In Utero Remodeling of the Fetal Lamb Ductus Arteriosus
The Role of Antenatal Indomethacin and Avascular Zone Thickness on Vasa Vasorum Proliferation, Neointima Formation, and Cell Death

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Background—The ductus arteriosus (DA) of newborn infants exposed in utero to indomethacin is resistant to postnatal indomethacin; we hypothesized that this is due to ductus constriction in utero, with subsequent remodeling of the vessel.

Methods and Results—Infusion of fetal lambs with indomethacin for 48 hours constricted the DA and increased the thickness of the avascular zone of the DA, which in turn induced the expression of vascular endothelial growth factor, endothelial nitric oxide synthase (due to ingrowth of vasa vasorum), neointima formation, and loss of smooth muscle cells; moderate degrees of DA constriction in utero increased NO production, which inhibited DA contractility. Marked degrees of DA constriction decreased tissue distensibility and contractile capacity.

Conclusions—DA patency is no longer controlled primarily by prostaglandins once it has been exposed to indomethacin in utero. (Circulation. 2001;103:1806-1812.)

Key Words: nitric oxide synthase ■ prostaglandins ■ endothelium-derived factors ■ nitric oxide ■ pregnancy

Prostaglandins (PGs) play a significant role in maintaining ductus arteriosus (DA) patency in the fetus and preterm newborn. Indomethacin produces constriction of both the fetal and newborn DA.1-3 Therefore, it is surprising that preterm newborn infants who have been exposed to indomethacin shortly before birth (during maternal tocolysis) have an increased incidence of patent DA (PDA) after delivery that is resistant to postnatal indomethacin treatment.4

We hypothesized that indomethacin induces DA constriction in utero, which produces DA wall hypoxia and remodeling, and that these changes would be analogous to those observed during postnatal constriction of the newborn DA. Postnatal constriction has been shown to produce a zone of profound hypoxia in the muscle media of the DA; hypoxia induces vascular endothelial growth factor (VEGF) expression or cell death, depending on its severity.5 Oxygen normally reaches the muscle media of the DA through either the lumen or the intramural vasa vasorum of the vessel. The depth to which vasa vasorum penetrate the muscle media depends on the thickness of the vessel wall.6 The muscle media of all arteries has an avascular zone adjacent to the lumen, which lacks vasa vasorum.6 The avascular zone thickness appears to be constant (0.47±0.6 mm).5,6 Normal arterial wall oxygenation is highest immediately adjacent to the vessel lumen, diminishes to a nadir in the middle of the avascular zone, and increases progressively again toward the vasa vasorum–rich outer muscle media.7,8 Therefore, the avascular zone, which depends on flow from both the lumen and the vasa vasorum to meet its nutrient needs, may be particularly vulnerable to changes in oxygen supply. Oxygen reserves can be exceeded if there is a decrease in luminal or vasa vasorum blood flow or if there is an increase in the distance that oxygen needs to diffuse (by an increase in the thickness of the avascular muscle media).5,7,9,10

In the present study, we tested the hypothesis that indomethacin-induced DA constriction in utero would increase the thickness of the DA avascular zone. We hypothesized that increases in avascular zone thickness would lead to progressive increases in VEGF and NO production and loss of medial smooth muscle cells and that these changes would decrease the ability of the DA to contract.

Methods

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

Pregnant sheep (mixed Western breed: 127 to 131 days gestation, term 145 days) were operated on under ketamine-diazepam anesthesia as previously described.11 The fetus was exposed through a uterine incision. The ascending aorta and superior vena cava were catheterized via the brachial artery and vein, respectively, and the main pulmonary artery through a thoracotomy. In 7 fetuses, a 4- to 6-mm Doppler flow transducer (Transonics Systems) was placed...
around the DA. The thoracotomy and skin incisions were closed, the fetus was returned to the uterus, and the laparotomy was closed. The vascular catheters were sealed with heparin and exteriorized.

One day after surgery, fetuses were infused for 48 hours intravenously with vehicle (50 mmol/L Tris-HCl, 10 mL/h) or indomethacin (0.2 mg · kg⁻¹·h⁻¹ estimated fetal weight); this dose produced stable plasma indomethacin concentrations (0.65±0.24 µg/mL, n=6). DA constriction was assessed in vivo by measurement of the pressure gradient across the fetal DA between the pulmonary artery and ascending aorta at 24 hours and 48 hours during the infusion and 24 hours after it was discontinued. In some fetuses, the DA was visualized 24 hours after start of the infusion with a 10F intravascular ultrasound catheter (Boston Scientific). The DA was considered to be widely patent if its narrowest diameter was >50% of the diameter of the main pulmonary artery (MPA), moderately constricted if it was <50% and >25% of the MPA diameter, and markedly constricted if it was <20% of the MPA diameter.

In Vitro Studies

The fetus was delivered by cesarean section and anesthetized with ketamine (30 mg/kg IM). The fetus was rapidly exsanguinated before spontaneous breathing. The DA was collected either at the end of the indomethacin/vehicle infusion (n=35) or 24 hours after it was discontinued (n=36).

Western Analysis of Endothelial NO Synthase and VEGF

Frozen tissue was homogenized in lysis buffer and centrifuged. Aliquots of the supernatant (20 µg/lane) were resolved by 9% SDS-PAGE and transferred to Immobilon P membranes (Millipore). Filters were blocked with 5% nonfat milk plus 0.01% Tween-20, followed by incubation with 0.25 µg/mL mouse monoclonal anti-endothelial NO synthase (eNOS) clone 3 antibodies (Transduction Laboratories) or 0.5 µg/mL rabbit polyclonal anti-VEGF antibodies (Santa Cruz Biotechnology) for 2 hours at room temperature and detected with a phosphatase-IgG–coupled detection system (Jackson Laboratories). Protein bands were visualized with the ECL kit (Pharmacia) and densitometry analyzed (NIH Image software and Adobe Photoshop).

Immunohistochemistry

Protocols for immunohistochemistry were similar to those reported. Briefly, DA were frozen immediately in liquid N₂ (for eNOS and von Willebrand factor [vWF] detection) or fixed for 10 hours at 4°C in fresh 4% paraformaldehyde before paraffin embedding (for VEGF and vWF detection). Frozen sections (6 µm) were incubated with either the anti-eNOS antibody (0.3 µg/mL) or rabbit anti-human vWF antibody (1 µg/mL, Dako) for 2 hours before detection. Paraffin-embedded sections (6 µm) were rehydrated and incubated overnight at 4°C with either anti-VEGF (0.6 µg/mL) or anti-vWF (1 µg/mL) antibody. Control sections were treated similarly and showed no staining (data not shown). Assays for any given antibody were reproduced on 3 separate occasions. Scoring for VEGF was as follows: 0, no staining; 1+, weak staining; 2+, moderate staining; and 3+, intense staining.

The avascular zone was defined as the region of the ductus wall without vasa vasorum between the endothelial cells lining the ductus lumen and the leading edge of the vasor vasorum into the muscle media from the adventitia. The neointimal zone was defined as the region between the luminal endothelial cells and the internal elastic lamina (identified by phase-contrast microscopy). A neointima was considered to be present when nonendothelial cells expanded the neointimal zone by >20 µm. Zone thicknesses were determined from 8 predetermined regions of the section by use of a template and NIH Image software.

Contraction Studies

The DA was divided into 1-mm-thick rings, and isometric tension was measured in a Krebs-bicarbonate solution. After the tension reached a plateau in 30% oxygen (Po₂ 175 to 200 mm Hg), indomethacin (5.6 µmol/L) was added. Once a new steady-state tension was reached, 100 µmol/L N'-nitro-L-arginine methyl ester (L-NAME) was added; maximal contraction was determined by the response to 100 µmol/L K⁺-Krebs solution. Minimal tension was determined by the response to the combination of sodium nitroprusside (SNP, 0.1 mmol/L) and EGTA (10 mmol/L).

The difference in tensions between the measured steady-state tension and the minimal tension produced by SNP plus EGTA was considered to be the active tension. The difference in tensions between the maximal contraction produced by K⁺-Krebs and the minimal tension was treated as the maximal active tension developed by the rings. The difference in tensions between the steady-state tension achieved with indomethacin and the steady-state tension achieved in 30% O₂ alone was considered the indomethacin-induced tension. The difference in tensions between the steady-state tension achieved after L-NAME and the steady-state tension after indomethacin was considered the L-NAME-induced tension.

Tissues were blotted dry and weighed after the experiments. The tension developed in the ring was expressed either as the force per unit cross-sectional area (g/cm²) or as a percentage of maximal active tension. In some experiments, bath solution was collected to measure PGE₂ and 6-keto-PGF₁α (PGI₂ metabolite) by radioimmunoassay.

Statistics

Statistical analyses of unpaired and paired data were performed with the appropriate t test or regression analysis. When >1 comparison was made, Bonferroni’s correction was used. Nonparametric data were compared by a Mann-Whitney test. Results are presented as mean±SD.

Results

Ductal Patency

Ductal patency was visualized in vivo 24 hours after the infusions were started. Among 8 indomethacin-infused fetuses, 5 were markedly constricted, 2 moderately constricted, and 1 widely patent; all control DA were patent (n=3). Indomethacin produced a greater change in the pressure gradient across the DA than in luminal blood flow (Figure 1). Luminal flow was still 64±28% of the preinfused level when the pressure gradient was ≥20 mm Hg (Figure 1).

After a 48-hour infusion, the pressure gradient across the DA was 14±7 mm Hg in indomethacin-infused animals (n=13) and 2±1 mm Hg in controls (n=12); Pao₂ (mm Hg)
was similar in the 2 groups (controls, 16±3; indomethacin-infused, 15±3).

DA Dimensions
The numbers of concentric muscle layers throughout the DA were similar in indomethacin- (48±6, n=13) and vehicle-infused (46±5, n=12) fetuses. Wall thickness, however, was greater in indomethacin-infused (1232±231 μm) than in control (1046±210 μm) fetuses, P<0.05; this difference was due to the substantial increase in avascular zone thickness in the indomethacin-infused fetuses (679±177 μm) compared with controls (497±99 μm), P<0.05. The number of muscle layers in the avascular zone did not differ between the indomethacin-infused (25±3) and control (26±3) animals. Although the thickness of the avascular zone often varied in the same DA (mean±SD, 18±8%), the maximal thickness was greater in the indomethacin-infused (753±159 μm) than in control (555±161 μm) fetuses. The thickness of the avascular zone was directly related to DA constriction (maximal thickness versus pressure gradient, r=0.77, P<0.01, n=25).

VEGF and eNOS
We hypothesized that increasing avascular zone thickness would lead to increased expression of the hypoxia-inducible growth factor, VEGF, in the DA wall. The DA from the indomethacin-infused fetuses expressed more VEGF (Figures 2, 3, and 4; P<0.05) than those from the controls. VEGF expression correlated with the thickness of the avascular zone (r=0.84, P<0.01, n=20; Figure 4). When the avascular zone was >500 μm, there was a progressive increase in VEGF expression in the muscle media (Figure 4). eNOS was expressed by endothelial cells lining the lumen and vasa vasorum of the DA (Figure 3). The increased expression of
VEGF in the indomethacin-infused animals was associated with an increase in the number of vasa vasorum, which, secondarily, increased the amount of eNOS in the DA (Figures 2 and 3).

Neointima Formation and Cell Loss
We hypothesized that increasing avascular zone thickness would lead to progressive DA remodeling. A neointima was present in 4 of 12 control fetuses; in each case, the neointima was restricted to less than half of the luminal circumference. In contrast, 10 of 13 indomethacin-infused fetuses had a neointima that either partially (n = 4) or completely (n = 6) encircled the lumen. The neointima (when present) was thinner in the control (40 ± 19 μm) than in the indomethacin-infused (79 ± 27 μm) fetuses (P < 0.05) (Figure 3D through 3F). Neointimal formation was significantly related (P < 0.01) to the thickness of the avascular zone (Figure 5) and was observed only when the avascular zone thickness was >600 μm.

We observed an extensive region of cell loss in the center of the DA wall in 5 of 13 indomethacin-infused fetuses (Figures 3C, 3F, and 5). Cell loss was detected only when the avascular zone was >800 μm (P < 0.01) (Figure 5).

In Vitro Contractions
We hypothesized that (1) moderate degrees of in utero constriction would lead to increased eNOS and NO production, which would oppose the effects of contractile agents on the DA, and (2) marked degrees of constriction would lead to smooth muscle cell loss, which would decrease the ability of the DA to contract. We used a separate group of 36 fetuses to examine the effects of in vivo constriction on in vitro contractility. The pressure gradient across the DA in the control fetuses was <4 mm Hg (mean 2 ± 1 mm Hg) during the 48 hours of infusion. We divided the indomethacin-infused fetuses into 2 groups according to their degree of constriction: in 1 group (moderate constriction, n = 14), the pressure gradient never exceeded 15 mm Hg (9 ± 4 mm Hg), whereas in the other (marked constriction, n = 10), it was >20 mm Hg (21 ± 2 mm Hg).

We waited 24 hours after stopping the infusions to allow indomethacin to be eliminated from the fetus (T 1/2 ± 0.07 hours) before removing the DA for our in vitro studies. By 24 hours, the pressure gradient returned to control values in the moderately constricted fetuses; in the markedly constricted group, it never dropped below 6 mm Hg (8.8 ± 2.5 mm Hg).

During an indomethacin infusion, PG production by the DA is reduced to 25% of control levels. By 24 hours after the infusion was stopped, PG production by indomethacin-exposed DA returned to control levels (Figure 6).

The distensibility (Figure 7) and maximal active tension (Figure 8) of DA from the indomethacin-exposed, moderately constricted fetuses were similar to control DA; active tension (35 ± 10 g/cm²) in response to 30% oxygen, however, was less than in control DA (55 ± 16 g/cm²) (P < 0.01) (Figure 8). We used indomethacin and L-NAME to uncover the role of endogenous PGs and NO in DA contractility. DA from both groups were equally responsive to exogenous PGE₂ and an NO donor, SNP (Figure 9). When indomethacin was added to the organ baths, DA rings from the moderately constricted...
fetuses developed an increase in tension (112±20 g/cm²) similar to that of controls (123±33 g/cm²) (Figure 8). In contrast, L-NAME produced a significantly greater tension (84±18 g/cm²) in the moderately constricted fetuses than it did in controls (43±17 g/cm²) (P<0.01) (Figure 8).

The distensibility (Figure 7) and maximal active tension (Figure 8) of the DA from the indomethacin-exposed, markedly constricted fetuses were significantly reduced compared with controls. As a result, their ability to contract in response to 30% oxygen, indomethacin, or L-NAME in vitro was significantly reduced (Figure 8).

Discussion

Six separate clinical studies16–20 have examined the incidence of PDA in preterm infants (<30 weeks gestation) who failed indomethacin tocolysis and were delivered shortly (<72 hours) after being exposed to indomethacin in utero. In this group of infants, the incidence of PDA that needed surgical ligation because of unresponsiveness to postnatal indomethacin therapy was significantly increased (meta-analysis odds ratio 5.9, 95% CI 3.3 to 10.8, P<0.01).

We found that VEGF and eNOS expression, neointima formation, and muscle media cell death were increased in the DA from fetuses that were infused with indomethacin in utero (Figures 2 through 5). We hypothesized that hypoxia of the DA wall due to increased avascular zone thickness was responsible for these changes. Preliminary studies from our laboratory suggest that indomethacin has only a limited effect on DA vasa vasorum blood flow in utero (unpublished observations). We also found that indomethacin causes only a modest decrease in DA luminal blood flow (Figure 1).

In the newborn DA, avascular zone thickness plays a critical role in determining the degree of hypoxia and remodeling of the DA wall; hypoxia and remodeling do not occur, even in the presence of a marked reduction in luminal blood flow, unless there is also a significant increase in avascular zone thickness.5 In our studies, exposure of the fetus to indomethacin caused a marked increase in the thickness of the avascular zone of the DA wall. The thickness of the avascular zone appeared to be determined by the degree of
DA constriction in utero. The increased avascular zone thickness was due to tissue compaction (caused by circumferential and longitudinal muscle contraction), rather than by endothelial or smooth muscle cell proliferation. In addition, we found that fetal DA remodeling was directly related to the thickness of the DA avascular zone (Figures 4 and 5). Although we did not measure the oxygen concentration in the DA wall, we hypothesize that the anatomic changes observed in the fetus are due to increasing degrees of smooth muscle hypoxia (a result of the increasing oxygen diffusion gradient across the avascular zone), as previously described in the newborn.

We found that control fetuses also had a wide variation in avascular zone thickness (Figure 5); this was directly related to the degree of DA constriction in utero (maximal avascular zone thickness [controls] versus pressure gradient [controls], r = 0.97, n = 12, P < 0.01). We hypothesize that the increase in fetal DA tone that normally occurs with advancing gestation increases the degree of DA constriction in utero. This would explain the increase in avascular zone thickness, VEGF expression, and neointima formation that occurs with advancing gestation.

We hypothesize that the hypoxia-inducible growth factor VEGF may play a pivotal role in vasa vasorum ingrowth and neointimal expansion, because it stimulates endothelial cell proliferation and permeability as well as NO production and monocyte chemotaxis. Previous studies have established an inverse relationship between endothelial cell integrity and neointima formation. Neo-intima formation may be due, in part, to the increased access of medial smooth muscle cells to blood-borne mitogens and chemoattractants.

We observed an extensive region of cell loss in the muscle media of the indomethacin-constricted DA when the thickness of the avascular zone increased beyond 800 μm (Figure 5). In other blood vessels, cell loss has been attributed to decreased luminal blood flow and decreased shear stress. This is an unlikely explanation for the extensive cell loss observed in the DA, however, because shear forces within the DA would be expected to increase, rather than decrease, during constriction. The pattern and location of cell loss in the DA wall suggest that the profound muscle media hypoxia that develops as the thickness of the avascular zone increases beyond the limits of its oxygen and nutrient supplies is responsible for this occurrence.

We found that the DA, in vitro, was less likely to constrict when exposed to either oxygen or the combination of oxygen plus indomethacin if it had previously been constricted with indomethacin in utero (Figure 8). It is unlikely that the decreased ability of the DA to constrict is due to residual effects of the in utero indomethacin infusion on PG production, because PG synthesis is similar in control and indomethacin-infused animals (Figure 6). Our findings suggest 2 distinct mechanisms that can explain the altered contractility: (1) increased NO production and (2) loss of smooth muscle cells. Moderate degrees of DA constriction in utero are associated with increased VEGF expression, ingrowth of vasa vasorum, and increased eNOS expression (Figures 2 and 3); inhibition of NO production produces a significantly greater contraction in the moderately constricted DA than in the control DA (Figure 8). These findings suggest that increased NO production contributes significantly to lower DA tensions. Unfortunately, we were unable to measure NO production in our tissue baths. We used a Sievers model 280 Nitric Oxide Analyzer to measure NO release by measuring its nitrate reaction products in the solution surrounding the DA. In our experiments, the rate of release of NO was below the limits of detection of the assay system (data not shown).

When indomethacin produces a marked degree of DA constriction in utero, there is a large increase in avascular zone thickness, which is associated with smooth muscle cell loss and smooth muscle cell migration into the neointima; this is associated with a significant decline in both tissue distensibility and maximal contractile capability (Figures 7 and 8). These experimental observations in fetal lambs help to explain the results of the meta-analysis of clinical trials reported above.

Acknowledgments

This study was supported in part by US Public Health Service, National Heart, Lung, and Blood Institute grants HL-46691 and HL-50601, and a gift from the Perinatal Associates Research Foundation. The authors thank Hensy Fernandez for technical assistance and Victoria Fontana, MSW, for editorial assistance.

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Circulation. 2001;103:1806-1812
doi: 10.1161/01.CIR.103.13.1806

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/103/13/1806