhibition of NO synthesis increases pulmonary vascular resistance in fetal lambs and attenuates the increase in pulmonary blood flow induced by maternal hyperbaric oxygen exposure, ventilation with air or oxygen (8–10, 16), or compression of the ductus arteriosus. Third, L-arginine, the precursor of NO, increases pulmonary blood flow in fetal and newborn lambs and augments endothelium-dependent pulmonary vasodilation (8,

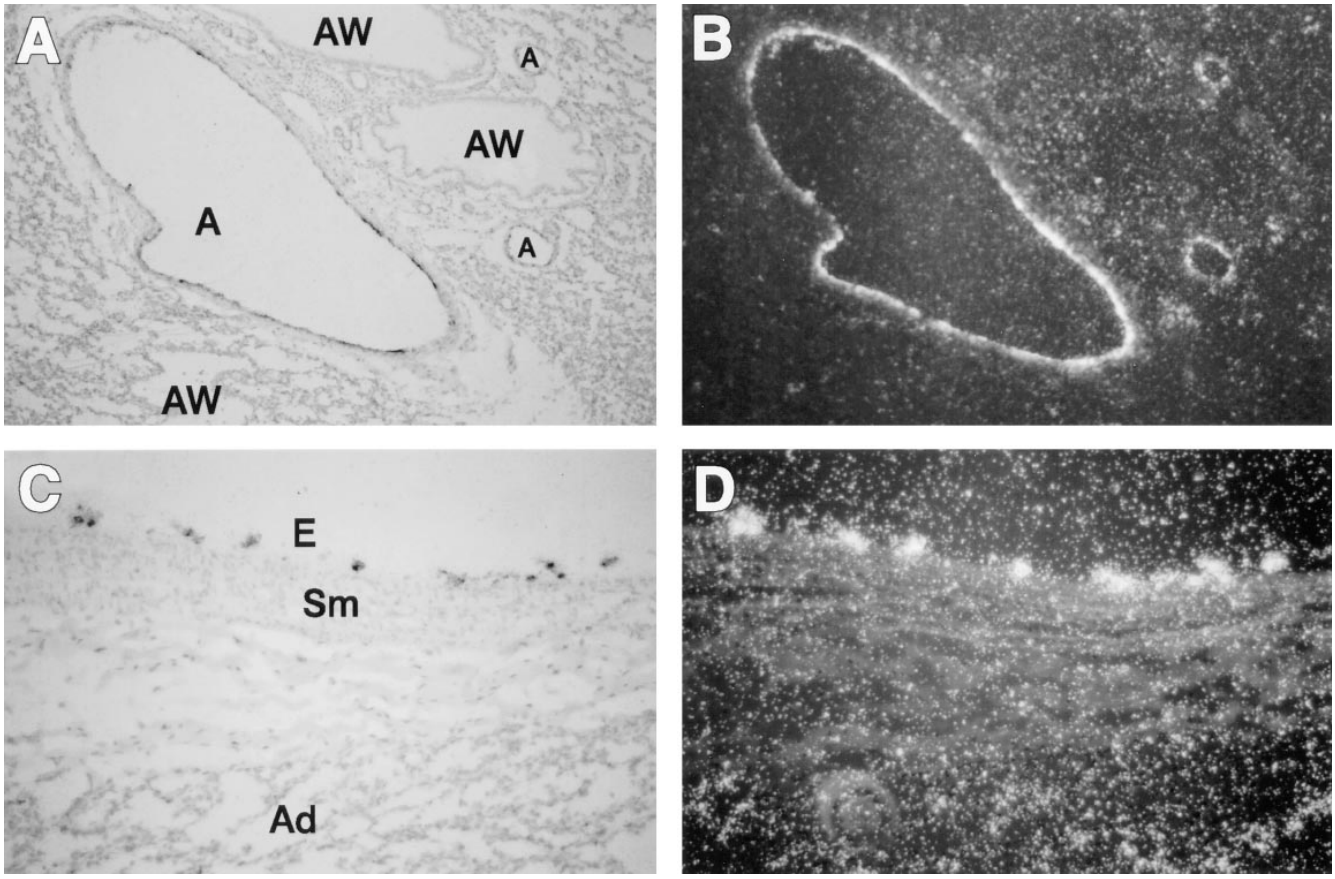

Figure 3. eNOS mRNA expression in the lung in vivo from near-term fetal lambs after 8 h of ventilation in utero with 100% oxygen. A cRNA probe for ovine eNOS was hybridized to tissue sections prepared from the lung of fetal lambs ventilated in utero for 8 h with 100% oxygen. Expression is seen as black grains in bright-field (*A* and *C*) and as silver grains in dark-field (*B* and *D*). *A* and *B*, ×200; *C* and *D*, ×400. Ovine eNOS mRNA is expressed only in the endothelium (*E*) of small and large pulmonary arteries (*A*) and veins but not in the smooth muscle (*Sm*) or adventitia (*Ad*), or in the airways (*AW*). The result shown is representative of results from three experiments using different fetal lungs after 8 h of ventilation with 100% oxygen.

42). Fourth, inhaled NO decreases pulmonary vascular resistance in fetal lambs and in newborn lambs with pulmonary hypertension (43, 44). Fifth, methylene blue, an inhibitor of soluble guanylate cyclase, increases pulmonary vascular resistance, while M&B 22948 and dipyridamole, inhibitors of cGMP phosphodiesterase, decrease pulmonary vascular resistance and augment endothelium-dependent pulmonary vasodilation in fetal and newborn lambs (45–47). Finally, there are also

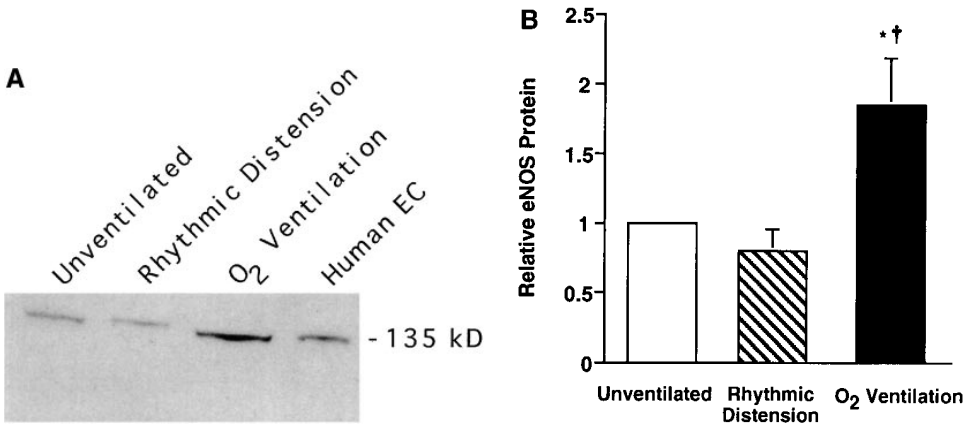

Figure 4. Western blot analysis for eNOS protein in lung tissue from near-term fetal lambs: unventilated, and after 8 h of in utero with a gas mixture which did not change fetal descending aortic blood gases or pH (rhythm distension) or with 100% oxygen (O_2 ventilation). (*A*) Protein extracts (100 μg), prepared from lung tissue from three near-term fetal lambs (unventilated, rhythmic distention, and O_2 ventilation), were separated on a 6% denaturing polyacrylamide gel, electrophoretically transferred to Hybond membranes, and analyzed using a specific antiserum raised against eNOS. Human endothelial cell protein (*Human EC*, 20 μg) was used as a control. There was no change in eNOS protein (135 kD) with rhythmic distension; there was an increase with O_2 ventilation. (*B*) The densitometric values for relative eNOS protein (normalized to unventilated) from six different experiments. Rhythmic distension did not change eNOS protein; O_2 ventilation increased eNOS protein by 1.9-fold ($P < 0.05$). Values are mean±standard error. $n = 6$. *$P < 0.05$ unventilated vs. O_2 ventilation. †$P < 0.05$ rhythmic distention vs. O_2 ventilation.

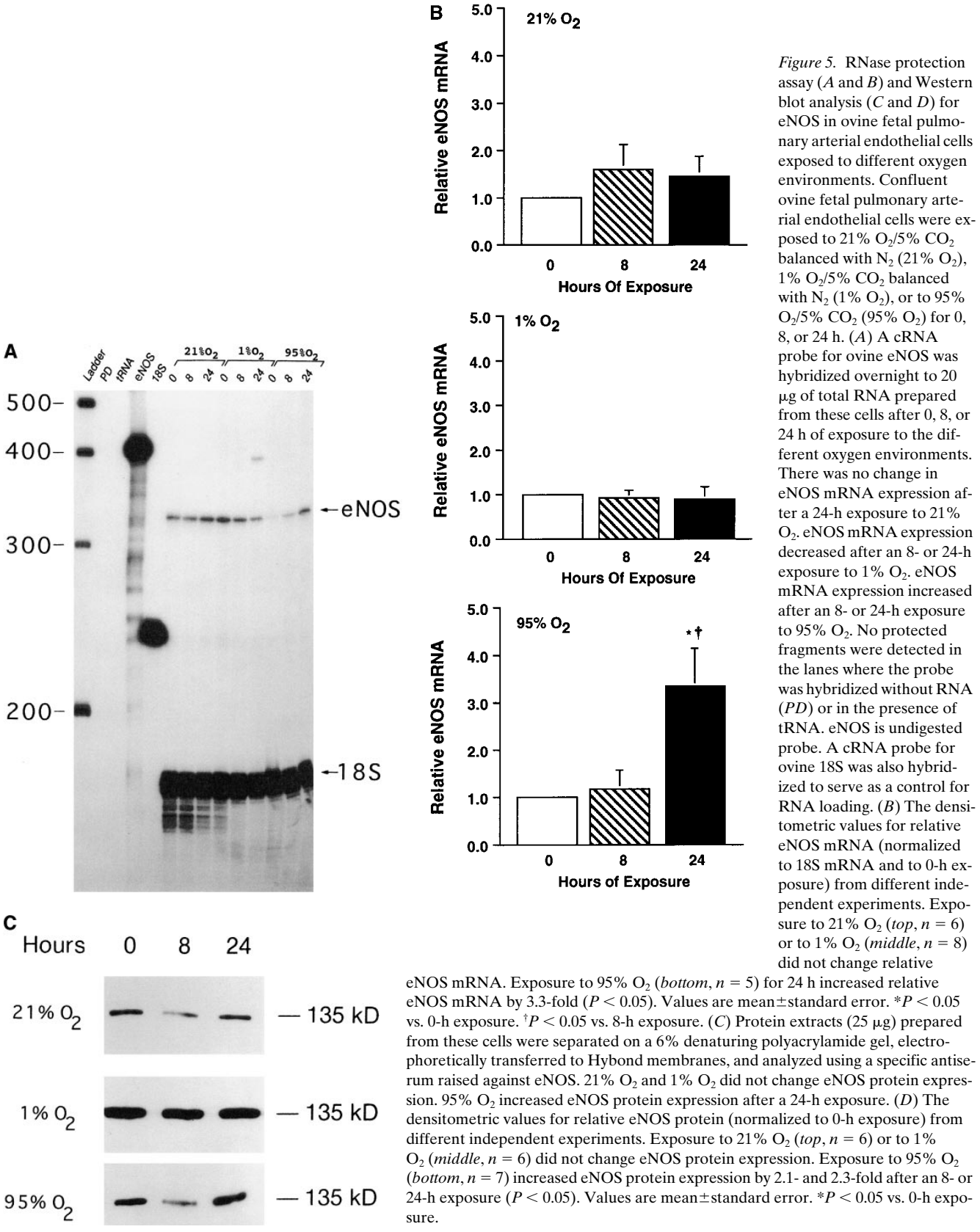

Figure 5. RNase protection assay (*A* and *B*) and Western blot analysis (*C* and *D*) for eNOS in ovine fetal pulmonary arterial endothelial cells exposed to different oxygen environments. Confluent ovine fetal pulmonary arterial endothelial cells were exposed to 21% O_2/5% CO_2 balanced with N_2 (21% O_2), 1% O_2/5% CO_2 balanced with N_2 (1% O_2), or to 95% O_2/5% CO_2 (95% O_2) for 0, 8, or 24 h. (*A*) A cRNA probe for ovine eNOS was hybridized overnight to 20 μg of total RNA prepared from these cells after 0, 8, or 24 h of exposure to the different oxygen environments. There was no change in eNOS mRNA expression after a 24-h exposure to 21% O_2. eNOS mRNA expression decreased after an 8- or 24-h exposure to 1% O_2. eNOS mRNA expression increased after an 8- or 24-h exposure to 95% O_2. No protected fragments were detected in the lanes where the probe was hybridized without RNA (*PD*) or in the presence of tRNA. eNOS is undigested probe. A cRNA probe for ovine 18S was also hybridized to serve as a control for RNA loading. (*B*) The densitometric values for relative eNOS mRNA (normalized to 18S mRNA and to 0-h exposure) from different independent experiments. Exposure to 21% O_2 (*top*, $n = 6$) or to 1% O_2 (*middle*, $n = 8$) did not change relative eNOS mRNA. Exposure to 95% O_2 (*bottom*, $n = 5$) for 24 h increased relative eNOS mRNA by 3.3-fold ($P < 0.05$). Values are mean±standard error. *$P < 0.05$ vs. 0-h exposure. †$P < 0.05$ vs. 8-h exposure. (*C*) Protein extracts (25 μg) prepared from these cells were separated on a 6% denaturing polyacrylamide gel, electrophoretically transferred to Hybond membranes, and analyzed using a specific antiserum raised against eNOS. 21% O_2 and 1% O_2 did not change eNOS protein expression. 95% O_2 increased eNOS protein expression after a 24-h exposure. (*D*) The densitometric values for relative eNOS protein (normalized to 0-h exposure) from different independent experiments. Exposure to 21% O_2 (*top*, $n = 6$) or to 1% O_2 (*middle*, $n = 6$) did not change eNOS protein expression. Exposure to 95% O_2 (*bottom*, $n = 7$) increased eNOS protein expression by 2.1- and 2.3-fold after an 8- or 24-h exposure ($P < 0.05$). Values are mean±standard error. *$P < 0.05$ vs. 0-h exposure.

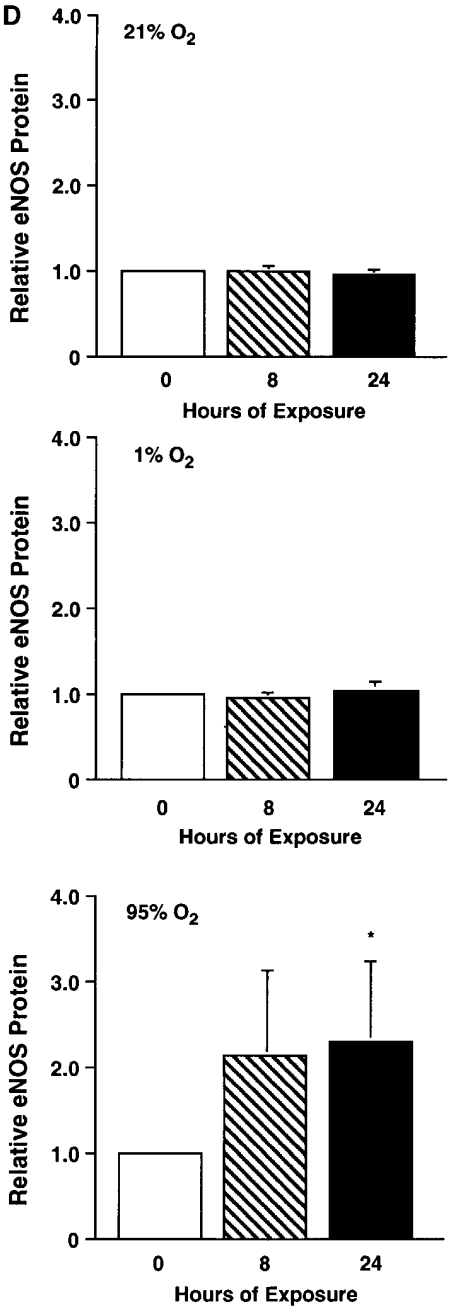

Figure 5 (Continued)

changes in NO production from isolated pulmonary arteries and endothelial cells with development. Basal and endothelial-dependent production of NO increase in newborn pulmonary arteries compared with near-term fetal pulmonary arteries, and in fetal pulmonary arterial endothelial cells exposed to hyperoxia; production is decreased by hypoxia (48–50). Therefore, changes in NOS activity and NO production likely mediate, in part, pulmonary vascular tone in the perinatal period.

eNOS differs in structure and regulation from the two other isoforms of NOS (51). eNOS is present on the cell membrane of endothelial cells and its activity is calcium and calmodulin dependent (11, 13–15, 23, 51). Human and bovine eNOS have been isolated and purified as a 135-kD protein whose clone identifies a 4.6–4.8-kb mRNA by Northern blot analysis (24, 33–35). eNOS has been described as a constitutive enzyme; however, it is developmentally regulated. In rat and sheep lungs, eNOS mRNA expression increases near term and decreases after birth as do eNOS protein levels (18, 19, 52). This increased eNOS expression near term leads to increased NO production resulting in pulmonary vasodilation at birth. The results of this study show that in the ovine fetal lung, the increase in pulmonary blood flow and oxygenation induced by ventilation increases eNOS mRNA and protein expression. These increases in eNOS mRNA and protein expression are necessary for the more gradual and progressive pulmonary vasodilation that occurs hours and days after birth. For example, mean pulmonary arterial pressure decreases to $\sim$ 50% of mean systemic arterial pressure by 24 h of age, and reaches adult values by 2–6 wk after birth (4).

eNOS is also regulated by changes in the oxygen environment. eNOS activity and NO production are increased by hyperoxia and decreased by hypoxia (10, 16, 48–50). In this study, eNOS mRNA and protein expression were increased in ovine fetal pulmonary arterial endothelial cells exposed to 95% oxygen for 24 h. In other studies, eNOS mRNA and protein expression were also increased to a similar degree in bovine adult and ovine fetal pulmonary arterial endothelial cells exposed to a high oxygen environment for 24 to 48 h (53–55). These increases in gene expression are mediated through transcriptional and posttranscriptional mechanisms. Since these oxygen-mediated increases in eNOS gene expression occur over 24 h, they likely will contribute to the gradual decrease in pulmonary vascular resistance after birth. In this study, eNOS mRNA and protein expression were unchanged in ovine fetal pulmonary arterial endothelial cells exposed to 1% oxygen for 24 h. However, in other studies, eNOS mRNA and protein expression were decreased in bovine adult and human umbilical vein endothelial cells exposed to a low oxygen environment for 24 to 48 h (53–55). Since ovine fetal pulmonary arterial endothelial cells are exposed to a very low oxygen environment in vivo, a lower oxygen tension in the culture medium than achieved in this study ($PO_2 < 40$ torr) may be needed to decrease eNOS gene expression.

In vivo, endothelial cells are exposed to fluid mechanical forces, including hydrostatic pressure, strain, and shear stress (25, 26, 38, 39). Shear stress is proportional to the velocity of blood and its viscosity and inversely proportional to the internal radius of the blood vessel to the third power (38, 39). Shear stress is increased in the pulmonary circulation of the fetus or newborn if: (*a*) increased pulmonary blood flow increases pulmonary blood flow velocity; (*b*) blood hematocrit is increased; and (*c*) there is underdevelopment of the pulmonary circulation. Increases in shear stress stimulate endothelial cells to produce several modulators of vascular tone, including NO (20–23). When endothelial cells are subjected to shear stress, a diverse set of responses is initiated, some of which occur within minutes, others which occur within hours or days (39). In vivo, the acute compression of the ductus arteriosus increases fetal pulmonary blood flow; this increase is blocked by inhibition of NO synthesis, suggesting that the increase in pulmonary blood flow has increased shear stress, increasing NOS activity and NO production (9). This increase in NO activity induced by shear stress is associated with rapid changes in endothelial cell calcium concentration, ionic conductance, adenylate cyclase activity, and inositol triphosphate generation (20–23, 39). If the shear stress is maintained, endothelial cells change their

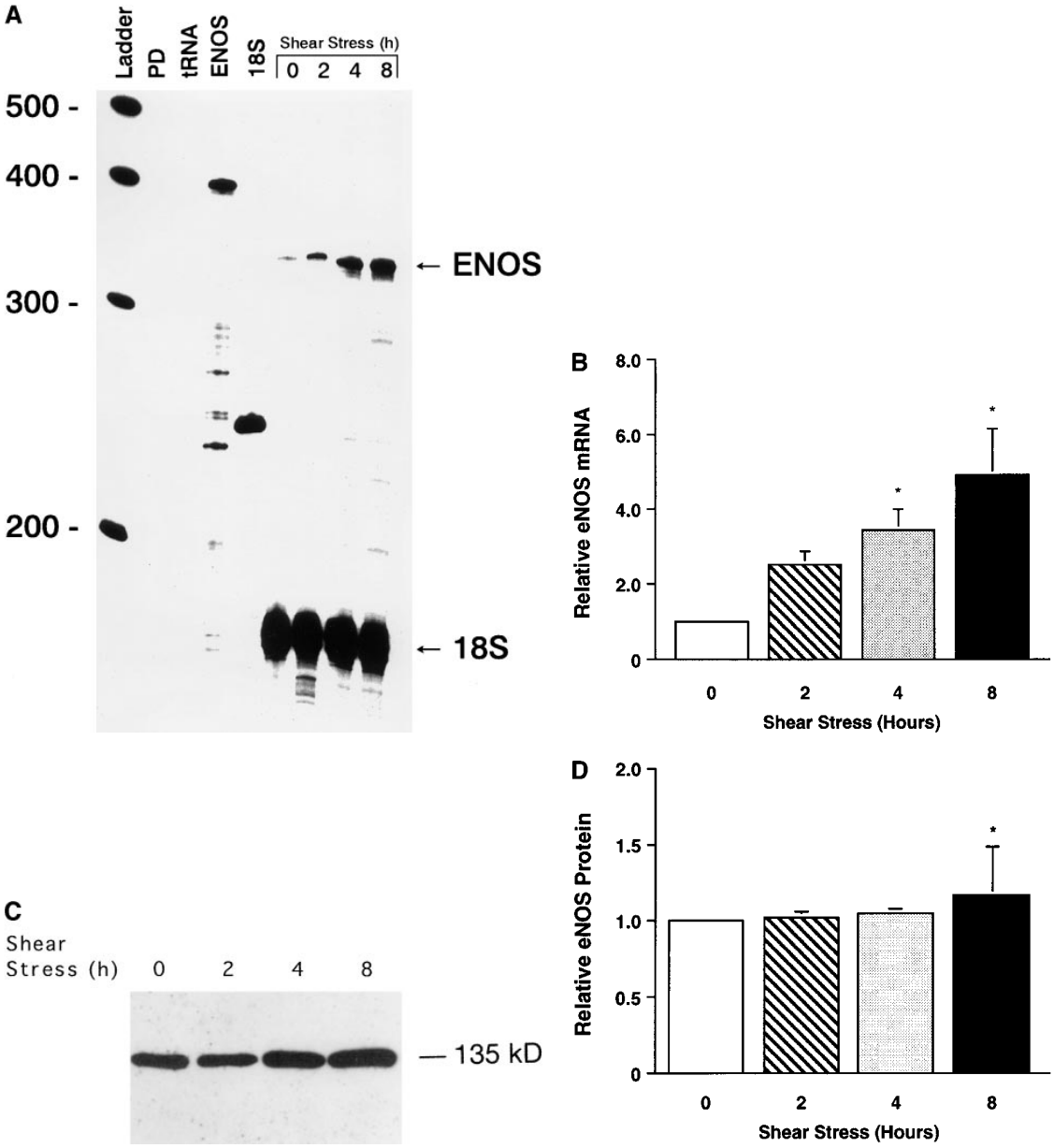

Figure 6. RNase protection assay (*A* and *B*) and Western blot analysis (*C* and *D*) for eNOS in ovine fetal pulmonary arterial endothelial cells exposed to fluid shear stress. Confluent ovine fetal pulmonary arterial endothelial cells were exposed for 0, 2, 4, and 8 h to radially constant fluid shear stress (20 dynes/cm^2). (*A*) A cRNA probe for ovine eNOS was hybridized overnight to 20 μg of total RNA prepared from these cells. From 0 h of exposure, eNOS mRNA expression was increased after 2 h of shear stress and continued to increase further after 4 and 8 h. No protected fragments were detected in the lanes where the probe was hybridized without RNA (*PD*) or in the presence of tRNA. ENOS is undigested probe. A cRNA probe for ovine 18S was also hybridized to serve as a control for RNA loading. (*B*) The densitometric values for relative eNOS mRNA (normalized to 18S mRNA and to 0-h exposure) from six different independent experiments. From 0 h of exposure, shear stress increased relative eNOS mRNA by 2.5-, 3.4-, and 4.9-fold after 2, 4, and 8 h ($P < 0.05$), respectively. Values are mean±standard error. $n = 6$. *$P < 0.05$ vs. 0-h exposure. (*C*) Protein extracts (25 μg), prepared from these cells, were separated on a 6% denaturing polyacrylamide gel, electrophoretically transferred to Hybond membranes, and analyzed using a specific antiserum raised against eNOS. Shear stress increased eNOS protein expression slightly after a 4- or 8-h exposure. (*D*) The densitometric values for relative eNOS protein (normalized to 0-h exposure) from four different independent experiments using different primary cultures. From 0 h of exposure, shear stress increased relative eNOS protein by 1.2-fold after an 8-h exposure ($P < 0.05$). Values are mean±standard error. $n = 4$. *$P < 0.05$ vs. 0-h exposure.

shape secondary to a structural reorganization of the cytoskeleton. There are also changes in gene expression. Shear stress induces PDGF-B mRNA and protein expression in endothelial cells (56–59). A 6-bp sequence (GAGACC) shear stress response element has been identified in the 5′-flank of the human PDGF-B gene and in other shear stress–inducible endothelial cell genes. The 5′-flank of the human NOS gene also contains the 6-bp shear stress response element (59). In this study, eNOS mRNA and protein expression were increased in ovine fetal pulmonary arterial endothelial cells ex-

posed to increased shear stress. In other studies, eNOS mRNA and protein expression were also increased to a similar degree and in a similar time course in bovine aortic and human umbilical vein endothelial cells exposed to increased shear stress. This shear stress–induced increase in eNOS gene expression in vitro was similar to the oxygen ventilation–induced increase in vivo, suggesting that the increase in pulmonary blood flow and the resulting increase in shear stress is more important than increased oxygenation in regulating eNOS gene expression.

There are multiple components contributing to the immediate decrease in pulmonary vascular resistance and increase in pulmonary blood flow with the initiation of ventilation and oxygenation at birth and the subsequent gradual (next hours) and progressive (days) transition to extrauterine life over the next hours to days. First, there is an immediate modest decrease in pulmonary vascular resistance and increase in pulmonary blood flow caused by physical expansion of the lung. This expansion produces partial pulmonary vasodilation independent of fetal oxygenation and NO production. This component of pulmonary vasodilation is not attenuated by inhibition of NOS activity. Next, there is further gradual pulmonary vasodilation associated with oxygenation, the initial increase in pulmonary blood flow, and the production of NO which results in a marked increase in pulmonary blood flow and decrease in pulmonary vascular resistance. This component of pulmonary vasodilation is attenuated by inhibition of NOS activity. Then, there is progressive pulmonary vasodilation induced by the increase in pulmonary blood flow and shear stress, and to a lesser degree, the increase in oxygenation. This is associated with increased eNOS mRNA and protein expression and further production of NO.

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