Patency of the Preterm Fetal Ductus Arteriosus is Regulated by Endothelial Nitric Oxide Synthase and is Independent of Vasa Vasorum in the Mouse

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Abstract

Patency of the fetal ductus arteriosus (DA) is maintained in an environment of low relative oxygen tension and a preponderance of vasodilating forces. In addition to prostaglandins, nitric oxide (NO), a potent vasodilator in the pulmonary and systemic vasculatures, has been implicated in regulation of the fetal DA. To further define the contribution of NO to DA patency, the expression and function of NO synthase (NOS) isoforms was examined in the mouse DA on days 17-19 of pregnancy and after birth. Our results show that endothelial NOS (eNOS) is the predominant isoform expressed in the mouse DA and is localized in the DA endothelium by in situ hybridization. Despite rapid constriction of the DA after birth, eNOS expression levels were unchanged throughout the fetal and postnatal period.

Pharmacologic inhibition of prostaglandin versus NO synthesis in vivo showed that the preterm fetal DA on day 16 is more sensitive to NOS inhibition than the mature fetal DA on day 19, whereas prostaglandin inhibition results in marked DA constriction on day 19 but minimal effects on the day 16 DA. Combined prostaglandin and NO inhibition caused additional DA constriction on day 16. The contribution of vasa vasorum to DA regulation was also examined. Immunoreactive PECAM and lacZ-tagged FLK1 localized to DA endothelial cells but revealed the absence of vasa vasorum within the DA wall. Likewise, there was no evidence of vasa vasorum by vascular casting. These studies indicate that eNOS is the primary source of NO in the mouse DA, and that vasomotor tone of the preterm fetal mouse DA is regulated by eNOS-derived NO and is potentiated by prostaglandins. In contrast to other species, mechanisms for DA patency and closure appear to be independent of any contribution of the vasa vasorum.
Introduction

The ductus arteriosus (DA) is a fetal vascular shunt that is interposed between the pulmonary artery and aorta. *In utero*, the DA conducts over 90% of right ventricular output to the aorta, bypassing the fetal pulmonary system. After birth, the transition from fetal to neonatal life is dependent on the newborn’s ability to establish its own respiratory gas exchange and redirect blood flow into the vessels of the newly inflated lungs. To accomplish this, simultaneous relaxation of the pulmonary vascular bed and constriction of the DA must occur soon after delivery. Many factors have been identified that affect fetal DA patency and postnatal closure (7, 47) but the molecular sequence of events that regulate this transition have not been fully resolved.

The preterm DA is morphologically and biochemically immature (4, 46, 49) and poorly responsive to contractile stimuli compared to the full term DA (11, 30, 31, 52). In contrast to the adjacent elastic great vessels, the DA has overlapping layers of vascular smooth muscle that facilitate rapid constriction of the DA lumen after birth. Relaxation of DA smooth muscle *in utero* occurs in an environment of low oxygen tension and the presence of vasodilators such as adenosine and certain prostaglandins (47). Nitric oxide (NO) is another mediator of vascular smooth muscle tone that is implicated in regulation of the DA (10). NO is a potent metabolite of L-arginine produced by the three known isoforms of NO synthase (NOS): type 1(neuronal or nNOS), type 2 (inducible or iNOS) and type 3 (endothelial or eNOS). Accordingly, NO is primarily found in neurons, monocytes/macrophages and vascular endothelia but is also active in the parenchyma of many other tissues. Compounds that inhibit NO synthesis constrict the lamb DA *in vitro* (9, 10, 13), providing indirect evidence of endogenous NO production. NOS inhibitors also constrict the DA of other species (3, 6, 32, 43) while NO donors can dilate the DA *in vitro* and *in vivo* (6, 9, 48, 53). Removal of the DA endothelium does not eliminate DA
constriction in response to NOS inhibitors (3, 9, 10) and eNOS has been localized in the vasa vasorum of the DA (9, 20). Both findings suggest that intramural sources of NO contribute to DA regulation. There is also recent evidence that NO mediates vasomotor tone of the immature DA more than the term DA (32, 51), whereas prostaglandins are more important for regulation of the term DA. Combined inhibition of both NO and prostaglandin synthesis has more impact on DA patency than inhibition of either system alone (32, 43, 50).

Currently, there is little information available to explain the differential sensitivity of the term and preterm DA to NO or prostaglandins. Controversy also exists regarding the source of NO since NO production in the lamb DA has been attributed to each of the three NOS isoforms (9, 27, 40). The newborn mouse DA is functionally closed by 3-4 hours of age and has structural features similar to larger species (49). Genetic and pharmacologic studies have also demonstrated the importance of mouse models of DA regulation (24, 34, 41, 42). A recent study shows that eNOS is expressed in the mouse DA at term gestation (2) but there is no information on NO actions in the DA during development. In addition, the levels of NOS expression in a given tissue may not correlate well with the levels of NO production. We hypothesized that NO would mediate DA relaxation in utero and that declining NO synthesis would accompany DA constriction after birth. Our results show that eNOS is the predominant NOS isoform in the mouse DA. In the preterm fetal DA, we observed that NO is the primary mediator of DA tone, and is augmented by the action of prostaglandins. Conversely, prostaglandins are the primary mediator of the term fetal DA, but the contribution of NO appears less significant at this time. Persistent expression of eNOS after DA closure suggests that NO may have additional roles in the DA remodeling process. Although the vasa vasorum are important for closure of the DA in some species, our data show that DA constriction can occur in the absence of vasa vasorum. These findings highlight the diverse functions of NO signaling in fetal vasculature during
development and provide additional insight into the mechanisms of DA regulation during vascular transition at birth.

**Materials and Methods**

**Animals and tissues**

All animals were housed in an AAALAC approved facility and were managed in accordance with NIH animal care standards. Adult female CD-1 mice (7-8 weeks old; Charles River, Raleigh, NC) were bred with fertile males for timed pregnancies (day 1 = presence of vaginal plug). Transgenic mice harboring a β-galactosidase reporter construct under the regulation of the flk-1 promoter were maintained as heterozygotes (flk-1lacZ/+ on a 129/Bl6 mixed genetic background (44). Flk-1lacZ/+ females were bred with wildtype males to generate wildtype and heterozygous littermates.

Tissue specimens were collected from pups after pregnant females received deep anesthesia by isoflurane inhalation (Baxter, Deerfield, IL) and were killed by cervical dislocation at 0900h on days 17-19 of pregnancy (mice in this colony typically deliver on the evening of day 19). Fetal DAs were isolated by sharp dissection immediately after delivery from non-breathing pups that were anesthetized by maternal isoflurane. Only the first 4-6 pups were used from each litter. Newborn DA samples were removed from isoflurane-anesthetized pups at 12-18 hours of age on postpartum day 1 (PP1). For each time point, 10-12 DA samples were obtained from 2-4 litters and pooled for analysis. To ensure sufficient starting material, the pooled sample of 10-12 DAs was considered as a single representative sample. Three separate groups of pooled DA samples were obtained at each time point; therefore, a minimum of 30 DAs and over 6 litters were represented for each gestational day. Separate groups of tissues were collected for
conventional PCR versus those analyzed by real-time, quantitative PCR. Tissues were placed in Trizol (Invitrogen, Carlsbad, CA), homogenized by repeated passage through a 27-gauge needle and total RNA was extracted per protocol. For localization studies, fetal and neonatal hearts were injected with a warmed solution of 4% low melting temperature agarose and 0.1% Chicago blue dye. The dissection field was rinsed with room temperature saline to set the agarose gel, and an anterior portion of the left and right ventricles were excised along with their respective outflow tracts. Tissues were mounted flat on a block of agarose gel, snap frozen, and stored at -80°C for subsequent analysis.

**Drug administration and estimation of DA patency**

Some pregnant CD-1 females received two doses of either indomethacin, 5.0 mg/kg/dose, i.p. (Merck & Co., West Point, PA), L-NG-nitroarginine methyl ester (L-NAME), 50 mg/kg/dose, i.p. (Cayman Chemical Co., Ann Arbor, MI) or saline, 0.15 ml i.p. Doses were given on day 15, 2100 h and day 16, 0900 h or on day 18, 2100 h and day 19, 0900 h. Four hours after the final dose, females were anesthetized and killed by cervical dislocation. Anesthetized, non-breathing fetal pups were removed for immediate assessment of DA status. Fetuses that attempted breathing or had any evidence of lung insufflation at the time of dissection were excluded from evaluation. Fetal DAs were individually inspected as soon as they were delivered from the uterus. DA constriction was estimated by visual observation of its luminal diameter, since intimal cushion formation is not required for mouse DA closure (49). The degree of constriction was scored by direct visualization in 25% increments under a dissecting stereomicroscope by a single observer who was blind to the treatment group (J.R.). The narrowest point of the DA luminal diameter was assessed in relationship to the internal
caliber of the main pulmonary artery below the outlet of the branch pulmonary vessels, as previously described (41).

Measurement of NO products

The effect of maternal L-NAME administration on fetal NO synthesis was determined in a separate group of mice from the above experiments. To examine the baseline fetal response, day 19 pregnant females were injected with a single dose of L-NAME, 50 mg/kg i.p., or 0.1 ml of saline at 0900 h. Blood samples were obtained from each pup immediately after cesarean section at 1300 h. To examine the LPS-stimulated fetal response, day 19 pregnant females received 100 μg of E.coli LPS (Sigma-Aldrich, St. Louis, MO), i.p., at 0900 h, followed by a single dose of L-NAME 50 mg/kg i.p., or 0.1 ml of saline at 1100 h. Pups were delivered by cesarean section at 1500 h, such that dams had 6 hours of LPS exposure and 4 hours of L-NAME or saline treatment prior to delivery. For both approaches, blood samples were collected in heparinized capillary tubes after cervical transection, separated by brief centrifugation, and the plasma fraction stored at -80 C for later analysis. Four to eight pups were analyzed per litter. NO products were measured in plasma after reduction to NO using a vanadium HCl catalyst. The NO generated was reacted in an ozone chamber and detected by chemiluminescence in a nitric oxide analyzer (Sievers 280i NOA, Boulder, CO). Sample quantification was performed by interpolation from standard curves generated from sodium nitrite injections.

Gene expression

Temporal changes in NOS isoform expression were analyzed by RT-PCR. Total RNA from day 17, 18, and 19, and PP1 DA specimens was reverse transcribed with oligo-dT and amplified with 28-30 cycles of PCR using isoform-specific primers for nNOS, iNOS, eNOS
(st97-678, st96-527, st97-689; Gene Expression Database (GXD), The Jackson Laboratory, Bar Harbor, ME), and the housekeeping gene ribosomal protein L7 (rpL7). Control samples included adult kidney and skeletal muscle RNA. RT-PCR products were electrophoresed, transferred onto nylon membranes and subjected to Southern hybridization with \(^{32}\text{P}\)-labeled internal oligonucleotides (nNOS: 5’-CGGTACCACGAGGACATCTT-3’; iNOS: 5’-
CCTCCTCCACCCCTACCAAGT-3’; eNOS: 5’-CACGAGGACATTTTCGGACT-3’). Southern blots were exposed from 1-24 hours. The intensity of NOS hybridization signal was analyzed by densitometry (Alpha Innotech, San Leandro, CA). Results were interpreted in relation to rpL7 expression because of the increased mass of the closed (PP1) versus the patent DA (days 17-19) and as an index of RNA loading. Experiments were repeated for each of the 3 groups of DA samples and averages obtained. Real-time quantitative PCR (Roche Diagnostics, Indianapolis, IN) was also performed on reverse-transcription products for the 3 groups from each time point. Serial dilution standards (100 pg-0.01 fg) for nNOS, iNOS, eNOS and rpL7 were derived from gel-purified RT-PCR products of positive control tissues. FastStart PCR reactions (Roche) were optimized to determine primer and template concentrations, magnesium concentration and resolve melting curve artifacts. Quantification of DA eNOS expression was determined in relation to serial dilutions of target sequence standards and normalized to rpL7 expression (as a loading control) according to the manufacturer’s recommendations (LightCycler software version 3.3).

**In situ hybridization**

To examine the cell-specific localization of NOS gene expression, *in situ* hybridization was performed as previously described (41). Briefly, 10 μm frozen sections of the right and left ventricles and outflow tracts were mounted onto polyL-lysine coated slides, fixed in cold 4%
paraformaldehyde, acetylated, and hybridized at 45 C for 4 hours in formamide hybridization buffer containing an $^{35}$S-labeled eNOS cRNA antisense probe (derived from the cDNA product above). RNase A-resistant hybrids were detected by autoradiography after 18-24 days of exposure using Kodak NTB-2 liquid emulsion. Parallel sections were hybridized with the corresponding sense probe. Sections were briefly poststained with eosin.

**Immunohistochemistry**

Frozen sections of ductal and control tissues were thaw mounted on glass slides then fixed in acetone at 4 C. Immunolocalization of platelet endothelial cell adhesion molecule (PECAM-1, rat anti-mouse monoclonal, BD Biosciences, San Diego, CA) was performed by serial washing steps, blocking non-specific staining with 10% nonimmune serum for 10 minutes, overnight incubation with 1:500 dilution of primary antibody at 4 C, 10-15 minute exposure to secondary antibody and incubation with chromagen/substrate according to the manufacturer’s recommendations (Zymed Laboratories, San Francisco, CA). Red-brown color indicated the sites of immunoreactive protein localization.

**LacZ staining**

Expression of the β-galactosidase reporter gene was assessed by staining for lacZ activity in day 19 and PP1 DA of wildtype or flk-1$^{lacZ/-}$ littermates. Briefly, 10 m frozen serial sections through the heart and outflow tracts of wildtype and transgenic pups were mounted on the same glass slide, fixed in 0.2% paraformaldehyde for 10 minutes, rinsed, stained with X-gal at 37 C for 2 hours and cleared with xylene per established protocol (28). Sections were counterstained with eosin.
**Vascular casting**

The vascular network surrounding the DA was examined by corrosion casts of day 19 fetuses and 1 and 3 hour old newborn pups. Fetal pups were surgically removed from anesthetized females at noon on day 19. Newborn pups were delivered by cesarean section on the afternoon of day 19 and maintained in room air until sacrifice under anesthesia. The chest wall was removed and 0.5 ml of phosphate buffered saline was injected into the right ventricle with a 32 gauge needle. A low viscosity acrylic resin used to visualize small (5 μm) capillary beds (Mercox, Ladd Research Industries, Williston, VT) was injected into the right and/or left ventricle under gentle pressure via 27 gauge needle and 1 ml syringe until the onset of polymerization (2-3 minutes). Carcasses were incubated in a 55°C oven for 30 minutes; extra tissues were cut away, then the thorax transferred to a 55°C water bath for 4 hours. Soft tissues were digested in 10% NaOH for 48 hours, cleared in distilled water at 60°C for three days, then dried and photographed.

**Statistics**

Student’s t-test was used to compare the level of eNOS expression relative to rpL7 at each stage of gestation, as determined by Southern hybridization or quantitative RT-PCR. Fetal NO production in saline versus L-NAME treated dams was also compared by t-test. Responses of the fetal DA to drug or saline exposure were compared by Chi square analysis on day 16 and between day 16 and day 19 groups. An extension of Fisher’s exact test was used to compare treatment groups on day 19 of gestation due to separation of data points between the groups. Analyses were performed using SAS version 8.2 (SAS Institute, Cary, NC).
Results

*eNOS is the predominant NOS isoform expressed in the mouse ductus arteriosus*

nNOS and iNOS expression were at the lower limits of detection by semi-quantitative RT-PCR. After 30 cycles of PCR, amplification products were only visible after Southern hybridization and 20-24 hour exposure times (Fig. 1A). The use of 40 amplification cycles showed only a marginal increase in detection (data not shown). In contrast, eNOS and rpL7 expression were detected after 28 cycles and brief exposure of the Southern blots. Densitometry revealed a trend toward increased expression from day 17 to day 19 but did not reach statistical significance (p=0.22). Real-time PCR showed linear amplification of DA samples during 28-32 cycles of PCR. Quantitative analysis showed consistent, low levels of eNOS expression from day 17 through the postpartum period (Fig 1B). There was no statistically significant difference between time points (p=0.14, d19 vs. PP1). Thus, despite the increased mass of DA tissue on PP1 after DA constriction, analysis of eNOS expression relative to the housekeeping gene rpL7 suggests that the thin and patent fetal DA has equivalent levels of eNOS expression to the thick, closed DA on PP1.

*eNOS mRNA is expressed in the ductal endothelium during fetal patency and postnatal closure*

The cell-specific expression pattern of eNOS was examined by *in situ* hybridization. On day 19, sparse eNOS autoradiographic signals were detected in the DA as well as the aorta and pulmonary artery. The compact nature of these vessels prevented distinction between signal localization in the vessel wall and endothelial surface (Fig. 2). Signals were increased over background but the accumulation was only somewhat higher than sense-labeled control slides. After constriction of the DA on PP1, eNOS signals were primarily restricted to the endothelial
cells in the closed DA lumen (Fig. 2). Lower levels of eNOS signal were noted in the DA wall or adventitia, but it was unclear whether this was localized to any particular structure. Specific, low level hybridization was also observed in the endothelial surface of the aorta and main pulmonary artery and throughout the myocardium.

*Developmental maturity confers differential sensitivity to NOS inhibition vs. prostaglandin inhibition*

Ductal patency was estimated by direct observation of DA caliber in comparison to the main pulmonary artery. Fetuses were immobile and not breathing at birth due to maternal anesthesia, thus cervical transection and exsanguination were avoided as a cause of inadvertent fetal DA constriction. The fetuses of saline-treated females on day 16 (n = 54, 4 litters) had some reduction of DA caliber using this approach, suggesting that mild DA constriction can occur with this technique. However, the DA of day 16 pups exposed to 2 doses of indomethacin (n = 39, 3 litters), a non-selective cyclooxygenase inhibitor, was no different than saline treated pups (Fig. 3). In contrast, day 16 fetuses that were exposed to L-NAME (n = 75, 7 litters), a non-specific inhibitor of all NOS isoforms, had more ductal constriction than either saline or indomethacin-treated mice \((\chi^2 = 17.4; p<0.01)\), suggesting that NOS inhibition has more impact than cyclooxygenase inhibition at this stage of gestation. Combined treatment with two doses of L-NAME and indomethacin resulted in fetal death and increased maternal mortality (6 litters). However, when a single dose of each inhibitor was given and c-section was performed four hours later (n = 41, 4 litters), day 16 fetuses were noted with significantly more constriction than was noted with L-NAME treatment alone \((\chi^2 = 39.7; p<0.01)\) (Fig. 3).

On day 19, there was marked reduction in the DA caliber of fetuses exposed to indomethacin (n = 33, 3 litters) versus either saline (n = 50, 4 litters) or L-NAME (n = 35, 3
litters) (p<0.0001). In the majority of these cases, the DA was completely closed (Fig. 3). DA constriction appeared somewhat more frequently in the aortic junction of the DA than the pulmonary. Pups with complete in utero DA closure were no different in external appearance than littermates with patent DAs. Despite severe DA constriction, all indomethacin-exposed fetuses were observed with regular heartbeats at the time of dissection. There was no difference in the number of fetal resorption sites between indomethacin and saline-treated females. In contrast to indomethacin treatment, there was no difference in DA caliber between L-NAME and saline-exposed fetuses on day 19. Compared to day 19 indomethacin treatment alone, combined administration of a single L-NAME and indomethacin dose did not result in further constriction of the day 19 fetal DA (p=0.12) (Fig. 3). The effect of maternal L-NAME on fetal NO production was measured in d19 fetuses (Table 1). There was no difference detected in fetal NO levels between saline and L-NAME treated dams. However, others have shown that L-arginine analogs such as L-NAME may be detected by NO assays that utilize ozone chemiluminescence or the modified Griess reaction (17). As an alternative approach, we measured NO production in the fetuses of LPS-stimulated dams. Compared to saline-treated females, NO levels were significantly reduced in the fetuses of L-NAME-treated females after LPS stimulation (Table 1). Taken together, these results suggest that maternally administered L-NAME inhibits fetal NO production but does not affect fetal DA patency on day 19.

Comparison of drug effects between day 16 and day 19 groups revealed that there was no difference in the degree of DA patency for saline-exposed fetuses on these days. However, a small but significant reduction in DA caliber was noted in L-NAME exposed fetuses on day 16 compared to day 19 (χ^2 = 24.3; p<0.001). In contrast, indomethacin treatment had little or no effect on the day 16 DA, but caused marked constriction in the day 19 fetuses (p<0.001). Overall, these results suggest that the preterm fetal DA is more sensitive to NOS inhibition than
cyclooxygenase inhibition, although the magnitude of DA constriction by L-NAME is small. This response is reversed in the term fetus, when the DA undergoes severe constriction in response to maternal indomethacin. Studies with combined inhibitors also suggest that NO actions are potentiated by prostaglandins in the day 16 DA. Conversely, combined inhibition of NOS and cyclooxygenase did not result in further DA constriction on day 19, possibly because the DA may already be maximally constricted.

Ductal closure in the mouse occurs in the absence of vasa vasorum

The vasa vasorum of the DA wall plays an important role in DA closure in baboons and sheep (9, 20, 43). Corrosion casts of the fetal and newborn mouse did not reveal the presence of vasa vasorum in the media of the patent or closing mouse DA, although the extensive vascular network within the fetal lung was easily demonstrated (Fig. 4A). In addition, immunostaining of serial sections of the DA for PECAM-1, a general marker of vascular endothelium, showed strong localization in the endothelium of the aorta, pulmonary artery, DA and in small vessels consistent with vasa vasorum in the tunica adventitia, but failed to demonstrate any staining within the DA wall (Fig 4B). To further evaluate a possible role of vasa vasorum in DA closure, mice with an flk reporter construct were examined, since flk is another established marker of vascular endothelial cells. Serial DA sections of flk-I\(^{lacZ/+}\) pups on day 19 (not shown) and PP1 (Fig. 4C) showed well demarcated flk-driven lacZ expression in the endothelium of the aorta, pulmonary artery and DA, as well as in muscular and thin-walled vessels in the tunica adventitia and pericardial membrane between the ascending aorta and main pulmonary artery, whereas lacZ expression was absent in the DA of wildtype littermates. Although PECAM and lacZ staining revealed the presence of vessels compatible with vasa vasorum in the tunica adventitia, there
were no such vessels in the wall of the constricted DA. Thus, in contrast to larger mammals, vasa vasorum in the tunica media do not contribute to DA regulation in the mouse.

**Discussion**

Patency of the fetal DA and rapid closure after birth are important aspects of fetal viability and the transition to newborn life. The present studies show that eNOS is the primary source of NO in the mouse DA and is restricted to endothelial cells in both the patent and constricted state. Inhibition of NO synthesis results in mild constriction of the preterm DA but no detectable effects at term. In contrast, inhibition of prostaglandin synthesis results in marked DA constriction at term but does not affect the preterm DA on its own. Combined inhibition of both NO and prostaglandin synthesis suggests that both vasodilatory systems are active in the preterm fetal DA, while patency of the term fetal DA is primarily regulated by prostaglandins. We hypothesized that NO would mediate DA relaxation *in utero* and that declining NO synthesis would accompany DA constriction at birth. However, our findings show that NO contributes to regulation of DA patency in the premature more than the term fetal mouse, despite persistent eNOS expression, even in the constricted, postnatal DA.

The relaxed state of the fetal DA was historically considered a passive process, but it is clear that vasodilating stimuli are required to keep the DA open *in utero* (47). NO contributes to fetal DA relaxation in baboons, sheep, rabbits and rats (6, 10, 13, 43), but its enzymatic source is not entirely clear. We observed uniform eNOS expression in the DA of mid- to late gestation fetal mice. nNOS mRNA was barely observed and iNOS expression was noted at the lower limits of detection and is unlikely to exert a physiologic contribution at these levels. Baragatti et. al., also detected eNOS but not nNOS or iNOS expression in the term mouse DA (2) but there
was no information on temporal or cell-specific expression patterns during development. The specificity of NO synthesis may be important, since strategies to manipulate DA patency could depend on inhibition of a single isoform. Contrary to our results, Bustamante et. al., reported that the heart and conotruncal vessels of the fetal rat express iNOS mRNA and showed that the fetal DA was constricted by maternal treatment with a selective iNOS inhibitor (6). Although the rat DA also constricts in response to non-specific NOS inhibition by L-NAME (32, 51), the presence and localization of specific NOS isoforms has not been determined in this species. The DA of fetal sheep constricts in response to NOS inhibition (9, 10, 13), but there is uncertainty about the role of each NOS isoform. Fox et al., demonstrated immunoreactive eNOS in the lamb DA endothelium (13). However, the lack of an eNOS-selective inhibitor prevents direct evaluation of eNOS effects on DA tone. In a related series of studies, western blot and selective inhibitors also demonstrated the expression and functional contribution of nNOS in late gestation fetal lambs (40). In those experiments, administration of an nNOS-specific inhibitor to chronically catheterized lambs \textit{in utero} induced a modest increase in pulmonary-aortic arterial pressure gradients, whereas similar experiments with selective iNOS inhibition did not affect transductal pressures or flow (39). In that study, there was no difference in the nNOS protein content of intact versus endothelium-denuded DA homogenates, suggesting nNOS localization in the muscular wall or adventitia. \textit{In vitro} studies of cultured lamb DA smooth muscle cells also established nNOS as the predominant source of ductal NO, although eNOS and iNOS were detected by western blot (27). These results are in contrast to other studies showing that the fetal lamb DA expresses mRNA and protein for eNOS and iNOS but not nNOS (9). The discrepancy over iNOS in the sheep DA may be related to localization techniques versus functional or \textit{in vitro} studies. However, the controversy over nNOS is more difficult to reconcile. It is possible that the development of selective inhibitors for each isoform and application to both cultured cells
and *in vivo* models will clarify their distinct contributions to DA tone. It is also known that eNOS regulates the baboon DA (20, 43) and there is some evidence to suggest that the human DA is influenced by NO (29) and expresses eNOS in the term and preterm endothelium (21) but a role for other isoforms has not been explored. Despite our results and the findings of other investigators, it is also possible that NOS expression levels do not correlate well with the actual production of NO in the DA. Thus, defining NOS isoform specificity in ductal tissues and developing methods to measure NO levels at the site of action remain important goals for the development of potential therapeutic interventions for the DA.

We observed differential responsiveness of the DA to NOS inhibition in preterm versus term fetal mice. There are previous reports that the preterm DA of rats and sheep are more sensitive to NOS inhibition than the term DA (20, 32, 51) but the mechanism of increased NO sensitivity is unknown. To address this, we speculated that the preterm DA might contain increased levels of NOS isoforms to actively maintain preterm DA patency and that NOS expression would decline as the time for rapid DA closure after birth approached. Instead, our observation that NOS expression is stable throughout late gestation and after birth suggests that regulation of preterm DA tone by NO may be overcome by other forces with advancing gestation. Prostaglandins are known to complement the role of NO in preserving fetal DA patency and combined inhibition has been shown to be more potent than inhibition of either system alone (32, 43, 50). Thus, one explanation for our results is that NO remains an active dilator of the term fetal DA, but it’s impact is minimal compared to prostaglandins and other vasodilators that take on a greater role near the time of delivery. Alternatively, our results are consistent with the possibility that NOS inhibition leads to a compensatory increase in dilatory prostaglandins in the preterm more than the term fetal DA. Although the term fetal DA is fully
constricted by prostaglandin inhibition, constriction of the day 16 DA by combined inhibitors is
greater than the response to either inhibitor alone, suggesting that synergistic effects exist
between these mediators and are important for regulation of the preterm DA. Withdrawal of
dilatory prostaglandin stimuli at birth is a common physiologic mechanism for DA constriction
across species (47). Pharmacologic inhibition of prostaglandin synthesis is a potent inducer of
DA constriction that is more pronounced in the term than the preterm DA (30, 31, 52). In
addition, we previously found that constriction of the mouse DA was less severe with selective
cyclooxygenase-2 inhibition than with indomethacin at term (41), but this is the first report we
are aware of on the differential effects of cyclooxygenase inhibition in term versus preterm mice.
Overall, our results concur with observations that prostaglandin signaling becomes increasingly
important for active relaxation of the DA as the time for delivery nears. On the other hand, our
data extends the concept that NO, or NO in combination with prostaglandins, contributes to
relaxation of the preterm more than the term DA despite stable eNOS expression over time.

The implication of sustained eNOS mRNA expression in the constricted DA is unclear.
eNOS protein also persists in the luminal endothelium of constricted DAs in sheep and baboons
(8, 20, 43). eNOS levels are regulated by physical stimuli, numerous transcription factors and
mRNA stability. It is possible that altered shear forces during DA closure influence the
expression or post-transcriptional modification of eNOS after birth. However, the DA lumen is
closed by 3-4 hours of age in mice and it is unlikely that increasing vascular resistance across the
constricting DA is the cause of continued eNOS gene expression 12-24 hours after it is closed.
Alternatively, vascular endothelial growth factor (VEGF), endothelin (ET-1), bradykinin,
calcium influx, and potassium channels are associated with various aspects of DA constriction
(47) but are also key regulators of eNOS transcription or function (12, 14, 45, 55). Persistent
eNOS expression in the constricted DA may therefore be secondary to the role these factors play in DA closure and vascular remodeling. eNOS itself may also be important for DA remodeling, since NO protects against oxidant injury, inhibits leukocyte and platelet adhesion, and can inhibit proliferation of smooth muscle cells and stimulate endothelial cell migration (1, 15, 33, 35, 56). Unfortunately, there is no DA-specific information on calcium/calmodulin interaction, estrogen/ERs, cytokines, HSP90, caveolin, or other intracellular signaling processes that regulate eNOS activity. NO exerts its actions in the cell occur through post-translational modification of other proteins or alterations in their expression, including growth factors, cytokines, inflammatory/anti-inflammatory mediators, adhesion molecules and matrix proteins (5, 38). In the sheep DA, NO acts on microtubule-associated proteins to induce fibronectin synthesis (27) which is critical for DA smooth muscle migration and obliteration of the DA lumen after birth (26). Thus, it is likely that eNOS has roles other than vasorelaxation in the postnatal DA. Further studies are necessary to determine whether persistent eNOS expression in the constricted DA is functionally active and whether these expression levels correspond to sustained production of NO in the DA wall.

We also found that DA closure in the mouse occurs without contribution of the vasa vasorum. Reports that vasa vasorum facilitate DA closure in other species prompted our interest in the mouse. Numerous studies have shown that vasa vasorum are not required for nourishment of the aorta or elastic vessels in smaller species with limited wall thickness (54). However, the DA is a muscular vessel with unique architecture and there is little information about vasa vasorum in the DA of smaller species. Our inability to demonstrate these vessels in the DA wall by fine vascular casting or with two established markers of vascular endothelium is not unexpected but raises the possibility that vasa vasorum in the tunica media may not be essential
for DA regulation. These findings are important since NO activity has been observed in the wall of denuded DA segments (3, 9, 10) and eNOS localizes to the vasa vasorum of sheep, baboons and humans (9, 20, 21). In these species, vasa vasorum are more prevalent in the term than preterm DA wall and increase with the onset of DA closure (9, 21, 43). It is unclear whether our results merely reflect variation between large and small species or if common mechanisms for DA closure exist despite the observed changes in vasa vasorum of larger vessels. For example, prostaglandin inhibitors typically have an acute constrictive effect on the DA in utero, but prenatal exposure to indomethacin can also unexpectedly result in persistent DA patency after birth (18, 36). In this circumstance, there is evidence that indomethacin treatment causes impaired wall perfusion and compensatory changes in the vasa vasorum, leading to postnatal DA insensitivity and increased DA patency in newborns (8, 16). However, prolonged in utero exposure to prostaglandin inhibitors also results in persistent DA patency in the newborn mouse (23), where the absence of vasa vasorum implies that this event must be mediated by another process. Persistent DA patency is also noted in knockout mice that lack prostaglandin synthesis or the EP4 prostaglandin receptor (24, 34, 41, 42), suggesting that there are alternative explanations for abnormal DA responsiveness that do not rely on changes in the vasa vasorum. Thus, mouse models of persistent DA patency may provide novel insights into general mechanisms of DA regulation that are independent of vasa vasorum effects.

Overall, our data show that gestational maturity has significant but opposite effects on NO and prostaglandin regulation of the mouse DA. eNOS is constitutively expressed in most vascular beds, but our results suggest that there may be different roles for eNOS-derived NO in the preterm and term DA. In addition, the impact of NOS inhibition does not seem to be as severe as inhibition of prostaglandin synthesis. Indeed, mice with targeted deletions of NOS
isoforms do not have overt DA abnormalities (19, 25, 37) whereas mice with disrupted prostaglandin signaling die on the first day of life with persistent DA patency (24, 34, 41, 42). The availability of mice lacking all three NOS isoforms may provide additional insight if compensatory upregulation of alternate NOS isoforms occurs in the DA as it does in the myocardium (22). Resolution of the mechanisms that regulate the DA will enhance our understanding of fetal transition after birth and may help prevent inadvertent constriction of the fetal DA in response to maternal drugs that are given during pregnancy.
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Disclosures

There are no conflicts of interest to disclose.
References


## Table 1. The effect of maternal L-NAME on fetal NO production

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<th>Fetus of saline-treated dam</th>
<th>Fetus of L-NAME-treated dam</th>
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<tr>
<td><strong>Baseline</strong></td>
<td>52.4 ± 6.2</td>
<td>58.3 ± 18.7</td>
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<td>(n)</td>
<td>(15/3)</td>
<td>(24/3)</td>
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<tr>
<td><strong>LPS-stimulated dam</strong></td>
<td>154.6 ± 30.6</td>
<td>107.5 ± 25.1*</td>
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<td>(n)</td>
<td>(16/4)</td>
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* *p*<0.05 compared to saline. Values are mean ± S.D.; n = no. pups/no. litters. NO products (M) determined on fetal plasma samples 4 hours after maternal saline or L-NAME administration (see Methods).
Figure Legends

Figure 1.  NOS expression in the mouse ductus arteriosus during gestation and after birth.
A, Southern hybridization of NOS isoform-specific RT-PCR in the mouse DA. RT-PCR products from day 17 of pregnancy through postpartum day 1 (PP1) were electrophoresed, blotted and hybridized with a $^{32}$P-labeled internal oligonucleotide. Assays were performed in the presence (+) or absence (-) of reverse transcriptase (RT). rpL7, ribosomal protein L7; kid, kidney; m, skeletal muscle.

B, Quantitative analysis of eNOS expression in the mouse DA. The temporal expression of eNOS relative to the housekeeping gene rpL7 was analyzed from day 17 of pregnancy through PP1. Open bars represent data based on densitometry of Southern blots. The solid line represents data based on quantitative PCR using serial dilutions of target sequence standards to quantitate cDNA products. Experiments were repeated 3 times with independent RNA samples. Data shown are mean ± SEM. There was no statistically significant difference between time points for either Southern hybridization or quantitative PCR approaches.

Figure 2. Distribution of eNOS mRNA during fetal patency and postnatal closure.
The cell-specific localization of eNOS mRNA in the mouse DA was analyzed by in situ hybridization. DA sections from day 19 of pregnancy and postpartum day 1 (PP1) were hybridized with $^{35}$S-labeled antisense and sense probes. Darkfield photomicrographs of representative longitudinal DA sections are shown (x100). AO, aorta; MPA, main pulmonary artery; DA, ductus arteriosus; BPA, branch pulmonary artery.
Figure 3. Effects of NOS and prostaglandin inhibition on fetal ductus arteriosus patency.
The status of day 16 or day 19 fetal DAs after maternal treatment with two doses of indomethacin (5.0 mg/kg/dose), L-NAME (50 mg/kg/dose), or saline is expressed as the number of pups with the observed degree of DA patency, as a proportion of the whole treatment group. Dams treated with combined L-NAME and indomethacin received a single dose of each drug, due to fetal and maternal toxicity. For each experiment, fetal DA status was determined four hours after the last drug dose. Open and hatched bars correspond to the degree of DA patency in relationship to the main pulmonary artery (e.g., open bar = 50% patency, dark hatched bar = 75% patency, solid bar = 100% patency). Inter-group comparisons were made for treatment groups and between gestational age groups. *p<0.01 for day 16 treatments compared to d16 saline or indomethacin; ** p<0.001 for day 16 versus d19 (comparing similar drug treatment groups); † p<0.001 for day 19 treatments compared to d19 saline or L-NAME. There was no significant difference in the degree of DA patency between saline and indomethacin on day 16, or between saline and L-NAME on day 19.

Figure 4. Absence of vasa vasorum in the media of the mouse ductus arteriosus.
A, Corrosion cast of the vascular network surrounding the DA. Low viscosity acrylic resin was injected into the right and/or left ventricle of newborn pups immediately after birth. Surrounding tissues were removed by dissection and maceration. B, Immunolocalization of PECAM in the mouse DA. Cardiac and conotruncal tissues collected on postpartum day 1 (PP1) were incubated with a rat anti-mouse monoclonal antibody. Red-brown deposits indicate positive immunostaining. Arrow, small muscular vessel consistent with vasa vasorum in the tunica adventitia. C and D, Expression of flk-driven lacZ in the mouse DA. Ductal sections from flk-1 lacZ+/+ and wildtype flk +/+ littermates were collected on PP1 and processed to observe lacZ activity.
Blue staining indicates lacZ expression under control of the \textit{flk} promoter. AO, aorta; DA, ductus arteriosus; MPA, main pulmonary artery. Arrow, thin walled vessels; arrowhead, small muscular vessels consistent with superficial vasa vasorum in the adventitia and aortico-pulmonary membrane around the DA.
Figure 1
Figure 3