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Chronic *In Utero* Cyclooxygenase Inhibition Alters PGE2-Regulated Ductus Arteriosus Contractile Pathways and Prevents Postnatal Closure

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ABSTRACT: Although prostaglandin E2 (PGE2) vasodilates the ductus arteriosus, tocolysis with cyclooxygenase (COX) inhibitors delays postnatal ductus arteriosus closure. We used fetal mice and sheep to determine whether PGE2 has a role in the development of ductus contractility that is distinct from its function as a vasodilator. Prolonged exposure of fetal ductus to PGE2 *in vitro* increased the expression of CaL- and K^+-channel genes (CaLα1c, CaLβ2, Kir6.1, and Kv1.5, which regulate oxygen-induced constriction) without affecting the genes that regulate Rho-kinase–mediated calcium sensitization. Conversely, chronic exposure to COX inhibitors *in utero* decreased expression of CaL- and K^+-channel genes, without affecting Rho-kinase–associated genes. Chronic COX inhibition *in utero* decreased the ductus' *in vitro* contractile response to stimuli that use CaL- and K^+-channels (like O_2 and K^+), whereas the response to stimuli that act through Rho-kinase–mediated pathways (like U46619) was not significantly affected. Phosphodiesterase expression, which decreases the ductus' sensitivity to cAMP- or cGMP-dependent vasodilators, was increased by PGE2 exposure and decreased by COX inhibition, respectively. These studies identify potential downstream effectors of a PGE2-mediated, developmental program, regulating oxygen-induced ductus closure. Alterations in these effectors may explain the increased risk of patent ductus arteriosus (PDA) after *in utero* COX inhibition. **(*Pediatr Res* 66: 155–161, 2009)**

Prostaglandins [especially prostaglandin E2 (PGE2)] vasodilate the fetal and neonatal ductus arteriosus. When nonselective inhibitors of cyclooxygenase (COX)-1 and -2, the rate-limiting enzymes for prostaglandin synthesis, are given to pregnant women to treat preterm labor, the ductus arteriosus constricts *in utero* (1). Surprisingly, some preterm infants, who are delivered after *in utero* exposure to indomethacin, have an increased, rather than decreased, incidence of patent ductus arteriosus (PDA) in the newborn period. The PDA in these infants fails to close with postnatal indomethacin treatment (2,3). Delayed closure of the newborn ductus arteriosus increases an infant's risk for pulmonary hemorrhage, necrotizing enterocolitis and chronic lung disease (4).

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Delayed closure of the newborn ductus also occurs in mice and sheep that have been exposed to nonselective COX inhibitors during the last 25% of gestation (5–7) and in mice lacking both COX genes (6–8). The basis for this paradoxical response is not entirely clear. Previous studies have suggested that *in utero* COX inhibition may contribute to delayed closure by increasing NO (5,9) or decreasing hyaluronic acid production in the ductus (10).

Postnatal closure of the ductus arteriosus requires the presence of specific components of ductus contractility: smooth muscle calcium channels, potassium channels (11–15), Rho-kinase–related calcium sensitizing pathways (11,16–18), mature myosin isoforms (19), and cytoskeletal proteins (20). Events or drugs that interfere with these pathways lead to delayed postnatal ductus closure (16,17,21–23). We speculated that prostaglandins may regulate the development of one or more of these contractile pathways and hypothesized that inhibition of COX activity *in utero* may alter the contractile apparatus' development, leading to a persistent PDA after birth.

In the following study, we examined the effects of prostaglandin exposure and inhibition on fetal mice and sheep ductus. We found that in addition to its known vasodilator effects, PGE2 plays an important role in the expression of specific pathways that are necessary for the ductus' oxygen-induced closure after delivery.

MATERIALS AND METHODS

All protocols were approved by the Vanderbilt University and the University of California San Francisco Institutional Animal Care and Use Committees.

Mouse studies. Wild-type female CD-1 mice (7- to 8-wk old; Charles River, Raleigh, NC) were bred to produce timed pregnancies (d 1, presence of vaginal plug; term, 19 d).

Pregnant females received either no drugs (control) or a combination of a selective COX-1 inhibitor (SC560, 30 mg/kg/dose, 0.2 mL gavage) and a selective COX-2 inhibitor (SC236, 15 mg/kg/dose, 0.2 mL gavage; Cayman Chemical Co., Ann Arbor, MI). Both the drugs cross the placenta (24). Pregnant mice received the inhibitors either on a single occasion (d 19 of gestation) or chronically from d 15 to d 19: SC560 (30 mg/kg/dose, twice daily) and SC236 (15 mg/kg/dose, every other day). Both fetuses and newborns were delivered by cesarian section, 4 h after the last drug dosage on d

Abbreviations: COX, cyclooxygenase; **PGE2,** prostaglandin E2; **PDA,** patent ductus arteriosus

19. Fetuses were euthanized at delivery. Newborn pups were placed in prewarmed cages in fraction of inspired oxygen (FiO_2), 0.8–1.0] to accelerate ductus closure, and the tissues were harvested 4 h later. Tissues were prepared for either RNA analysis or histology as previously described (6,25).

Determination of vessel caliber. Serial sections of fetal and newborn mouse thoraces were examined by an observer, who was blinded to the treatment group (R.I.C.). The inner diameters of the ductus arteriosus lumen (DA) and the transverse aortic arch lumen (AO) were determined at their narrowest points. DA diameter was expressed as a ratio of the ductus diameter to the diameter of the transverse aortic arch (DA/AO ratio) (6).

Mouse pressure myography studies. Fetal mouse ductus were isolated and mounted in 4-mL chambers as previously described (26). Distending pressure (in mmHg) was generated by a column of deoxygenated Krebs buffer. Nonrecirculating, deoxygenated Krebs buffer (36.5–37.5°C) perfused the chambers at 6 mL/min. The lumen diameter was measured, at the point of maximum constriction, using an inverted microscope and a video image capture system; during full vessel closure, measurement was obtained at the optically dense regions of the internal elastic lamina.

Vessels were initially pressurized to 20 mm Hg (in 5 mm Hg increments). The vessels were then exposed to 50 mM K^+- deoxygenated Krebs buffer (with KCl substituted for NaCl) for 3–5 min to stimulate ductus contractility. After this, *N(G)-nitro-l-arginine methyl ester (l-NAME)* (10^{-4} M; Cayman Chemical Co.) and indomethacin (5.6×10^{-5} M; Sigma-Aldrich Chemical Co., St. Louis, MO) were added to the perfusion solution, and the distending pressure was increased to 25 mm Hg for the duration of the experiment. This eliminated any differences between the groups in ongoing NO and prostaglandin production *in vitro* (9,27). After a 60-min equilibration period with L-NAME and indomethacin at 25 mm Hg, the vessels were exposed to 50 mM K^+- deoxygenated Krebs buffer for 3–5 min to stimulate ductus contractility.

Vessels from littermates were then exposed to one of the three study protocols: 1) increasing oxygen concentrations (0, 2, 5, 12, and 21% O_2; 5% CO_2, balance N_2), 2) increasing K^+ concentrations in deoxygenated (95% N_2, 5% CO_2) Krebs buffer, or 3) increasing concentrations of U-46619, a thromboxane receptor agonist (Cayman Chemical Co.) in deoxygenated Krebs buffer. Each change in concentration was maintained until a stable new diameter was established (10–30 min). At the completion of each study protocol, vessels were exposed to papaverine (10^{-4} M; Sigma-Aldrich Chemical Co.) to determine the vessel caliber at maximal relaxation.

Sheep studies:* in vitro *PGE2 incubation. The small size of the mouse ductus presented a great challenge in attempting to acquire sufficient tissue to perform the required number of biochemical analyses from individual ductus incubation studies. Therefore, we used the much larger fetal sheep ductus to examine the effects of PGE2 on isolated ductus arteriosus RNA expression. Immature sheep fetuses (mixed western breed: 103- ± 2-d gestation; term, 145 d) were delivered by cesarian section and anesthetized with i.v. ketamine HCl (30 mg/kg) before rapid exsanguination. The ductus arteriosus was divided into 1-mm thick rings (2 rings per animal), which were stretched to 5.0 mm in separate 20 mL organ chambers. Throughout the 23-h experiment, the rings were perfused at 10 mL/h with nonrecirculating modified Krebs buffer (pH, 7.4; 37.5°C) containing indomethacin (5×10^{-6} M; to inhibit endogenous prostaglandin production; 28). Because of the tissue thickness, the chambers were continuously aerated with 30% O_2, 5% CO_2, and balance N_2. After a 3-h equilibration period, 10^{-8} M PGE2 [similar to fetal plasma concentrations at late gestation (29)] was added to the indomethacin-containing Krebs buffer perfusing one of the rings. The other ring continued to be perfused with the indomethacin-containing buffer without PGE2. Both rings were incubated for an additional 20 h before being snap frozen in liquid nitrogen for RNA analysis.

Preparation of total RNA, reverse transcription, and quantitative polymerase chain reaction. Total RNA was isolated from the mouse and sheep ductus, and the TaqMan Universal PCR master mix of PE Applied Biosystems (Foster City, CA) was used to quantify the expression of the genes as described elsewhere (25). Taqman probes were designed using the Primer Express program and labeled with fluorophores FAM (6-caboxy-fluorescein) and TAMRA (6 carboxy-tetramethyl-rhodamine) as reporter and quencher dyes, respectively. An ABI PRISM 7700 sequence detection system was used to determine the cycle threshold (CT). Malate dehydrogenase (MDH) was used as an internal control to normalize the data (11).

Statistics. Statistical analyses of unpaired and paired data were performed by the appropriate *t* test and by analysis of variance. Scheffe's test was used for *post hoc* analysis. Values are expressed as mean ± SD. Drug concentrations refer to their final molar concentration in the bath.

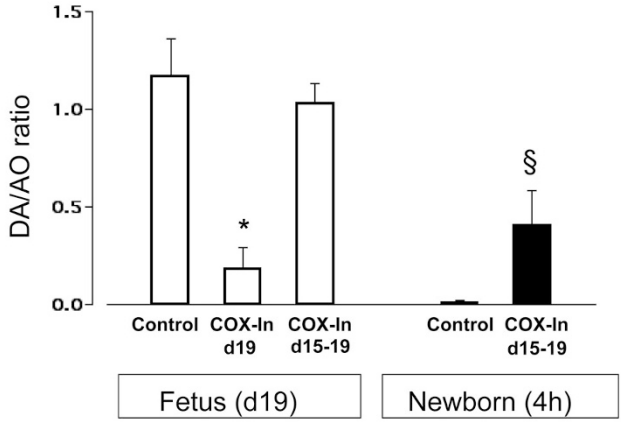

Figure 1. Response of the fetal and newborn ductus arteriosus to acute (COX-In d 19) and chronic (COX-In d 15–19) COX inhibition *in vivo*. DA, ductus and AO, transverse aorta lumen diameters. Acute COX inhibition constricted the fetal ductus at term gestation ($n = 17$ fetuses; 7 litters) compared with control fetuses ($n = 9$; 6 litters). Chronic COX inhibition ($n = 17$; 6 litters) did not constrict the fetal ductus. Ductus closure in 4-h-old newborn pups (chronically exposed to COX inhibitors *in utero* ($n = 26$; 11 litters) was significantly reduced (resulting in a PDA) compared with control newborns ($n = 12$; 4 litters). $*p < 0.05$ compared with fetal controls; $§p < 0.05$ compared with newborn control.

RESULTS

A one-time exposure (on d 19 gestation) to the two selective COX inhibitors (SC560 plus SC236) produced marked constriction *in vivo* of the fetal mouse ductus arteriosus 4 h after administering the drugs (Fig. 1).

We wanted to examine the effects of chronic COX inhibition, which were independent of the initial acute constriction. Therefore, we started the chronic treatment (SC560 plus SC236) at a point in gestation (d 15) when the inhibitors had no acute contractile effect on the fetal mouse ductus (6) and continued the treatment regimen to d 19 of gestation. The ductus of fetuses that were exposed to the inhibitors (SC560 plus SC236) from d 15 to 19 of gestation did not constrict *in utero*, and they had the same degree of patency as control fetuses on d 16, 17, 18 (data not shown), and 19 (Fig. 1). Chronic exposure to the COX inhibitors *in utero* resulted in incomplete closure of the newborn ductus after birth (Fig. 1).

We next examined the effects of chronic COX inhibition on the ductus contractility using isolated pressurized ductus from control fetal mice and from mice exposed to the combined COX inhibitors (from d 15 to 19) *in utero*. Ductus smooth muscle contractility is determined by developmentally regulated pathways that control both the concentration of intracellular calcium and the sensitivity of the contractile proteins to changes in intracellular calcium. Intracellular calcium concentrations are determined by calcium influx through L-type calcium channels; similarly, Rho or Rho-kinase activity plays an important role in regulating calcium sensitization during the sustained phase of ductus contraction (11–17). Stimuli like oxygen and elevated K^+ affect ductus contractility primarily by increasing calcium entry through voltage-dependent CaL channels (11,15,16,18,30,31). However, most agonists that alter vascular tone affect both aspects of ductus contractility.

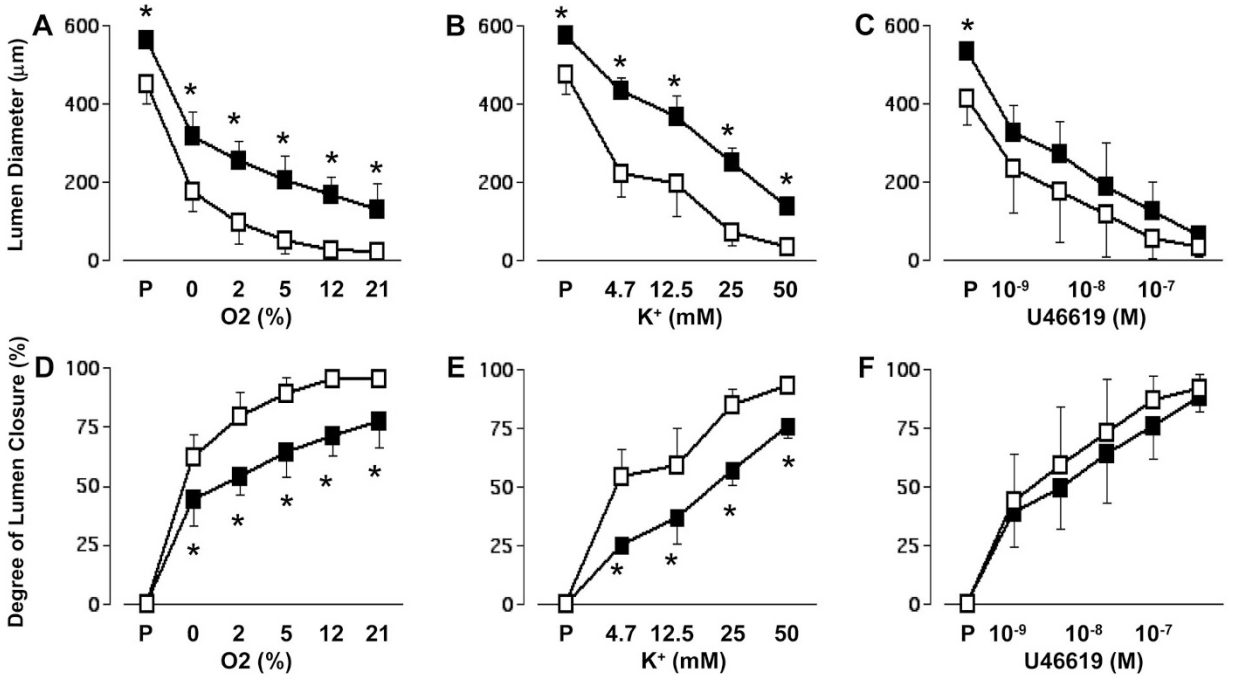

Figure 2. Contractile response of isolated ductus from19-d-old fetal mice treated with or without chronic COX inhibitors. Ductus were incubated in deoxygenated buffer containing L-NAME and indomethacin and exposed to increasing oxygen (*A*, *D*), K^+ (*B*, *E*), or U-46619 (*C*, *F*) concentrations. P, maximal relaxation with papaverine. Steady-state oxygen tensions were 42.5 ± 2, 57.3 ± 2.1, 75.1 ± 1.8, 118.8 ± 1.7, and 181.0 ± 5.6 mm Hg when the buffer was bubbled with 0, 2, 5, 12, and 21% oxygen, respectively. The K^+ and U-46619 dose-response experiments were performed in 0% oxygen. Degree of lumen closure, 1-(measured diameter/diameter after papaverine relaxation). *Open squares*, controls; *solid squares*, chronic COX inhibition. Animal numbers: O_2 (*A*, *D*): control, 6; COX inhibition, 6; K^+ (*B*, *E*): control, 9; COX inhibition, 5; U-46619 (*C*, *F*): control, 7; COX inhibition, 6. *$p < 0.05$ compared with control.

For example, in other vessels, U46619, a thromboxane-mimetic, seems to affect both calcium entry and Rho-kinase–mediated calcium sensitization (32,33).

When incubated at the same pressures, under baseline conditions (buffer bubbled with 0% O_2), ductus obtained from term fetuses that were chronically exposed to COX inhibitors *in utero* had significantly larger lumina than control ductus (lumen diameter: COX-inhibitor exposed, 360 ± 70 μm, $n = 17$; control, 223 ± 74 μm, $n = 22$, $p < 0.05$; Fig. 2). Similarly, when the ductus were maximally relaxed by papaverine, the lumina of the chronically COX-inhibited ductus were significantly larger than those of the control ductus (lumen diameter: COX-inhibitor exposed, 559 ± 31 μm; control, 449 ± 61 μm, $p < 0.05$). Ductus from both the groups constricted with increasing concentrations of O_2, K^+, and U46619 (Fig. 2). However, O_2 and K^+ produced a lesser degree of constriction in ductus that were chronically exposed to COX inhibitors *in utero* than they did in the control ductus (Fig. 2). On the other hand, the thromboxane-mimetic U46619 constricted both the groups of ductus to a similar degree (Fig. 2).

In the control ductus, peak concentrations of O_2 (21%), K^+ (50 mM), and U46619 (10^{-7} M) produced the same maximal degree of constriction (% closure: 95 ± 2%, 93 ± 4%, and 92 ± 6%, respectively). In contrast, after chronic COX inhibition, U46619 (10^{-7} M) produced a significantly greater degree of ductus closure (% closure, 88 ± 5) than either 21% O_2 (% closure, 77 ± 11; $p < 0.05$) or 50 mM K^+ (% closure, 77 ± 5; $p < 0.05$; Fig. 2).

We examined the ductus obtained from 19-d gestation mice fetuses to determine whether chronic *in utero* COX inhibition altered the developmental expression of genes known to affect ductus contractility. Chronic treatment with SC560 plus SC236 altered the expression of genes that regulate CaL and K^+ channels (CaLα1c, CaLβ2, CaLβ3, Kir6.1, and Kv1.5), actin or myosin interactions (caldesmon, myocardin, and tropomyosin), matrix production (tenascin C and TGFβ), and vasodilator activity (hemeoxygenase-1, PDE 1A, PDE 3A, and VEGF; Table 1).

As chronic COX inhibition decreased the expression of genes that facilitate ductus closure late in gestation, we hypothesized that prolonged PGE2 exposure (early in gestation) may have the opposite effect and promote their developmental expression. To test this hypothesis, we used isolated ductus from immature fetal sheep, mounted in organ culture baths. The isolated ductus were incubated in media containing indomethacin to inhibit the increase in tissue prostaglandin production that follows ductus dissection and manipulation (28). Parallel rings from the same ductus were incubated either with or without PGE2 (10^{-8} M) for 20 h. *In vitro* PGE2 exposure increased the expression of genes that regulate CaL channels (CaLα1c and CaLβ2), K+ channels (Kir6.1 and Kv1.5), and phosphodiesterase activity (PDE 1A and PDE 3A; Table 2). This is consistent with PGE2 having a direct, positive effect on the *in utero* expression of these genes. On the other hand, PGE2 exposure did not affect the expression of genes that regulate actin or myosin interactions (caldesmon, myocardin,

Table 1. *Real-time PCR measurements of genes involved with ductus closure and remodeling: ductus obtained from 19-d gestation fetuses*

	δCT (MDH-gene)				
	Control		COX inhibition		
Gene	Mean	SD	Mean	SD	*p*
Ca^{++} L-channels					
CaLα1c	−2.33	±0.52	−3.09	±0.31	*
CaLβ2	−2.52	±0.34	−3.26	±0.53	*
CaLβ3	−2.71	±0.41	−3.48	±0.52	*
K^+ channels					
BKCa	−9.34	±0.93	−7.99	±1.31	
BKCaβ1	−3.70	±0.52	−4.30	±0.36	*
Kir6.1	−2.44	±0.72	−3.52	±0.87	*
SUR2	−3.21	±0.43	−3.63	±0.61	
Kv1.2	−4.68	±0.53	−4.99	±0.76	
Kv1.5	−3.11	±0.36	−3.99	±0.48	*
Kv2.1	−3.53	±0.83	−3.69	±0.71	
Kv9.3	−1.47	±0.41	−2.18	±0.53	
Kvβ1.2	−3.96	±0.63	−4.36	±0.36	
Kvβ1.3	−1.96	±0.36	−2.29	±0.27	
Contractility					
Caldesmon	−0.03	±0.21	−0.76	±0.41	*
Calponin	−1.58	±0.55	−2.01	±0.49	
Myocardin	−3.17	±0.38	−3.97	±0.31	*
MYH1	0.41	±0.68	0.05	±0.81	
MYH2	−1.97	±0.93	−2.34	±0.48	
RhoA	−0.94	±0.74	−0.80	±0.44	
RhoB	−2.36	±0.27	−2.87	±0.51	
ROCK1	−4.02	±0.81	−4.27	±0.64	
NCX1	−4.29	±0.95	−4.92	±0.60	
PHLBN	−2.88	±0.68	−2.98	±0.51	
SERCA3	−6.58	±0.42	−6.64	±0.23	
Tropomyosin	−3.00	±0.39	−3.80	±0.54	*
Endothelin					
ECE1	−1.18	±0.68	−1.86	±0.26	
ET1	−4.43	±0.95	−4.55	±0.62	
ETAr	−5.99	±1.52	−6.27	±1.67	
ETBr	−2.66	±0.79	−2.77	±0.37	
Vasodilation					
COX1	−5.34	±0.37	−5.29	±0.69	
COX2	−5.53	±0.58	−4.75	±0.89	
eNOS	−8.86	±0.91	−9.68	±1.43	
EP2	−8.16	±1.00	−7.76	±0.36	
EP3	−5.46	±0.27	−5.63	±0.62	
EP4	−0.20	±1.20	−0.71	±0.87	
HO1	−3.52	±0.31	−1.48	±0.97	*
HO2	−2.51	±0.42	−2.51	±0.28	
PDE1A	−5.22	±0.40	−5.89	±0.19	*
PDE1B	−2.52	±1.03	−2.83	±0.48	
PDE3A	−2.38	±0.28	−3.41	±0.54	*
PDE3B	−2.80	±0.52	−3.05	±0.13	
PDE4D	−6.22	±0.69	−6.51	±0.30	
PDE5A	−2.75	±0.36	−3.26	±0.55	
Remodeling					
Ang-1	−4.44	±0.54	−4.76	±0.36	
Ang-2	−5.91	±0.47	−5.82	±0.74	
ATIIR-1	−4.96	±0.72	−5.08	±0.45	
HAS2	−4.69	±0.45	−4.99	±0.19	
HIF1α	−3.75	±0.87	−3.99	±0.25	
HIF2α	−2.71	±0.66	−2.76	±0.44	
IFNγ	−3.07	±0.54	−3.18	±0.25	
IL6	−3.66	±0.37	−4.06	±0.26	
PDGF-B	−5.06	±0.45	−5.19	±0.54	
Tenascin-C	−1.60	±0.30	−2.13	±0.36	*
TFAP2b	−4.81	±1.34	−5.36	±1.05	
TGFβ1	−4.17	±0.37	−4.67	±0.29	*
TGFβ3	−2.71	±0.23	−3.43	±0.21	*
TNFα	−2.38	±1.00	−2.69	±0.29	
TRAF1	−5.96	±1.05	−6.31	±0.53	
VEGF	−3.57	±0.50	−2.78	±0.42	*

δCT (MDH-gene), difference in cycle threshold between the expression of the housekeeping gene Malate dehydrogenase (MDH) and the gene of interest. Each unit of δCT (MDH-gene) represents a 2-fold increase in a gene's mRNA. The more negative the δCT (MDH-gene), the fewer the number of starting copies of a gene (mRNA). Number of separate litters used: control (d 19), 9; COX-inhibition (d 15–19), 7. From each litter of mice, we obtained and pooled between 10 and 12 ductus.

* $p < 0.05$.

MYH, myosin heavy chain; ROCK1, Rho kinase 1; NCX1, Na^+/Ca^{++} exchanger; PHLBN, phospholamban; SERCA, sarcoplasmic reticulum Ca^{++}-ATPase; ECE, endothelin converting enzyme; ET, endothelin; ETAr, endothelin receptor A; eNOS, endothelial nitric oxide synthase; EP, prostaglandin E receptor; HO, heme oxygenase; PDE, phosphodiesterase; ang, angiopoietin; ATIIR-1, angiotensin II receptor; HAS, hyaluronic acid synthase; HIF, hypoxia inducible factor.

and tropomyosin) or matrix production (tenascin C and TGFβ). We also examined the expression of several genes that were not affected by chronic COX inhibition *in utero* (large conductance BKCa channel, endothelin-1, platelet derived growth factor-B chain, Rho A, Rho B, and ROCK-1). As might be anticipated, PGE2 exposure did not affect their expression.

DISCUSSION

Although acute COX inhibition in near-term mice led to fetal ductus constriction (Fig. 1), prolonged COX inhibition during the last 25% of gestation led to an impaired contractile response and an incomplete closure in the newborn (Fig. 1). The effects of prolonged COX inhibition *in utero* are similar to the effects observed after deletion of both COX genes *in utero* (6–8). Similar findings have also been observed in larger species [*e.g.* humans (2) and sheep (5)] after indomethacin exposure *in utero*. In larger species, however, the loss of ductus contractility, after COX inhibition, seems to be due to ischemia of the ductus wall secondary to *in utero* ductus constriction and loss of vasa vasorum blood flow to the muscle media (5). Ductus wall ischemia does not seem to be the explanation for our findings in mice. The mouse ductus is so thin that luminal flow sustains all of its nutrient needs. As a result, the mouse ductus has no need for muscle media vasa vasorum (34,35). In our study, COX inhibition was started on d 15, when COX inhibitors do not contract the mouse ductus (6). Chronic COX inhibition (between d 15 and 19) does not affect the *in utero* dimensions of the ductus lumen (6) (Fig. 1). Consistent with these findings, surrogate markers of hypoxia, like HIF1α and HIF2α, whose expression increases during ductus hypoxia (36), are unaffected by chronic COX inhibition *in utero* (Table 1).

Table 2. *Real-time PCR measurements in sheep ductus*

	δCT (MDH-gene)				
	Control		PGE2 incubation		
Gene	Mean	SD	Mean	SD	*p*
Genes affected by chronic COX inhibition in utero					
CaLα1c	−0.24	±0.49	0.59	±0.54	*
CaLβ2	−2.31	±1.01	−1.33	±0.83	*
CaLβ3	−1.28	±0.38	−1.12	±0.31	
Caldesmon	1.12	±0.78	1.11	±0.63	
BKCaβ1	−4.40	±0.26	−4.06	±0.44	
HO1	−2.08	±0.38	−1.59	±0.47	*
Kir6.1	−5.21	±1.00	−4.31	±0.92	*
Kv1.5	−1.99	±1.40	−0.96	±0.87	*
Myocardin	−3.40	±0.47	−3.29	±0.77	
PDE1A	−5.79	±1.02	−4.72	±0.95	*
PDE3A	−2.58	±0.66	−1.62	±0.96	*
Tenascin-C	1.00	±0.81	0.64	±1.18	
TGFβ1	−0.61	±0.34	−0.35	±0.31	
TGFβ3	−4.09	±0.76	−3.76	±0.64	
Tropomyosin	−0.25	±1.01	−0.50	±0.58	
VEGF	0.31	±0.72	1.61	±0.86	*
Genes un-affected by chronic COX inhibition in utero					
BKCa	−7.13	±0.98	−7.27	±0.55	
COX2	0.77	±0.96	1.06	±1.36	
EP2	−1.86	±0.43	−1.96	±0.85	
EP3	−5.44	±1.03	−4.99	±0.98	
ET1	−4.52	±1.33	−4.74	±1.22	
PDGF-B	−3.95	±0.83	−3.91	±0.71	
RhoA	1.87	±0.41	2.07	±0.39	
RhoB	2.42	±0.61	2.80	±0.78	
ROCK1	−2.82	±0.75	−2.49	±0.59	
SUR2	−1.04	±0.41	−0.78	±0.55	

Rings from the same sheep ductus were incubated with or without PGE2 (10^{-8} M) for 20 h. We examined the expression of genes that were altered by chronic COX inhibition *in utero* in Table 1. We also examined the expression of several of the genes that were not affected by chronic COX inhibition (see Table 1). Number of separate animals used, 7.

* $p < 0.05$.

We hypothesized that PGE2 may play a unique role in the development of ductus contractility that is distinct from its function as a vasodilator. Previous studies found that gene deletion of the PGE2 receptor EP4 produced a persistent PDA phenotype in newborn mice (37) that was similar to what occurs after COX gene deletion or chronic *in utero* COX inhibition. Our findings are consistent with PGE2 having a direct effect on ductus contractility by increasing the developmental expression of genes that regulate calcium availability. The developmentally regulated, oxygen-induced constriction of the ductus seems to be due in large part to the increased expression of CaL channels (11,15) and oxygen-sensitive K^+ channels (11,14,15). In our experiments, *in vitro* exposure to PGE2 increased the expression of CaL channel (CaLα1c and CaLβ2) and K^+ channel (Kir6.1 and Kv1.5) genes without affecting genes that regulate Rho-kinase–mediated calcium sensitization (Table 2). Conversely, inhibition of prostaglandin production, by chronic *in utero* COX inhibition, decreased the expression of the same CaL and K^+ channel genes, without affecting Rho-kinase–associated genes (Table 1).

The effects of chronic *in utero* COX inhibition on gene expression are consistent with the effects we found on ductus contractility. Contractile stimuli, which act primarily on ductus K^+ and CaL channels [like O_2 and K^+ (11–14,17,32)], have a diminished contractile effect, whereas U46619, which has been shown to affect both calcium entry and Rho-kinase–mediated calcium sensitization in other vascular tissues (32,33), constricts control and COX-inhibited ductus to a similar degree (Fig. 2).

Several other genes were also affected by chronic COX inhibition and may contribute to the delayed ductus closure after birth (Table 1). Previous studies have shown that a developmental increase in phosphodiesterase (PDE 1A and PDE 3A) expression and activity decreases the sensitivity of the late gestation ductus to vasodilators, like PGE2 (38). We found that the phosphodiesterase genes were downregulated by chronic COX inhibition *in utero* (Table 1) and upregulated by PGE2 exposure *in vitro* (Table 2). This is consistent with endogenous PGE2 having a direct, positive effect on PDE 1A and PDE 3A expression *in utero*.

Conversely, the effects of chronic COX inhibition on other genes (like caldesmon, myocardin, tropomyosin, tenascin C, TGFβ, hemeoxygenase-1, and VEGF) may not be due to the direct effects of PGE2 on the ductus. For example, genes that regulate actin or myosin interactions (caldesmon, myocardin, and tropomyosin) and matrix production (tenascin C and TGFβ; which were decreased by chronic COX inhibition *in utero*) were not affected by incubation with PGE2 *in vitro*; and the hemeoxygenase-1 and VEGF genes, which were increased by chronic COX inhibition *in utero*, were not decreased by

incubation with PGE2 *in vitro*. The effects of chronic COX inhibition on these genes may be due to the distal effects of COX inhibition on other maternal, placental, or fetal organs. The fact that PGE2 incubation did not produce the opposite effect as chronic COX inhibition for this set of genes may also be due to differences in the experimental design (*in vitro versus in vivo*) or the species used (sheep *versus* mice).

Increased NO production has been implicated in delayed ductus closure after prolonged COX inhibition *in utero* (9). Functional coupling of COX and NOS systems is a well-recognized phenomenon (5,9). In the mouse ductus arteriosus, eNOS is the predominant isoform for NO production (35). We did not observe a change in ductus eNOS expression after chronic COX inhibition; however, we did observe changes in PDE1A and VEGF expression, which could contribute to increased NO activity or production (Table 1). Our contractility experiments were not designed to examine the effects of chronic COX inhibition on the production of NO or other vasodilators. We were primarily interested in examining the effects of COX inhibition on the contractile apparatus. Therefore, we specifically incubated the isolated ductus with inhibitors of prostaglandin and NO production to eliminate any differences between the groups in *in vitro* prostaglandin and NO production.

Our studies examine the chronic effects of COX inhibition and PGE2 stimulation on ductus gene expression and contractility. They do not identify the mechanism(s) by which PGE2 is able to affect these changes. Previously, cAMP has been shown to regulate K^+ channel activity and expression in excitable cells (39); a similar mechanism may mediate the effects of PGE2 on CaL and K^+ channel gene expression in these experiments. Future studies, designed to measure protein expression and intracellular ion fluxes, will be necessary to identify the exact pathways that have been altered by our pharmacologic manipulations.

In summary, we speculate that the paradoxical effects of acute and chronic COX inhibition are consistent with the existence of two complementary roles for PGE2 during ductus development: one that promotes the expression of pathways necessary for its oxygen-induced closure after delivery, and second that maintains ductus patency for fetal wellbeing. A better understanding of these two processes will be important for the development of new strategies to treat preterm labor without affecting fetal vascular development.

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