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Regulation of the fetal mouse ductus arteriosus is dependent on interaction of nitric oxide and COX enzymes in the ductal wall

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Abstract

Nitric oxide (NO) and cyclooxygenase (COX)-derived prostaglandins are critical regulators of the fetal ductus arteriosus. To examine the interaction of these pathways within the ductus wall, the ductus arteriosus of term and preterm fetal mice was evaluated by pressurized myography. The isolated preterm ductus was more sensitive to NOS inhibition than at term. Sequential NOS and COX inhibition caused 36% constriction of the preterm ductus regardless of drug order. In contrast, constriction of the term ductus was dependent on the sequence of inhibition; NOS inhibition prior to COX inhibition produced greater constriction than when inhibitors were given in reverse order (36±6% versus 23±5%). Selective COX-1 or COX-2 inhibition prior to L-NAME induced the expected degree of constriction. However, NOS inhibition followed by selective COX-2 inhibition caused unexpected ductal dilation. These findings are consistent with NO-induced activation of COX in the ductus arteriosus wall and the production of a COX-2-derived constrictor prostanoid that contributes to the balance of vasoactive forces that maintain fetal ductus arteriosus tone.

Introduction

The ductus arteriosus is a prostaglandin-sensitive fetal vascular shunt. Maintenance of ductus arteriosus patency *in utero* is required for adequate perfusion and oxygen delivery to fetal tissues. Timely closure of the ductus arteriosus after birth is critical for successful postnatal circulatory adaptation. Failure of postnatal ductal constriction with persistent patency of the ductus arteriosus (PDA) has particularly harmful consequences in premature newborns, who are placed at increased risk for pulmonary over circulation, congestive heart failure, intracranial hemorrhage, compromised blood flow to the brain and systemic organs and development of chronic lung disease¹⁻³.

Patency of the fetal ductus is primarily attributed to low oxygen tension and active vasodilation by endogenous prostaglandins and nitric oxide (NO). Prostaglandins from both cyclooxygenase-1 (*ptgs-1* or COX-1) and COX-2 actively relax the fetal ductus arteriosus

while falling prostaglandin levels facilitate its closure after birth. The relative contribution of each COX isoform and whether the prostaglandins that act on the ductus are derived from the circulation or from the ductal wall are not fully resolved. In mice, COX-2 appears to contribute more to ductal relaxation than does COX-1. Our lab and others have shown that treatment of pregnant dams with indomethacin fully constricts the fetal ductus^{4–6} but selective COX-1 inhibition causes less fetal ductus constriction than does COX-2 inhibition^{6,7}. Although COX genes are expressed at low levels in the ductus compared to surrounding tissues⁸, the mouse ductus contains both COX-1 and COX-2 mRNA and the PGE synthetic enzymes for local prostaglandin production⁹. Additionally, COX-2 gene expression is reported to increase with advancing gestation, and immunoreactive COX-2, but not COX-1, is localized in the wall of the mouse ductus^{4,10}.

Examination of knockout mice has also shed light on the relative contributions of COX isoforms to ductal tone. Deletion of COX-2 has more impact on ductus function than deletion of COX-1^{4,6,7,10}. However, prostaglandin deficiency throughout gestation in COX null mice paradoxically results in PDA, not ductal closure, suggesting that prolonged prostaglandin exposure *in utero* is necessary for normal development of the postnatal contractile response^{6,8}. *Ex vivo* studies on isolated fetal mouse ductus rings from COX null mice seemed to implicate a role for NO or other COX-independent vasodilators in the etiology of PDA in these mice⁹. In contrast, our recent *in vivo* studies showed that serial injections of an NO synthase inhibitor did not constrict the PDA of COX deficient mice⁶. These discrepancies highlight the need to better understand the role of local versus circulating prostaglandins and the interplay between NO and prostaglandin synthesis within the ductus wall.

Previous *in vivo* studies have established that the effects of NO and prostaglandins on the ductus are developmentally regulated, such that NO plays a more significant role than prostaglandins in patency of the preterm fetal ductus, while prostaglandins assume greater importance at term^{11–13}. However, these studies fail to distinguish between intrinsic and circulating sources of NO and prostaglandins that influence ductus tone. In addition, NO and prostaglandin interactions may be functionally coupled within the ductus wall^{9,14}. Thus, the purpose of this study was to examine responses of the *ex vivo* term and preterm mouse ductus arteriosus to NO and prostaglandin inhibition using a pressurized myography technique. We hypothesized that: 1) the response of the isolated ductus to NOS and COX inhibition would differ from that of the *in vivo* ductus, 2) NO and prostaglandin interactions change with advancing gestation, and 3) interactions between NO and COX are isoform-specific. We chose to study term (day 19) and preterm (day 15) isolated mouse ductus arteriosus because our previous *in vivo* studies showed that the mouse fetal ductus requires prostaglandin exposure beginning at day 15–16 of gestation⁶ and is sensitive to NOS inhibition at this developmental stage¹².

Materials and Methods

Animals and tissues

Experiments were conducted in accordance with National Institutes of Health animal care standards and were approved by the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center. Adult female CD-1 mice (Charles River, Raleigh, NC) were bred to produce timed pregnancies. The morning of finding a vaginal plug was designated as day 1 of pregnancy. Pregnant females were anesthetized by i.p. injection of 0.4 ml of 2.5% avertin (2,2,2-tribromoethanol in *tert*-amyl alcohol; Sigma-Aldrich, St. Louis, MO), followed by isoflurane inhalation (Baxter, Deerfield, IL) to facilitate fetal anesthesia. Dams were killed by cervical dislocation at 0830–0900h on day 15 or day 19 of pregnancy. Any fetuses that displayed spontaneous respiratory efforts after delivery or had signs of lung inflation at the time of dissection were excluded from further study.

Fetuses were submerged in ice-cold, deoxygenated (95% N₂, 5% CO₂) Krebs buffer and secured in supine position. Krebs buffer was modified (in mM: 109 NaCl, 34 NaHCO₃, 4.7 KCl, 0.9 MgSO₄, 1.0 KH₂PO₄, 11.1 dextrose, and 2.5 CaCl₂) to maintain a stable pH (7.30–7.35) in the presence of the vigorous gas bubbling rate required to maintain relative hypoxia in the vessel bath (dissolved oxygen content = 1.5–1.8%; measured PaO₂ = 38–45 Torr). Thoracic contents were exposed under stereomicroscopy. Isolation of the fetal ductus was performed by first dissecting a portion of cardiac tissue below the pulmonary valve. The pulmonary outflow tract was freed from the aortic root, and proximal sections of the right and left branch pulmonary arteries were dissected free, leaving sufficient length to secure the distal ends during vessel mounting. The transverse aorta was cut on either side of the ductal isthmus, resulting in isolation of the main pulmonary artery-ductus arteriosus-transverse aorta segment of vascular tissue (Fig. 1A).

Vessel studies

Isolated tissues were transferred to custom microvessel perfusion chambers (Instrumentation and Model Facility, University of Vermont) filled with chilled, deoxygenated Krebs buffer (4 ml capacity). Glass micropipettes (1.2 mm, World Precision Instruments, Sarasota, FL) were pulled to 120 or 170 μ m diameter for preterm and term vessels, respectively. The excised vessel preparation was positioned on pipette tips with care taken to prevent movement into the ductus arteriosus lumen. Tissues were tied in position using a single frayed strand of braided nylon suture. Micromanipulators were used to position the preparation, resulting in isolation of the ductus arteriosus between the pipette tips (Fig. 1A, inset).

Once mounted, vessels and perfusion chambers were transferred to an inverted microscope equipped with a video camera and computer-assisted image capture system (IonOptix, Milton, MA) to continuously record intra-luminal diameter (100x). Optical markers used to detect the lumen diameter were positioned at the endothelial-smooth muscle interface at the narrowest point of the constricted vessel. Due to endothelial approximation during vessel constriction, an –85 to –90% change from baseline reflected complete closure of the ductus lumen. Bath temperature was held constant at 36.5–37.5°C and was continuously monitored by a thermal microprobe tip placed adjacent to the mounted vessel. Chambers were perfused with warmed deoxygenated Krebs buffer at 6 ml/minute via low gas permeability Tygon tubing.

Oxygenation of the bath solution was determined by dissolved oxygen content (*inO*₂; Innovative Instruments, Tampa, FL) and by periodic blood gas determination. Vessels were pressurized by a column of deoxygenated Krebs buffer at 5 mmHg (term) or 2 mmHg (preterm) and allowed to equilibrate at 37°C for 30–40 minutes. Stepwise increases in pressure were applied to determine the appropriate working pressure. Vessels were exposed to 50 mM KCl for 3–5 minutes (with 1–2 repetitions) to determine the contractile response for each vessel. After return to baseline, the bath was changed to a recirculating 100 ml buffer circuit for treatment with various drugs or changes in environmental parameters.

Drugs and compounds

The isolated ductus arteriosus was exposed to increasing oxygen tension (2, 5, 12, 21 or 95% O₂), or increasing concentrations of ethanol, dimethyl sulfoxide (DMSO) or KCl (Sigma-Aldrich, St. Louis, MO). The sodium content of the Krebs buffer was concomitantly reduced to maintain isotonicity with increasing KCl concentrations. Vessel studies were performed with established dosages for the non-specific NOS inhibitor, N(G)-nitro-L-arginine methyl ester (L-NAME) (10^{–4} M; Cayman Chemical, Ann Arbor, MI), the nonspecific COX inhibitor, indomethacin (10^{–5} M; Sigma-Aldrich, St. Louis, MO), the selective COX-1 inhibitor, SC-560 (10^{–6} M; Cayman), the selective COX-2 inhibitors, SC-236 (10^{–6} M) or CAY10404 (10^{–6} M; Cayman), the synthetic thromboxane receptor agonist, U-46619 (10^{–7} M; Cayman), the calcium ionophore, A23187 (10^{–6}–10^{–5} M; Sigma-Aldrich), acetylcholine (10^{–7}–10^{–5} M;

Sigma-Aldrich), sodium nitroprusside (SNP) (10^{-5} M; Sigma-Aldrich), or papaverine (10^{-4} M; Sigma-Aldrich). Acetylcholine, SNP, and papaverine were dissolved in water; all other drugs were dissolved in ethanol except A23187 (DMSO). Final solvent concentration in the bath was limited to 0.04%. Vessels were exposed to each agent until a stable baseline diameter was reached (typically 20–30 minutes). In some cases, a stable baseline diameter was not achieved until 40–60 minutes after treatment with L-NAME or indomethacin. The general experimental order was L-NAME, indomethacin, U-46619, and then 12% oxygen. A final KCl stimulation confirmed vessel responsiveness at the end of the study, followed by exposure to either SNP or papaverine, to determine maximum vessel diameter. In some studies, vessels were exposed to indomethacin prior to L-NAME. A subset of U-46619-precontracted vessels was treated with acetylcholine or A23187 to examine endothelial-dependent responses. Vessels were snap frozen, sectioned and stained for histological analysis. An additional series of studies examined the effects of selective COX-1 or COX-2 inhibition in combination with L-NAME. Exposure to L-NAME and COX inhibitors was additive, whereas oxygen, U-46619, the final KCl exposure, and SNP or papaverine were administered after wash off with deoxygenated Krebs.

Statistical analysis

Data were plotted as mean $\pm$ SEM. Alterations in vessel dimensions were expressed as percent change from baseline diameter. One-way ANOVA with repeated measures was used to detect differences in vessel caliber in response to increasing concentrations of KCl, oxygen, ethanol, or DMSO, or to compare sequential treatments with L-NAME, indomethacin, U-46619, and oxygen in the same vessel. Two-way ANOVA was used to compare response curves between d15 and d19 vessels. Bonferroni post-hoc analysis was performed when significant differences were found. A paired, two-tailed Student's t-test was used to examine differences between two different treatments in the same vessel (selective COX inhibitor studies); unpaired analysis was performed for comparison between preterm and term vessels or between treatments in different sets of vessels. A P value < 0.05 was considered significant.

Results

Establishment of baseline conditions

Stepwise increases in distending pressure produced a consistent myogenic response, defined as a pressure-induced increase in vessel tone and mild dilation in response to reduction in distending pressure (Fig. 1B). Convergence of the plots for pressure-induced tone and 50 mM KCl-induced constriction occurred near 40 mmHg (Fig. 1C). The maximal difference between pressure-induced and KCl-induced tone occurred at 20–25 mmHg, suggesting that this pressure would provide optimal conditions for *in vitro* studies. A distending pressure of 20 mmHg was chosen for subsequent experiments, similar to the mean arterial pressure of newborn mice^{15–17}. The pressurized ductus was stable at 20 mmHg for prolonged periods of study (Supplement, Fig. 1).

The cannulated preterm (d15) fetal ductus also demonstrated myogenic tone and pressure-dependent KCl-induced constriction (Fig. 1C) with optimal pressure-diameter response conditions at 6–8 mmHg. A distending pressure of 6 mmHg was chosen for subsequent *in vitro* studies, in agreement with estimates of fetal blood pressure at this gestation¹⁸. In contrast to the term ductus, the preterm ductus did not fully close its lumen in response to 50 mM KCl (Fig. 1C). Preterm vessels frequently had small dilatatory and contractile oscillations with a magnitude of approximately 1–3% of baseline lumen diameter (Supplement, Fig. 1). The presence of oscillations had no effect on the ability to constrict or dilate to various experimental stimuli.

Increased oxygen tension induced constriction of the cannulated, pressurized d19 ductus (Fig. 1D). The shift from 0 to 12% oxygen produced an increase in PaO₂ from 38–45 to 90–120 Torr in the perfusion bath. This change caused approximately 120 μ m reduction in vessel diameter (43% of resting baseline), and resulted in a lumen diameter that was similar to vessels at 5 mmHg under deoxygenated conditions. An additional 8–10 mmHg of pressure was required before the diameter of the oxygen-exposed ductus reached the diameter of the ductus in deoxygenated conditions at the working pressure of 20 mmHg. Constriction of the cannulated ductus occurred at the aortic end of the vessel in response to either oxygen or KCl, similar to *in vivo* ductus closure in the newborn mouse ¹⁹.

Characteristics of the isolated ductus arteriosus

Both the preterm and term fetal ductus demonstrated concentration-dependent constriction in response to KCl with maximal constriction at 50 mM KCl (Fig. 2A). Compared to resting baseline in normal (4.7 mM KCl) Krebs buffer, exposure to Krebs containing 1 mM KCl induced a small dilation in the term but not the preterm ductus. The preterm fetal ductus was more constricted at lower KCl concentrations than the term ductus, but did not constrict as tightly as term vessels at the highest KCl concentrations, similar to pressure optimization studies (Fig. 1D).

Exposure of the isolated ductus to solvents used for the drugs in this study showed that ethanol and DMSO had little effect on ductus tone at concentrations of 0.01 to 0.1%. At higher concentration, ethanol induced constriction of the ductus in a dose-dependent manner (Fig. 2B) while DMSO induced ductus dilation. Constriction of the ductus in response to ethanol was more prominent at the pulmonary end of the vessel.

Oxygen-induced constriction of the term fetal ductus was reversible; repeated cycles of exposure to Krebs buffer bubbled with 12% oxygen caused ~50–60% constriction followed by dilation under deoxygenated conditions (Fig. 2C). Brief vessel spasm and luminal closure was typically noted after several oxygen exposures, but vessels routinely returned to a stable, partially constricted baseline after 20–30 minutes. Oxygen content of the bath solution correlated with increasing oxygen concentration in the gas mixture: 0% oxygen (42.5 $\pm$ 2.1 Torr), 2% oxygen (57.3 $\pm$ 2.1 Torr), 5% oxygen (75.1 $\pm$ 1.8 Torr), 12% oxygen (118.8 $\pm$ 1.7 Torr), 21% oxygen (181.0 $\pm$ 5.6 Torr), and 95% oxygen (773.0 $\pm$ 61.3 Torr) (n=8). Both the term and preterm ductus demonstrated oxygen dose-dependent constriction (Fig. 2D). The lumen of the term ductus was fully closed when exposed to 21% oxygen, although additional muscular constriction occurred in 95% oxygen. In comparison, the preterm ductus was significantly less constricted at each level of oxygen exposure and maintained an open lumen even in 95% oxygen (Fig. 2D).

Response to NO and prostaglandin inhibition

A stable response to L-NAME or indomethacin often required 40–60 minute exposures, similar to ductal rings ⁹, whereas oxygen induced more rapid, intense constriction (Fig. 3A). Inhibition of NO synthesis by L-NAME induced more potent constriction in the preterm than term ductus (Fig. 3B). The addition of indomethacin to L-NAME-constricted vessels induced further constriction in both the preterm and term ductus (Fig. 3B). Although L-NAME alone had more potent effects on the preterm ductus, L-NAME in combination with indomethacin induced an equivalent degree of constriction at both gestational ages. On rare occasions, combined treatment with L-NAME and indomethacin resulted in vessel spasm, irreversible constriction, or loss of vessel responsiveness, leading to termination of the study.

Response to U-46619 or increased oxygen tension

The preterm ductus constricted 48% from baseline in response to the thromboxane mimetic, U-46619 (Fig. 3C), similar to the response to 50 mM KCl, and significantly greater than the response of the preterm ductus to increased oxygen tension. U-46619 also induced a strong contractile response in the term ductus, resulting in 77% constriction from baseline lumen diameter, significantly greater constriction than induced by L-NAME or L-NAME plus indomethacin. Wash off of U-46619 resulted in prompt return to baseline diameter. A sub-maximal dose of U-46619 (10^{-8} M) was used to pre-constrict some d19 vessels to examine endothelial-dependent vasodilation to acetylcholine or the receptor-independent, endothelial-dependent vasodilator, A23187. Acetylcholine had little or no effect on the U-46619 pre-constricted ductus, whereas 10^{-5} M A23187 induced transient dilation (Supplement Fig. 2). Histologic analysis of these vessels demonstrated an intact endothelium.

Increased oxygen tension in the perfusion bath resulted in 28% and 65% constriction of the preterm and term ductus, respectively (Fig. 3C). Oxygen-induced constriction was similar to L-NAME-induced constriction in the preterm ductus. Constriction of the term ductus by oxygen was significantly greater than its response to L-NAME or L-NAME plus indomethacin. Oxygen-induced constriction was a reversible process, as vessels returned to baseline diameter after 10–30 minutes in deoxygenated buffer.

Response to vasodilators

Vessels were exposed to the NO donor, SNP, or the direct-acting vasodilator papaverine at the end of each study. Preterm and term vessels dilated similarly in response to SNP. Papaverine induced a significantly greater dilation than SNP, up to 70% of resting baseline dimensions in vessels at both gestational ages (Fig. 3D). Exposure of preterm and term vessels to calcium-free Krebs buffer did not result in relaxation of the isolated ductus (not shown).

Order- and isoform-specific responses to NOS and COX inhibition

In the preterm ductus, L-NAME alone induced significantly greater constriction than did indomethacin alone. Sequential inhibition of NOS and then COX enzymes in d15 vessels produced 31% constriction after L-NAME alone, increasing to 36% following the addition of indomethacin. Indomethacin alone produced only 10% constriction of the preterm ductus. Addition of L-NAME increased constriction to 36%, similar to the constriction observed when inhibitors were given in the opposite order (Fig. 4A). The term ductus constricted similarly in response to L-NAME or indomethacin alone (Fig. 4B). L-NAME-exposed vessels further constricted to 36% of baseline after the addition of indomethacin. Vessels that were exposed to indomethacin prior to L-NAME constricted significantly less than vessels that were exposed to these inhibitors in the reverse order (Fig. 4B). Because this effect was not observed in d15 vessels, additional studies were performed in d19 vessels to determine whether NOS-COX interactions were specific for COX-1 or COX-2 in the isolated ductus.

Exposure of the term ductus to the selective COX-1 inhibitor, SC-560, or the selective COX-2 inhibitor, SC-236, resulted in similar mild degrees of vessel constriction (Fig. 5A, 5B). A ten-fold higher dose did not constrict the vessel further. Selective inhibitors induced relatively less constriction than indomethacin (Fig. 4B). The addition of L-NAME augmented ductus constriction after selective inhibition of either COX isoform, but was not as potent as combined inhibition with indomethacin and L-NAME (Fig. 5A, 5B).

Reciprocal experiments were performed to examine the impact of NOS inhibition prior to selective COX inhibition. Term vessels exposed to L-NAME followed by the selective COX-1 inhibitor SC-560 demonstrated 16% constriction from baseline (Fig. 5C). COX-1 inhibition did not significantly increase the constriction already induced by L-NAME. Contrary to

expectation, vessels exposed to the selective COX-2 inhibitor SC-236 after incubation with L-NAME underwent relaxation and reversal of L-NAME-induced constriction (+12% change from L-NAME baseline; Fig. 5D). To determine whether these results were due to a drug-specific effect, a second, more highly selective COX-2 inhibitor, Cay10404, was used. Addition of 10^{-6} M Cay10404 to vessels precontracted by L-NAME induced a small relaxation (+2.8% change from L-NAME baseline, n=7), while addition of 10^{-5} M Cay10404 induced +12% change in vessels previously exposed to L-NAME (n=3), similar to the effects of SC-236.

Discussion

Infants with PDA present a daunting clinical dilemma. Indomethacin and ibuprofen are the only available non-surgical treatments but may fail in 30–40% of small preterm infants^{20, 21}. One hindrance to improved medical management of this problem is insufficient understanding of the interactive roles of COX-1, COX-2, and nitric oxide in the ductus wall. The novel findings of this study are that the isolated term ductus only modestly constricts to COX or combined COX and NOS inhibition, in stark contrast to the effects of systemic COX inhibition. In comparison, the isolated term ductus is exquisitely sensitive to an increase in oxygen tension analogous to the physiologic changes in oxygenation that occur after birth. The d15 preterm ductus constricts less than the term ductus to KCl, oxygen, and U-46619, but L-NAME-induced constriction is greater in preterm than in the term ductus, corroborating prior observations from *in vivo* animal studies^{11–13}. Combined NOS and COX inhibition is more potent than inhibition of either pathway alone. However, the degree of vessel constriction is dependent on the order in which the enzymes are inhibited. This cannot be explained on the basis of COX isoform specificity, since COX-1 or COX-2 inhibition prior to NOS inhibition yielded the expected degree of partial constriction. Interestingly, NOS inhibition followed by selective COX-2 inhibition results in relaxation of the ductus from its L-NAME-constricted baseline, suggesting that NO has unique interactions with the COX-2 enzyme in the ductus wall. Dual inhibition appears to uncover the production of a COX-2-derived vasoconstrictor that contributes to basal ductal tone.

To our knowledge, this is the first study to apply the pressurized myography technique to investigations of the mouse ductus arteriosus. Coceani and colleagues first demonstrated the use of wire myography to study isolated ring preparations of the term fetal mouse ductus arteriosus²². The use of pressurized vessel myography to study the mouse ductus offers the advantage of maintaining anatomical connections to the pulmonary artery and aorta, as well as observation of the entire vessel, where regional differences along the length of the ductus have been noted²³. Cannulated vessels also retain their native anatomical shape and experience transmural wall pressure without contact of opposing endothelial surfaces. This paper demonstrates that the term and preterm *ex vivo* cannulated mouse ductus arteriosus is viable and responsive to vasoactive stimuli for extended periods in a pressurized myography system. This approach enabled us to study the preterm ductus at d15 of gestation. The use of this technique also revealed that exposure to high concentrations of ethanol and DMSO produced opposite changes in vessel tone, and that acetylcholine and A23187 have minimal effects in the precontracted mouse ductus despite an intact endothelium. One drawback of this approach is the finite limitation on contraction measurements. Contractile force in a cannulated preparation cannot be measured beyond the point when the vessel lumen has completely closed (Fig. 2). Isometric tension in a ring preparation is not limited by such anatomic parameters as vessel closure, although the physiological significance of contractile forces exceeding those required for obliteration of the vessel lumen is uncertain.

The extraordinary sensitivity of the ductus arteriosus to oxygen tension is one of its defining characteristics. Oxygen can condition the response of the ductus to other vasoactive mediators, including prostaglandins and NO²⁴. In rabbits, increasing oxygen tension modifies

prostaglandin actions such that the ductus is desensitized to vasodilating prostaglandins, facilitating its closure²⁵. In fetal lambs, the preterm ductus is less sensitive to oxygen than at term and demonstrates altered sensitivity to prostaglandins²⁶. Paradoxically, elevated oxygen tension may also result in a significant increase in local prostaglandin production^{27–29}. In our experiments, the preterm mouse ductus was less sensitive than the term ductus to oxygen-induced constriction, coinciding with the greater incidence of PDA in the most immature preterm infants²¹ and a reduction in oxygen-sensitive potassium channels in the premature human ductus³⁰. Constriction of the isolated term ductus by 65% in response to oxygen in our *in vitro* studies paralleled the *in vivo* effects of increasing the oxygen content of the fetus to newborn levels noted by Coceani et al., who placed pregnant dams in hyperoxic chambers with a resulting ~50% reduction in the fetal ductus lumen²². In that study, the effects of oxygen were equipotent to the effects of indomethacin in isolated ductus rings. In contrast, our results and those in ring studies of the fetal rat ductus³¹ suggest that COX inhibition is less potent than oxygen-induced constriction in the isolated ductus.

Our finding that indomethacin causes only mild constriction in the isolated term ductus, in contrast to near-complete closure of the *in vivo* mouse fetal ductus upon *in utero* exposure to indomethacin^{4–7, 12} suggests that circulating prostaglandins play a more prominent role in regulation of the fetal ductus than locally-produced prostaglandins in the ductus wall^{26, 32}. Using isolated ductus rings from COX deficient mice, Baragatti et al. showed that indomethacin caused prompt constriction of the wild type and COX-1 null ductus, while COX-2 null rings had slower, less potent and more irregular constriction⁹. We previously showed that COX-1 and COX-2 gene expression in the ductus wall is low compared to high levels of expression in placenta, lung, liver and surrounding vascular tissues⁸. Moreover, our current studies demonstrate that selective inhibition of either COX-1 or COX-2 only weakly constricted the isolated ductus and combined isoform inhibition was similar to the modest degree of indomethacin-induced constriction. Because the same selective COX inhibitors significantly constrict the fetal ductus upon *in utero* exposure⁶, our results support and extend the concept that prostaglandins from systemic sources are important in ductus regulation.

It is increasingly clear that COX-1 and COX-2 have different roles within the ductus wall. Loftin et al. reported that immunoreactive COX-2 but not COX-1 localizes to the wall of the mouse ductus⁴. More recent studies using quantitative PCR showed that ductus COX-2 levels increase with advancing gestation while COX-1 levels are unchanged¹⁰. Reduced COX-2 expression was noted in the ductus of prematurely delivered mice and in the PDA of mice lacking the EP4 receptor for PGE₂, again supporting a predominant role for COX-2 in the ductus wall. On the other hand, Coceani and colleagues found differential responses of the isolated COX-1 and COX-2 null ductus to indomethacin and an excess of cytosolic COX-1 in the vessel wall, favoring a prominent role for COX-1. However, a more significant role was attributed to COX-2 based on the intimate co-localization of COX-2 and PGES enzymes⁹. Our data corroborate a pivotal role for COX-2 within the ductus wall in mediating overall ductus tone, expanding that role by identifying its capacity to produce both dilator and constrictor prostaglandins under the control of nitric oxide.

Our studies add to a growing body of literature linking NOS activity and prostaglandin vascular actions, including in the ductus arteriosus^{9, 33}. Salvemini first demonstrated that NO regulates COX activity³⁴. NO can activate both COX-1 and COX-2, depending on the cellular context and the inciting stimulus, and is able to increase the activity of purified recombinant COX enzymes^{35, 36}. NO stimulates prostaglandin production in cultured endothelial and vascular smooth muscle cells^{37, 38} as well as in isolated vessels^{39–41}. NO donors induce COX-2 and inflammation, while NSAIDs inhibit NO effects. These findings suggest that NO-driven prostaglandin production may play a significant pathophysiological role³⁵. Multiple mechanisms for NO-induced prostaglandin synthesis have been proffered, including NO acting

as an antioxidant and interfering with superoxide-mediated COX autoinactivation, NO-based generation of either nitrosothiols or peroxynitrite, or nitrosylation of cysteine residues in the COX enzyme, in each case leading to activation of COX reviewed in: ^{35, 36}. Kim et al. demonstrated specific binding of iNOS to COX-2 and activation of prostaglandin synthesis by S-nitrosylation of C526, the only one of thirteen different cysteine residues where NO stimulated COX-2 activity ⁴². NO also S-nitrosylates and activates cPLA2, the first critical enzyme in prostaglandin synthesis ⁴³, providing another mechanism whereby NOS inhibition leads to decreased prostaglandin production. In addition, NO can upregulate COX-2 and is required for maintenance of prolonged COX-2 expression ^{44–46}. “Cross-talk” between NOS and COX pathways may also take place at the cGMP and cAMP levels ⁴⁷.

The sole source of NO production in the mouse ductus arteriosus is eNOS ^{12, 14}. Our findings suggest that within the microenvironment of the ductus wall, eNOS-derived NO interacts differently with COX-2 than COX-1. Paradoxical dilation of the ductus after sequential NOS and then COX-2 inhibition suggests that COX-2 produces a contractile prostanoid. Therefore, “steady-state” COX-2 activity may be responsible for both dilatory and contractile prostaglandin products, striking a balance that is dependent on the available NO. Administration of NOS and then COX inhibitors also causes vasodilation in other vascular beds ^{48, 49}, again suggesting a role for COX-derived vasoconstrictors that are under the influence of NO. Endogenous constrictor prostanoids within the ductus were originally demonstrated in fetal rabbits ²⁵. More recently, Loftin et al. suggested that COX-2-derived prostaglandins have a contractile role in the mouse ductus ^{7, 10}. In addition, we previously reported that eNOS expression persists in the closed and remodeling ductus, beyond the period when NO would play its anticipated role in maintenance of ductus patency ¹². Prolonged expression of eNOS and the postnatal increase in COX-2 expression within the ductus ¹⁰ may be linked. Combined with results showing that COX activity is functionally coupled with NOS in the mouse ductus ⁹, our current data indicate that NO-driven prostaglandin production via COX-2 mediates an underlying contractile tone that was previously unappreciated.

In summary, these studies demonstrate that relative to circulating sources, products of local NOS and COX enzymes make only a modest contribution to ductus arteriosus tone. Interactions between NO and COX determine the balance of dilator and constrictor prostaglandin production that regulates ductal tone in the fetus. The dynamics of NO-COX balance may have an important role in patency or closure of the ductus arteriosus, and should be considered in the development of new therapeutic strategies, including the use nitro-NSAIDs or NO-releasing COX inhibitors for management of PDA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Pressure myography of the isolated fetal mouse ductus

Pressure-diameter relationships under basal conditions and following KCl-induced constriction are shown. **A.** Representative pulmonary outflow tract from a d19 fetal mouse secured with 100 μ m dissecting pins, then mounted on 80 μ m glass pipette tips in a myography chamber (inset). **B.** Representative tracing of baseline and KCl-induced diameter changes over increasing distending pressures demonstrating a myogenic response. **C.** Comparison of baseline (BL) and KCl-constricted curves for the d15 ($n = 13$) and d19 ($n = 15$) fetal ductus. **D.** Response of the d19 fetal ductus ($n = 12$) to bubbling 12% oxygen in the perfusate. Oxygen-induced ductal constriction was overcome by 9 – 10 mmHg of additional distending pressure.

Figure 2. Response of the isolated ductus arteriosus to vasoactive stimuli

A Vessels from d15 (n = 11) or d19 (n = 15) fetuses were exposed to cumulative increases in KCl in isotonic buffer under deoxy conditions. Results are plotted as cumulative % change from the initial resting baseline. **B.** Response of the d19 fetal ductus to increasing concentrations of ethanol (n = 10) or DMSO (n = 10) under deoxygenated conditions. **C.** Representative tracing of the d19 fetal ductus in response to repetitive exposure to increased oxygen tension. The bath solution was drawn from Kreb's buffer bubbled with either 0% or 12% oxygen. **D.** Vessels from d15 (n = 6) or d19 (n = 10) fetuses were exposed to sequential increases in oxygen concentration bubbling the perfusate. Results are plotted as cumulative % change from the initial resting baseline. †p < 0.01, d15 versus d19. *p < 0.05, compared to baseline within treatment group.

Figure 3. Response of the isolated ductus arteriosus to inhibitors of nitric oxide and prostaglandin synthesis, and to vasoactive stimuli

A Representative tracing of a d19 fetal ductus arteriosus to 10^{-4} M L-NAME, 10^{-5} M indomethacin (Indo), and shift from 0 to 12% oxygen in the buffer reservoir. **B.** Response of d15 and d19 vessels to L-NAME alone, followed by the addition of indomethacin. **C.** Response of vessels previously exposed to L-NAME and indomethacin to the addition of the thromboxane mimetic, U-46619 (10^{-7} M). After wash and return to baseline, the response to an increase from 0% to 12% oxygen is shown. **D.** The dilatory response of isolated vessels to 10^{-5} M SNP or 10^{-4} M papaverine (PAP). Results are plotted as cumulative % change from the initial resting baseline. Bars are mean $\pm$ SEM. *p < 0.05, term versus preterm vessels.

**p < 0.05, SNP versus PAP within gestation group.

Figure 4. Constriction of the term, but not preterm, ductus arteriosus is dependent on the order of NOS and COX inhibition

A Exposure of d15 vessels to L-NAME alone induced significant ductal constriction compared to indomethacin (Indo) alone. Combined NOS and COX inhibition produced similar degrees of ductal constriction regardless of the order of drug administration. **B**. Exposure of the isolated d19 ductus to L-NAME or Indo alone resulted in similar, modest degrees of constriction. L-NAME given prior to Indo caused more ductal constriction than when Indo was given prior to L-NAME. Results from Fig. 3 are represented here for comparison (left panels). Results are plotted as cumulative % change from the initial resting baseline. Bars are mean $\pm$ SEM. * $p < 0.05$, d19 versus d15. ** $p < 0.05$, Indo versus L-NAME within gestation group. # $p < 0.05$, single

versus combined inhibitors within the same vessel. † $p < 0.05$, L-NAME+Indo versus Indo+L-NAME.

Figure 5. Differential interactions of COX-1 and COX-2 with nitric oxide in the isolated term ductus arteriosus

A Modest constriction of vessels exposed to the selective COX-1 inhibitor, SC-560, followed by L-NAME. **B.** Vessels exposed to the selective COX-2 inhibitor, SC-236, followed by L-NAME also developed modest, progressive constriction. Combined COX-1 or COX-2 + NOS inhibition was similar to indomethacin + L-NAME in the d19 ductus (Fig 4). **C.** Vessels exposed to L-NAME followed by the COX-1 inhibitor, SC-560, constricted modestly. **D.** Vessels exposed to L-NAME followed by the COX-2 inhibitor, SC-236, underwent dilation (n=11). Results are plotted as cumulative % change from the initial resting baseline. Bars are mean $\pm$ SEM. *p < 0.05, compared to initial treatment.