Ontogeny of cyclooxygenase-1 and cyclooxygenase-2 gene expression in ovine lung

TIMOTHY S. BRANNON,1 AMY N. MACRITCHIE,1 MARINA A. JARAMILLO,2 TODD S. SHERMAN,1 IVAN S. YUHANNA,1 LINDA R. MARGRAF,2 AND PHILIP W. SHAUL1
Departments of 1Pediatrics and 2Pathology, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235-9063

Brannon, Timothy S., Amy N. MacRitchie, Marina A. Jaramillo, Todd S. Sherman, Ivan S. Yuhanna, Linda R. Margraf, and Philip W. Shaul. Ontogeny of cyclooxygenase-1 and cyclooxygenase-2 gene expression in ovine lung. Am. J. Physiol. 274 (Lung Cell. Mol. Physiol. 18): L66–L71, 1998.—Prostacyclin is a key mediator of pulmonary vascular and parenchymal function during late fetal and early postnatal life, and its synthesis in whole lung increases during that period. The rate-limiting enzyme in prostacyclin synthesis in the developing lung is cyclooxygenase (COX). We investigated the ontogeny and cellular localization of COX-1 (constitutive) and COX-2 (inducible) gene expression in lungs from late-gestation fetal lambs, 1-wk-old newborn lambs (NB1), and 1- to 4-mo-old newborn lambs (NB2). COX-1 mRNA abundance rose progressively from fetal to NB1 to NB2, increasing 12-fold overall. In parallel, immunoblot analysis revealed a progressive increase in COX-1 protein, rising fourfold from fetal lambs to NB2. COX-2 mRNA levels increased fivefold from fetal to NB1 but were similar in NB1 and NB2. However, COX-2 protein was not detectable by immunoblot analysis in any age group. Immunohistochemistry for COX-1 showed intense immunostaining in endothelial cells at all ages. COX-1 was also expressed in airway epithelium at all ages, with a greater number of epithelial cells staining positively in NB2 compared with fetal and NB1 groups. In addition, COX-1 was expressed in airway smooth muscle from NB1. COX-2 immunostaining was absent in all age groups. These findings indicate that there is differential expression of COX-1 and COX-2 in the developing lung and that the enzymes are expressed in a cell-specific manner. The developmental upregulation in COX-1 may optimize the capacity for prostaglandin-mediated vasodilation, bronchodilation, and surfactant synthesis in the newborn lung.

Methods

Animal model. Lung tissue was studied from three groups of mixed-breed lambs. They were fetal lambs at 125–135 days of gestation (term = 144 ± 4 days), 1-wk-old newborn lambs (NB1), and 1- to 4-mo-old lambs (NB2). The pregnant ewes used were multiparous and had singletons, twins, or triplets. Before death, the animals were housed in the Animal Resources Center of the University of Texas Southwestern Medical Center and were given standard animal chow and water ad libitum. The ewes and fetus(es) were euthanized with 120 mg/kg pentobarbital sodium given intravenously to the ewe, and the fetuses were delivered by cesarean section. The newborn lambs were killed in a similar manner. Distal lung tissue was immediately removed and frozen in liquid N2. The procedures followed in the care and euthanasia of the study animals were approved by the Institutional Review Board for Animal Research.

Northern analysis. Total cellular RNA was obtained and purified from the lungs by a single-extraction method with an acid guanidinium thiocyanate-phenol-chloroform mixture (15). The total RNA was subjected to oligo(dT) affinity chromatography to obtain poly(A)+ RNA (15), and 10 µg of poly(A)+ RNA were size fractionated on 1.0% agarose formaldehyde gels and were transferred to nylon membranes. The RNA was cross-linked to the membranes by ultraviolet irradiation. After prehybridization, the membranes were hybridized overnight at 42°C in the presence of random-primed 32P-labeled COX-1 or COX-2 cDNA probes (Cayman Chemical, Ann Arbor, MI).
After hybridization, the blots were washed, and autoradiography was performed. Northern analysis was also performed for the housekeeping gene malate dehydrogenase (MDH) using a 0.5-kb random-primed [32P]cDNA probe. Previous studies in the rat have demonstrated that pulmonary MDH expression is not developmentally regulated (15). Levels of COX mRNA expression were normalized for the abundance of MDH mRNA to verify equivalent RNA stability and loading between samples.

Immunoblot analysis. Isolation of membrane-bound COX was carried out using methods modified from those of Van der Ouderaa et al. (27). Lung tissue was thawed and homogenized on ice in 50 mM KH2PO4 buffer containing 250 mM mannitol, 5 mM disodium-EDTA, 0.3 mM diethyldithiocarbamate, and 0.1 mM indomethacin (pH 7.8) using a Polytron blender (Brinkmann Instruments, Westbury, NY). The homogenate was centrifuged at 12,000 × g at 4°C for 10 min, and the supernatant was centrifuged at 110,000 × g at 4°C for 2 h. The pellet was resuspended in ice-cold 50 mM KH2PO4 buffer containing 0.1 mM diethyldithiocarbamate and 1% Tween 20 and was ultrasonically disrupted (Branson Ultrasonics, Chicago, IL). The protein content of the microsomal preparation was determined by the method of Bradford (5) using bovine serum albumin as the standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed with 7% acrylamide, and the proteins were electrophoretically transferred to nitrocellulose filters overnight (15). The nitrocellulose filters were blocked for 1 h in a buffer containing 100 mM NaCl, 20 mM NaH2PO4, and 80 mM Na2HPO4 (PBS; pH 7.5) with 1% Tween 20 and 5% dried milk and then were incubated with 1:1,000 COX-1 antiserum (Cayman Chemical) or 1:500 COX-2 antiserum (Oxford Biochemical Research, Oxford, MI) for 1 h at room temperature. After incubation with primary antisera, the nitrocellulose filters were washed in PBS with 1% Tween 20 and were incubated for 1 h with a 1:10,000 dilution of an anti-rabbit immunoglobulin antibody-horseradish peroxidase conjugate raised in donkey (Amersham). The filters were washed in PBS with Tween 20, and the bands for COX were visualized by chemiluminescence (ECL Western Blotting Analysis System, Amersham) and were quantitated by densitometry. Purified COX-1 and COX-2 proteins (Cayman Chemical) were used as positive controls. There was no cross-reactivity between the antisera for COX-1 and COX-2. In selected experiments, both tissue homogenates and microsomal preparations were examined.

Immunohistochemistry. The section preparation and immunohistochemical staining methods followed standard procedures (14). After thawing, lung tissue was fixed for 10–12 h in 10% buffered Formalin, processed through graded alcohols, and paraffin embedded. Deparaffinized sections (5-µm) were incubated for 2 h with a 1:50 primary antisera specific for COX-1 or COX-2 at room temperature after inhibition of endogenous peroxidases and trypsin digestion (1.0 mg/ml). Immunostaining was performed using standard strepavidin-biotin detection methodology with hematoxylin counterstaining. Preliminary studies revealed that freshly harvested tissue and specimens stored in liquid N2 had comparable histology and immunostaining. The latter approach made it possible to process and stain specimens from the different age groups simultaneously. Negative controls consisting of lung tissue incubated only with preimmune serum were run concurrently with each group of slides stained. Sheep placenta obtained after the onset of parturition, which expresses COX-2 protein (28), was evaluated simultaneously as a positive control for that isofrom. Lungs from a minimum of four sheep per age group were studied.

Statistical analysis. Analysis of variance (ANOVA) with Newman-Keuls post hoc testing was employed to compare mean values between more than two groups. Nonparametric ANOVA was used when indicated. Significance was accepted at the 0.05 level of probability. All results are expressed as means ± SE.

RESULTS

COX mRNA expression. The ontogeny of COX-1 mRNA expression in fetal and newborn lungs is shown in Fig. 1. A, single 2.8-kb mRNA species was identified. COX-1 mRNA was minimally detectable in fetal lung, and it increased progressively in abundance in NB1 and NB2. Quantitative densitometry from four experiments are given in B. COX-1 mRNA abundance corrected for MDH mRNA is expressed as a percentage of abundance in fetal lung. Values are means ± SE. *P < 0.05 vs. fetal lambs. †P < 0.05 vs. NB1.
independent experiments (Fig. 1B) concurred with these observations. Compared with fetal lung, steady-state levels of COX-1 mRNA were increased 6.2-fold in NB1 lung. COX-1 mRNA abundance increased further in NB2 lung, reaching levels that were 11.5-fold greater than those in fetal lung.

Developmental changes in COX-2 mRNA expression are shown in Fig. 2. A single 4.3-kb mRNA species was identified (Fig. 2A). COX-2 mRNA was minimally evident in fetal lung, and its abundance was greater in newborn compared with fetal lung. Comparable findings were obtained in four separate studies, which revealed 4.5-fold and 5.8-fold greater COX-2 mRNA abundance in NB1 and NB2 lungs, respectively, compared with fetal lung. There was no discernable difference in COX-2 mRNA abundance between NB1 and NB2.

COX protein expression. The ontogeny of COX-1 protein expression in fetal and newborn lungs is revealed in Fig. 3. In the representative immunoblot shown (Fig. 3A), COX-1 protein was detectable in fetal lung, and its abundance increased progressively from fetal to NB1 to NB2. Quantitative densitometry of four independent experiments concurred with these findings (Fig. 3B), revealing a 2.2-fold increase in COX-1 abundance from fetal to NB1 and an additional 2.2-fold increase from NB1 to NB2.

Immunoblot analysis for COX-2 protein in the fetal and newborn lungs is shown in Fig. 4. Despite detection of the purified COX-2 standard, the protein was not detected in lungs from any age group. Equivalent results were obtained with lung homogenate and microsomal preparation (data not shown), and similar findings were made in three separate experiments.

Cell specificity of COX expression. To determine the cell specificity of COX-1 protein expression in the developing lung, immunohistochemistry was performed us-
ing COX-1 antisera. In fetal lung, strong immunostaining for COX-1 was evident in vascular endothelial cells, but no COX-1 was detected in VSM at all levels of the vasculature (Fig. 5A). Similar observations were made in NB1 and NB2 (not shown). COX-1 protein was also detected in airway cells. In fetal airways, positive immunostaining was present in isolated epithelial cells and it was absent in airway smooth muscle (Fig. 5B). In the airways of NB1 lambs, immunostaining was evident in isolated epithelial cells and it was also present in airway smooth muscle (Fig. 5C). In the airways of NB2 lambs, a greater number of epithelial cells stained positively for COX-1 protein than in fetal or NB1 lung (Fig. 5D). However, immunostaining was not detectable in NB2 airway smooth muscle. In all age groups, COX-1 immunostaining was greatest in bronchioles and was less evident in central bronchi. There was minimal COX-1 identified in terminal and respiratory bronchioles, in alveolar wall cells, and in fibroblasts and connective tissue stroma. The findings for COX-1 immunostaining in the different age groups, which were confirmed in four independent experiments, are summarized in Table 1.

To determine if COX-2 protein is expressed in individual cell types in the developing lung, immunohistochemistry was performed using COX-2 antisera. Consistent with the observations made by immunoblot analysis, COX-2 immunostaining was not detected in four independent experiments in any pulmonary cell type at any age examined. However, immunostaining for COX-2 was readily apparent in the cotyledons of the sheep placenta (not shown), which served as a positive control for that isoform.

DISCUSSION

In the present study, we have delineated the ontogeny of COX gene expression in the lungs of fetal and newborn sheep. We have demonstrated that pulmonary COX expression is enhanced in the early newborn period compared with in late fetal life and that COX expression increases further during newborn life. The upregulation in COX expression may underlie the increase in the synthesis of PGI₂ and other vasodilatory PGs in whole lung during late gestation and in the newborn (18, 19).

We first evaluated steady-state levels of COX-1 mRNA in the developing lung and demonstrated that they increase progressively from the fetal to the NB1 and to the NB2 age groups. In parallel with the developmental increase in COX-1 mRNA, pulmonary COX-1 protein expression also rose progressively during the late fetal and early postnatal period. The present findings for COX-1 protein in the lung parenchyma are in agreement with the results we previously obtained specifically in fourth-generation intrapulmonary arteries (6).
The present observations also suggest that the abundance of pulmonary COX-1 is developmentally upregulated by either enhanced transcription of the gene or increases in COX-1 mRNA stability. To our knowledge, these are the only studies demonstrating developmental regulation of COX-1 gene expression.

Previous studies in cultured endothelial cells and fibroblasts have demonstrated that COX-1 expression is regulated by a variety of factors. There is evidence of COX-1 upregulation by phorbol 12-myristate 13-acetate, transforming growth factor-β, and interleukin-1β (12, 23, 29), and downregulation has been demonstrated in response to acidic fibroblast growth factor-1 (11). Because transforming growth factor-β and acidic fibroblast growth factor-1 are produced by the developing lung mesenchyme (25), they may be involved in the ontogenic regulation of pulmonary COX-1 expression.

In addition to potential regulation by growth factors, there is evidence that COX-1 gene expression may also be under hormonal control. In aortic vascular endothelium and smooth muscle, physiological concentrations of estradiol-17β cause marked increases in PGI2 and PGF2α production (7, 24). The effect of estradiol is evident after 2–3 days of exposure, suggesting that it may involve an increase in COX expression (7, 24). In addition, fetal plasma estrogen levels increase dramatically with advancing gestation due to increased placental production of the hormone (22), and estrogen has been implicated in maturational changes in pulmonary endothelial cell morphology in the late fetus (13). As such, we postulate that estrogen may play a role in pulmonary COX-1 upregulation during late fetal life. In contrast to estrogen-mediated upregulation, studies in cultured VSM cells indicate that glucocorticoids attenuate COX-1 expression (2). Because there is a dramatic fall in plasma cortisol levels postnatally (4, 10), we further postulate that the release of this inhibitory effect may play a role in the continued upregulation in pulmonary COX-1 expression in the newborn.

In the present investigation, we also examined COX-2 mRNA abundance in the developing lung and demonstrated that it also increases from the fetal to the NB1 age group. However, in contrast to COX-1, there was no difference in COX-2 mRNA abundance between the NB1 and NB2 groups. In addition, COX-2 protein was not detected in either fetal or newborn lung by either immunoblot analysis or immunohistochemistry. Because COX-2 protein was readily detectable in the cotyledons of the sheep placenta by immunohistochemistry, the lack of detection of the protein in pulmonary cells is not due to an inability to detect ovine COX-2. Instead, it more likely reflects the presence of minimal, if any, COX-2 protein in the fetal and newborn lungs. The detection of COX-2 mRNA in the absence of discernible COX-2 protein suggests that either the gene is transcribed but not translated in the developing lung or the COX-2 protein is highly unstable. It is now evident that there is differential expression of the two major COX isoforms in the developing lung.

The cell specificity of COX-1 expression was evaluated by immunohistochemical analysis. In the intrapulmonary arteries at all levels and in all age groups, strong immunostaining for COX-1 was evident in endothelial cells and no COX-1 was detected in VSM. These observations are consistent with our previous finding that PGI2 production in fourth-generation intrapulmonary arteries from fetal and newborn sheep is primarily derived from the endothelium (6). The other major site of COX-1 localization was in bronchial and bronchiolar epithelium and smooth muscle. In the epithelial cell layer, positive immunostaining was present in occasional cells in the fetal lung and in NB1 lung. In contrast, a larger number of epithelial cells stained positively for COX-1 protein in NB2 lung. This may reflect a developmental increase in the expression of the enzyme in individual epithelial cells, or it may reflect an age-related difference in the distribution of specific epithelial cell subtypes. In the airway smooth muscle, COX-1 immunostaining was absent in fetal lung, it was present in the lungs of NB1 lambs, and it was undetectable in NB2 lung. This observation suggests that there is developmental regulation of COX-1 expression in the airway smooth muscle, maximizing the abundance of the enzyme in the early postnatal period.

Previous studies of pulmonary COX expression have been limited primarily to evaluations of whole lung. Experiments in mature rat lung have shown that the abundance of COX-1 mRNA is greater than that of COX-2 mRNA, whereas studies of mature human lung have shown the opposite (9, 16). However, the abundance of COX-1 or COX-2 protein in normal lung parenchyma has not been previously evaluated in any species. Studies of adult human bronchial biopsy specimens have revealed positive immunostaining for both isoforms in the epithelial cell layer (8), but the present studies are the first to evaluate the cell specificity of COX isoform expression in the lung parenchyma.

There are numerous physiological implications of the cellular distribution and maturational changes in COX expression elucidated in the present investigation. Within the vasculature, the developmental increase in COX-1 expression and the resulting increase in PGI2 synthesis (6) may play a role in the normal fall in pulmonary vascular resistance occurring immediately after birth and also more gradually in the early postnatal period (21). In addition, because PGI2 and PGF2α inhibit DNA synthesis and cell proliferation in VSM cells (17), COX-1 upregulation may also be involved in the decline in pulmonary arterial muscularity that

### Table 1. Cyclooxygenase-1 immunostaining in developing lung

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<th>Fetal</th>
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<td>Endothelium</td>
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<td>Vascular smooth muscle</td>
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<td>Airway epithelium</td>
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<td>Airway smooth muscle</td>
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NB1, 1-wk-old lambs; NB2, 1- to 4-mo-old lambs; 0, absent; +, rare; ++, minimal; +++, moderate; ++++, intense.
occurs from fetal to postnatal life (21). In the airway, COX-1 may serve numerous functions in the developing lung because there is evidence that epithelium-derived PGs modulate airway smooth muscle tone, ion transport, mucus secretion, and sympathetic neurotransmission (20). Finally, because PGs regulate surfactant apoprotein expression, phosphatidylcholine production, and alveolar luminal and lamellar body volume density in the fetal lung (1, 3), the developmental upregulation in pulmonary COX-1 expression may also be necessary for optimal alveolar function in the newborn. Future studies of the mechanisms underlying the ontogenic changes in pulmonary COX expression may increase our understanding of the various roles of PGs in pulmonary vascular, airway, and parenchymal development and function.

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Present addresses: T. S. Brannon, Neonatology Dept., Baylor Univ. Medical Center, 3500 Gaston Ave., Dallas, TX 75246; A. N. MacRitchie, Dept. of Pediatrics, Univ. of Utah School of Medicine, 50 N. Medical Drive, Salt Lake City, UT 84132.

Address for reprints requests: P. W. Shaul, Dept. of Pediatrics, Univ. of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235-9063

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