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IN UTERO INDOMETHACIN ALTERS O_2 DELIVERY TO THE FETAL DUCTUS ARTERIOSUS: IMPLICATIONS FOR PERSISTENT POSTNATAL PATENCY.

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Abstract

Exposure of the fetus to indomethacin produces constriction of the fetal ductus arteriosus (DA) and hypoxia in the avascular muscle media of the vessel wall. Hypoxia induces cell death, which increases the incidence of patent DA in the newborn period. We used a fetal sheep model to determine the factors that were responsible for indomethacin-induced hypoxia at various degrees of DA constriction. Indomethacin produced DA constriction in all fetuses studied in vivo. Cell death in the DA wall was directly related to the degree of indomethacin-induced DA constriction and was present at both moderate (pressure gradient across DA <16 mmHg) and marked (≥16 mmHg) degrees of constriction. Indomethacin did not alter oxygen consumption in DA rings studied in vitro, indicating that oxygen demand in the constricting tissue is not significantly increased by indomethacin. Both moderate and marked degrees of DA constriction reduced vasa vasorum flow to the ductus (moderate = 69±25%; marked = 30±16% of pre-indomethacin exposure values) and increased the thickness of the ductus wall. In contrast, DA luminal blood flow was not affected by moderate degrees of constriction and was reduced only after marked constriction. Our findings suggest that changes in vasa vasorum blood flow and muscle media thickness are the primary contributors to hypoxia-induced cell death at moderate degrees of indomethacin-induced constriction. Diminished luminal blood flow only appears to contribute to the induction of cell death following the development of marked degrees of constriction. These findings help to explain why in utero exposure to indomethacin in late gestation fetuses, which depend on vasa vasorum to supply O_2 to the DA muscle media, leads to hypoxia-induced remodeling and and increased incidence of postnatal patent DA.

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Introduction

The ductus arteriosus (DA) is a fetal blood vessel that connects the pulmonary artery to the aorta. In utero, it provides a shunt from the right side of the heart to the aorta, allowing blood to bypass the high resistance pulmonary circulation. After birth, it is essential for the ductus to constrict so that the systemic and pulmonary circulations are separated.

The ductus arteriosus normally closes within 24 hours of birth in the full-term newborn. Postnatal constriction results in an initial functional closure of the DA. Functional closure causes a decrease in DA luminal blood flow and leads to a decrease in the diffusion of blood from the lumen into the muscle media (1). DA constriction may also cause a decrease in blood flow through the vasa vasorum, small adventitial vessels that provide an important blood supply to the DA muscle media in the full-term fetus. Decreased vasa vasorum blood flow may occur through physical occlusion as the DA muscle media constricts, or by independent constriction via the action of locally produced vasoconstrictive substances. The loss of luminal and vasa vasorum blood supply leads to a decreased P_{O2}, one that becomes insufficient to fulfill the oxygen needs of the vessel wall. The resultant tissue hypoxia is associated with the local production of hypoxia-inducible growth factors and cell death in the muscle media (2) (see diagram 1). Hypoxia appears to be a necessary step in initiating the anatomic remodeling that leads to permanent closure (3, 4).

Although the sequence of events leading to development of tissue hypoxia in the constricting ductus is not known, some of the cellular and biochemical pathways leading from tissue hypoxia to permanent DA closure in the newborn have been examined and

deserve a brief review. Anatomic remodeling in the full-term DA occurs through complex changes in expression of hypoxia-inducible growth factors, production of extracellular matrix components, and migration of cells into the subendothelium. Increased expression of growth factors including VEGF and TGF-ß are detectable in the closing DA. VEGF is an hypoxia-inducible growth factor that is present only in areas of lowest oxygen tension in the closing ductus (4, 5, 6). TGF-ß, which is upregulated in the outer media and adventitia of the ductus within hours of delivery, may help to sustain contracture of the DA by increasing smooth muscle cell adhesion to the extracellular matrix (6). Endothelial cells migrate away from the internal elastic lamina as the subendothelium begins to widen. Accumulation of glycosaminoglycans and migration of smooth muscle cells causes intimal thickening in the subendothelium (7). Changes in the extracellular matrix occur as the subendothelium expands; laminin and type I collagen are no longer expressed while production of type III collagen is upregulated (8). Endothelial cells and smooth muscle cells express an increased range of integrins, some of which are essential for the migration of smooth muscle cells into the subendothelial region (9). The process of neointimal formation resulting from these changes is essential for permanent closure of the DA.

Oxygen reaches the muscle media of the DA through its lumen and its vasa vasorum. Oxygen requirements of the arterial wall can surpass supply when oxygen consumption is increased or oxygen delivery is decreased. Decreased delivery can result from either a decrease in luminal or vasa vasorum blood flow or an increase in the region of the DA muscle media that lacks vasa vasorum (see diagram 2). This area, termed the avascular zone, is defined as the region of the arterial media adjacent to the lumen into

which vasa vasorum do not penetrate. Large arteries appear to have an avascular zone of constant thickness (0.47 ± 0.6 mm) (10, 11). The depth of penetration of the vasa vasorum into the media increases with the thickness of the arterial wall such that, under normal conditions, the avascular zone does not exceed this maximal thickness.

Oxygen tension in the walls of large arteries varies continuously, decreasing from the luminal side to the avascular media and then increasing towards the outer muscle media and adventitia (12). The diffusion distance to the inner (avascular) media is greater than that to either the luminal media or adventitial media (13). Under normal conditions, tissue oxygenation in the arterial wall reaches its lowest point at the center of the avascular zone. This region of lowest oxygen tension, which depends on nutrient supply from both the lumen and vasa vasorum, may be especially susceptible to alterations in blood flow. Several studies have illustrated the dependence of the avascular zone on the oxygen provided by vasa vasorum flow. Within a week of completely occluding the vasa vasorum in the aorta, intimal thickening and loss of muscle media cells can be seen (14). Ligation of the intercostal arteries, which supply the vasa vasorum of the thoracic aorta, leads to medial necrosis, indicating that vasa vasorum are critical for nutrient supply to the avascular arterial wall (11).

In contrast to the full-term newborn, the preterm DA frequently fails to achieve permanent closure (15). In the human fetus, the ductus does not develop vasa vasorum until the 28th week of gestation (16). Luminal blood flow is sufficient to meet the oxygen demands of the thin-walled ductus before the 28th week. Even with functional ductus constriction induced by indomethacin, the premature ductus, which lacks dependence on vasa vasorum blood flow, frequently fails to remodel and does not

permanently close. Of infants <27 weeks gestation who received prophylactic indomethacin within 15 hours after birth, 21% failed to permanently close their ductus and required surgical ligation (15). After 3 doses of indomethacin within 36 hours of birth, 23% of immature infants (gestational age 23-33 weeks) had residual luminal flow on echocardiogram, which was associated with a higher rate of clinical reopening compared to infants with a closed ductus diagnosed by echocardiagram (1). The development of dependence on vasa vasorum for oxygen supply appears to be critical for achieving permanent closure.

Like preterm newborns, fetuses that have been exposed to indomethacin in utero often fail to achieve permanent ductus closure in the newborn period (17, 18, 19). In utero, the fetal ductus remains patent primarily as a result of prostanoids, which are produced locally in the tissue and circulate in high concentrations. Indomethacin, a frequently used tocolytic agent, blocks prostaglandin production. Its primary clinical use is inhibiting the synthesis of maternal prostaglandins that are associated with uterine irritability and premature labor. However, indomethacin crosses the placenta and inhibits prostaglandin production in the fetal ductus. This inhibition induces contraction in the fetal DA, which is associated with tissue hypoxia of the DA wall and loss of smooth muscle cells from the muscle media (2, 20, 21) (see diagram 3). The initial indomethacin-induced constriction often fails to completely occlude the lumen and does not generate the levels of tissue hypoxia that are required to induce the anatomic remodeling necessary for closure (4). However, the tissue hypoxia, cell loss, and partial remodeling that occurs following indomethacin-induced constriction impairs the future ability of the DA to constrict and results in an increased risk of postnatal patent ductus

arteriosus (2). Premature infants who have been exposed to indomethacin in utero have an increased incidence of patent DA after birth (17, 18, 19).

Statement of Purpose

The relative effects of changes in oxygen supply and demand on tissue hypoxia in the constricting ductus are unknown. Indomethacin may have a direct effect on oxygen consumption in DA tissue. On the one hand, indomethacin induces muscular constriction, which would be expected to increase the metabolic oxygen demands of the tissue. On the other, prostaglandin production utilizes oxygen as a substrate, and an indomethacin-induced inhibition of prostaglandin production would be expected to decrease oxygen utilization. The experiments in which we measured in vitro oxygen consumption in the constricting ductus were designed to test whether metabolic oxygen demand changed during indomethacin-induced ductus constriction.

Oxygen supply may also be affected by constriction of the near-term DA. Decreased luminal flow, decreased vasa vasorum flow, and increased thickness of the avascular zone may contribute to tissue hypoxia by varying degrees depending on the degree of constriction. The studies examining ductus flow during indomethacin-induced constriction were designed to quantify the relationship between luminal blood flow and the degree of DA constriction, as measured by the pressure gradient across the ductus. We made use of microsphere experiments to understand how vasa vasorum blood flow was affected by varying degrees of indomethacin-induced ductus constriction. We also set up an experiment that compared the thickness of the DA muscle media with the pressure gradient that developed across the DA to study the association between avascular zone thickness and degree of DA constriction.

We hypothesized that indomethacin-induced constriction of the near-term fetal DA causes tissue hypoxia primarily by compromising vasa vasorum blood flow. Clinical

evidence has shown that infants <28 weeks gestation are less likely to be affected by in utero indomethacin exposure than infants >28 weeks, the age at which the DA develops a dependence on vasa vasorum blood flow (18). Initial constriction of the DA causes an increase in the thickness of the avascular zone via circumferential and longitudinal muscular constriction, which could cause a physical occlusion of the vasa vasorum running through the muscle media (22). Partial indomethacin-induced constriction of the fetal DA may decrease luminal blood flow only nominally (2). We hypothesized that greater degrees of DA constriction would cause a relative increase in the thickness of the avascular zone in the DA wall. We suspected that the factors contributing to tissue hypoxia would be dependent on the degree of constriction that developed in the DA.

Methods

In Vivo Studies

Near-term fetal sheep were used to measure DA luminal and vasa vasorum blood flow during indomethacin-induced constriction. To measure luminal flow, we placed a Doppler flow probe around the fetal DA (experiments performed by R. Clyman). Microspheres were used to examine vasa vasorum blood flow, which required injection of spheres into the superior vena cava (SVC) and inferior vena cava (IVC), as well as blood sampling from the ascending and descending aorta following injection (see below). A pulmonary artery (PA) catheter was placed to measure pulmonary artery pressure so that a pressure gradient across the ductus could be determined.

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

Surgical Approach

Pregnant mixed Western breed sheep of 131-137 days gestation were operated under intravenous (0.001 mg/kg/min diazepam and 0.24 mg/kg/min ketamine hydrochloride) anesthesia. A sterile field was prepared over the anterior abdomen and a midline incision made under local (2% lidocaine hydrochloride) anesthesia. Fetal anesthesia consisted of local injection of 2% lidocaine hydrochloride and intramuscular ketamine hydrochloride (20 mg/kg). The fetus was exposed via a uterine incision. Both fetal hindlimbs were exposed and polyvinyl catheters inserted into the pedal artery and vein on each limb and advanced to the descending aorta and inferior vena cava, respectively. The fetal forelimb was mobilized and the pedal artery and vein catheterized to access the ascending aorta and superior vena cava, respectively. The internal thoracic

artery was reached through a left lateral thoracotomy in the fourth intercostal space. The pericardium was incised along the main pulmonary trunk and a polyvinyl catheter inserted into the pulmonary artery (see diagram 4). In 7 fetuses, the DA was isolated and a 4-6 mm Doppler flow transducer (Transonics Systems, Ithaca, NY) was placed around the DA. In 7 fetuses, no transducer was placed. The thoracotomy was closed. A polyvinyl catheter was placed in the amniotic cavity. Intrauterine warm saline and antibiotics (2×10^6 U penicillin G procaine and 200 mg gentamicin sulfate) were added before the uterine incision was closed. The catheters were flushed with heparin sodium, plugged, exteriorized, and placed in a pouch along the ewe's flank. The ewe was returned to the cage after recovery from anesthesia.

Arterial pressures were measured using with pressure transducers on the pulmonary and aortic catheters, and hemodynamic variables were recorded on a multichannel electrostatic recorder (Gould Electronics, Pleasanton, CA). Electrical integration was used to obtain mean pressures. Hemoglobin was measured on an OSM 2 Hemoxymeter (Radiometer Copenhagen, Denmark). Lactate was measured with a 1500 Sport Lactate Analyzer (YSI, Yellow Springs, OH). Systemic arterial blood gases and pH were measured on an ABL 5 Blood Gas Analyzer (Radiometer Copenhagen, Denmark).

Luminal Flow

The day following surgery, the chronically catheterized group of fetuses with a Doppler flow transducer (group 1, n=7) were given indomethacin (0.2 mg/kg/h given estimated fetal weight) over 4 h. This indomethacin infusion produces stable fetal plasma concentrations of 0.65 ± 0.24 μg/ml plasma (2). DA constriction was assessed by

continuous measurement of the pressure gradient between the ascending aorta and pulmonary artery. With a patent DA, blood flows readily between the ascending aorta and pulmonary artery. With DA constriction, the high PA pressure resulting from the high resistance fetal pulmonary circulation can no longer be unloaded through the narrowed DA, and a pressure gradient develops across the ductus. Moderate constriction was defined as a pressure gradient (ΔPress[DA]) < 16 mmHg; marked constriction was defined as (ΔPress[DA]) $\geq$ 20 mmHg.

Vasa Vasorum Flow

Flow to vasa vasorum has been measured using the microsphere technique. Rudolph and Heymann first used radioactive microspheres to measure fetal blood flow to placenta and organs in 1967 (23). They provided evidence of several critical characteristics of microspheres: the distribution of spheres is proportional to blood flow; recirculation of spheres is not significant; and injection of spheres does not alter hemodynamics. Calculations using microspheres also depend on two assumptions: the microspheres are completely mixed with blood near the site of injection such that streaming does not occur; and withdrawals occur during first pass circulation through the bloodstream (11, (23). The first microsphere experiments with vasa vasorum determined that small (15μm) microspheres could be used to accurately assess vasa vasorum flow because they do not undergo shunting through arteriovenous anastamoses (24).

With this background, we set up a microsphere experiment to measure vasa vasorum blood flow in the second group of chronically catheterized fetuses. Blood flow to the vasa vasorum of the DA is derived from the ascending and descending aorta but not from the pulmonary artery (Clyman, pers. comm.). Blood flows from the SVC and

IVC into the right atrium and ventricle. In the fetus, blood supplying the vasa vasorum then flows through either the pulmonary artery and the DA to the aortic isthmus, or through the foramen ovale to the left ventricle and ascending aorta. We used simultaneous microsphere injections of differing fluorescence in the SVC and IVC because the blood from the IVC streams preferentially through the foramen ovale, and may contribute differentially to vasa vasorum blood supply.

The vasa vasorum flow group of chronically catheterized fetuses (group 2, n=7) received an intravenous infusion of indomethacin (0.2 mg/kg/h) the day after surgery; infusion rate was titrated to achieve significant ductal constriction without compromising the fetus (5-10 ml/min). DA constriction was assessed at 30 minute intervals. Microsphere measurements were made before the indomethacin infusion began and during the first 6 hr of the infusion (between 2 and 6 hr). Injections consisted of two sets of 2-4 million microspheres (approximately $3x10^6$, 15μm; Interactive Medical Technologies Ltd., Irvine, CA) of differing fluorescence which were simultaneously released into the SVC and IVC catheters over 10s and followed by a saline flush. Exact time of injection was based on the magnitude of the pressure gradient across the DA. Reference blood samples were withdrawn from ascending and descending aorta catheters at a constant rate (4ml/min) over the following two minutes using an automated withdrawal system (Harvard Apparatus, South Natick, MA) (see diagram 4). Blood withdrawals were weighed. Oxygen saturation, hemoglobin, blood pH, P_{CO_2}, P_{O_2}, and lactate were monitored at 30 minute intervals. Arterial oxygen content (ml O_2/ml blood) was determined as the product of Hb concentration, oxygen saturation, and an oxygen binding capacity of 1.34 ml O_2/g Hb. Vasa vasorum oxygen delivery (ml O_2/min/gm

tissue) was calculated as the product of arterial oxygen content and vasa vasorum blood flow (ml blood/min/gm tissue).

Vasa Vasorum Blood Flow Determination

The vessels and blood samples were weighed, digested in alkali, and the released fluorescent microspheres were quantified by flow cytometry (25). Process control microspheres were added to each tissue or blood sample to determine the number of microspheres lost during tissue 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).

Systemic arterial blood gas measurements were taken every 30 minutes. Hemodynamic variables (mean systemic and pulmonary pressures) were continuously recorded throughout the study period. THAM was given to decrease acidosis as appropriate. After the third microsphere injection, the lamb was given a lethal dose of pentobarbital solution. The uterine incision was reopened and the fetus weighed. The DA and portions of the ascending and descending aorta were removed from the fetus. The adventitia was carefully stripped from the arterial wall.

Cell Death

Indomethacin infusion in the DA would be expected to produce a variable degree of cell death depending on the severity of constriction and resultant tissue hypoxia that it produced. Tissue hypoxia of the muscle wall is necessary for successful closure of the ductus. Previous work has shown that tissue hypoxia is directly associated with cell loss. The EF5 technique detects regions of hypoxia using nitroimidazoles that bind to cysteine

residues on intracellular proteins after they have been reduced by hypoxia-dependent enzymes (26). It can be used to make a quantitative estimate of oxygen concentration within a tissue using a stain specific for EF5 tissue adducts. Experiments in the baboon ductus have shown that areas of intense EF5 staining in the hypoxic zone also contained large numbers of cells with evidence of nuclear fragmentation and cell death, as assayed by the TUNEL technique. Regions with less intense EF5 binding contained no TUNEL-positive cells. This previous work indicates that a significant association exists between hypoxia and cell death in the ductus, and allows us to use cell death as an indicator of tissue hypoxia (4).

With the presumption that areas of cell death in the constricting DA wall correspond to regions of hypoxia, we attempted to determine how the degree of DA constriction affects the magnitude of cell death. In a third group of 17 fetuses, indomethacin or vehicle (50mM Tris-HCl, 10ml/h) was infused onto the fetus 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 buffered salt solution at 4°C, embedded in Tissuetek (Miles, Inc., Elkhart, IN) and frozen in liquid nitrogen.

We used the terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) technique to detect cells in the early stages of DNA fragmentation and cell death in the indomethacin and vehicle infused fetuses (4). This technique identifies cells undergoing necrosis (27) as well as apoptosis (28). DNA breaks were detected with the Apoptag Peroxidase detection system (Intergen, Purchase, NY). There was no nonspecific binding of reagents to nuclei when deoxynucleotidyl transferase was omitted from the assay (data

not shown). The number of TUNEL-positive nuclei per 100 nuclei was scored in the middle of the muscle media.

To detect endothelial cells, we incubated frozen sections with a mouse monoclonal antibody against endothelial cell nitric oxide synthase (eNOS: Clone 3, Transduction Lab, Lexington, KY) as previously reported (2, (4). Histologic measurements of were made at the level of minimal luminal area, which was determined from serial sections made through the tissue. Vasa vasorum penetrate the outer muscle media of the DA. The muscle media of all arteries has an avascular zone, adjacent to the lumen, which lacks vasa vasorum (10). We defined the avascular zone of the DA as the region of the DA wall between the endothelial cells lining the DA lumen and the leading edge of the vasa vasorum. Tissue dimensions and zone thickness were determined by averaging measurements made from 8 predetermined regions of the section, using a Template and NIH Image software.

In Vitro Oxygen Consumption of DA Rings

Indomethacin may change oxygen consumption in DA tissue independent of alterations in oxygen supply. We designed an apparatus that would allow for the simultaneous determination of O_2 consumption rate and active isometric tension system in DA rings during indomethacin-induced constriction. Fetal lambs were delivered by Cesarean section and anesthetized with ketamine HCl before rapid exsanguination. The ductus was divided into rings (37±7 mg wet weight, n=6) and mounted in a 37°C, water-jacketed glass chamber equipped with an O_2 electrode (see Hellstrand P, Acta Physiol. Scand. 1977: 100: 91) at an optimal length for tension development (7.0±0.6 mm) (29). 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 O_2 into or out of the chamber (30, (31). Sterile buffer solution (in mM: NaCl 120, KCl 4, glucose 10, $MgCl_2$ 1.2, KH_2PO_4 1, $CaCl_2$ 2.6, HEPES 25, pH 7.45 that had been pregassed with 21% O_2) passed through a 0.22μ filter before perfusing the chamber (1.2ml volume) at 0.5 ml/min. A Teflon coated magnetic stirrer ensured adequate mixing.

The oxygen electrode (YSI Model 53 Biological Oxygen Monitor, Yellow Springs, OH) was calibrated with 21% O_2 saturated buffer. The solubility of O_2 in buffer solution was assumed to be 0.20 μmol/ml at 37°C, equilibrated with air (30). Oxygen consumption rate was measured during a 10 min interval during which the tissue chamber was closed. The background oxygen consumption rate of the system (without tissue) was determined in each experiment. The mean background O_2 consumption was 13.3% of the mean measured tissue oxygen consumption rate, which can be attributed primarily to the oxygen consumption of the electrode itself.

The rings were allowed to incubate for 4 h at 37°C during which time basal oxygen consumption rate and isometric tensions stabilized. At 4 h, indomethacin (5.6 μM) was added and the isometric tension and oxygen consumption rate were measured during the next hour. The difference in tensions between the measured tension and the 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 the force per unit cross-sectional area (g/mm^2) (29).

Flow Model

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 (Q_{DA}) can be represented as the sum of the vasa vasorum flow from both the ascending aorta (Q_{aAo}) and descending aorta (Q_{dAo}) to the DA:

$$Q_{DA} = Q_{aAo} + Q_{dAo}$$

Similarly, the number of microspheres in the DA ($microspheres_{DA}$) is:

$$microspheres_{DA} = R_{aAo}\, Q_{aAo} + R_{dAo}\, Q_{dAo} \qquad \text{(eqn. A)}$$

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

Because these are the only sources of DA flow,

$$Q_{dAo} = (x)(Q_{DA}) \qquad \text{(eqn. B)}$$

$$Q_{aAo} = (1\text{-}x)(Q_{DA}) \qquad \text{(eqn. C)}$$

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

Substituting eqn. B and eqn. C in eqn. A and then solving for x:

$$x = \frac{microspheres_{DA} - R_{aAo}Q_{DA}}{R_{dAo}Q_{DA} - R_{aAo}Q_{DA}} \qquad \text{(eqn. D)}$$

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

$$\frac{microspheres_{DA1} - R_{aAo1}Q_{DA}}{R_{dAo1}Q_{DA} \quad R_{aAo1}Q_{DA}} = \frac{microspheres_{DA2} - R_{aAo2}Q_{DA}}{R_{dAo2}Q_{DA} \quad R_{aAo2}Q_{DA}} \qquad \text{(eqn. E)}$$

Solving eqn. E for Q_{DA} gives:

$$Q_{DA} = \frac{\frac{\text{microspheres}_{DA1}}{[R_{dAo1} - R_{aAo1}]} - \frac{\text{microspheres}_{DA2}}{[R_{dAo2} - R_{aAo2}]}}{\frac{R_{aAo1}}{[R_{dAo1} - R_{aAo1}]} - \frac{R_{aAo2}}{[R_{dAo2} - R_{aAo2}]}}$$

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

We used 56 fetal sheep to determine which factors were responsible for indomethacin-induced DA tissue hypoxia. 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 hr, an extensive region of cell loss (absent nuclei) was already apparent in the avascular zone of four of the DA (Fig 1,2). 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 ($\leq$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).

We determined luminal blood flow using Doppler flow transducers around the DA (n=7). Indo constricted the DA in all fetuses (pressure gradient, ΔPress[DA] (mm Hg): preinfusion = 1±1, 4 h = 14±6). Indomethacin altered DA luminal blood flow; however, this was observed only after marked degrees of constriction had been achieved. Moderate constriction (ΔPress[DA] <16 mmHg) did not affect DA luminal flow. Marked constriction ($\geq$16 mmHg) reduced luminal flow to 64±28% of preinfusion levels.

Vasa vasorum flow was determined with fluorescent microspheres (n=7). In contrast to luminal flow, vasa vasorum flow to the DA media was inversely related to the degree of DA constriction ($r=-0.79$, $p<.0001$)(Fig 5). At moderate constriction, vasa vasorum flow was 69±25% of preinfusion levels; at marked constriction, it fell to

30±16%. Oxygen delivery through the vasa vasorum fell in parallel with vasa vasorum flow (in ml O_2/min/gm: 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/gm: preinfusion = 0.027± 0.008; indomethacin = 0.022± 0.010); nor was it affected by the degree of DA constriction (in ml/min/gm: pressure gradient ≤4 mmHg = 0.027± 0.007 versus pressure gradient >16 mmHg = 0.024± 0.014). Individual DA response to indomethacin varied significantly. When experimental flow values for each animal were normalized to control flow values, the relationships to pressure gradient were unchanged. Blood flow to the DA adventitia was not related to the pressure gradient across the ductus.

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

The present study attempts to identify the factors that contribute to tissue hypoxia in the fetal ductus undergoing indomethacin-induced constriction. As the ductus constricts initially, resistance to blood flow through the DA increases, and the pressure gradient between the PA and aorta increases. As the pressure gradient increases, constriction of the muscle media of the DA may cause a decrease in blood flow through the vasa vasorum that course into the muscle media from the adventitia. The increase in muscle media thickness during constriction may increase the distance required for oxygen diffusion into the avascular zone. The decrease in DA luminal blood flow that occurs with marked degrees of initial functional closure leads to a decrease in the diffusion of blood from the lumen into the muscle media (1). The loss of luminal and vasa vasorum blood supply leads to a decreased P_{O2}, one that becomes insufficient to fulfill the oxygen needs of the widened vessel wall. The tissue hypoxia that develops seems to be a critical initiator of the anatomic remodeling that leads to permanent closure (3, 4).

The sequence of events leading from ductus constriction to permanent closure is complex. Ductus constriction is initiated by a decrease in prostaglandin production and sensitivity. The ductus is extremely sensitive to the vasodilating effects of prostanoids, particularly PGE_2, which appears to play a primary role in maintaining ductus patency (32). Prostaglandins are produced primarily by the cyclooxygenase enzyme, which exists in 2 isoforms, COX-1 and COX-2. COX-1 is found in endothelial and smooth muscle cells of the ductus wall, while COX-2 is found exclusively in endothelium. In the fetus, selective inhibition of the COX-1 or COX-2 enzymes independently produces

constriction in the fetal lamb ductus (33). In contrast, the newborn ductus has a much more attenuated response to both COX-1 and COX-2 inhibition, indicating a decrease in expression of both of the COX enzymes soon after delivery (3). The decreased postnatal production of PGE_2 leads to a lower level of circulating vasodilator, which helps to initiate the process of DA constriction.

In addition to a decreased production of prostaglandins by cyclooxygenase, the full-term newborn also has a decreased vasodilatory response to PGE_2. PGE_2 acts through four receptors, EP_1, EP_2, EP_3, and EP_4, each of which is coupled to a distinct second messenger system (34). The fetal ductus expresses three of the four receptors, EP_2, EP_3, and EP_4. In contrast, ligand binding studies and selective EP-receptor stimulated cAMP production experiments have both shown that the newborn ductus undergoes complete loss of EP_3 and EP_4 receptors, leaving only EP_2 receptor concentration unchanged (35). Both decreased prostaglandin production and altered receptor expression decrease the vasodilatory stimuli in the newborn ductus. These changes initate the process of newborn DA constriction and closure.

It should be noted that nitric oxide (NO) also appears to play a role in regulating the postnatal ductus. NO regulates tone in many vascular beds. RT-PCR and immunohistochemistry have been used to show that two of the three NO synthase (NOS) isoforms are present in the DA, ecNOS and iNOS. The former appears to exert the greatest effect on contractility (36). Experiments with competitive inhibitors of NO synthase have shown that blocking NO production in the oxygen-constricted ductus produces a significant further constriction. In contrast, at low P_{O_2}, competitive NO synthase inhibitors had little effect (36). In the DA, NO may play an important

regulatory role at high but not low P_{O_2}. NO release appears be minimal at fetal P_{O_2}, but may be upregulated and have significant vasodilatory effects at neonatal P_{O_2}s. The constriction resulting from decreased prostaglandin production and sensitivity may lower P_{O_2} sufficiently to decrease nitric oxide synthesis.

Constriction of the DA leads to tissue hypoxia in the muscle media. Tissue hypoxia may result from a decrease in DA luminal or vasa vasorum blood flow, or an increase in the thickness of the muscle media. Previous work has shown that regions of tissue hypoxia in the constricting DA muscle media closely correspond with areas of cell death (4). Cell death and anatomic remodeling are required for the DA to achieve permanent closure. As previously discussed, permanent closure occurs through changes in expression of hypoxia-inducible growth factors, production of extracellular matrix components, and cell migration.

Indomethacin has been shown to induce constriction of the fetal and newborn DA in clinical studies, primarily through inhibition of PGE_2 (20). This mechanism has led to the use of indomethacin as a treatment for patent ductus arteriosus (PDA) in newborns. However, indomethacin also leads to constriction of the fetal DA when used maternally as a tocolytic agent. Indomethacin-induced constriction of sufficient severity leads to hypoxia of ductus tissue and initiation of anatomic remodeling, as well as an increase in avascular zone thickness (2).

Indomethacin induced constriction in all the study fetuses. We verified that exposure of the fetus to indomethacin produced nuclear fragmentation and cell death in smooth muscle cells of the DA muscle media (Fig 1,2). We have previously found that decreased cell viability in the DA muscle media (in addition to a reduction in NO

production) leads to a significant decline in both tissue distensibility and contractile capacity and explains why the newborn DA remains patent and fails to close after indomethacin exposure in utero (2). The DA that has been exposed to indomethacin in utero may retain some constrictive capacity, but its ability to constrict sufficiently to induce the tissue hypoxia and subsequent anatomic remodeling needed for permanent closure is compromised. 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 is responsible for this reduced response to indomethacin (2, 4). We found that the extent of cell death is directly related to the degree of DA constriction (Fig 1). Cell death occurred even at moderate degrees of constriction, suggesting that tissue hypoxia becomes significant even before the DA constricts completely.

Moderate indomethacin-induced constriction produces cellular changes associated with tissue hypoxia, which appears to be the critical initiator of the anatomical changes that lead to failed constriction. However, the specific mechanisms leading from constriction to tissue hypoxia have not been clearly delineated. In the maturing fetus, oxygen delivery to the ductus wall is tightly regulated. Vasa vasorum appear to invade the fetal DA only when the developing muscle media attains a certain minimal thickness, presumably outgrowing diffusion of nutrients from the luminal flow. Although vessel wall thickness varies markedly between species, the thickness of the inner avascular zone of the muscle media remains constant at 0.47 ± 0.06 mm (10, 11, 37, 38). 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 examined which contributors to oxygen supply (luminal blood flow, vasa vasorum blood flow, and avascular zone thickness) and oxygen demand (indomethacin-induced changes in oxygen consumption) were affected by indomethacin-induced DA constriction. Our findings indicate that the increased incidence of muscle media cell death associated with indomethacin-induced constriction was not due to an increase in tissue oxygen consumption *in vitro* (Fig 3). Indomethacin had no effect on oxygen consumption in the DA; this finding is consistent with other studies in both noncontractile (hepatic, jejunal, gastric, and cerebral) and contractile (myocardial) tissues (39, 40, 41, 42, 43). Indomethacin is known to cause DA constriction by inhibiting prostaglandin production and unmasking the intrinsic tone in the ductus. While overall oxygen consumption does not appear to change during indomethacin-induced constriction, opposing pathways may be at work and could help to explain this finding. An increased metabolic need for oxygen during constriction could be balanced by a decreased requirement for oxygen as a substrate for prostaglandin synthesis. Conversely, decreasing oxygen concentration may also have a direct influence on DA prostaglandin production, suggesting that a more complex feedback loop may exist (44, 45). In addition, small overall changes in oxygen consumption may not have been detectable with the apparatus that we developed. Although indomethacin requires oxygen to produce constriction in DA rings in vitro, it does not appear from our experiment to alter net DA oxygen consumption independently (36). This novel finding suggests that oxygen demand is not a driving force for tissue hypoxia in indomethacin-induced DA constriction.

On the other hand, indomethacin had a profound effect on the blood flow and muscle media 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 (Fig 4,5,6).

Indomethacin induced DA constriction in all fetuses, leading to an increased resistance to luminal flow. In the fetus, high pulmonary vascular resistance opposes the increase in pulmonary blood flow that occurs during DA constriction in the newborn. At moderate degrees of DA constriction, the pressure generated by right ventricular contraction was sufficient to overcome the increasing resistance to flow through the contracting DA. Not until the pressure gradient across the DA exceeded 20 mmHg did luminal flow begin to decrease in comparison to preinfusion levels. The right ventricular pressure generated at moderate levels of constriction appears to be sufficient to preserve DA luminal blood flow. Our experiments establish that luminal blood flow is compromised only at marked degrees of constriction, and may not contribute to hypoxia during moderate constriction.

Tissue compaction during constriction of the DA is responsible for the initial increase in avascular zone thickness in the DA wall (22). We hypothesized 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). The novel finding in this experiment is that vasa vasorum blood flow to the DA media was reduced even at moderate levels of constriction, and further reduction occurred as DA constriction grew more severe. Physical occlusion could

explain the decreased flow through vasa vasorum, although other factors may play a role. The vasa vasorum are lined with smooth muscle cells and may also respond independently to local changes in oxygen supply, glucose level, and soluble mediators of constriction. An initial compression and reduction in blood flow could subsequently alter local conditions that might contribute to constriction. Neurohumoral reflexes might also contribute to the constrictive response to tissue hypoxia (46). We noted no decrease in vasa vasorum flow in the adjacent aorta during ductus constriction, indicating that in general vasa vasorum do not respond independently to indomethacin. However, it is possible that, like the ductus luminal endothelium, ductus vasa vasorum may have a unique sensitivity to prostaglandins which contributes to constriction during indomethacin infusion. Reduced blood supply from vasa vasorum during moderate constriction would contribute to decreased oxygen tension in the susceptible avascular zone. Marked constriction appears to generate tissue hypoxia through both a further decrease in vasa vasorum flow and compromised luminal blood flow.

The notion that tissue compaction physically occludes the vasa vasorum in the muscle media is reinforced by the finding that adventitial vasa vasorum flow did not consistently decline during DA constriction. Adventitia generally receives a relatively higher blood flow via vasa vasorum than does the muscular media in vascular tissue. Our data did not show a consistent relationship between ductal constriction and adventitial flow. The vasa vasorum in the adventitia may escape the constrictive effect that contraction of the muscular media has on medial vasa vasorum. Adventitial vasa vasorum did not appear to constrict predictively with indomethacin, which would have suggested that another mechanism at work. It should be noted that fetal brown fat, which

is highly vascularized and difficult to completely remove from adventitial tissue, may have affected the accuracy of our adventitial flow data.

In addition to its effects on blood flow to the DA, indomethacin caused a marked increase in the thickness of the muscle media and the oxygen diffusion gradient across 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) (4, 22). Particularly intriguing from this study is that even moderate degrees of DA constriction produced an increase in avascular zone thickness (Fig 6). The thickness of the avascular zone was directly related to the degree of DA constriction. The resulting increase in diffusional distance may decrease oxygen tension in the center of the unperfused media; at moderate levels of constriction, avascular zone thickness was 151% of control values. The combination of increased avascular zone thickness and decreased blood flow through vasa vasorum at moderate levels of constriction facilitate tissue hypoxia generation in the muscle media.

Our findings allow us to speculate on some of the previosly noted clinical effects of indomethacin on the DA. Wall thickness in the preterm DA is less than that of the full-term DA, and the preterm ductus may not be as dependent on vasa vasorum flow for oxygenation. With more immature infants, permanent ductus closure becomes increasingly difficult (15). Even with functional constriction, the preterm DA is less able to generate the levels of tissue hypoxia required for anatomic remodeling (4). Because the preterm ductus may not be dependent on vasa vasorum flow, moderate levels of constriction, which our experiments have shown do not affect luminal flow, may not be sufficient to produce the levels of hypoxia necessary for DA closure. Marked

constriction, which begins to compromise luminal flow, may be needed for permanent remodeling and closure. The sustained levels of tissue hypoxia required for permanent remodeling in the preterm ductus may only be reached with the most marked levels of constriction.

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 (18). The presence of vasa vasorum in the muscle media depends on the thickness of the arterial wall (10, 47). In the human fetus, vasa vasorum usually enter the ductus wall after the 28th week of gestation (16). Before 28 weeks, luminal blood flow is sufficient to meet the oxygen demands of the thin-walled ductus. Previous reports have shown that infants >28 weeks gestation are much more likely to be affected by in utero indomethacin exposure than infants <28 weeks gestation (18). 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 (2) (Fig. 1). An increase in avascular zone thickness and a decrease in vasa vasorum flow appear to be responsible for the tissue hypoxia and cell death that occur in the late gestation ductus. These novel findings help to explain why infants <28 weeks 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, while fetuses >28 weeks gestation who are exposed to indomethacin have a higher incidence of postnatal patent ductus arteriosus.

Although newborns >28 weeks gestation who have received indomethacin in utero are at greater risk than premature infants, both groups have an increased incidence of postnatal patent ductus arteriosus. The preterm newborn may respond to both earlier

pharmacologic intervention and an increased number of indomethacin doses with more severe constriction, increasing the likelihood of permanent closure (15). Because the preterm DA has not undergone cellular remodeling and hypoxic change, its constrictive capacity would be expected to remain intact. In contrast, the newborn exposed to indomethacin in utero after 28 weeks gestation is far more likely to have sustained hypoxia-induced injury to the DA wall, which makes it less responsive to prostaglandin inhibition as a newborn. Intensive treatment with indomethacin may increase closure rates in premature infants, but other forms of treatment for PDA might be more appropriate in patients who have received indomethacin after 28 weeks gestation. Clinical studies would help to better define the effect of postnatal indomethacin on different groups at risk for patent ductus arteriosus.

Further work is needed to more completely characterize the relationship between indomethacin-induced DA constriction, tissue hypoxia, cell death, and ductus closure. Oxygen consumption is difficult to measure in the ductus because of the multiple oxygen utilization pathways present and the small size of the tissue. Further experiments might use a substance that induced constriction independent of prostaglandins, since prostaglandin production requires oxygen as a substrate. Measuring oxygen consumption after potassium administration, which induces intense ductus constriction, might allow for better characterization of metabolic vs. substrate-driven oxygen consumption (36).

More precise characterization of the hypoxic threshold for apoptosis and cell death is currently underway. Using an in vitro tissue bath system in which the oxygen and glucose concentration of the media can be carefully controlled, we have begun to study TUNEL staining in both the near-term and premature fetal ductus under a variety of

conditions. Using rings of DA from mature and immature lamb fetuses suspended in organ culture baths, we incubated tissue for 24 h at high and low concentrations of both O_2 and glucose. Initial findings indicate that the percentage of TUNEL stained cells increases with either hypoxia or hypoglycemia, but that extensive cell death, as normally occurs in the full-term newborn in vivo, was seen exclusively in rings exposed to both hypoxic and hypoglycemic conditions. Mature and immature DA tissue response was similar, indicating that the preterm DA is equally susceptible to cell death when hypoxia and hypoglycemia become sufficiently severe.

Our data suggested that the DA vasa vasorum have a critical role in inducing tissue hypoxia at moderate degrees of constriction, even in the presence of persistent luminal blood flow. We designed an experiment to determine whether loss of vasa vasorum flow alone is sufficient to produce tissue hypoxia and remodeling of the DA wall. We performed a thoracotomy on 6 fetal lambs and cauterized the adventitial surface of the main pulmonary artery, aortic isthmus, and descending aorta. The vasa vasorum travel through the adventitia of these vessels before perfusing the DA. Initial findings indicate a marked reduction in perfusion of cells in the regions supplied by the cauterized vasa vasorum. There was an increase in neointimal thickness and cell death in the affected areas of the DA wall. These findings support our contention that vasa vasorum occlusion in the late gestation DA is sufficient to produce tissue hypoxia and anatomic remodeling. The premature DA, which lacks vasa vasorum, would need to completely obliterate its lumen before generating the same degree of tissue hypoxia.

This study establishes a critical role for vasa vasorum as a regulator of tissue oxygenation in the near-term ductus undergoing indomethacin-induced constriction.

Even moderate degrees of constriction are sufficient to decrease vasa vasorum blood flow and increase wall thickness and oxygen diffusion distance to the avascular zone. These findings explain why infants exposed to indomethacin in utero after 28 weeks gestation (the time at which vasa vasorum begin to provide a vital oxygen supply to the ductus wall) have an increased incidence of postnatal patent DA. Luminal flow, which provides oxygenation to the DA wall prior to 28 weeks, is only reduced at the most severe levels of indomethacin-induced constriction, and may not be as frequent a contributor to tissue hypoxia near-term ductus. Further studies are underway to more precisely define the hypoxic thresholds for cell death and anatomic remodeling in the DA, and to establish the dependence of the near-term ductus on the vasa vasorum for oxygen supply.

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Figure Legends

Figure 1. Moderate and marked degrees of constriction cause an increase in 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 prior to necropsy. Filled circles and squares represent 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 a closed circle), an extensive region (> 20,000 μm^2) 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.

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

Figure 3. Indomethacin increases active tension in DA rings in vitro but has no effect on oxygen consumption (n = 6). DA rings were incubated in buffer solution for 4 hr (control period) prior to indomethacin exposure. See Methods for details. Arrows indicate time of indomethacin (5.6 μM) addition. Values = mean ± SD, n = 6. * $p<0.05$ versus control (–15 min) period. $p<0.05$: tension at 60 min versus tension at 30 min.

Figure 4. Only marked degrees of ductus constriction decrease ductus luminal blood flow during indomethacin infusion. Panel A) Ductus luminal blood flow and pressure gradient across the ductus were monitored continuously during an indomethacin infusion into 7 fetal sheep. See Methods. Open diamonds = values prior to indomethacin infusion

(n = 7); closed diamonds = values during indomethacin infusion. Values represent the mean (± SD) luminal flow at each pressure gradient across the ductus; all 7 fetuses developed pressure gradients up to 16 mm Hg during the indomethacin infusion; only 3 fetuses had pressure gradients that also went up to 24 mm Hg. Panel B) Columns represent the mean (± SD) flows before indomethacin (preinfusion, open column) and during the indomethacin infusion (closed columns), when the pressure gradient was either ≤4 mm Hg, between 4 and 16 mm Hg, or >16 mm Hg. *$p<0.05$ versus preinfusion values.

Figure 5. Both moderate and marked degrees of ductus constriction decrease ductus vasa vasorum blood flow during indomethacin infusion. Panel A) A preinfusion microsphere measurement of ductus vasa vasorum blood flow was performed in each of the 7 fetuses prior to starting the indomethacin infusion. Following the preinfusion measurements, a total of 13 measurements were performed during the indomethacin infusions in the 7 fetuses (see Methods). Pressure gradients across the ductus were measured during each microsphere measurement. Panel B) Columns represent mean (±SD) vasa vasorum flow prior to indomethacin (open column, n = 7) and during the indomethacin infusion (closed columns) when the pressure gradient was either ≤4 mm Hg (n = 2), between 4 and 16 mm Hg (n = 7), or >16 mm Hg (n = 4). *$p<0.05$ versus preinfusion values; $p<0.05$ versus values when pressure gradient was between 4 and 16 mm Hg.

Figure 6. Both moderate and marked degrees of ductus constriction increase the thickness of the avascular zone and total muscle media of the ductus. (see Methods for definitions). Fetuses were infused with either vehicle control (open columns, n = 8) or indomethacin (closed columns, n = 9) for 24h. Pressure gradient across the ductus was

measured prior to necropsy. Histologic measurements obtained from indomethacin-infused fetuses with a moderately constricted ductus (>4 and ≤16 mmHg, n = 6) and those with a markedly constricted ductus (>16 mmHg, n = 3) were compared with control fetuses (≤4 mmHg, n = 8). *$p<0.01$, $p<0.05$ versus control values. #$p<0.05$ versus values when pressure gradient across ductus was >4 and ≤16.

Diagram 1

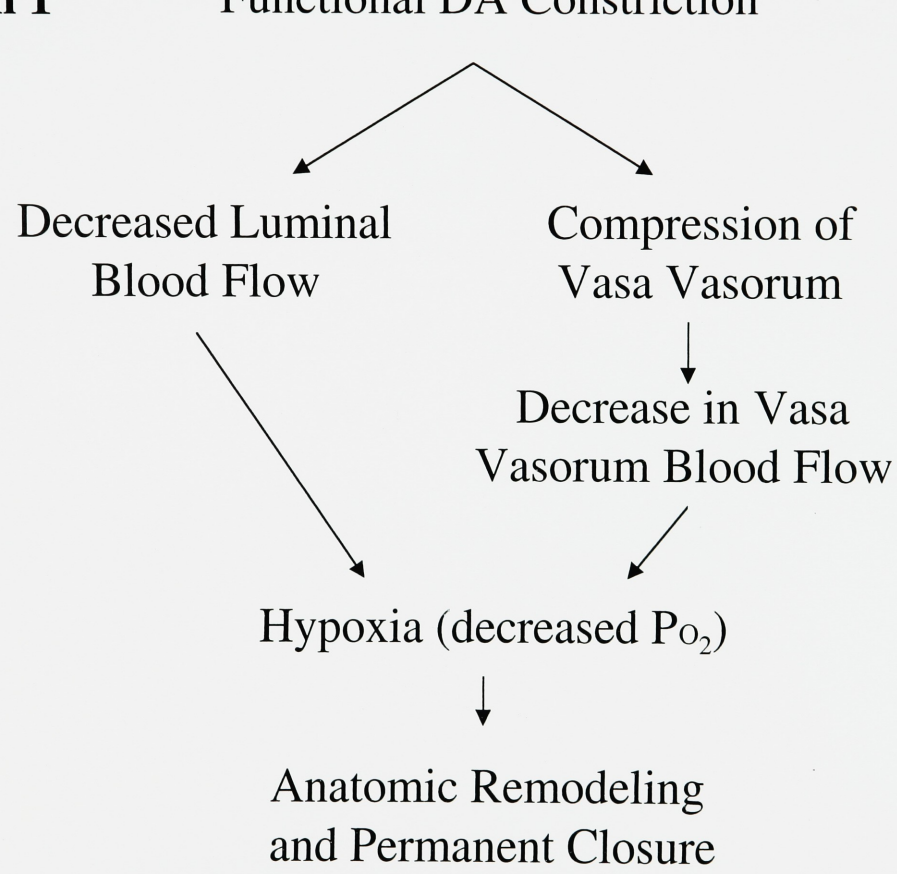
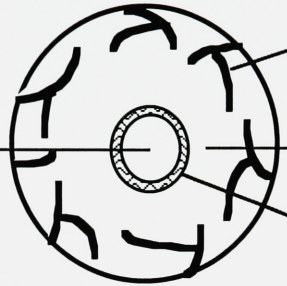

Functional DA Constriction
Decreased Luminal Blood Flow
Compression of Vasa Vasorum
Decrease in Vasa Vasorum Blood Flow
Hypoxia (decreased P_{O_2})
Anatomic Remodeling and Permanent Closure

Diagram 2



Vasa Vasorum

Lumen

Avascular Zone

Endothelium

Decreased Luminal Flow

Decreased Vasa Vasorum Flow

Increased Diffusion Distance

Increased Oxygen Consumption

Hypoxia

Diagram 3

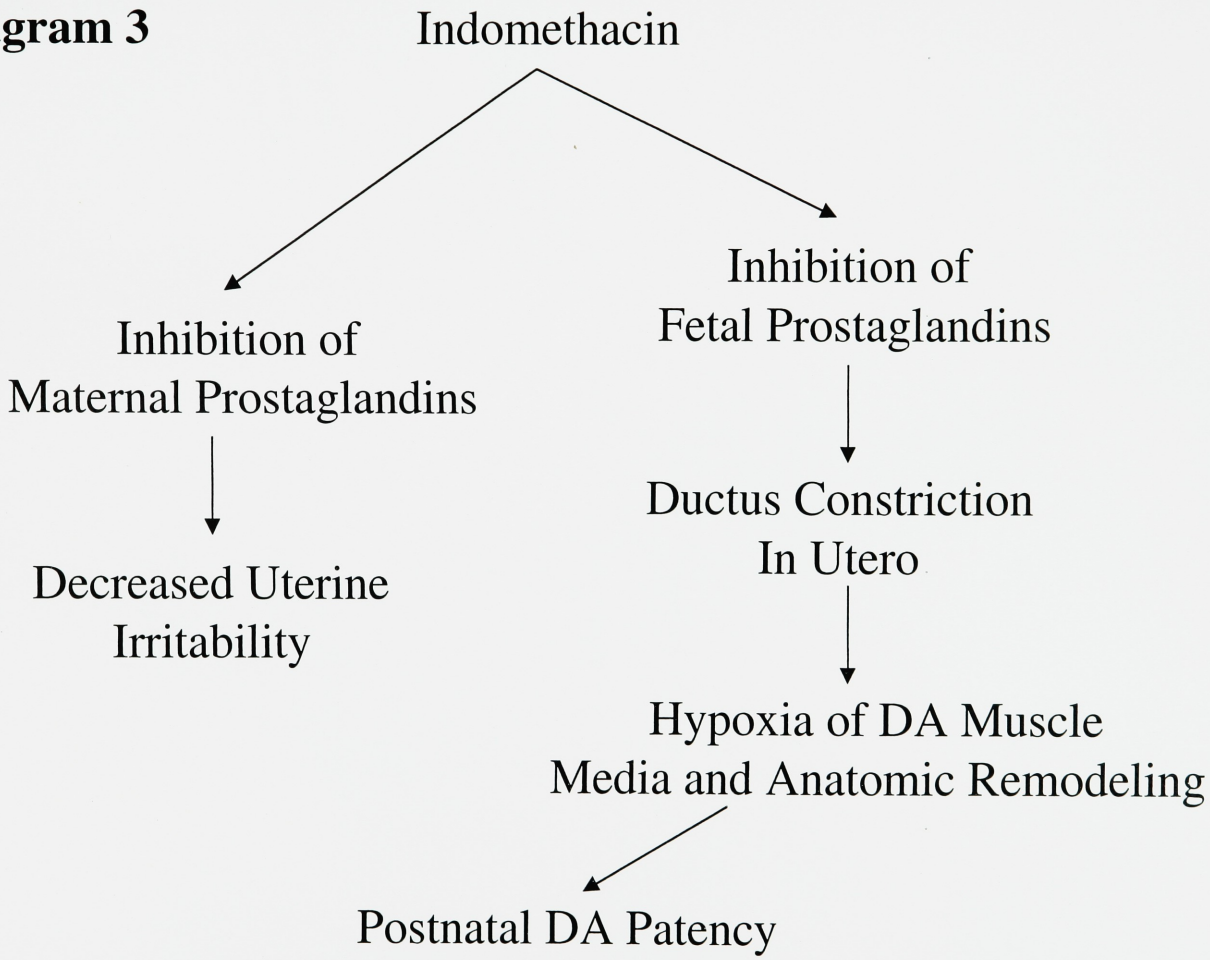

Indomethacin
Inhibition of
Maternal Prostaglandins
Decreased Uterine
Irritability
Inhibition of
Fetal Prostaglandins
Ductus Constriction
In Utero
Hypoxia of DA Muscle
Media and Anatomic Remodeling
Postnatal DA Patency

Diagram 4

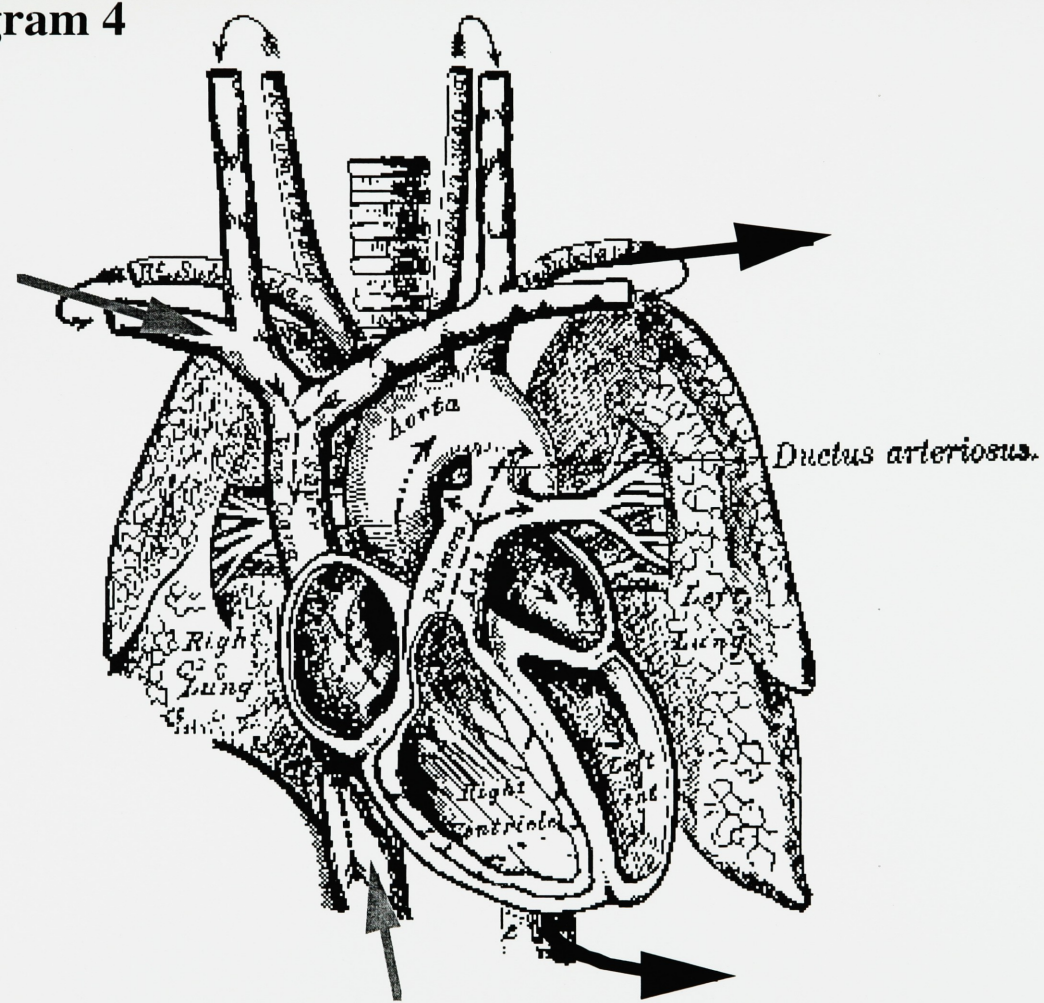


Figure 1

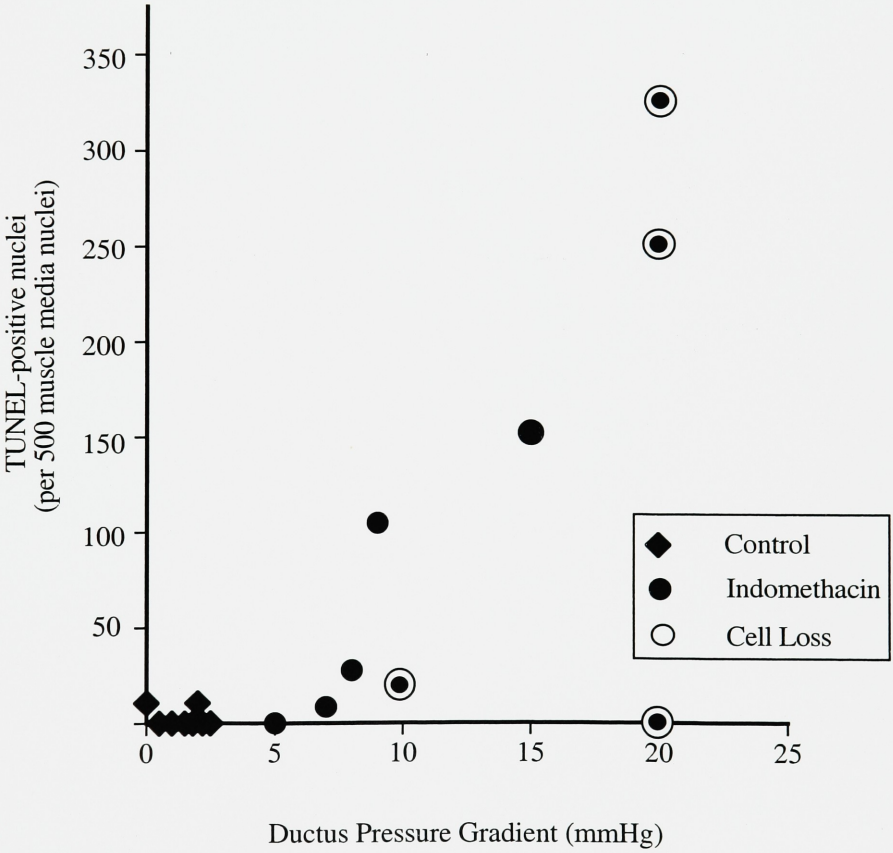

TUNEL-positive nuclei
(per 500 muscle media nuclei)
350
300
250
200
150
100
50
0
5
10
15
20
25
Ductus Pressure Gradient (mmHg)
Control
Indomethacin
Cell Loss

Figure 2

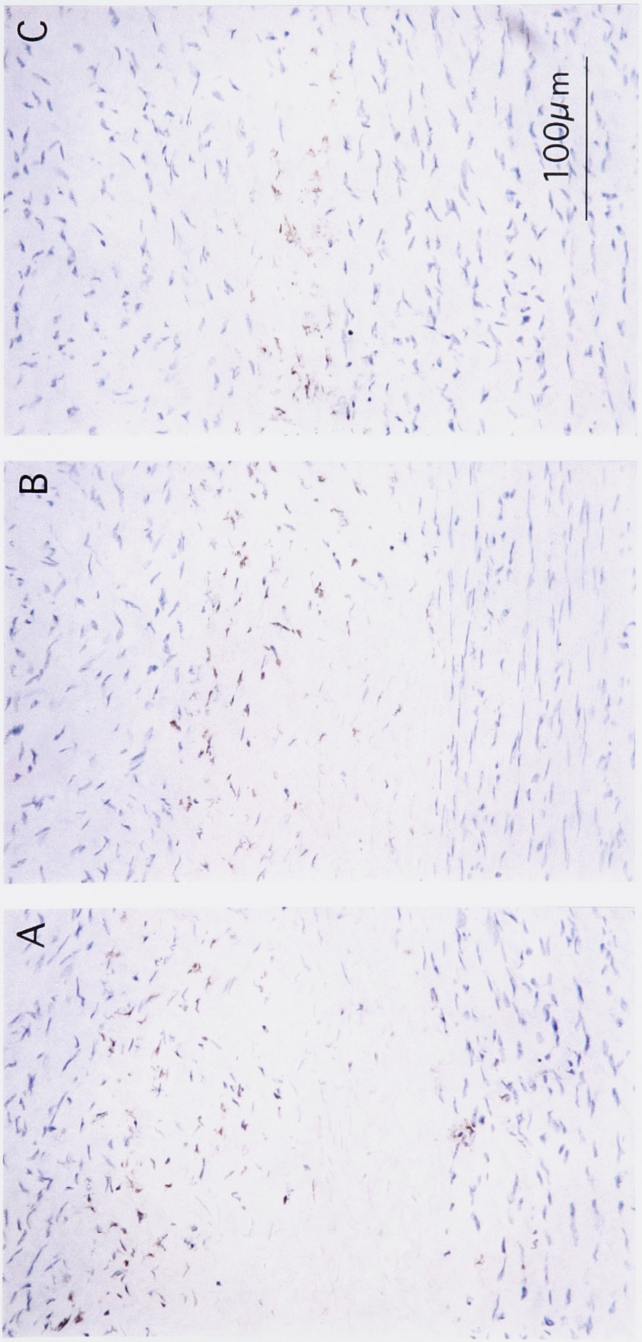


Figure 3

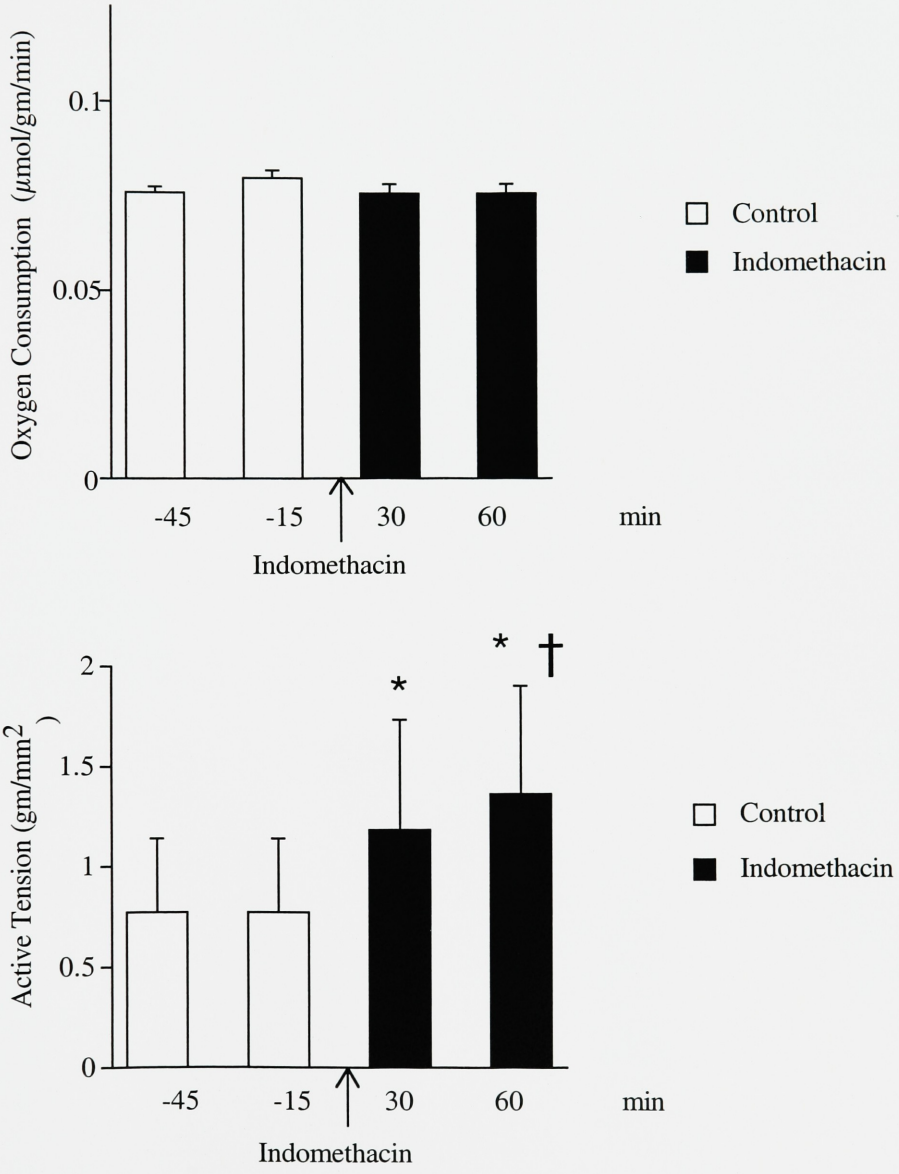

Oxygen Consumption (μmol/gm/min)
0.1
0.05
0
-45
-15
30
60
min
Indomethacin
Control
Indomethacin
Active Tension (gm/mm2)
2
1.5
1
0.5
0
*
*
†
-45
-15
30
60
min
Indomethacin
Control
Indomethacin

Figure 4

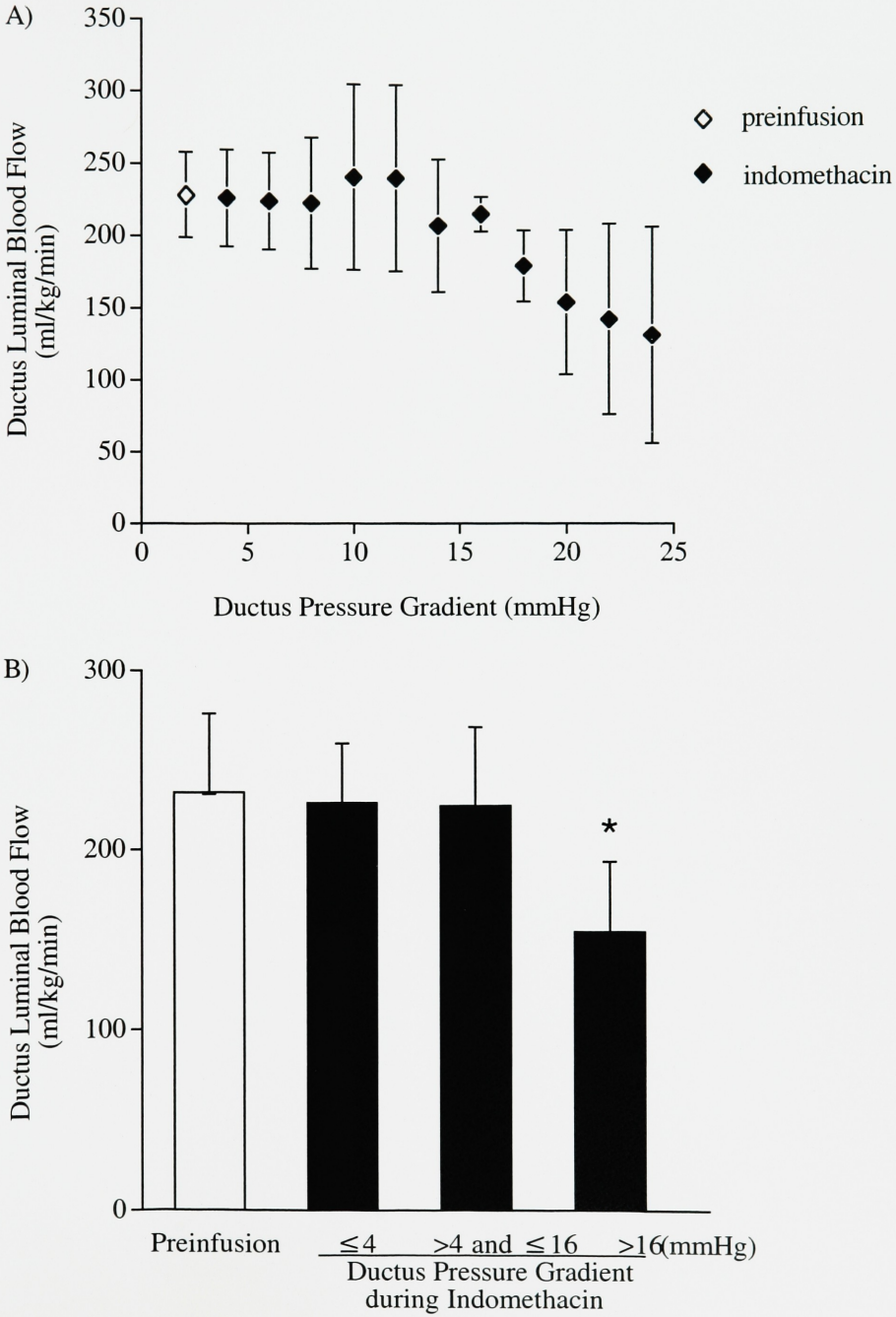
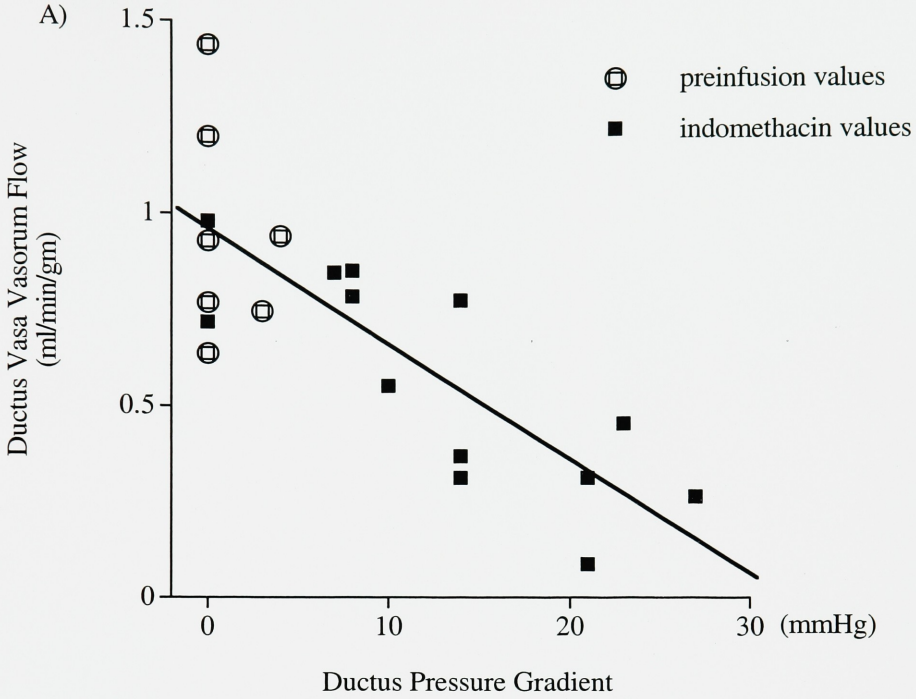

A)
350
300
250
200
150
100
50
0
Ductus Luminal Blood Flow (ml/kg/min)
0
5
10
15
20
25
Ductus Pressure Gradient (mmHg)
preinfusion
indomethacin
B)
300
200
100
0
Ductus Luminal Blood Flow (ml/kg/min)
*
Preinfusion
≤4
>4 and ≤16
>16(mmHg)
Ductus Pressure Gradient during Indomethacin

Figure 5


A)
1.5
1
0.5
0
preinfusion values
indomethacin values
Ductus Vasa Vasorum Flow
(ml/min/gm)
0
10
20
30
(mmHg)
Ductus Pressure Gradient

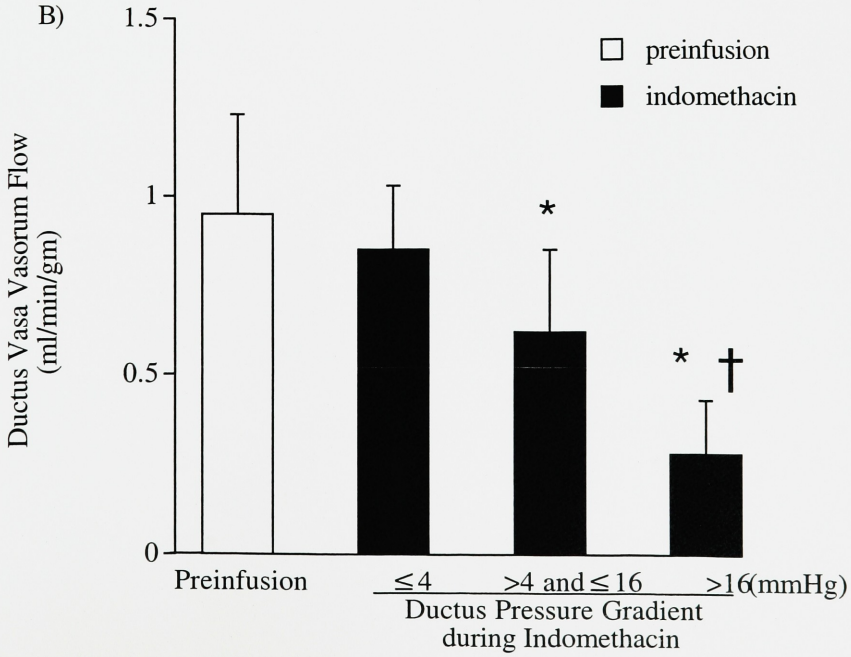
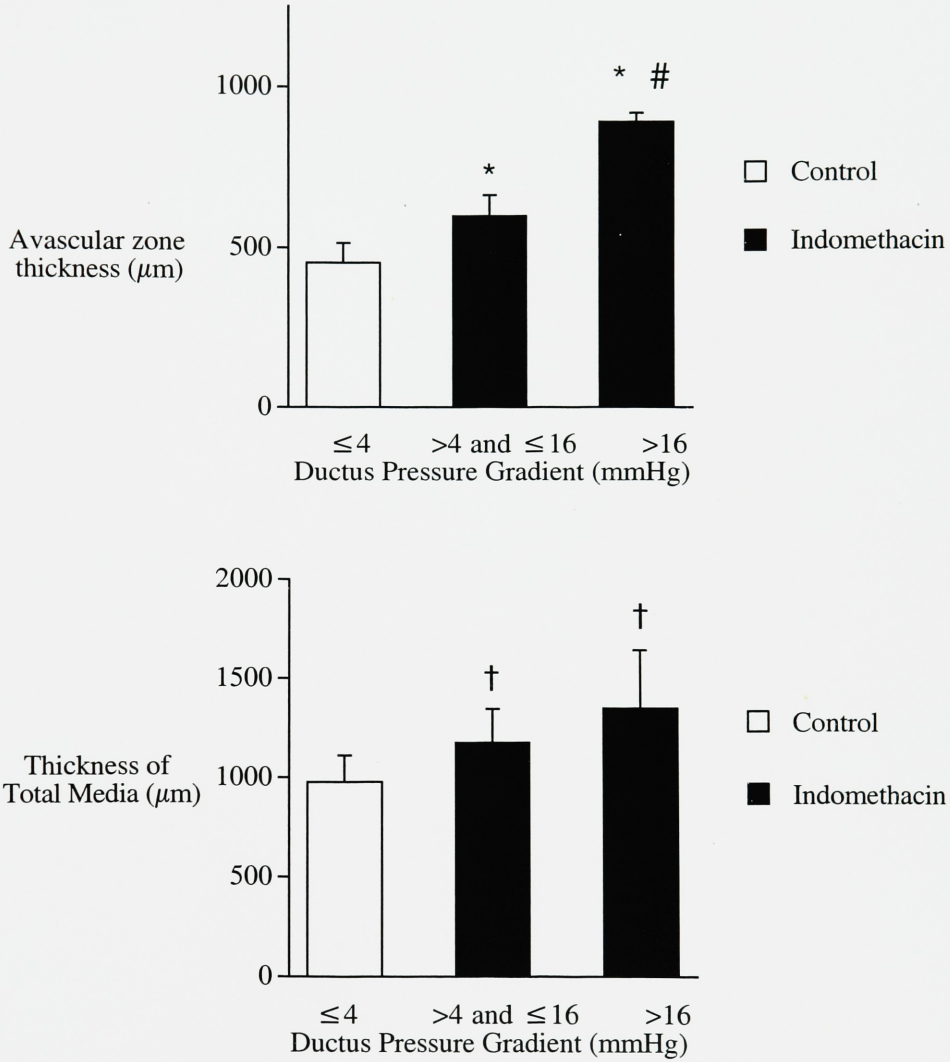

B)
1.5
1
0.5
0
preinfusion
indomethacin
Ductus Vasa Vasorum Flow
(ml/min/gm)
*
*
†
Preinfusion
≤4
>4 and ≤16
>16
(mmHg)
Ductus Pressure Gradient
during Indomethacin

Figure 6


Avascular zone thickness (μm)
1000
500
0
* #
*
Control
Indomethacin
≤4
>4 and ≤16
>16
Ductus Pressure Gradient (mmHg)
Thickness of Total Media (μm)
2000
1500
1000
500
0
†
†
Control
Indomethacin
≤4
>4 and ≤16
>16
Ductus Pressure Gradient (mmHg)

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