Cyclooxygenase-2 plays a significant role in regulating the tone of the fetal lamb ductus arteriosus

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Clyman, Ronald I., Pierre Hardy, Nahid Waleh, Yao Qi Chen, Françoise Mauray, Jean-Claude Fouron, and Sylvain Chemtob. Cyclooxygenase-2 plays a significant role in regulating the tone of the fetal lamb ductus arteriosus. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R913–R921, 1999.—Nonselective cyclooxygenase (COX) inhibitors are potent tocolytic agents but have adverse effects on the fetal ductus arteriosus. We hypothesized that COX-2 inhibitors may not affect the ductus if the predominant COX isofrom is COX-1. To examine this hypothesis, we used ductus arteriosus obtained from late-gestation fetal lambs. In contrast to our hypothesis, fetal ductus arteriosus expressed both COX-1 and COX-2-immunoreactive protein (by Western analysis). Although COX-1 was found in both endothelial and smooth muscle cells, COX-2 was found only in the endothelial cells lining the ductus lumen (by immunohistochemistry). The relative contribution of COX-1 and COX-2 to PG synthesis was consistent with the immunohistochemical results: in the intact ductus, PGE2 formation was catalyzed by both COX-1 and COX-2 in equivalent proportions; in the endothelium-denuded ductus, COX-2 no longer played a significant role in PGE2 synthesis. NS-398, a selective inhibitor of COX-2, was 66% as effective as the selective COX-1 inhibitor valeryl salicylate and the nonselective COX inhibitor indomethacin in causing contraction of the ductus in vitro. At this time, caution should be used when recommending COX-2 inhibitors for use in pregnant women.

A patent ductus arteriosus is essential for fetal well-being because it allows 90% of the right ventricular output to bypass the high-resistance pulmonary vascular bed in utero (16). PGs play a major role in maintaining ductal patency in utero (4). The fetal ductus arteriosus synthesizes two important vasoactive PGs: PGE2 and prostacyclin (PGI2) (31). PGE2 appears to be the most important prostanoiogen regulating ductus patency, because it is 1,000 times more potent than PGI2 in relaxing the vessel in vitro (4, 8). Inhibition of PG synthesis, by inhibiting the enzyme cyclooxygenase (COX), produces constriction of the ductus in both animals (26) and humans (25) in vivo.

COX converts arachidonic acid to PGH2, which is then further metabolized to various PGs and thromboxanes (38). COX exists in two isofoms: COX-1 and COX-2. COX-1 is constitutively expressed by most tissues and seems to be responsible for the majority of PG production in the adult (29). The well-known gastric and renal "side effects" of nonselective COX inhibitors appear to be attributable to their inhibition of COX-1 (21). COX-2 is an inducible form of the COX enzyme, which is stimulated by proinflammatory agents (22, 23). In contrast to COX-1, selective inhibition of COX-2 does not seem to be associated with adverse effects to the gastrointestinal tract and kidneys (2, 12, 13). On the basis of these observations, it has been hypothesized that the antiinflammatory effects of currently available, nonselective COX inhibitors may be due primarily to their action on COX-2, whereas their effect on COX-1 may explain their unwanted side effects (40).

Recent studies have found that COX-2 may be involved in the process of parturition (17). This has led some investigators to use selective COX-2 inhibitors in the management of preterm labor (33). Unfortunately, there is limited information about the effects of COX-2 inhibition on the fetus. Although COX-2 is induced by cytokines, it is also constitutively expressed by certain organs during fetal development (15, 17, 32). Information is limited regarding the relative contributions of COX-1 and COX-2 to PG production by the fetal ductus. We have recently observed that the ductus arteriosus of the fetal pig expressed predominantly COX-1-immunoreactive protein; COX-2 was barely detectable. Similarly, PG production by the fetal pig ductus was due entirely to COX-1 activity; COX-2 inhibitors had no effect (14). To see whether these findings could be generalized to other species, we studied the expression of COX-1 and COX-2 in the ductus arteriosus of the fetal lamb. We also assessed the ability of selective COX-1 and COX-2 inhibitors to affect PG production and contractility of the fetal lamb ductus. Surprisingly, we observed that both COX-1 and COX-2 are expressed by the fetal lamb ductus arteriosus; in addition, a substantial proportion of the PG production and active tone of the ductus is regulated by COX-2.

METHODS

Tissue collection. Fetal lambs (mixed Western breed, between 125 and 137 days of a 145-day-term gestation) were delivered by caesarean section. The ewe was anesthetized with a constant intravenous infusion of ketamine HCl and diazepam throughout the procedure. The fetus was given ketamine HCl (30 mg/kg im) before rapid exsanguination. These procedures were approved by the Committee on Animal Research at the University of California, San Francisco. The ductus arteriosus was dissected free of loose adventitial...
Western analysis of COX-1 and COX-2. We examined the expression of COX-1 and COX-2 proteins in pieces of ductus arteriosus and aorta from four fetal sheep. After dissection, the tissues were frozen with liquid N₂ and stored at −80°C until assayed. Frozen ground tissue (~10 mg) was homogenized with 100 µl lysis buffer consisting of 50 mM Tris·HCl, pH 7.4, 0.1 M NaCl, 1% Triton X-100, 5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml pepstatin, 1.9 µg/ml aprotinin, and 0.5 µg/ml leupeptin and centrifuged for 5 min at 12,000 rpm at 4°C. Equal amounts of supernatant (100 µg protein/lane) were resolved by 10% SDS-PAGE. The proteins were transferred to Immobilon P membranes (Millipore, Bedford, MA) by electroblotting. The filters were blocked with 5% nonfat milk plus 0.01% Tween-20 overnight followed by incubation with the mouse monoclonal IgG primary antibodies [anti-ovine COX-1 (5 µg/ml) or anti-human COX-2 (0.5 µg/ml); Cayman Chemical, Ann Arbor, MI] for 2 h at room temperature. The primary antibodies were detected with a horse anti-mouse IgG-biotin conjugate (Vector Laboratories, Burlingame, CA) coupled to an alkaline phosphatase detection system (Vector Laboratories). Purified COX-1 (ovine) and COX-2 (ovine) electrophoretic standards were obtained from Cayman Chemical.

Immunohistochemistry. After dissection, the ductus arteriosus was fixed for 16 h at 4°C in fresh 4% paraformaldehyde, embedded in Tissuetek (Miles, Elkhart, IN) and frozen in liquid nitrogen. Specimens were stored at −80°C until analyzed. The protocol for immunohistochemical studies of COX-1 and COX-2 was similar to methods we have reported previ-
PGE₂ synthesis was determined on the supernatants by radioimmunoassay as described previously (6). Throughout the experiment, the rings were suspended between two stainless-steel hooks at 38°C in a modified Krebs solution (in mM: 118 NaCl, 4.7 KCl, 2.5 CaCl₂, 0.9 MgSO₄, 1 KH₂PO₄, 11.1 glucose, 23 NaHCO₃) equilibrated with 5% CO₂ (pH 7.4), balanced 30% O₂-65% N₂. The bath solution was changed every 20 min. Isometric responses of circumferential tension were measured by Grass FT03C force transducers (Quincy, MA). Each of the rings was stretched to an initial length, which results in a maximal contractile response to increases in oxygen tension (5). Initially the rings were stretched during a 15-min interval in medium equilibrated with fetal PO₂ (20–34 mmHg, 0.15–0.26 kPa) (starting tension). The bath solution then was bubbled with 30% O₂-65% N₂-5% CO₂ (a PO₂ of 175–200 mmHg, 1.31–1.50 kPa) until the tension reached a new plateau (approximately 90–120 min). Inhibitors of COX (indomethacin (19, 24), NS-398 (12, 19), and VSA (1, 19)) then were added to the bath solution. The specific COX-1 inhibitor (VSA) and the specific COX-2 inhibitor (NS-398) were added in concentrations that were specific for their targeted enzymes (1, 12, 19). Indomethacin was prepared in ethanol (42 × 10⁻³ M). NS-398 was prepared in DMSO (51 × 10⁻³ M). The final concentration of ethanol or DMSO in the bath solution was never greater than the concentration of the nonselective COX inhibitor ibuprofen (0.1 mM), the specific COX-1 inhibitor valeryl salicylate (VSA), 0.1 mM (1, 19), and L-745,337, 0.5 µM (2)); all concentrations used were shown to inhibit the targeted enzymes specifically. The difference in tensions between the COX inhibitor-induced contraction and the steady-state tension achieved in 30% oxygen was considered the COX-inhibitor tension. Changes in tension for each experimental condition were expressed as a percent of net active tension. The net active tension was always greater than the difference in tension between the maximal tension and the starting tension by 12 ± 8%, mean ± SD (P < 0.01, n = 39). This indicates that the ductus...
Fig. 4. Inhibitors of both COX-1 and COX-2 contract the fetal lamb ductus arteriosus. Ductus arteriosus rings from 8 fetuses were incubated in 30% oxygen. Each ring was exposed to increasing concentrations of either the COX-1 inhibitor (VSA; B), the COX-2 inhibitor (NS-398; A), or the nonspecific COX inhibitor (indomethacin; Indo; C). Maximal tension was determined with 100 mM KCl-Krebs solution (K+); minimal tension was determined by 10^{-5} M sodium nitroprusside (SNP). All tensions expressed as a percentage of net active tension, means ± SD, n = 8. Starting tensions (in g): A, 6 ± 1; B, 6 ± 1; C, 5 ± 2. Ring weight (in mg): A, 58 ± 20; B, 71 ± 36; C, 54 ± 32. *P < 0.01, COX-inhibitor-induced tension vs. O_2-induced tension.
rings were actively contracting even at the time of their initial mounting in the organ bath. In some experiments, PG production by the rings of ductus arteriosus was measured. For these experiments, the bath solution was changed every 40 min. The rings were exposed to two changes of bath solution for each experimental condition. The second change in bath solution was collected for PGE2 or 6-keto-PGF1α analysis (see below). After the experiment, the rings were removed from the baths and blotted dry and their wet weights were determined.

PGE2 and 6-keto-PGF1α (PGI2 metabolite) radioimmunoassay. To determine the amount of PGE2 and 6-keto-PGF1α released into the organ bath by rings of ductus arteriosus, samples of bath solution were acidified to pH 3 with glacial acetic acid and applied to octadecylsilyl silica columns. The columns were washed with 15% aqueous ethanol followed by petroleum ether and then eluted with methyl formate. Methyl formate was dried (Speed Vac), and PGs were reconstituted in PBS for radioimmunoassay (15, 32). The recovery of radioactive PGE2 and 6-keto-PGF1α was >96%, and the interassay variability during the radioimmunoassay was <5%.

Chemicals. L-745,337 was provided by Merck-Frosst (Pointe-Claire, PQ, Canada). The following compounds were used: pepstatin A, dimethyl sulfoxide, arachidonic acid, ibuprofen, indomethacin, EDTA, aprotinin, PMSF, Triton X-100, diaminobenzidine, and β-mercaptoethanol (Sigma, St. Louis, MO); leupeptin (Boehringer Manheim, Montreal, PQ, Canada), VSA and NS-398 (Cayman Chemical); protein determination assay and SDS-polyacrylamide gel (Bio-Rad, Richmond, CA); radioimmunoassay kit for PGE2 (Advanced Magnetics, Boston, MA).

Statistics. Statistical analysis was performed by the appropriate Student's t-test and by analysis of variance. Scheffé's test was used for post hoc analyses. Nonparametric data were compared with a paired sign test. Values are expressed as

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**Fig. 5.** COX-1 regulates ductus tension to a greater extent than COX-2. See Fig. 4 legend for tissue preparation and definitions. *P < 0.05, values from 1 experimental condition compared with prior experimental condition. Starting tensions (in g): A, NS-398/VSA ring, 6 ± 2; B, VSA/NS-398 ring, 7 ± 1; C, NS-398/Indo ring, 6 ± 1; D, VSA/Indo ring, 6 ± 1. Ring weights (in mg): A, NS-398/VSA, 62 ± 24; B, VSA/NS-398, 88 ± 50; C, NS-398/Indo, 61 ± 21; D, VSA/Indo = 59 ± 18.
means ± SD. Drug doses refer to their final molar concentration in the bath.

RESULTS

Expression of COX-1- and COX-2-immunoreactive protein. Ductus arteriosus and aorta obtained from the late-gestation fetal lamb expressed both COX-1- and COX-2-immunoreactive proteins (Fig. 1). Immunohistochemical findings were consistent with those observed by Western analysis (Fig. 2). COX-1 was detectable in both the endothelial cells lining the ductus lumen and the smooth muscle cells in the muscle media (Fig. 2, A and B). The expression of COX-2, on the other hand, appeared to be restricted to the luminal endothelial cells (Fig. 2D). In the late-gestation fetal lamb, vasa vasorum arborize throughout the outer half of the ductus arteriosus (7); COX-1 and COX-2 were not detectable in the proliferating endothelial cells (3) of the vasa vasorum that penetrated the muscle media (Fig. 2, B and E). In contrast, both COX isoforms were present in endothelial cells lining the occasional large muscularized vasa vasorum found in the outer adventitia (data not shown).

The pattern of COX expression in the fetal aorta differed from that in the ductus. COX-1 and COX-2 were expressed only in endothelial cells of the fetal aorta (Fig. 2, C and F). Neither the COX-1 nor COX-2 isoform was detectable in smooth muscle cells of the fetal aorta (Fig. 2, C and F).

Relative contributions of COX-1 and COX-2 in PGE2 synthesis. We determined the effects of COX-1 and COX-2 inhibitors on the rate of PGE2 synthesis by homogenates of intact or endothelium-denuded ductus arteriosus. In the intact ductus, the nonselective COX inhibitor ibuprofen reduced PGE2 synthesis by ~60% (Fig. 3). As might be anticipated from the immunohistochemical findings, both the selective COX-1 inhibitor VSA and the selective COX-2 inhibitors NS-398 and L-745,337 decreased PGE2 synthesis; each selective COX inhibitor reduced PGE2 production by ~40–50%. It thus appears that COX-1 and COX-2 contribute equivalently to PGE2 production by homogenates of intact ductus arteriosus.

Removal of the luminal endothelium decreased the rate of PGE2 synthesis to less than half of that of the intact ductus (Fig. 3). After endothelial denudation, PGE2 synthesis was minimally affected by either of the COX-2 inhibitors; in contrast, the COX-1 inhibitor VSA blocked PGE2 synthesis to the same degree as seen with the nonselective COX inhibitor ibuprofen. Consistent with the immunohistochemical findings (Fig. 2D), it appears that the majority of COX-2 activity is localized in the luminal endothelial cell compartment.

Contractile effects of COX-1 and COX-2 inhibitors. In the presence of 30% O2 (PO2 175–200 mmHg, 1.31–1.50 kPa), rings of ductus arteriosus contracted spontaneously to a tension that was 30% of the rings' net active tension (Fig. 4). The nonselective COX inhibitor indomethacin caused an additional dose-dependent increase in tension (Fig. 4C). Both the selective COX-1 inhibitor VSA and the selective COX-2 inhibitor NS-398 caused dose-dependent increases in ductus tension at concentrations that were specific for their respective enzymes (1, 12, 19, 24). At the highest concentrations tested, the contraction caused by NS-398 (5 × 10^-6 M) was ~66% of the contraction induced by VSA (3 × 10^-3 M) (Fig. 4). VSA contracted the ductus to the same extent as the nonselective COX inhibitor indomethacin (Figs. 4 and 5D). Neither NS-398 nor indomethacin caused any additional increase in tension after a VSA-induced contraction (Fig. 5, B and D). In contrast, after a contraction induced by the selective COX-2 inhibitor NS-398, both VSA and indomethacin produced an additional increase in tension (Fig. 5, A and C).

Inhibitors of both COX-1 and COX-2 decreased the rate of PGE2 release into the surrounding bath solution by rings of ductus arteriosus (Fig. 6). The rate of release was diminished comparably by VSA, NS-398, and indomethacin. Similarly, the rate of release of the stable metabolite of prostacyclin 6-keto-PGF1α was decreased by both COX-1 and COX-2 inhibitors (Fig. 6).

DISCUSSION

The results of the current study in fetal lambs are markedly different from those in the fetal pig (14). We
found that the fetal lamb ductus arteriosus expressed both COX-1 and COX-2 (Figs. 1 and 2). Accordingly, inhibitors of both COX-1 and COX-2 decreased PG production by homogenates of the lamb ductus arteriosus (Fig. 3). In addition, inhibitors of both COX-1 and COX-2 caused significant constriction of the lamb ductus arteriosus (Figs. 4 and 5). Thus COX-2 exerts a significant contribution to PG formation and vasomotor tone in the fetal lamb ductus arteriosus.

We found COX-2 primarily in the endothelial cells lining the ductus lumen, whereas both endothelial and smooth muscle cells expressed COX-1 (Fig. 2). This latter observation was supported by the finding that inhibitors of both COX-1 and COX-2 affected PGE2 production when the ductus had an intact endothelium; however, when the luminal endothelium was removed, the COX-1 inhibitor became the major selective inhibitor that affected PGE2 production. Similarly, we found that removal of the ductus luminal endothelium caused a marked reduction in the contractile effects of the COX-2 inhibitor NS-398 (n = 2, data not shown).

Although the contraction produced by NS-398 was only two-thirds that observed with the selective COX-1 inhibitor (VSA) or the nonselective COX inhibitor (indomethacin) (Figs. 4 and 5), the inhibitory effects on PGE2 release were comparable among the three COX inhibitors (Fig. 6). Therefore, the magnitude of the reduction in PGE2 release, caused by the COX inhibitors, did not correlate with the effects of the inhibitors on ductus contractility. One possible explanation for this discrepancy is that PGI2 (rather than PGE2) may play a more important role in regulating ductus patency than had been appreciated previously (4, 8). Interestingly, the effects of VSA, NS-398, and indomethacin on 6-keto-PGF1α release parallel their effects on ductus contractility (Figs. 5 and 6). PGI2 is the main product of PGH2 metabolism in the lamb ductus. It exceeds PGE2 production by 10-fold (30, 34). However, PGI2 is 100–1,000 times less potent than PGE2 in causing relaxation of the ductus arteriosus in vitro (4, 8, 35, 36); this makes PGI2 less likely to play a major role in ductus relaxation.

Another possible explanation for the discrepancy between the COX inhibitors’ effects on PGE2 concentrations in the organ bath and their effects on tissue contractility is that bath concentrations may not reflect the actual intramural PGE2 production rate. Most of the PGE2 synthesized by the intact ductus ring is made within the muscle media (9). As a result, the PGE2 released from the muscle cells must diffuse through the tissue’s interstitium before it can get into the bath solution. It has recently been found that most cells take up PGs via a specific transporter (20). The affinity of the transporter for PGE2 is >100 times that for 6-keto-PGF1α; this makes PGE2 the most readily transported PG (18). We speculate that most PGE2 made by the muscle media is taken up by neighboring cells before it reaches the bath solution. If this scenario is correct, then the bath solution more accurately reflects PGs released by the surface of the ductus ring. Because COX-1 and COX-2 are not equally divided between the muscle media and the luminal endothelium (Fig. 2), the PGE2 released into the bath may not be an accurate predictor of either the relative contributions of the two COX-isozymes to the overall production of PGE2 or the contraction produced by selective COX inhibitors.

We also observed that COX-1 and COX-2 blockers exerted different inhibitory effects on PGE2 and 6-keto-PGF1α release from ductal rings (Fig. 6). For example, the selective COX-1 inhibitor VSA blocked 6-keto-PGF1α and PGE2 release by 98 and 61%, respectively; therefore, the effectiveness ratio of VSA for blocking 6-keto-PGF1α:PGE2 was 1.6:1.0. In contrast, the effectiveness ratio for the selective COX-2 inhibitor NS-398 was only 0.99:1.00 and that for the nonselective COX inhibitor indomethacin lay in between (1.32:1.00). A possible explanation for such differential effects of COX inhibitors on PGE2 and PGI2 release (reflected by 6-keto-PGF1α) is that PGI2 synthesis is more tightly linked to COX-1 than to COX-2. In support of this inference, both COX-1 and PGI2 synthase are cell membrane-bound microsomal enzymes (27, 39), whereas COX-2 is mainly localized in the nuclear envelope at a site more distant from PGI2 synthase (37). Because PGE2 is either synthesized nonenzymatically from PGH2 (28, 34) or by PGE2 isomerase in the cytosol (9), its rate of production may not be as tightly linked to the specific location of COX-1 or COX-2 (37).

The presence of both COX-1 and COX-2 in the ductus is not unique to the lamb. We have recently found that the fetal ductus arteriosus of the baboon also expresses both COX-1 and COX-2. The distribution and relative quantities of the two enzymes in the baboon ductus appear to be similar to our findings in the fetal lamb (R. I. Clyman and S. Seidner, unpublished results).

**Perspectives**

Altogether, our studies reveal an important role for both COX-1 and COX-2 in PG production and vasomotor tone of the fetal ductus in vitro. The relative roles of COX-1 and COX-2 in vivo remain to be addressed. It may be that the activity of either isozyme is sufficient to maintain ductus patency; e.g., genetically engineered fetal mice lacking COX-1 (21) or COX-2 (11) do not have ductus-related problems in utero. On the other hand, these knockout mice may not reflect acute conditions where compensation by the alternate COX isozyme has not had a chance to occur. In fact, preliminary observations in our laboratories reveal that administration of selective COX-1 or COX-2 inhibitors to the fetal lamb produces significant ductus constriction in vivo (Y. Takahashi, C. Roman, and R. I. Clyman, unpublished results). At this time, caution should be used when recommending COX-2 inhibitors for use in pregnant women.

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