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Function of cyclo-oxygenase-1 and cyclo-oxygenase-2 in the ductus arteriosus from foetal lamb: differential development and change by oxygen and endotoxin

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1 Prenatal patency of the ductus arteriosus is maintained mainly by prostaglandin(PG) E_2. Here we have examined the relative importance of cyclo-oxygenase-1 (COX1) and cyclo-oxygenase-2 (COX2) for PGE_2 formation in the foetal lamb ductus (0.65 gestation onwards).

2 Using fluorescence microscopy and immunogold staining, COX1 appeared more abundant than COX2 in endothelial and smooth muscle cells, and this difference was greater before-term. Inside muscle cells, COX1 and COX2 immunoreactivity was located primarily in the perinuclear region. Endotoxin, given to the lamb *in utero* ($\sim$0.1 μg kg^{-1}), caused COX2 upregulation, while an opposite effect with disappearance of the enzyme followed endotoxin treatment *in vitro* (100 ng ml^{-1}). COX1 immunoreactivity remained virtually unchanged with either treatment; however, this isoform as well as any induced COX2 migrated towards the outer cytoplasm.

3 The COX2 inhibitor L-745,337 (1–10 μM) contracted the isolated ductus at term, the response being almost as high as that to indomethacin (dual COX1/COX2 inhibitor) over the same dose-range. Conversely, L-745,337 was relatively less effective in the premature.

4 Pretreatment of the premature *in vivo* with endotoxin enhanced the contraction of the ductus to L-745,337, while *in vitro* endotoxin had a variable effect.

5 The premature ductus exhibited a stronger contraction to L-745,337 following exposure to oxygen. On the other hand, the oxygen contraction, which is modest before-term, was enhanced by L-745,337.

6 We conclude that COX1 and COX2 develop unevenly in the ductus. While both enzymes contribute to PGE_2 formation at term, COX1 is the major isoform in the premature. COX2, however, may acquire greater importance before-term following physiological and pathophysiological stimuli.

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Abbreviations: BSA, bovine serum albumin; COX, cyclo-oxygenase; ET-1, endothelin-1; FITC, fluorescein isothiocyanate; L-745,337, 5-methanesulphonamido-6-(2,4-difluorothiophenyl)-1-indanone; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; OCT, optimum cutting temperature compound; PBS, phosphate buffer solution; PG, prostaglandin

Introduction

It is now established that a key enzyme in the prostaglandin (PG) synthetic pathway, cyclo-oxygenase (COX), exists in two isoforms, COX1 and COX2, differing in tissue distribution, inducibility and location inside cells (Murakami *et al*., 1994; Smith *et al*., 1996; Spencer *et al*., 1998; Vane *et al*., 1998). While COX1 is constitutively expressed and does not require priming for full activity, typically COX2 becomes evident in response to certain agents, including pyrogens (Pritchard *et al*., 1994; Murakami *et al*., 1994; Smith *et al*., 1996). There are exceptions, however, to this rule. For example, COX1 is upregulated by oxygen in the foetal pulmonary vasculature (Shaul *et al*., 1993; Brannon *et al*., 1994; North *et al*., 1994), and both COX isoforms are found naturally in the amnion and chorion (Slater *et al*., 1995; Gibb & Sun, 1996). In fact, COX2 becomes the predominant enzyme in foetal membranes during the period immediately preceding labour (Slater *et al*., 1995; Gibb & Sun, 1996). A similar arrangement, with COX2 overriding COX1 as the PG-forming enzyme, is also seen in foetal and adult brain (Kaufmann *et al*., 1997).

Knowledge of the organization of the COX system is particularly important when dealing with processes which are critically PG-dependent. One such process is prenatal patency of the ductus arteriosus, with ample evidence implicating PGE_2, both locally formed and blood-borne, as the prime effector (Clyman, 1987; Coceani & Olley, 1988; Smith, 1998). This PGE_2-based relaxing mechanism develops early in gestation – indeed, its intraductal component is relatively more effective before term (Clyman *et al*., 1978; Coceani *et al*., 1979) – and in the premature is potent enough to

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override the constrictor action of oxygen (Smith, 1998). Hence, characterization of the functional COX(s) in the ductus is important not only for conceptual but also for practical reasons. Specifically, with the development of isoform-specific COX inhibitors (Warner *et al*., 1999), one may aim to a targeted treatment of infants with persistent ductus or the safer management of conditions in the pregnant mother, such as premature labour, still being treated with conventional nonsteroidal anti-inflammatory drugs lacking enzyme selectivity (Norwitz *et al*., 1999). Available data afford a partial view of this subject. The pig ductus is apparently endowed only with COX1 at 0.75 gestation and acquires both enzymes shortly after birth (Guerguerian *et al*., 1998). The lamb ductus, on the other hand, has both COX isoforms at term gestation, but they are reportedly confined to endothelial cells (Clyman *et al*., 1999). Muscle cells have instead only COX1 (Clyman *et al*., 1999). In neither species, however, has the ductus been studied through successive stages of gestation, nor has the impact of potential COX inducers been assessed at any age.

The purpose of our investigation was to gain a better insight into the functional organization of the COX system in the ductus. This was achieved by examining the distribution of COX1 and COX2 in the preterm vs the term ductus and by concomitantly assessing the relative contribution of the two isozymes to the PG-based relaxing mechanism. In addressing the latter point, we compared the action of compound L-745,337 (a COX2 inhibitor) and indomethacin (a dual COX1/COX2 inhibitor) (Chang *et al*., 1995; Warner *et al*., 1999), bearing in mind that any contraction to either agent reflects primarily, if not exclusively, interference with PGE_2 formation. PGE_2 is, in fact, the most potent among the COX-derived relaxant products in the vessel (Clyman, 1987; Coceani & Olley, 1988; Smith, 1998). Lastly, we studied the effect on the PGE_2 mechanism of physiological (i.e. oxygen) and pathophysiological (i.e. pyrogen) stimuli which, in the clinical context, may influence the course and management of persistent patency of the ductus.

Methods

General procedure

Experiments were performed on pre-term (two age groups: 103–107 days, 0.7 gestation; 94–97 days, 0.65 gestation) and near-term (134–139 days gestation; term, 145 days) pregnant sheep of pure Southdown or Dorset breed, or Southdown/Dorset crossbreed. The procedures for anaesthesia, Caesarean delivery of the foetuses, and isolation of the ductus arteriosus have been described previously (Coceani *et al*., 1986). In certain cases, however, foetuses, whether premature (103–107 days gestation) or near-term (134–139 days gestation), were treated with endotoxin at the time of surgery. For this purpose, the head of the animal was exteriorized while taking care to cover the snout with a glove to prevent breathing, and endotoxin at the intended dose of 0.1 $\mu g\ kg^{-1}$ was administered over 5 min *via* the external jugular vein (total volume, 1.2 and 4.5 ml with premature and near-term foetuses, respectively). This dose, which changed slightly after being corrected for the weight of the animal at the end of the experiment (0.099 ± 0.004 and 0.102 ± 0.005 $\mu g\ kg^{-1}$ respectively for the premature and near-term foetus), has been selected from data in the literature (Snapper *et al*., 1998) and after noting the absence of distress signs in preliminary trials. The treated animal was then placed back in the uterus, which was repositioned within the abdomen, and 3 h were allowed to elapse before proceeding with Caesarean delivery and the actual procedure. The ductus arteriosus, whether untreated or endotoxin-primed, was removed and placed in ice-cold Krebs solution gassed with 5% CO_2 in N_2. No difference was noted between vessels belonging to the two groups, except for a modest constriction being evident at times after endotoxin treatment. All specimens were freed of loose connective tissue before further work up.

Morphological analysis

Freshly dissected blood vessels from untreated foetuses were processed immediately or, at certain ages (near-term and 94–97 days gestation), were incubated first (2 h; 37°C) in Krebs medium containing endotoxin (100 ng ml^{-1}). The medium was gassed with 2.5% O_2 : 5% CO_2 in N_2, and incubation without endotoxin served as a control. No prior incubation was used instead with vessels from animals (near-term and 103–107 days gestation) treated with endotoxin *in utero*. Being relatively small, the youngest foetuses (94–97 days gestation) did not appear suitable for *in vivo* treatment with endotoxin.

For immunofluorescence microscopy, specimens were flash frozen in embedding medium (Tissue-Tek optimum cutting temperature compound (OCT), Sakura Finetek, Torrance, U.S.A.) by use of liquid nitrogen and were then cut in a cryostat. In all cases, the plane of cutting was perpendicular to the main axis of the vessel. Cryostat sections were mounted on salinated slides and were fixed for 30 min with 4% paraformaldehyde in 0.1 M phosphate buffer (PBS, pH 7.2). Before the labelling, the fixative was removed by thorough rinsing with PBS (four times, 5 min each), and any residual aldehyde was neutralized by treatment with 0.15% glycine-0.5% bovine serum albumin (BSA) in PBS. After an additional rinsing in PBS, tissues were incubated (1 h; room temperature) with antiserum against COX1 or COX2 (dilution, 1 : 200 for both) with BSA-supplemented PBS as a vehicle. The antiserum was subsequently washed off with PBS, and another incubation was carried out in the dark (1 h; room temperature) with a secondary antibody (goat antirabbit IgG) conjugated to FITC (dilution, 1 : 50). Controls included the use of an irrelevant antiserum (goat antimouse IgG), preabsorption of the primary antiserum with enzyme protein, or omission of the primary antiserum. In addition, as a positive control for the visualization of muscle, certain specimens were treated with an antiserum against α-actin (dilution, 1 : 600). Sections were then examined with a Leica TCDS confocal microscope and representative images were saved. Three sections were taken from each preparation and about five fields were viewed per section.

For transmission immunoelectron microscopy, specimens were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). The fixed specimens were then divided into 1-mm^3 blocks and were kept in fixative for 2 h. Afterwards, they were stored in PBS containing sodium azide (20 mM) and, before being cut, they were infused with 2.3 M sucrose overnight. Tissue blocks were mounted on aluminum pins

and plunge frozen in liquid nitrogen. Cryosections (−100°C) of approximately 100-nm thickness were prepared with glass knives in a Reichert Jung Ultracut E microtome equipped with a FC4D cryochamber. These ultrathin sections were transferred to glow-discharged carbon-formvar nickel grids by use of a loop of molten sucrose. After any residual aldehyde had been neutralized by treatment with 0.15% glycine – 0.5% BSA in PBS, sections were washed repeatedly with BSA-supplemented PBS (0.5%) and were then incubated with antiserum against either COX1 or COX2 (dilution, 1 : 200 for both). The antiserum was subsequently removed by repeated rinsing with BSA-supplemented PBS, and specimens were treated for 60 min with gold-labelled goat antirabbit immunoglobulin (dilution, 1 : 20). After another thorough wash with PBS and distilled water in succession, the sections were embedded in a thin film of methylcellulose containing 0.2% uranyl acetate and then were viewed and photographed in a JEOL model 1200 Ex II transmission electron microscope. Specificity of binding was confirmed in the same way as in the immunofluorescence study (see above). Gold particles were counted in myocytes either at random within the main immunoreactive regions (perinuclear and peripheral cytoplasm, see Results; 3 fields $cell^{-1}$ at 25,000 × magnification) or in the whole cell (average 3.8 fields $cell^{-1}$ at 25,000 × magnification). The number of cells examined in each specimen varied from a minimum of 25, when the plan of section crossed the perinuclear cytoplasm, to a maximum of 50, and three specimens were used for each condition. Negative images were digitized and quantification of particles in the entire cell (expressed as particles $cell^{-1}$) as well as in discrete regions of each cell (expressed as particles μm^{-2}) was obtained with a morphometric programme (NIH Image 1.53).

Pharmacological analysis

As previously reported (Coceani *et al*., 1986), the ductus arteriosus was opened and then cut perpendicularly to the main axis to yield one to three strips, depending on the age of the foetus and the attendant length of the vessel. Ductal strips were used intact in all, but few, experiments in which the endothelium was removed. Endothelium-free strips were prepared by rubbing filter paper (Whatman No. 41) over the intimal surface. Preparations were mounted in individual 10-ml baths between a stationary glass rod and an isometric tension transducer (Grass FT-03C) coupled to a Grass polygraph. The initial load was applied in a single step (term ductus, 1.55 ± 0.01 g; 1 g weight = 9.8 mN) or a series of steps (preterm ductus, 1.39 ± 0.03 and 1.06 ± 0.01 g respectively at 103 – 107 and 94 – 97 days gestation), and preparations were stretched by about 50% of the original length to obtain an optimal tension output (Somlyo & Somlyo, 1964). However, ductal strips from endotoxin-treated lambs were less compliant to stretch, specifically at term, and the required load was generally greater (1.83 ± 0.08 g). Throughout this procedure care was taken not to damage the endothelium in preparations of the whole ductus.

Tissues were equilibrated in Krebs solution gassed with 2.5% O_2 : 5% CO_2 in N_2, and the same gas mixture or mixtures containing a higher oxygen content (15, 30, and 95%) were employed in the actual experiment. The 2.5% O_2 mixture mimicked the condition *in utero*, while higher oxygen concentrations duplicated the neonatal condition (Coceani *et al*., 1986). The latter concentrations, exceeding the normal range for the neonate, were selected as the ductus is thick and, consequently, there is a steep oxygen gradient across its wall (Fay, 1973). The study included several protocols with both control and endotoxin-treated preparations. Unless otherwise specified, experiments were carried out at 2.5% O_2. In all cases, the room was kept darkened.

Control ductus In protocol 1, compound L-745,337 was added to the perfusion fluid in cumulative doses (1, 2.8, and 10 μM), and its effect was assessed on the basal tone. Indomethacin was used for comparison at the same doses. An equivalent experiment was performed in protocol 2, except for the fact that the COX2 inhibitor was tested on the vessel both before and after exposure to increasing concentrations of oxygen (1, 5, 30, and 95%). The interval between oxygen priming and the second series of L-745,337 tests varied between 52 and 78 min (mean, 62), while oxygen exposure lasted 52 – 110 min (mean, 72). Individual differences in the development and reversal of the oxygen contraction explain this variable timing. In protocol 3, the sequence of oxygen tests was carried out before, during, and after treatment with an intermediate dose of L-745,337 (2.8 μM). Pretreatment with the inhibitor lasted 1 h. While protocol 1 was used with both premature (0.7 and 0.65 gestation) and term preparations, protocols 2 and 3 were limited to the premature (0.7 and 0.65 gestation) when, characteristically, the contraction to oxygen is incompletely developed (Smith, 1998). The idea in studying the oxygen/L-745,337 interaction in the premature was to ascertain whether oxygen, despite its modest constrictor effect, may alter COX function.

Endotoxin-treated ductus Endotoxin was employed to obtain COX2 induction as it may occur in the clinical situation. The pyrogen was administered *in vitro* (all ages) and *in vivo* (103 – 107 days gestation) (see above), using a dose schedule that, judging from available data (O'Sullivan *et al*., 1992; Cao *et al*., 1997; 1999), should ensure upregulation of the enzyme. The reason for limiting the *in vivo* treatment with endotoxin to premature animals was 2 fold: the practical need to be conservative with a protocol that is intrinsically complex and the expectation, based on preliminary results, that this age group would have shown the greatest impact of COX2 induction. For the *in vitro* treatment, endotoxin (100 ng ml^{-1}) was added to the perfusion fluid in two periods, a priming period of 1 h followed by a period, coinciding with the L-745,337 test, in which the pyrogen was kept in contact with the tissue for as long as it was required to the inhibitor to develop a full effect. The interval between the two treatment stages was 10 min, and L-745,337 was applied to the bath 9 – 11 min after the start of the second stage. Regardless of whether endotoxin treatment was carried out *in vitro* or *in vivo*, L-745,337 was tested on the baseline tone, hence duplicating the protocol 1 used with the control ductus (see above). However, certain preparations from preterm, endotoxin-treated (treatment *in vivo*) foetuses were also tested with L-745,337 before and after being primed with oxygen (i.e. according to protocol 2, see above). In this case, exposure to oxygen lasted 55 – 87 min (mean, 69) and the second set of L-745,337 applications was carried out 29 – 55 min (mean, 37) after oxygen.

To rule out any damaging effect of endotoxin, specifically on the endothelium (Brigham & Meyrick, 1986), in separate experiments bradykinin (1 pM – 1 μM) was tested on term ductal strips that had been pretreated with the pyrogen *in vitro* and *in vivo*. In either case, the tone of preparations had also been raised with indomethacin (2.8 μM) to avoid any confounding effect of prostaglandins (conceivably PGE_2) originating from both endothelial and extra-endothelial sources in response to bradykinin (Coceani *et al*., 1986; Bateson *et al*., 1999). The compound had a relaxant effect, expectedly endothelial nitric oxide synthase (eNOS)-linked, similar in all respects to that seen with control preparations (Coceani *et al*., 1994). This confirmed the structural integrity of the endothelium (results not shown). Concentrations of added endotoxin were also verified in the bathing fluid by the Limulus assay and results proved the absence of any loss through incubation (results not shown).

Solutions and drugs

The Krebs solution had the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1, $MgSO_4$ 0.9, dextrose 11.1 and $NaHCO_3$ 25. Potassium-Krebs solution (55 mM) was prepared by substituting NaCl with an equimolar amount of KCl. The pH of the solution was 7.4 after equilibration with gas mixtures containing 5% CO_2. Purified seminal vesicle COX1 and placental COX2, both from sheep, were supplied by Cayman (Ann Arbor, U.S.A.). COX1 and COX2 antisera, obtained from Merck Frosst (Montreal, Canada), were polyclonal and had been generated in the rabbit. Colloidal gold-labelled (particle diameter, 10 nm) goat antirabbit immunoglobulin and an antibody against α-actin were purchased from Amersham (Piscataway, U.S.A.), while FITC-conjugated goat antirabbit immunoglobulin and goat antimouse immunoglobulin were supplied by, respectively, Molecular Probes (Eugene, U.S.A.) and Miles Pharmaceuticals (Mississauga, Canada).

The following compounds were used: 5-methanesulphonamido-6-(2,4-difluorothiophenyl)-1-indanone (compound L-745,337; Merck Frosst); indomethacin (Sigma, St. Louis, U.S.A.); bradykinin acetate (Sigma) and endotoxin (lipopolysaccharide W, *E. coli*, serotype 055:B5) (Difco, Detroit, U.S.A.). L-745,337 and indomethacin were dissolved in distilled ethanol (10 mg ml^{-1}) before the preparation of the final solution in Krebs medium. Other substances dissolved readily in saline or Krebs medium. Doses of all compounds are given in molar concentrations and refer to their final concentration in the bath. Vehicle alone, without or with ethanol (maximum concentration in bath, 0.03 – 0.04%), had no effect on the vessel tone.

Analysis of data

Baseline contractile tension, which varied with the preparation and the experimental condition (see Results), refers to the net active tension (i.e., total tension minus applied tension) developed by the preparation prior to any treatment. Responses to test agents are given as the fractional change from baseline.

Data are expressed as the mean $\pm$ s.e. mean. Statistical comparison of two means was done by Student's *t*-test for paired or unpaired observations. Multiple comparisons were made with a two-way, repeated measures analysis of variance (ANOVA) followed by an F-test for simple effect. Differences are considered significant for $P<0.05$.

Results

Localization of cyclo-oxygenases 1 and 2

Epifluorescence microscopy demonstrated COX1 immunolabelling in the ductus arteriosus from the untreated term foetus, its intensity being high in endothelial and smooth muscle cells and faint instead in the fibroblasts (Figure 1B). Conversely, COX2 labelling was uniformly weak in all cells (Figure 1D). Endotoxin, given to the foetus *in utero*, did not produce any obvious change in COX1 immunoreactivity (Figure 1F). In contrast, COX2 immunoreactivity increased diffusely and became particularly strong in smooth muscle cells (Figure 1H). Treatment of the term ductus with endotoxin *in vitro* (results not shown), as in *in vivo*, had no effect on COX1 staining. However, COX2 staining, from being weak across the vessel wall, became undetectable. No such enzyme loss was observed when preparations were incubated in Krebs medium alone (results not shown). The ductus arteriosus from the premature foetus, whether untreated or endotoxin-treated, had a comparable immunoreactivity pattern, except for the fact that COX2 was barely expressed constitutively and showed only a modest upregulation after treatment with endotoxin *in vivo* (Figure 1A, C,E,G). Significantly, whatever the age no immunostaining could be detected for the enzymes when omitting the primary antibody or after treating tissues with either an irrelevant antibody (goat antimouse IgG) or an antibody that had been preincubated with the appropriate antigen (COX1 or COX2 protein).

When viewed by transmission immunoelectron microscopy, muscle cells from the term ductus exhibited greater immunogold reactivity for COX1 than COX2, regardless of whether the tissue had been processed fresh (77 ± 6 and 43 ± 8 gold particles $cell^{-1}$ for COX1 and COX2, respectively; $n=38$ and 36, $P<0.005$) or after incubation in Krebs medium (74 ± 11 and 37 ± 10 gold particles $cell^{-1}$ for COX1 and COX2, respectively; $n=26$ and 41, $P<0.005$). *In vivo* treatment with endotoxin resulted in a modest, albeit significant, increase in the number of COX1-linked gold particles (from 76 ± 4 to 89 ± 9 particles $cell^{-1}$; $n=48$ and 37, $P<0.05$) and a definitely greater enrichment in COX2-linked gold particles (from 45 ± 6 to 68 ± 8 particles $cell^{-1}$; $n=45$ and 29, $P<0.01$). No such change was seen in muscle cells following treatment with endotoxin *in vitro* and, in this case, COX1 immunogold reactivity remained constant (82 ± 7 particles $cell^{-1}$, $n=33$) and COX2 reactivity, instead of increasing, became unmeasurable. A difference in the relative expression of the two enzymes was also noted in ductal muscle cells of the premature, although the number of gold particles was consistently lower (particles $cell^{-1}$ at 0.7 gestation: COX1 50 ± 12, COX2 30 ± 5, $n=25$ and 39, respectively; particles $cell^{-1}$ at 0.65 gestation: COX1 48 ± 7, COX2 22 ± 4, $n=41$ and 36, respectively). However, COX2 was still measurable by the immunogold labelling procedure, despite its near absence on immunofluorescence (Figure 1C). This apparent incongruence originates from the fact that no

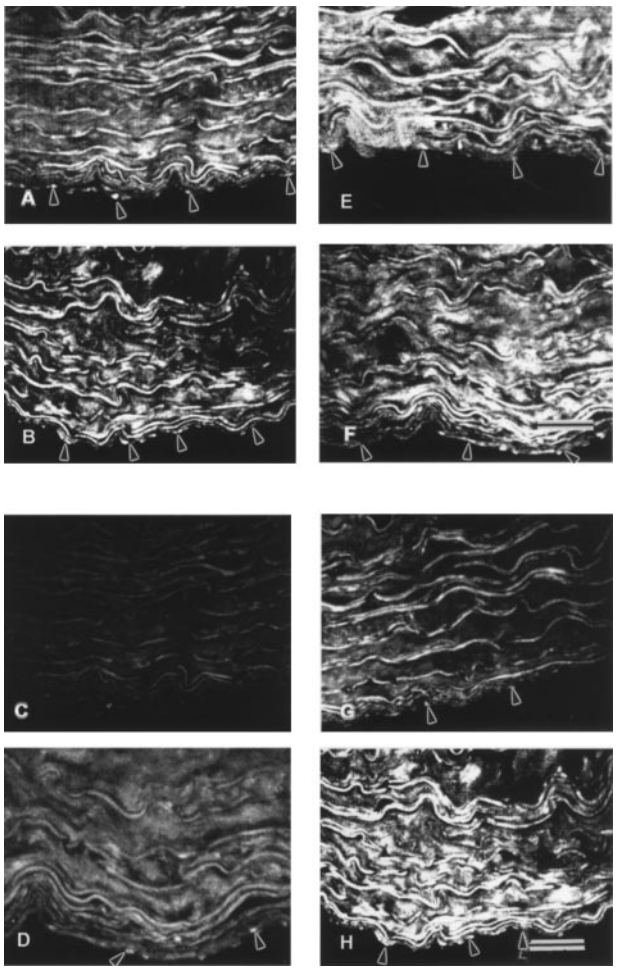

Figure 1 Immunofluorescence micrographs of the ductus arteriosus from control vs endotoxin-treated (treatment *in vivo*) foetal lambs at 0.7 gestation and at term. (A) Control preparation at 0.7 gestation, section incubated with COX1 antiserum; note the labelling of endothelial (arrowheads in this and the following panels) and smooth muscle cells. (B) Control preparation at term gestation, section incubated with COX1 antiserum. (C) Sequential section to (A) incubated with COX2 antiserum; note the faint staining compared to COX1. (D) Sequential section to (B) incubated with COX2 antiserum; COX2 immunoreactivity increases with gestation age, although it still appears weak compared to COX1 immunoreactivity. (E) Endotoxin-treated preparation at 0.7 gestation, section incubated with COX1 antiserum; note that immunolabelling is essentially the same as in the control preparation. (F) Endotoxin-treated preparation at term gestation, section incubated with COX1 antiserum; immunolabelling virtually unchanged compared to controls. (G) Sequential section to (E) incubated with COX2 antiserum; note the increased immunoreactivity relative to control of the same age. (H) Sequential section to (F) incubated with COX2 antiserum; immunoreactivity is stronger compared to both the control of the same age and the endotoxin-treated premature. Note that under control conditions immunoreactivity for both COX1 and COX2 showed no obvious difference at 0.65 vs 0.7 gestation (results not presented). Bar represents 250 μm.

antigenicity at all occurred between the perinuclear and peripheral regions of the cytoplasm, thus making the cells virtually indiscernible by fluorescence analysis. Otherwise, in general accord with immunofluorescence data endotoxin *in vivo* had no major effect on either immunogold label, while endotoxin *in vitro* caused disappearance of COX2 without altering COX1. Whether effective or not in promoting enzyme upregulation, endotoxin treatment led to a redistribution of both COX1 (*in vivo* and *in vitro* treatment) and COX2 (*in vivo* treatment) inside cells. As exemplified by Figures 2A,B and 3A,B, in the untreated tissue antigenicity was predominant in the perinuclear cytoplasm and related organelles. However, after tissues had been exposed to endotoxin, both COX1- and COX2-linked gold particles were found in the outer rather than the inner region of the cytoplasm, often clustered along the sarcolemma (Figures 2C,D and 3C,D,E). A modest particle enrichment, compared to findings with endotoxin, was also seen in the peripheral cytoplasm following incubation with medium alone. Table 1 reports the actual number of particles in different regions of the cell before and after treatment with endotoxin at the two foetal ages. In addition, it shows that with either control condition (i.e. no incubation and incubation) COX1 and COX2 immunoreactivity increased with age ($P<0.05$ or better by ANOVA or Student's *t*-test, as applicable), the only

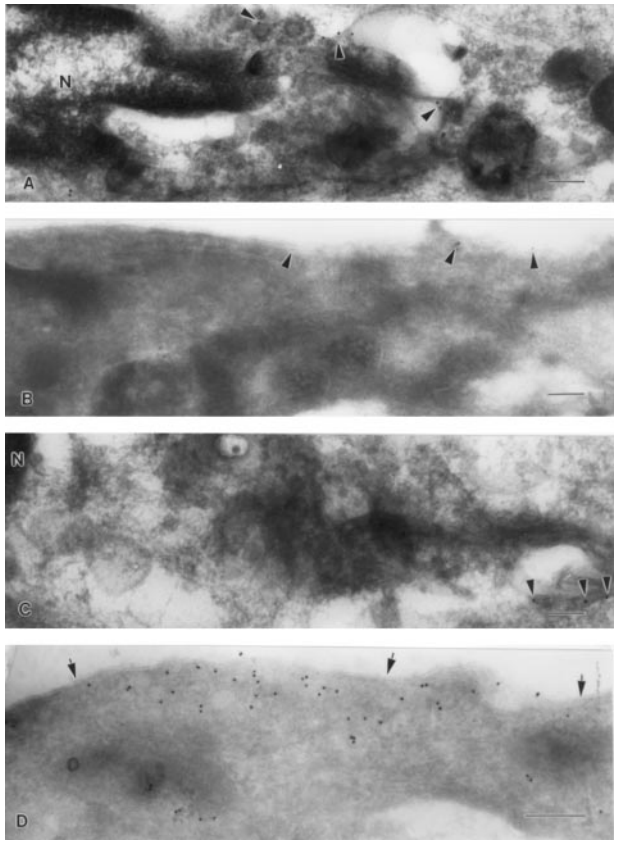

Figure 2 Transmission electron micrograph of ultrathin cryosections from the muscle layer of the foetal ductus arteriosus at term (untreated animal vs animal treated with endotoxin *in vivo*). In all panels, sections have been reacted with antiserum against COX1. (A) Immunogold labelling of the perinuclear region in the untreated tissue; note that label is found close to the nucleus (N) in Golgi apparatus and Golgi-derived vesicles (arrowheads). (B) Immunogold labelling in the untreated tissue; note the sparse label in the peripheral cytoplasm along the plasma membrane (arrowheads). (C) Immunogold labelling in the endotoxin-treated tissue; label is found in vesicular and membraneous structures distant from the nucleus (N). (D) Immunogold labelling in the endotoxin-treated tissue; note the abundance of label in the peripheral cytoplasm in proximity of the plasma membrane (indicated with arrows). Bar represents 0.2 μm.

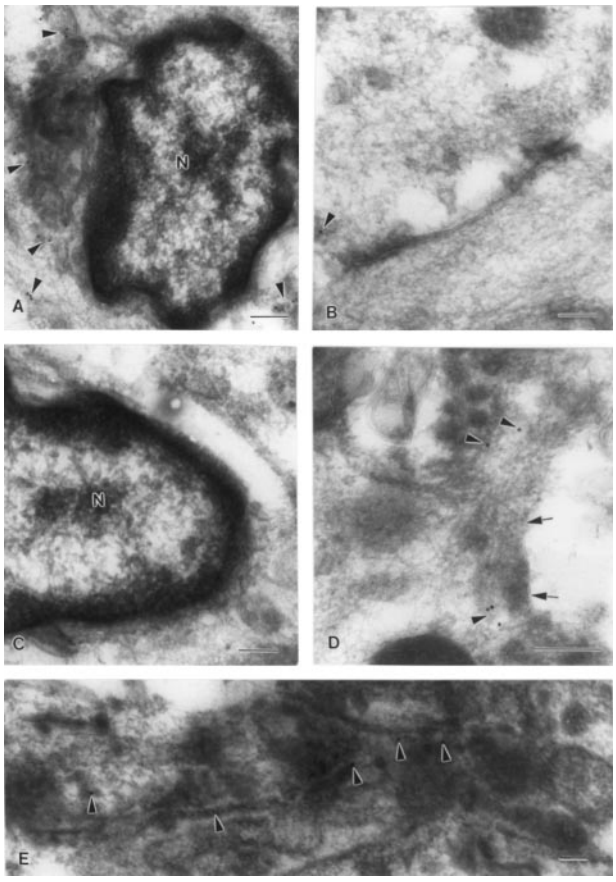

Figure 3 Transmission electron micrograph of ultrathin cryosections from the muscle layer of the foetal ductus arteriosus at term (untreated animal vs animal treated with endotoxin *in vivo*). In all panels, sections have been reacted with antiserum against COX2. (A) Immunogold labelling of the perinuclear region in the untreated tissue; note the abundance of label (arrowheads) in the Golgi apparatus, Golgi-derived vesicles and sarcoplasmic reticulum surrounding the nucleus (N). (B) Immunogold labelling in the untreated tissue; note the distribution of sparse label (arrowhead) in the peripheral cytoplasm of two adjacent muscle cells. (C) Immunogold labelling in the endotoxin-treated tissue; the cytoplasm adjacent to the nucleus (N) is void of any label. (D) Immunogold labelling in the endotoxin-treated tissue; note label (arrowheads) in the peripheral cytoplasm close to the plasma membrane (indicated with arrows). (E) Immunogold labelling in the endotoxin-treated tissue; note the high label density in the sarcoplasmic reticulum (arrowheads). Bar represents 0.2 μm.

exception being COX1 over the interval between 0.7 gestation and term where the change did not reach significance.

Effect of L-745,337 on vascular tone

Ductal strips developed active tension during equilibration in Krebs medium gassed with 2.5% O_2. Typically, their tone rose gradually to a peak and then abated to a stable level. A further contraction occurred upon exposure to higher oxygen concentrations and a maximum was reached with 95% O_2. Likewise, tissues contracted maximally to excess potassium. No change in this general pattern was noted with either the age of the animal or prior exposure of the foetus to endotoxin. However, while tension values were lower with pre-term than term preparations, at any age endotoxin-primed preparations settled at a higher baseline compared to controls. Table 2 provides a breakdown of findings under different experimental conditions.

Term foetus Near-term ductal preparations contracted to compound L-745,337. The contraction was slow in development and its magnitude correlated with the concentration over the range between 1 and 10 μM (Figure 4A). L-745,337 was as effective as indomethacin at all, but the lowest, concentrations tested (Figure 4B). Compared to the intact ductus, the endothelium-denuded ductus developed a smaller contraction to L-745,337, the peak response being on the average reduced by 35% (10 μM) to 73% (1 μM) ($n=3$). When exposed to endotoxin in the bath, preparations lost completely their tone but, this notwithstanding, contracted more strongly to L-745,337 (Figure 4A).

Premature foetus, 103–107 days gestation Compound L-745,337 (1–10 μM) was far less potent as a constrictor on the pre-term than the term ductus (Figure 5A), and this lesser effectiveness became particularly evident when using the response to excess potassium as a reference. While at term the contractions to L-745,337 (10 μM) and potassium were similar in magnitude (Figure 4A,C), at 0.7 gestation the contraction to the former agent was a fraction (about 37%) of that to the latter (Figure 5A,C). Conversely, no such age-related loss in efficacy *vis-à-vis* potassium was noted with indomethacin (Figure 5B,C). Furthermore, in the premature as at term, indomethacin elicited a maximal response over the entire dose-range (Figure 5B). L-745,337 tended to be more effective on preparations that had been exposed to oxygen (Figure 6A) as, on the other hand, the oxygen contraction was enhanced by the COX2 inhibitor (Figure 6B).

Endotoxin altered unevenly the response of the ductus to L-745,337 depending on whether treatment was carried out *in vitro* or *in vivo*. When added to the organ bath, endotoxin relaxed the vessel and the magnitude of the relaxation was comparable to that observed at term (see above). Once fully relaxed, however, the premature vessel, unlike the term vessel, contracted less (Figure 5A), and not more (Figure 4A), to L-745,337. Conversely, an enhanced response to L-745,337 was observed with preparations from animals that had been treated with endotoxin *in vivo* (Figure 5A). This enhancement became even greater if tissues, besides being primed with endotoxin, had also been exposed temporarily to oxygen *in vitro* (results not shown).

Premature foetus, 94–97 days gestation The efficacy of L-745,337 was also reduced at this early stage of gestation and, as evident from Figure 7A, the attendant contraction was barely measurable. However, the susceptibility of the ductus to the inhibitor increased upon exposure to oxygen (Figure 7B) and this effect, unlike that seen at 0.7 gestation (Figure 6A), attained clear significance. Nevertheless, the enhanced L-745,337 contraction was still far from matching that of the untreated preparation at term. The enhancing effect of L-745,337 on the oxygen response, which was seen with the 0.7-gestation group (Figure 6B), did not reach instead significance (results not shown). Furthermore, treatment with endotoxin *in vitro*, while causing complete reversal of tone in these as in more mature preparations, had a variable and insignificant effect on the response to L-745,337 (Figure 7A).

Table 1 Cyclo-oxygenase (COX)1 and COX2 immunogold labelling of muscle cells of the foetal lamb ductus arteriosus: gestational changes and effect of endotoxin

		No. of gold particles (particles μm^{-2})					
Region of the cell	*Condition of tissue*	*0.65 gestation*		*0.7 gestation*		*Term gestation*	
		COX1	*COX2*	*COX1*	*COX2*	*COX1*	*COX2*
Cytoplasm, perinuclear	No incubation, control	8.9±2.6	8.4±2	13±3.5	11±2.4	14.2±3.1	14.8±1.6
	No incubation, endotoxin *in vivo*	–	–	2.6±1.2*	3.2±0.8*	2.6±0.9*	2.7±1.9*
	Incubation, control	7.5±1.7	7.2±3.2	–	–	15.2±1.5	14.2±2.9
	Incubation, endotoxin *in vitro*	5.5±1.5†	ND	–	–	2.6±0.5††	ND
Cytoplasm, peripheral	No incubation, control	2.3±0.8	0.9±0.04	2.9±0.7	2.5±0.8	3±0.9	1.9±0.5
	No incubation, endotoxin *in vivo*	–	–	13.3±3.2*	7.3±1.4*	10.2±1.8*	11.4±2.4*
	Incubation, control	2.4±0.4	$2.8\pm0.05^{++}$	–	–	2.4±1.3	$2.4\pm0.8^{++}$
	Incubation, endotoxin *in vitro*	11.1±3††	ND	–	–	8.6±1.3††	ND

Gold particles (mean±s.e.mean) were counted in transmission electron micrograph of cells at different stages of gestation, before and after treatment with endotoxin *in vitro* or *in vivo* (for details, see Methods). ND, not detectable. * vs no incubation, control, $P<0.01$ (Student's *t*-test); † vs incubation, control, $P<0.05$ (Student's *t*-test); †† vs incubation, control, $P<0.01$ (Student's *t*-test); $^{++}$ vs no incubation, control, $P<0.05$ (Student's *t*-test).

Table 2 Contractile tension of the ductus arteriosus from untreated vs endotoxin-treated (treatment *in vivo*) foetal lambs at different gestation ages

		Contractile tension (g)			
		2.5% O_2			
Age (d)	*Condition*	*Maximal*	*Steady-state*	*95% O_2*	*K^+, 55 mM*
134–139	Control	2.5±0.2 (24)	0.48±0.08 (24)	3.9±0.2 (3)	3.6±0.3 (7)
	Endotoxin	1.9±0.1 (9)	1±0.19 (9)*	4.1±0.6 (3)	–
103–107	Control	0.38±0.04 (26)	0.15±0.03 (26)	1.5±0.1 (11)	2.3±0.2 (23)
	Endotoxin	0.72±0.15 (11)*	0.38±0.11 (11)*	1.8±0.2 (7)	2±0.2 (10)
94–97	Control	0.43±0.04 (17)	0.36±0.05 (17)	1.2±0.1 (12)	1.8±0.1 (17)
	Endotoxin	–	–	–	–

Data are mean±s.e.mean for the number of experiments given in parentheses. Note that values of tension at 2.5% O_2 refer to the entire tension developed by the preparation (i.e. total tension minus applied tension), while responses to 95% O_2 and excess potassium (at 2.5% O_2) are expressed as the increase in tension over baseline (i.e. the steady-state tension at 2.5% O_2; for details, see Methods). * vs control at the same age, $P<0.02$ (Student's *t*-test).

Discussion

This study, while confirming that the ductus arteriosus responds to oxygen and other agents in an age-related fashion (Smith, 1998), documents an uneven development of COX isoforms. COX1 is the dominant enzyme throughout gestation and, consequently, is primarily responsible for the operation of the PGE_2-based relaxing mechanism. Conversely, COX2 is weakly expressed in the premature and only at term becomes active enough to complement COX1 in this role. At any age, COX2 is constitutively expressed and, in this respect, the ductus departs from vessels studied by other investigators in which the enzyme is marginally evident or absent altogether (Habib *et al*., 1993; Pritchard *et al*., 1994). In addition, this native COX2 is liable to induction by endotoxin and, to a lesser degree, by oxygen. The latter finding is unexpected and extends to COX2 a property, i.e. the susceptibility to oxygen upregulation, which until now has been linked exclusively to COX1 (Shaul *et al*., 1993; Brannon *et al*., 1994; North *et al*., 1994). The mechanism of action of oxygen remains to be elucidated. However, we would assume that enzyme activation does not take place directly but involves an intermediary agent that could be identified with endothelin-1 (ET-1). Supporting this possibility is the fact that oxygen promotes ET-1 formation in the ductus (Coceani *et al*., 1999; Coceani & Kelsey, 2000) and that the peptide may, in turn, upregulate selectively COX2 (Kester *et al*., 1994). Leaving aside this point, the concurrence of COX2 upregulation by oxygen with an enhancement of the oxygen contraction during L-745,337 treatment points to a mutual control between oxygen and COX2-derived PGE_2 in adjusting muscle tone.

COX isozymes were found in both endothelial and smooth muscle cells of the ductus but, considering the marked loss in L-745,337 effectiveness upon removal of the endothelium, the former site is ascribed greater importance in sustaining COX2 function. Even with this differential enzyme distribution, however, our results depart from those of Clyman *et al*. (1999), obtained in the lamb too, showing a strictly endothelial location for COX2. The reason for this inconsistency is not clear, but methodological differences may be a factor. COX antisera were obtained from different sources in the two investigations. Furthermore, in the approach taken by Clyman *et al*. (1999) the relative

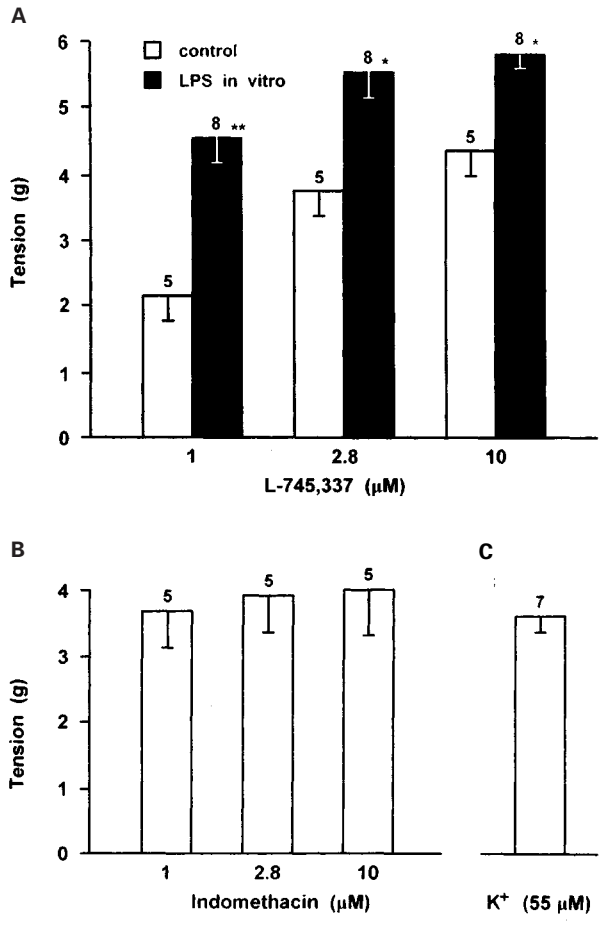

Figure 4 Intact strip of ductus arteriosus from near-term foetal lamb. Contractile responses to L-745,337 (A), indomethacin (B), and excess potassium (C). L-745,337 effect was measured before and during treatment with endotoxin (LPS) *in vitro* (for details, see Methods). For each group, number of experiments are given above the columns, and a significant difference in panel (A) between control and treatment groups is indicated with $*P<0.05$ or $**P<0.01$ (ANOVA).

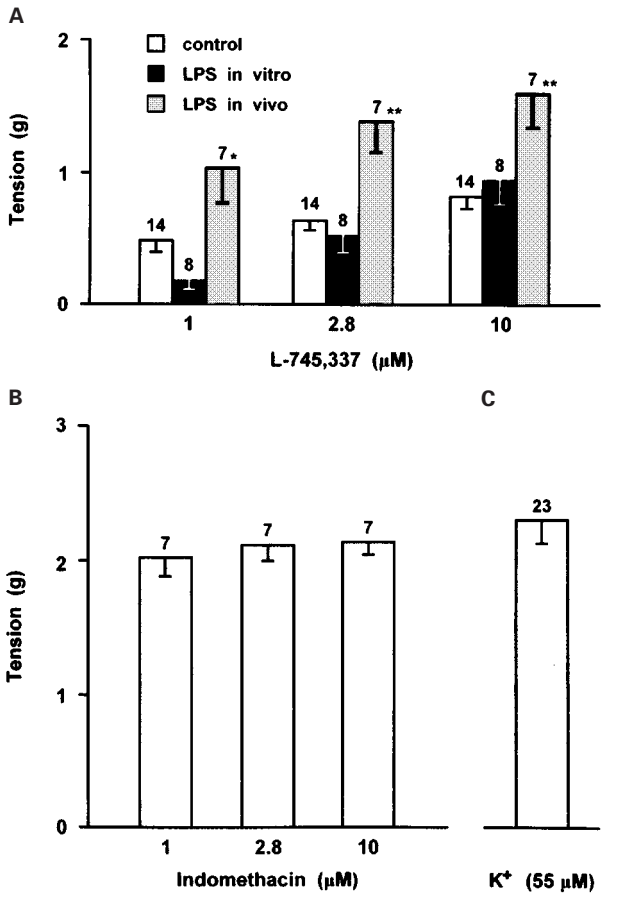

Figure 5 Intact strip of ductus arteriosus from foetal lamb at 103–107 days gestation. Contractile responses to L-745,337 (A), indomethacin (B), and excess potassium (C). L-745,337 effect was measured in control tissues and tissues treated with endotoxin (LPS) *in vitro* and *in vivo* (for details, see Methods). For each group, number of experiments are given above the columns, and a significant difference in panel (A) between control and treatment groups is indicated with $*P<0.05$ or $**P<0.01$ (ANOVA). Note that the inhibitory effect of *in vitro* endotoxin became significant when the analysis was limited to the subgroup of experiments in which responses were measured in the same tissue before and during treatment (1 μM, $P<0.01$; 2.8 μM, $P<0.05$; $n=8$).

contribution of the endothelium vs smooth muscle to the generation of COX-2-linked PGE_2 was deduced from the metabolism of added arachidonic acid. An exogenous substrate may or may not behave as the endogenous substrate in gaining access to competing enzymes. Likewise, a methodological factor could account for the apparent absence, also reported by the same group (Guerguerian *et al.*, 1998), of COX2 in the ductus arteriosus from the foetal pig. The latter study, unlike the study in the foetal lamb (Clyman *et al.*, 1999), was carried out at 0.75 gestation, that is at an age in which COX2 is conceivably underexpressed and may be straddling detectability. In fact, we have found that the pig ductus shows COX2 immunoreactivity close to term, though its intensity is weak compared to COX1 (C. Ackerley and F. Coceani, unpublished observations).

By showing a differential expression of COX isozymes depending on the stage of development and intervening challenges, our study adds a new degree of complexity to the mechanism sustaining patency of the ductus *in utero*. Not only are several agents potentially involved in this process (Smith, 1998), but also PGE_2, the most important among them, may be formed through separately controlled pathways. Earlier in gestation, PGE_2 formation appears to be dependent on COX1 rather than COX2. However, at term the two enzymes operate jointly and, if necessary, may substitute each other. Indeed, ductus function is outwardly normal in mutant mice lacking either enzyme (Langenbach *et al.*, 1999; Loftin *et al.*, 1999). Only after removing both enzymes the vessel becomes dysfunctional and loses, in particular, its capacity to close postnatally (Langenbach *et al.*, 1999; Loftin *et al.*, 1999). This abnormal behaviour, resulting in all likelihood from the loss of a potent stimulus for closure (i.e. the rebound contraction coinciding with the subsidence of the relaxing influence of PGE_2 at birth; Coceani *et al.*, 1999), is also seen after deleting the appropriate PGE_2 receptor (Nguyen *et al.*, 1997). Endotoxin modifies this arrangement, but the pattern of changes differs depending on whether treatment is performed *in vivo* or *in vitro*. In the former case, there is, at least at the foetal age examined (i.e. 0.7 gestation), an enhancement of the L-

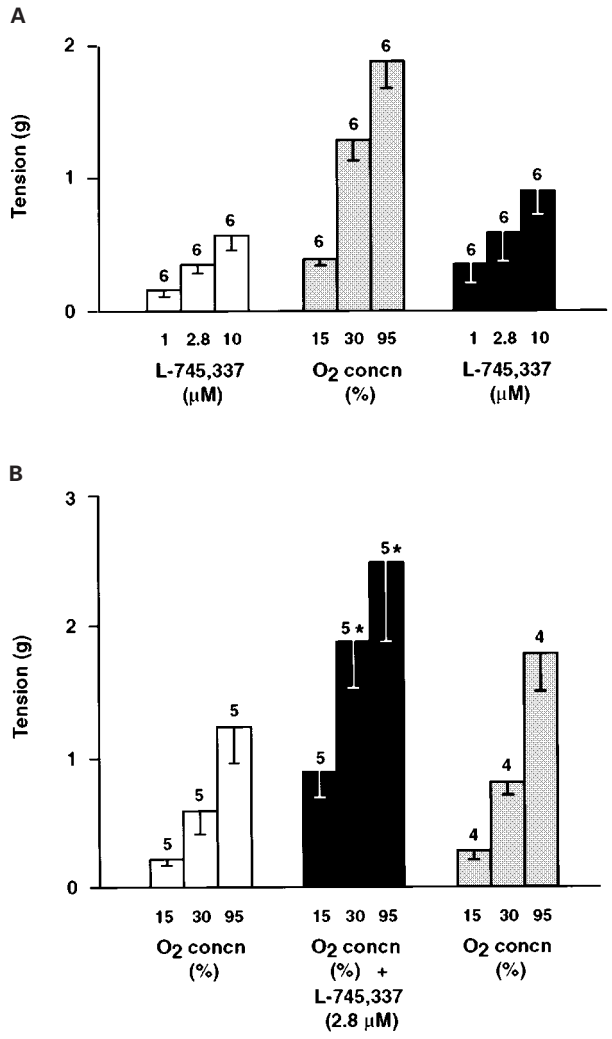

Figure 6 Intact strip of ductus arteriosus from foetal lamb at 103–107 days gestation. (A) Contractile response to L-745,337 before and after exposure to oxygen (for details, see Methods). (B) Contractile response to oxygen before, during, and after treatment with L-745,337 (2.8 μM) (for details, see Methods). Note that in panel (B) L-745,337 alone caused a contraction of 0.78 ± 0.2 g and that the response to oxygen is measured from this new baseline. For each group, number of experiments are given above the columns, and a significant difference relative to control is indicated with $^{*}P < 0.01$ (ANOVA).

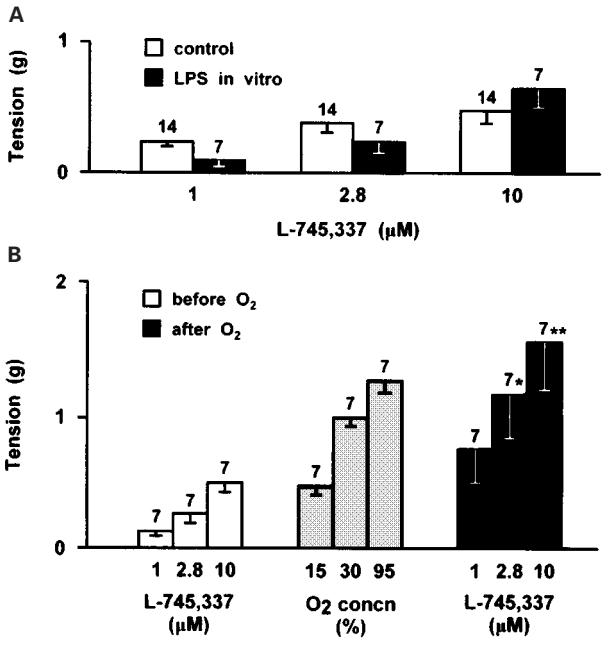

Figure 7 Intact strip of ductus arteriosus from foetal lamb at 94–97 days gestation. (A) Contractile response to L-745,337 before and during treatment with endotoxin (LPS) *in vitro* (for details, see Methods). (B) Contractile response to L-745,337 before and after exposure to oxygen (for details, see Methods). For each group, number of experiments are given above the columns, and a significant difference in panel (B) relative to the control before oxygen exposure is indicated with $^{*}P < 0.05$ or $^{**}P < 0.01$ (ANOVA). Note that in this age group, unlike the 103–107 days group (see Figure 5), the inhibitory effect of endotoxin did not reach significance even when comparing responses to L-745,337 in the same tissue ($n = 7$).

745,337 contraction and, by inference, greater efficiency of COX2. Conversely, the treatment *in vitro* leads to disappearance of COX2, and this peculiar phenomenon is associated with uneven changes in the response to the inhibitor. Specifically, at term gestation there is potentiation of the response, while the premature shows a reduction or no difference from controls. An explanation for these seemingly incongruous findings is best sought by considering the mode of action of endotoxin on the tissue. Our study did not address this particular point; however, it is reasonable to assume that disappearance of COX2 is linked to mechanisms that control, directly or through the transcription factor NF-κB, the level of expression of the enzyme. In particular, one may surmise that the endotoxin stimulus, if sufficiently strong, exerts a biphasic effect on the enzyme, consisting first of upregulation and then downregulation. The actual mechanism responsible for the secondary phase of downregulation remains speculative. One possibility is that pyrogen promotes the formation of a substance, such as nitric oxide (NO), interfering with the expression of COX2 but not COX1 (Swierkosz *et al*., 1995; Habib *et al*., 1997). Indeed, Swierkosz *et al*. (1995) have proposed a dual effect of NO on COX2 depending on its concentration. Alternatively, or concomitantly, PGE_2 may attain levels high enough to exert a receptor-mediated inhibitory influence on COX2 expression (Bishop-Bailey *et al*., 1998). Complicating further this scheme is the fact that endotoxin may or may not activate simultaneously PGE_2 synthase (Brock *et al*., 1999; Jakobsson *et al*., 1999). Any such activation could compensate, in part, for the downregulation of COX2. If taking place together, these events can well explain the variable susceptibility of the endotoxin-treated ductus to L-745,337. In addition, one must note that endotoxin pretreatment *in vitro* lasted less with the L-745,337 test than the immunohistochemical analysis (70 vs 120 min; see Methods). Hence, the inhibitor might have been applied to the tissue when COX2 was still upregulated or was straddling the threshold for downregulation.

The reason for the endotoxin-induced translocation of COX1 (*in vitro* treatment) and COX2 (*in vitro* and *in vivo* treatment) from the inner to the outer regions of muscle cell cytoplasm remains unsettled. In all likelihood, however, this process is linked to changes in the synthetic activity, promoted by pyrogen, which favour the generation of PGE_2 at the expense of other prostanoids (Brock *et al*.,

1999). Specifically, after moving across the cytosol COX enzymes may interact more efficiently with PGE_2 synthase. The importance of an appropriate interaction between COXs and terminal enzymes for optimal product formation has already been documented with endothelial PGI_2 (Liou *et al*., 2000). In this connection, the observation that in the endotoxin-treated (*in vivo* treatment), premature ductus the contraction to L-745,337 is significantly enhanced despite a modest increase in COX2 immunoreactivity is unlikely fortuitous and may, in fact, denote the impact of a better functional coupling.

Our findings have important implications if translated to the clinical situation. Specifically, they validate the use of a dual COX1/COX2 inhibitor, such as indomethacin, in the management of a persistent ductus in the prematurely born infant. This conclusion is based not only on the presence of COX2 in the premature vessel, however modest this might be relatively to COX1, but also on the notion that the enzyme is liable to upregulation by a host of physiological and pathophysiological stimuli. They include the postnatal rise in blood oxygen tension which may affect COX2 (this study) without being able to induce ductus closure (Smith, 1998), the shear stress on the vessel wall resulting from a change in the direction of blood flow after birth (Topper *et al*., 1996), and any intervening infectious state (this study). Conversely, a selective COX2 inhibitor appears better suited than currently used nonselective COX1/COX2 inhibitors for the prevention of premature labour. The advantage in choosing the former drug is that the labour sequence, which is COX2-based (Slater *et al*., 1995; Gibb & Sun, 1996; Wu *et al*., 1999) would be suppressed, while a marginal effect, or no effect at all, is expected on the pre-term ductus (this study) and the pulmonary vasculature (Brannon *et al*., 1994). In addition, a COX2 inhibitor may not curtail (Guerguerian *et al*., 1998), or may curtail only in part, the high blood level of PGE_2 typical of the foetal condition (Challis *et al*., 1976; Jones *et al*., 1993) thus allowing a full-fledged drop of the compound at birth. This transitional fall in blood PGE_2 is regarded as a powerful stimulus for ductus closure (Nguyen *et al*., 1997; Coceani *et al*., 1999) and its lesser magnitude, rather than any vessel damage resulting from a transient constriction (Norton *et al*., 1993), may account for the higher incidence of persistent ductus in infants from mothers treated antenatally with indomethacin (Norton *et al*., 1993).

In conclusion, the present study demonstrates that COX1 and COX2 develop unevenly in the ductus arteriosus. While the two enzymes sustain PGE_2 formation at term gestation, COX2 contributes little to this process in the premature. COX2 function, however, may increase upon exposure to a physiological stimulus, such as oxygen, and during treatment with endotoxin. Translated to the clinical situation, these findings reaffirm the usefulness of a dual COX1/COX2 inhibitor for the management of a persistent ductus in the premature infant. Conversely, a selective COX2 inhibitor is expected to be a better choice in the prevention of pre-term labour, insofar as it may combine greater safety with effectiveness.

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