In utero indomethacin alters \( \text{O}_2 \) delivery to the fetal ductus arteriosus: implications for postnatal patency

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Goldbarg, Seth H., Yasushi Takahashi, Carolyn Cruz, Hiroki Kajino, Christine Roman, Bao Mei Liu, Yao Qi Chen, Francoise Mauray, and Ronald I. Clyman. In utero indomethacin alters \( \text{O}_2 \) delivery to the fetal ductus arteriosus: implications for postnatal patency. Am J Physiol Regulatory Integrative Comp Physiol 282: R184–R190, 2002.—Indomethacin produces constriction and hypoxia of the fetal ductus arteriosus. This is associated with death of smooth muscle cells in the ductus wall and an increased incidence of patent ductus arteriosus in the newborn period. We used fetal sheep to determine which factors are responsible for indomethacin-induced hypoxic cell death. Cell death in the ductus wall is directly related to the degree of indomethacin-induced ductus constriction and is present at both moderate and marked degrees of constriction. Both moderate and marked degrees of ductus constriction reduce vasa vasorum flow to the ductus (moderate = 69 ± 25%; marked = 30 ± 16% of preinfusion values) and increase the thickness of the ductus wall. In contrast, ductus luminal blood flow is not affected by moderate degrees of constriction and is reduced only after marked constriction. Although indomethacin increases ductus tone, it has no effect on ductus oxygen consumption. These findings suggest that the hypoxic cell death that occurs during the early stages of indomethacin-induced constriction is primarily due to changes in vasa vasorum blood flow and muscle media thickness.

vasa vasorum; microspheres; cell death; oxygen consumption; tocolysis

PREMATURE INFANTS who have been exposed to indomethacin in utero have an increased incidence of patent ductus arteriosus (DA) when the exposure occurs after the 28th wk of gestation (19). Indomethacin increases the contractile tone of the fetal DA in utero. Functional constriction of the DA is associated with hypoxia of the vessel wall. Hypoxia induces the expression of VEGF, endothelial nitric oxide synthase (eNOS), and loss of smooth muscle cells from the DA muscle media (6). These changes impair the future ability of the DA to constrict (6) and lead to an increased incidence of patent DA in the newborn (12, 19, 23).

Oxygen normally reaches the muscle media of the fetal DA through either the vessel’s lumen or its vasa vasorum. The muscle media adjacent to the lumen of the fetal DA normally lacks any vasa vasorum (6). Vasa vasorum are present in the adventitia and may be present in the outer muscle media when the vessel wall exceeds a certain size (6, 26). The avascular muscle media depends on flow through both the lumen and vasa vasorum to meet its nutrient needs (2, 5, 27). The oxygen concentration of the arterial wall is highest immediately adjacent to the lumen, diminishes to a nadir in the middle of the avascular zone, and increases progressively again toward the vasa vasorum rich outer vessel wall (2, 5, 27). Oxygen reserves in the avascular zone can be exceeded if there is 1) a decrease in luminal blood flow, 2) a decrease in vasa vasorum blood flow, 3) an increase in the diffusion distance (or thickness) of the avascular zone, or 4) an increase in the oxygen consumption of the DA wall.

In the following study we examined how each of these factors is altered by indomethacin exposure in utero. We hypothesized that the degree of DA constriction is the primary factor responsible for regulating each of the individual determinants of oxygen balance. We performed our studies in fetal lambs, because the changes in DA wall thickness and the presence of vasa vasorum are similar to those found in the human DA.

METHODS

Fetal studies in vivo. All studies were approved by the Committee on Animal Research at the University of California San Francisco.

Pregnant mixed Western breed sheep \([n = 37, 133 ± 2\; \text{days\; gestation\; (±SD); term = 145\; days}]\) were operated on under intravenous ketamine HCl and diazepam anesthesia (6). Our purpose was to examine the effects of varying degrees of DA constriction on nutrient supply to the DA wall. We used near-term fetuses because they develop a greater contractile force in their DA than less mature fetuses (16).

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We hoped that late gestation fetuses would allow us to observe the changes that occur after ductus constriction with greater consistency than if we had chosen less mature fetuses. Fetuses were exposed through a uterine incision. The ascending aorta, superior vena cava, descending aorta, and inferior vena cava were catheterized through the forelimb and hindlimb pedal artery and vein, respectively. The pulmonary artery was catheterized directly through a thoracotomy. In seven fetuses, a 4–6 mm Doppler flow transducer (Transonics Systems, Ithaca, NY) was placed around the DA to measure luminal blood flow. The thoracotomy was closed and the fetus was returned to the uterus for subsequent experimentation.

On the day after surgery, fetuses were infused with indomethacin (0.2 mg·h⁻¹·kg⁻¹ estimated fetal wt⁻¹). This infusion rate produces stable fetal plasma indomethacin concentrations (0.65 ± 0.24 μg/ml; Ref. 6). DA constriction was assessed by continuously measuring the pressure gradient across the DA (between the ascending aorta and pulmonary artery). Moderate constriction was defined as a pressure gradient of DA <16 mmHg. Severe constriction was defined as DA Press(DA) ≥16 mmHg.

In the first group of fetuses (n = 17), indomethacin or vehicle (50 mM Tris·HCl, 10 ml/h) was infused into the fetus via a hindlimb pedal vein for 24 h to determine the incidence of cell death in the DA. At the end of the infusion, the fetus was anesthetized with ketamine HCl and the DA was collected. The DA was dissected in Dulbecco’s phosphate-buffered saline solution at 4°C, embedded in Tissuetek (Miles, Elkhart, IN), and frozen in liquid nitrogen for subsequent immunohistochemistry.

In the second group of fetuses (n = 7), continuous Doppler measurements of DA luminal blood flow were recorded during the first 4 h of the indomethacin infusion.

In the third group of fetuses (n = 7) fluorescent microspheres (Interactive Medical Technologies, Irvine, CA) were used to determine vasa vasorum blood flow by methods similar to those published previously (18). Microsphere measurements were made both before and during the first 6 h of the indomethacin infusion (between 2 and 6 h). The time of measurement was based on the pressure gradient across the DA. For each microsphere measurement, two separate sets of fluorescent microspheres (~3 × 10⁶, 15 μm) were injected simultaneously into the superior and inferior vena cava, respectively. Reference blood samples were withdrawn from the ascending and descending aorta. We previously found that blood flow to the vasa vasorum of the DA is derived from the ascending and descending aorta but not from the pulmonary artery (data not shown). After the experiment, the DA and ascending aorta were removed from the fetus and the loose adventitia was carefully stripped from each artery. The vessels and reference blood samples were weighed, digested in alkali, and the released fluorescent microspheres were counted by flow cytometry (1). Process control microspheres were added to each tissue or blood sample to determine the number of microspheres lost during sample processing. Vasa vasorum blood flow was calculated from the number of microspheres in the vessel divided by the number of microspheres in the appropriate reference arterial blood sample(s) and multiplied by the reference blood flow(s) (see APPENDIX).

Arterial pH, PO₂, and PO₂ were measured on a Radiometer Blood Gas Analyzer (Radiometer, Copenhagen, Denmark); oxygen saturation and Hb concentration were measured on an OSM-2 Hemoximeter (Radiometer). Arterial oxygen content (ml O₂/ml blood) was determined as the product of Hb concentration, oxygen saturation, and an oxygen binding capacity of 1.34 ml O₂/g Hb. Vasa vasorum oxygen delivery (ml O₂·min⁻¹·g tissue⁻¹) was calculated as the product of arterial oxygen content and the vasa vasorum blood flow (ml blood·min⁻¹·g tissue⁻¹).

Histochemistry. We used the terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) technique to detect cells in the early stages of DNA fragmentation and cell death as we have described previously (5). This technique identifies cells undergoing necrosis (8) as well as apoptosis (10). DNA breaks were detected with the Apoptag peroxidase detection system (Intergen, Purchase, NY). There was no nonspecific binding of reagents to nuclei when terminal deoxynucleotidyl transferase was omitted from the assay (data not shown). The number of TUNEL-positive nuclei per 500 nuclei was measured in a circumferentially oriented, 67-μm-wide region around the middle of the muscle media that contained the largest number of TUNEL-stained cells.

We used a mouse monoclonal antibody against eNOS (Clone 3, Transduction Lab, Lexington, KY) to identify endothelial cells in the frozen sections as previously reported (5, 6). Histologic measurements were made at the level of minimal luminal arterial area, which was determined from serial sections made along the main axis of the ductus. The muscle media of all arteries has an avascular zone, adjacent to the lumen, which does not contain any of the vasa vasorum that supply the outer muscle media (6, 26). We defined the avascular zone of the DA as the region of the DA wall between the endothelial lining of the DA lumen and the leading edge of the vasa vasorum. The total media thickness was defined as the region between the luminal endothelium and the outer layer of smooth muscle cells in the muscle media. Tissue dimensions and zone thickness were determined by averaging measurements made from eight predetermined regions of the section, using a template and National Institutes of Health Image software (6).

Oxygen Consumption in vitro. Fetal lambs were anesthetized with ketamine HCl before rapid exsanguination. The ductus was divided into rings (37 ± 7 mg wet wt, n = 6) and mounted at an optimal length for tension development (7.0 ± 0.6 mm) (7) in a 37°C, water-jacketed glass chamber equipped with an O₂ electrode (see Ref. 15). A conically ground, 4-cm-tall plastic plug sealed the chamber and contained a small channel through which a freely moveable stainless steel hook connected the ductus ring to an isometric force transducer. The ductus ring was stretched between the moveable hook and a fixed hook within the chamber. The long diffusion path through the small bore hole effectively prevented the leakage of O₂ into or out of the chamber (15, 20). Sterile buffer solution (in mM: 120 NaCl, 4 KCl, 10 glucose, 1.2 MgCl₂, 1 K₂HPO₄, 2.6 CaCl₂, 25 HEPES, pH 7.45 that had been pregressed with 21% O₂) was passed through a 0.22-μm filter before the chamber (1.2 ml volume) was perfused at 0.5 ml/min. A Teflon-coated magnetic stirrer ensured adequate mixing. This apparatus allowed the simultaneous determination of O₂ consumption rate and active isometric tension. We used buffer solution equilibrated with 21% O₂ to perfuse the chamber, because this produced average tissue oxygen concentrations in the rings similar to those observed in the fetal ductus in vivo (17).

The oxygen electrode (YSI model 53 Biological Oxygen Monitor, Yellow Springs, OH) was calibrated with 21% O₂ saturated buffer. The solubility of O₂ in buffer solution that was equilibrated with 21% O₂ at 37°C was assumed to be 0.20 μmol/ml (16). Oxygen consumption rate was measured during a 10-min interval during which the tissue chamber was closed. Background oxygen consumption rate (without tissue) was determined in each experiment and was 13.5% of the measured tissue oxygen consumption rate.
The DA rings were initially incubated for 4 h at 37°C. During this time interval oxygen consumption rates and isometric tensions stabilized. At 4 h, indomethacin (5.6 μM) was added to the buffer solution, and changes in isometric tension and oxygen consumption rate were monitored over the next hour. The difference in tensions between measured tension and passive tension produced by stretching the ring at the start of the experiment was considered to be the active tension. Tissues were blotted dry and weighed after the experiments. The tension developed in the rings was expressed as the force per unit cross-sectional area (g/mm²) (7).

**Statistics.** Comparison of unpaired data was performed by the appropriate t-test or regression analysis. When more than one comparison was made, Bonferroni’s correction was used. Nonparametric data were compared with a Mann-Whitney test. Results are presented as means ± SD.

**RESULTS**

An infusion of indomethacin was associated with DA constriction and evidence of cell death (as demonstrated by the increased incidence of TUNEL-positive cells) in the middle of the DA muscle media (the region of the avascular zone adjacent to the leading edge of the vasa vasorum; Fig. 1). By 24 h, an extensive region of cell loss (absent nuclei) was already apparent in the center of four of the DA (Figs. 1 and 2). The incidence of TUNEL-positive cells in the DA wall was directly related to the degree of DA constriction (r = 0.91, n = 17, P < 0.0001; Fig. 1). Even moderate degrees of DA constriction (≥16 mmHg gradient) were associated with an increase in the incidence of cell death.

Although indomethacin produced a significant increase in active tension of DA rings in vitro, it had no effect on tissue oxygen consumption (Fig. 3).

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**Fig. 2.** Three contiguous regions (A-C) from the middle of a single ductus showing TUNEL-positive nuclei (brown) bordering a region of extensive cell loss. A: a large area of cell loss bordered by TUNEL-positive nuclei. C: mostly TUNEL-positive cells before the cell nuclei disappear. Hematoxylin (blue) counterstain.

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**Fig. 1.** Moderate and marked degrees of constriction cause an increase in terminal deoxynucleotidyl transferase nick-end labeling (TUNEL)-positive cells and cell loss in the ductus. Fetuses were infused with either vehicle (control, n = 8) or indomethacin (n = 9). Pressure gradient across the ductus was measured just before necropsy. • and ◆, the number of TUNEL-positive nuclei (per 500 nuclei) found in a 67-μm-wide, circumferentially oriented region around the middle of the muscle media (see METHODS). In 4 indomethacin-infused ductus (indicated by a ring around ◆), an extensive region (>20,000 μm²) of cell loss was observed in the middle of the muscle media. One ductus no longer had evidence of TUNEL staining and had only an extensive region of cell loss at the time of necropsy.
Indomethacin altered DA luminal blood flow; however, this was observed only after marked degrees of constriction had been achieved. Moderate (≤16 mmHg gradient) degrees of DA constriction had no effect on DA luminal flow. Marked degrees of DA constriction (>16 mmHg) reduced luminal flow to 64 ± 24% of preinfusion levels (Fig. 4).

Indomethacin reduced DA vasa vasorum blood flow. The decrease in DA vasa vasorum flow was directly related to the degree of DA constriction (r = 0.79, P < 0.0001; Fig. 5). Even moderate degrees of DA constriction produced a significant decrease in DA vasa vasorum flow (Fig. 5B). Oxygen delivery through the vasa vasorum fell parallel with vasa vasorum flow [in ml O2·min⁻¹·g⁻¹; preinfusion = 0.059 ± 0.015; moderate ductus constriction (>4 to ≤16 mmHg gradient) = 0.024 ± 0.014; marked constriction (>16 mmHg) = 0.017 ± 0.005]. In contrast, vasa vasorum flow to the ascending aorta was not affected by indomethacin (in ml·min⁻¹·g⁻¹; preinfusion = 0.027 ± 0.008; indomethacin = 0.022 ± 0.010), nor was it affected by the degree of DA constriction (in ml·min⁻¹·g⁻¹; pressure gradient ≤4 mmHg = 0.027 ± 0.007 vs. pressure gradient >16 mmHg = 0.024 ± 0.014).

Indomethacin-infused fetuses had a marked increase in the thickness of their muscle media when compared with vehicle-infused fetuses (Fig. 6). The number of concentric muscle layers throughout the DA was similar in indomethacin (46 ± 6, n = 9) and vehicle-infused (44 ± 3, n = 8) fetuses. The difference in wall
thickness was due to a substantial increase in avascular zone thickness in the indomethacin-infused fetuses. The thicknesses of both the avascular zone \((r = 0.95, n = 17, P < 0.01)\) and the total muscle media \((r = 0.64, n = 17, P < 0.01)\) were directly related to the degree of DA constriction; both were increased significantly even at moderate degrees of DA constriction (Fig. 6).

**DISCUSSION**

Exposure of the fetal lamb to indomethacin produced nuclear fragmentation and cell death in smooth muscle cells of the DA muscle media (Figs. 1 and 2). We previously found that changes in cell viability (in addition to changes in NO production) lead to a significant decline in tissue distensibility and contractile capacity (6). These changes explain why the DA remains patent and fails to close after indomethacin exposure in utero. The pattern and location of cell death in the avascular zone of the DA wall suggest that the profound muscle media hypoxia that develops during DA constriction (5, 6) is responsible for this occurrence. The extent of cell death is directly related to the degree of DA constriction (Fig. 1). We found that cell death occurs even at moderate degrees of constriction.

Oxygen delivery to the arterial wall is tightly regulated. Although vessel wall thickness varies markedly between species, the maximal thickness of the inner...
vascular zone of the muscle media remains constant at <0.5 mm (3, 11, 14, 26). The avascular zone, which depends on flow from both the lumen and the vasa vasorum, is particularly vulnerable to changes in oxygen demand or supply.

We found that the increased cell death associated with indomethacin-induced constriction was not due to an increase in tissue oxygen consumption (Fig. 3). Indomethacin had no effect on oxygen consumption in the DA; this finding is consistent with other studies in myocardial, hepatic, cerebral, gastric, and jejunal tissues (9, 13, 21, 22, 25).

On the other hand, indomethacin had a profound effect on the oxygen supply to the ductus. Indomethacin decreased both the DA luminal and vasa vasorum blood flow and at the same time increased the thickness of the DA avascular zone (Figs. 4–6).

Indomethacin produced an increase in DA tone that ultimately decreased DA luminal blood flow; however, the reduction in luminal flow occurred only with marked degrees of DA constriction. At moderate degrees of DA constriction, the increased pressure gradient across the DA, generated by the high fetal pulmonary vascular resistance, was sufficient to maintain DA luminal blood flow. In contrast, the changes in vasa vasorum flow and avascular zone thickness occurred even at moderate degrees of DA constriction.

We hypothesize that indomethacin’s effect on vasa vasorum blood flow was due to physical compression of the thin-walled vasa vasorum during constriction of the DA outer muscle media; the reduction in DA vasa vasorum blood flow was directly related to the degree of DA constriction (Fig. 5). Although indomethacin might have a direct effect on vasa vasorum tone, it did not appear to alter flow in the vasa vasorum of an adjacent, noncontracting vessel, the aorta. It is possible that the ductus vasa vasorum may have unique properties that differ from those in the aorta.

In addition to its effects on flow to the DA, indomethacin caused a marked increase in the thickness of the avascular zone of the DA wall (Fig. 6). The increased avascular zone thickness was due to tissue compaction (caused by circumferential and longitudinal muscle constriction; Refs. 5, 24). Even moderate degrees of DA constriction produced an increase in avascular zone thickness (Fig. 6) and an increase in oxygen diffusion distance across the muscle media.

Our findings suggest that there are several mechanisms responsible for the tissue hypoxia that accompanies in utero DA constriction. Moderate degrees of constriction decrease vasa vasorum blood flow and increase wall thickness. Following the development of marked degrees of DA constriction, luminal blood flow starts to decline and further contributes to the induction of cell death.

Perspectives

When late gestation fetuses are exposed to indomethacin in utero, ischemic changes in the muscle wall occur even before there are any changes in ductus luminal flow (Fig. 1, Ref. 6). We found that avascular zone thickness and vasa vasorum perfusion are the determinants of oxygen balance that are most affected by indomethacin-induced ductus constriction. An increase in avascular zone thickness and a decrease in vasa vasorum flow appear to be responsible for the hypoxia and cell death that occur in the late gestation ductus. This phenomenon probably accounts for the higher incidence of patent ductus arteriosus that occurs in neonates that have been exposed to indomethacin in utero.

It is interesting to note that indomethacin exposure in utero is more likely to be associated with a patent ductus arteriosus when the exposure occurs later in gestation (19). The presence of vasa vasorum in the muscle media depends on the thickness of the arterial wall (6, 26). In the human fetus, vasa vasorum usually enter the ductus wall after the 28th wk of gestation (4).

Before 28 wk, luminal blood flow is sufficient to meet the oxygen demands of the thin-walled ductus. Previous reports have shown that infants >28 wk gestation are much more likely to be affected by in utero indomethacin exposure than infants <28 wk gestation (19). Our findings help to explain why infants <28 wk gestation (who do not depend on vasa vasorum flow to provide nutrition to their ductus wall) are much less likely to be affected by indomethacin exposure in utero.

APPENDIX: DETERMINATION OF VASA VASORUM FLOW TO THE DUCTUS ARTERIOSUS

Vasa vasorum flow to the muscle media of the DA is derived from the ascending and descending aorta, but not from the pulmonary artery (data not shown). Vasa vasorum flow (ml blood/min) to the DA (QDA) can be represented as the sum of the vasa vasorum flow from both the ascending aorta (QaAo) and descending aorta (QdAo) to the DA

\[ Q_{DA} = Q_{aAo} + Q_{dAo} \]

Similarly, the number of microspheres in the DA (microspheres\(_{DA}\)) is

\[ \text{microspheres}_{DA} = R_{aAo}Q_{aAo} + R_{dAo}Q_{dAo} \]  

where \( R \) is the concentration of microspheres (microspheres/ml-1·min-1) in the corresponding ascending aorta or descending aorta reference samples.

Because these are the only sources of DA flow

\[ Q_{aAo} = (x)(Q_{DA}) \]

\[ Q_{dAo} = (1-x)(Q_{DA}) \]

where \( x \) is the proportion of flow to the DA from the descending aorta.

Substituting Eq. 2 and Eq. 3 in Eq. 1 and then solving for \( x \)

\[ x = \frac{\text{microspheres}_{DA} - R_{aAo}Q_{DA}}{R_{aAo}Q_{DA} - R_{aAo}Q_{DA}} \]

To measure \( Q_{DA} \) we use two different sets of microspheres (#1, #2) that are injected simultaneously. Because \( x \) is the same for microspheres #1 and #2

\[ \frac{\text{microspheres}_{DA} - R_{aAo}Q_{DA}}{R_{aAo}Q_{DA} - R_{aAo}Q_{DA}} = \frac{\text{microspheres}_{DA} - R_{aAo}Q_{DA}}{R_{aAo}Q_{DA} - R_{aAo}Q_{DA}} \]

\[ \frac{\text{microspheres}_{DA} - R_{aAo}Q_{DA}}{R_{aAo}Q_{DA} - R_{aAo}Q_{DA}} = \frac{\text{microspheres}_{DA} - R_{aAo}Q_{DA}}{R_{aAo}Q_{DA} - R_{aAo}Q_{DA}} \]
Solving Eq. 5 for $Q_{DA}$ gives

$$Q_{DA} = \frac{\text{microspheres}_{DA1}}{(R_{DA1} - R_{DA2})} - \frac{\text{microspheres}_{DA2}}{(R_{DA1} - R_{DA2})}$$

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