Factors that increase the contractile tone of the ductus arteriosus also regulate its anatomic remodeling

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Kajino, Hiroki, Yao-Qi Chen, Steven R. Seidner, Nahid Waleh, Françoise Mauray, Christine Roman, Sylvain Chemtob, Cameron J. Koch, and Ronald I. Clyman. Factors that increase the contractile tone of the ductus arteriosus also regulate its anatomic remodeling. Am J Physiol Regulatory Integrative Comp Physiol 281: R291–R301, 2001.—Permanent closure of the full-term newborn ductus arteriosus (DA) occurs only if profound hypoxia develops within the vessel wall during luminal obliteration. We used fetal and newborn baboons and lambs to determine why the immature DA fails to remodel after birth. When preterm newborns were kept in a normoxic range (PaO2: 50–90 mmHg), 86% still had a small patent DA on the sixth day after birth; in addition, the preterm DA was only mildly hypoxic and had only minimal remodeling. The postnatal increase in PaO2 normally induces isometric contractile responses in rings of DA; however, the excessive inhibitory effects of endogenous prostaglandins and nitric oxide, coupled with a weaker intrinsic DA tone, make the preterm DA appear to have a smaller increment in tension in response to oxygen than the DA near term. We found that oxygen concentrations, beyond the normoxic range, produce an additional increase in tension in the preterm DA that is similar to the contractile response normally seen at term. We predicted that preterm newborns, kept at a higher PaO2, would have increased DA tone and would be more likely to obliterate their lumen. We found that preterm newborns, maintained at a PaO2>200 mmHg, had only a 14% incidence of patent DA. Even though DA constriction was due to elevated PaO2, obliteration of the lumen produced profound hypoxia of the DA wall and the same features of remodeling that were observed at term. DA wall hypoxia appears to be both necessary and sufficient to produce anatomic remodeling in preterm newborns.

In the full-term infant, closure of the ductus arteriosus (DA) occurs in two phases: 1) initial “functional” closure of the DA lumen by surrounding smooth muscle constriction and 2) “anatomic” occlusion of the lumen, due to extensive neointimal thickening, and loss of smooth muscle cells (SMC) from the inner muscle media (2). The initial “functional” constriction appears to be required for the ultimate “anatomic” closure of the DA. Loss of luminal blood flow produces a zone of hypoxia in the DA’s muscle media. Hypoxia induces vascular endothelial growth factor (VEGF) expression, angiogenesis, neointima formation, and cell death, in proportion to its severity (2). Failure to develop hypoxia of the muscle media, despite initial constriction, leads to failure of DA remodeling (2).

The initial “functional” constriction of the DA depends on a balance between dilating and contracting forces. In the late-gestation fetus, the DA has a high level of intrinsic tone (24). After delivery, the increase in arterial oxygen concentration leads to a further increase in DA tone. The DA also produces several vasodilator substances that oppose the intrinsic and oxygen-induced contractile forces. Both vasodilator prostaglandins (PG), especially PGE2, and nitric oxide (NO) play a significant role in opposing DA closure and maintaining its patency during fetal life (10, 13, 19, 24, 32). After delivery, there is an increase in arterial oxygen concentration, a decrease in circulating PGE2 concentrations, and a decrease in intraluminal blood pressure (due to the drop in pulmonary vascular resistance); all of these events promote DA constriction in the full-term neonate (4, 6).

In contrast with the full-term infant, the DA of the preterm infant frequently remains open for many days after birth. Even when it does constrict, profound hypoxia in the DA wall and anatomic remodeling often fail to develop (2). This leads to subsequent DA reopening (34). Prior studies have attributed the preterm infant’s weaker “functional” constriction to either 1) its blunted response to oxygen (1, 30, 36, 37) or 2) its increased sensitivity to PGE2 and NO (5, 7, 10). The contribution
of intrinsic tone (24) to DA contractility has not been examined in the preterm newborn. In the following study, we examined the relative importance of the factors that regulate DA tone in the preterm newborn. We hypothesized that if we could make the preterm DA constrict more tightly, it might completely obliterate its luminal blood flow and develop the degree of tissue hypoxia needed to initiate the remodeling process.

METHODS

In vitro studies: fetal lambs. Fifty-two late-gestation, near-term lambs (mixed Western breed: 134 ± 6 days gestation, term = 145) and 25 preterm lambs (105 ± 2 days) were delivered by cesarean section and anesthetized with ketamine HCl (30 mg/kg iv) before rapid exsanguination. These procedures were approved by the Committee on Animal Research at the University of California, San Francisco.

The DA was divided into 1-mm-thick rings (3–4 rings/animal), and isometric tension was measured in a Krebsbicarbonate solution (8, 10, 24). An oxygen electrode (YSI Model 53 Biological Oxygen Monitor, Yellow Springs, OH), placed in the 10-ml organ bath, measured the oxygen concentration. The bath solution was equilibrated with gas mixtures containing 5% CO2 and was changed every 30 min. Rings were stretched to an initial length (preterm: 5.2 ± 0.4 mm; late gestation: 6.6 ± 0.4 mm) that produced a maximal contractile response to increases in oxygen tension (8) and were exposed to one of two experimental protocols. In all experiments, we allowed the tension in the rings to reach a new steady-state plateau (30 min-1 h) before another experimental change was made.

Protocol 1: effects of oxygen, PG, and NO on near-term and preterm DA. Rings were equilibrated with a solution containing one of the following oxygen concentrations: 2, 6, 16, 30, or 95%. After the tension reached a plateau, indomethacin (5.6 × 6-⁷ M) was added; this was followed by N⁶-nitro-L-arginine methyl ester (L-NAME; 10⁻⁴ M). Maximal tension was determined by the response to 100 mM K⁺-Krebs solution (equilibrated with 95% O₂-5% CO₂). After the maximal tension had been determined, minimal tension was determined by the response to sodium nitroprusside (SNP; 10⁻⁴ M).

The difference in tensions between the measured steady-state tension, at a particular oxygen concentration, and the minimal tension produced by SNP was considered the net state tension, at a particular oxygen concentration, and the minimal tension produced by SNP was considered the net state tension.

Protocol 2: effects of oxygen on near-term and preterm DA pretreated with indomethacin and L-NAME. Rings of DA that were used in protocol 2 were first preconditioned (1.5 h) with solution containing indomethacin and L-NAME and 6% oxygen. Next, the rings were exposed sequentially to 95, 30, 16, 6, and 2% oxygen. The difference in tensions between the steady-state tension at 2% oxygen and either 6, 16, 30, or 95% oxygen was considered the O₂-induced tension due to that particular oxygen concentration.

Tissues were blotted dry and weighed after the experiments (preterm: 35 ± 8 mg; late gestation: 59 ± 17 mg).

Tensions developed in the rings were expressed as force per unit cross-sectional area (g/cm²) (8) or as percentage of maximal active tension.

Detection of hypoxia with [2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl) acetamide]. To determine the degree of hypoxia within the rings of DA, we used the [2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl) acetamide] (EF5) detection system that we have described previously (24). EF5 binds to cysteine residues of intracellular proteins after it has been acted on by hypoxia-dependent nitroreductases (25). When it has been studied in different cell lines, different tissues, and different species, both in vivo and in vitro, EF5 has been found to have a similar oxygen dependency for its rate of binding (25). We have shown previously that EF5 binding can be assessed quantitatively using either radioactive EF5-binding, immunofluorescent flow cytometry or immunoblot techniques (24, 25).

Rings of DA were suspended in the organ baths and incubated in Krebs solution containing EF5 (10⁻⁴ M). The bath was cooled to 4°C and filled with 1 ml fresh medium containing EF5 (10⁻⁴ M) in its unlabeled or labeled form ([¹⁴C]-position: 43 μCi/mg; synthesized by Dr. M. Tracy, SRI International, Palo Alto, CA). Dishes were placed in leak-proof aluminum chambers that were connected to a manifold allowing the gas phase of the chambers to be exchanged for the desired oxygen concentration (24, 25). The chambers were immersed in a 37°C water bath for rapid warming and shaken gently. After a 3-h incubation, the cells were rinsed three times and processed for either radioactive counting (25), flow cytometry (25), or immunoblot assay (24). A mouse monoclonal IgG primary antibody, ELK 3–51, which is highly specific for EF5 tissue adducts (25), was used to detect bound EF5 in the flow cytometry and immunoblot assays (24, 25).

Fig. 1. Direct comparison of dot immunoblot assay, relative median fluorescence intensity (flow cytometric analysis [FACS]) assay, and radioactive [2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl) acetamide] (EF5) uptake (radiolabel) assay for detection of EF5 binding in ductus arteriosus (DA) smooth muscle cells (SMC). SMC were incubated with 10⁻⁴ M EF5 for 3 h at the indicated oxygen concentrations. Data from each assay were normalized to the values obtained at the lowest oxygen concentration, 0.01%.
solution was preequilibrated with the desired oxygen concentration. After a 3-h incubation, unbound EF5 was rinsed out of the baths and the rings were frozen with liquid N₂ (24). Immunoblot analyses of EF5 binding in DA rings were compared with measurements made in SMC that were incubated in known oxygen concentrations (24). Lysates from both the tissue samples and the SMC standard curve were blotted onto the same membrane and processed together. Because the oxygen dependence of EF5 is linearly related to drug exposure (16, 25), the relative binding of EF5 to a tissue lysate was corrected for its drug exposure. The in vitro drug exposure to EF5 was the same in both the tissue samples and the SMC standard curve (3 × 10⁻⁴ M/h). The oxygen concentration in the tissue was determined by comparing the EF5 binding in the tissue with the EF5 binding in the SMC standard curve lysates. Two types of control tissues were used in the DA ring experiments: 1) tissue from rings that were not given EF5, and 2) tissue from EF5-treated rings that were incubated in 95% oxygen for 3 h. There was no antibody (ELK 3–51) binding to either of the control tissues (data not shown).

In vivo studies: preterm baboons. We used preterm baboons Papio sp. (140 days gestation; full term: 185 days) to examine the effects of arterial oxygen tension on ductus closure. Animal care, surgery, and necropsy were performed at the Southwest Foundation for Biomedical Research (San Antonio, TX). Animals were killed at the end of the study with an overdose of pentobarbital sodium. Preterm fetal baboons were delivered by cesarean section and euthanized before breathing. Preterm newborns were delivered by cesarean section and cared for in the primate intensive care nursery for the first 6 days after delivery (2). Ventilator management was designed to maintain the arterial PO₂ (PaO₂) in either a normoxic range (50–90 Torr) or a hyperoxic range (>200 Torr) [with a fractional inspired oxygen concentration (FiO₂) = 1.0]. At 6 days after delivery, immediately before necropsy, the baboons still required mechanical ventilation, and the baboons lost weight at a rate of 1% per day. The fractional inspired oxygen concentration (FiO₂) was 0.25 ± 0.14 (normoxia) or 1.0 ± 0.0 (hyperoxia). There were no differences in PaCO₂, pH, or blood pressure between the groups during the first 6 days (data not shown).

Pulsed Doppler flow studies were performed before necropsy, using an 8-MHz transducer interfaced with a Biosound ND256 echocardiographic system to confirm the presence, direction, and timing of ductus flow (2).

EF5 was given to the baboons (10⁻⁴ mol/kg iv over 10 min) 36 h before necropsy. Blood samples for EF5 determination were collected at 1, 6, 12, 24, and 36 h after the dose and analyzed as previously described (2). The in vivo exposure of tissues to EF5 was calculated from the area under the curve of the EF5 serum concentrations and was similar in the two groups (data not shown). We compared the measurements of EF5 binding in the two groups of 6-day-old, premature baboons with measurements made in 2-day-old, full-term baboons (n = 3). The full-term baboons were delivered by the normal spontaneous vaginal route and were given EF5 36 h before necropsy.

Immunohistochemistry. Protocols for the immunohistochemistry of endothelial cell nitric oxide synthase (eNOS), von Willebrand factor (vWF), VEGF, and EF5 were similar to those reported previously (2, 10). Briefly, DA was frozen in liquid N₂. Frozen sections were incubated with either mouse monoclonal anti-eNOS (Clone 3, Transduction Lab, Lexington, KY) or rabbit anti-vWF (Dako, Carpinteria, CA) to detect endothelial cells, or with mouse monoclonal antibody ELK 3–51 conjugated with the fluorescent dye Cy3 to detect EF5 binding.

Some DA were divided in two before freezing. One-half was fixed for 5 h in fresh 4% paraformaldehyde at 4°C before paraffin embedding. Paraffin-embedded sections were dehydrated and incubated with rabbit anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Scoring for VEGF was as follows: 0, no staining; 1+, mild staining; and 2+, moderate staining. All sections were stained in the same assay. Assays were reproduced on three separate occasions. Control sections were treated similarly and had no staining (data not shown).

Histological measurements were made at the level of minimal luminal area that was determined from serial sections made through the tissue. Tissue dimensions were determined by averaging measurements made from eight predetermined regions of the section, using a template and National Institutes of Health Image software. The neointimal zone was defined as the region between the luminal endothelial cells and the internal elastic lamina (identified by phase contrast microscopy). The depth of vasa vasorum ingrowth, into the muscle media, was based on the presence of vasa vasorum within predetermined concentric regions that partitioned the DA wall between the intima and the adventitia (10).

In some DA, regions of cell loss (absent nuclei) were observed in the intima/inner muscle media. The region of cell loss was defined as minimal if its area was >3,000 and <15,000 μm² and moderate if it was >20,000 μm².

We have shown previously that EF5 binding in vivo can be assessed quantitatively using immunofluorescent techniques (2, 25). Direct digital image acquisition was performed with a Photometrics “Quantix” CCD camera, and the digitized images were analyzed with National Institutes of Health Image software and Adobe Photoshop. The microscope was calibrated for constant light output as previously described (2). For digital analysis, the exposure time was set by the camera’s automatic exposure system. We assigned a “relative intensity” to a microscopic field by considering the range of pixel intensities and the total exposure. These procedures allowed us to adjust the exposures to provide visually acceptable images while preserving the absolute intensity information. The absolute intensity values were corrected for the in vivo EF5 drug exposure.

Statistics. Statistical analysis was performed by the appropriate Student’s t-test and by analysis of variance. Scheffé’s test was used for post hoc analysis. Nonparametric data were compared with a chi square analysis. Results are presented as means ± SD.

RESULTS

In vitro studies: fetal lamb DA. We used isolated rings of lamb DA to determine how oxygen concentration affected the contractility of the preterm and late-gestation DA. We used five specific bath-solution oxygen concentrations and measured the corresponding average tissue oxygen concentration by the EF5 immunoblot technique (Table 1). As the bath-solution oxygen concentration increased from 2 to 95%, the tissue oxygen concentration increased from <0.2 to >10% (Table 1). Preterm and late-gestation DA rings had the same average tissue oxygen concentration at each of the five bath-solution oxygen concentrations.

The maximal active tension produced by K⁺ in the preterm DA (236 ± 36 g/cm²) was similar to the maximal active tension in the late-gestation DA (271 ± 84 g/cm²).
In protocol 1, as the bath oxygen concentration increased from 6 to 95%, there was a progressive increase in isometric tension in both preterm and late-gestation DA rings (Fig. 2A). However, the net active tension of the preterm DA was significantly (P < 0.01) less than that of late-gestation DA (Fig. 2A); in addition, the preterm DA appeared to be less sensitive to increases in bath oxygen concentration than the late-gestation DA (EC50 (bath oxygen concentration that produces 50% of the change in tension observed between 6 and 95% oxygen): preterm, 50% oxygen; late gestation, 28% oxygen).

When the bath oxygen concentration dropped to 2% (which corresponds to a tissue oxygen concentration of 0.2%), the severely hypoxic rings developed a tension that was greater than that achieved when the oxygen concentration in the bath solution was 30% (Fig. 2A). The net active tension achieved by the preterm rings, which were severely hypoxic, was significantly less than that achieved by late-gestation rings (P < 0.0001).

We used indomethacin and L-NAME to uncover the role of endogenous PG and NO in opposing DA contractility. Indomethacin had no contractile effect on DA rings that were severely hypoxic (bath oxygen concentration <2%) (Fig. 2B); this is due to the marked inhibition of endogenous PG production that occurs when tissue oxygen concentration is <0.2% (Fig. 3) (24). In contrast, indomethacin caused an increase in DA tension when the oxygen concentration was ≥6% (Fig. 2B). Although PG production in the preterm DA was less than or equal to the production in the late-gestation DA (Fig. 3), the tension induced by indomethacin, in the preterm DA, represented a significantly greater percent of the active tension (net + indomethacin-induced + L-NAME-induced tension) than it did near term (P < 0.01).

L-NAME also had no effect on DA rings that were severely hypoxic (Fig. 2C); however, when the bath oxygen concentration was ≥6%, L-NAME increased DA tension. The tension induced by L-NAME represented a significantly greater percent of the active tension in the preterm DA than it did near term (P < 0.01).

In protocol 2, ductus rings were pretreated with indomethacin and L-NAME before oxygen concentrations in the rings were varied. DA rings had an intrinsic vasoconstrictor tone even at negligible oxygen concentration.
centrations (bath concentration 2% oxygen) (Fig. 4). This oxygen-independent tone was equal to 64 ± 7% of the maximal active tension in the late-gestation DA, but only 34 ± 10% in the preterm DA (P < 0.001). In the absence of endogenous PG and NO production, the preterm DA no longer had a blunted response to oxygen, and it was more sensitive to changes in bath oxygen concentration (EC50: 16 ± 2% oxygen) than was the near-term DA (EC50: 30 ± 7%) (P < 0.05) (Fig. 4).

In vivo studies: preterm baboons. Premature newborn baboons do not constrict their DA as tightly and do not remodel their DA to the same degree as full-term baboons (2). On the basis of our in vitro experiments, we would predict that increasing the arterial PaO2 from the normoxic range (50–90 mmHg) to the hyperoxic range (250–350 mmHg) would more than double the net active tension produced by the premature DA (Fig. 2A). We hypothesized that if we kept the arterial PaO2 in the hyperoxic range, we would produce a greater degree of DA constriction in vivo, which would increase the likelihood of anatomic remodeling.

Twenty-seven premature newborn baboons were kept in the normoxic range for the first 6 days after birth (Fig. 5A). Although 74% had no evidence of a patent DA by Doppler examination on day 6 (Fig. 5B), a small patent lumen (>0-mm diameter) was still perceptible at the time of necropsy in 86% of the DA (Fig. 5C). In contrast, 96% of the premature newborns that were kept in the hyperoxic range had a closed DA by Doppler examination (P < 0.05) (Fig. 5B), and only 14% had a patent lumen at necropsy (P < 0.01) (Fig. 5C).

**Fig. 3.** Severe hypoxia inhibits prostaglandin E2 (PGE2) and 6-keto-PGF1α released during a 30-min collection period and expressed per 100 mg tissue wet wt. Tissue wet weight (in mg): 60 ± 22 near term; 36 ± 10 preterm. *P < 0.01, †P < 0.05 vs. amount released at 6% bath oxygen concentration. Note that 95% oxygen concentration also decreased PGE2 release. Number of DA rings in parentheses.

**Fig. 4.** Effects of oxygen concentration on DA contractility after PG and nitric oxide (NO) production has been inhibited. Rings of DA were first equilibrated with indomethacin and L-NAME; next they were exposed sequentially to the following bath-solution oxygen concentrations: 95, 30, 16, 6, and 2%. Some of the rings were not exposed to all of the oxygen concentrations. Number in parentheses = number of DA rings, from separate animals, exposed to each oxygen concentration. Height of column represents active tension (means ± SD) at a particular oxygen concentration. The difference in tension between 2% bath oxygen concentration and either 6, 16, 30, or 95% oxygen concentration is considered the oxygen-induced tension. *P < 0.01, †P < 0.05.
We injected the hypoxia indicator EF5 into full-term and premature baboons 36 h before necropsy. The DA of the full-term baboons was closed by Doppler examination at the time of necropsy. We compared photometric analyses of EF5 binding in the DA with measurements made in the ascending aorta. We found negligible EF5 binding in sections from the ascending aorta (data not shown) (2). We anticipated this result because the aorta is a well-oxygenated vessel, with intramural PO2 values (20–30 mmHg) (2, 23, 35) that lie at the low end of the EF5 dynamic binding curve (Fig. 1). In contrast, sections of DA, from the full-term baboons, had intense EF5 binding when compared with sections from the ascending aorta (median EF5 binding: 25-fold greater than aorta; range: 14- to 50-fold greater).

DA from the preterm baboons, whose PaO2 had been kept in the normoxic range, had significantly less EF5 binding than those at term. Even when the DA was closed on Doppler examination, the intensity of EF5 binding was only 20% (median; range: 10–37%) of the EF5-binding intensity at term. In contrast, when preterm baboons were ventilated with 100% oxygen, the intensity of EF5 binding was similar to the intensity at term (median: 60%; range: 30–110%) (Fig. 6).

We looked at VEGF expression during DA closure because VEGF is induced by hypoxia (21) and plays an important role in endothelial cell proliferation and migration (39). DA from preterm newborn baboons, ventilated with 100% oxygen, expressed moderate levels of VEGF in the region of the muscle media that had intense EF5 binding. The DA from the baboons ventilated with 100% oxygen expressed significantly more VEGF than the DA from normoxic baboons (P < 0.05) (Figs. 7, A and B, and 8). VEGF expression was significantly related to the degree of luminal constriction (VEGF vs. diameter of DA lumen, P < 0.01; Fig. 8).

In the full-term DA, proliferation of luminal endothelial cells and migration of medial SMC produce a neointima that occludes the DA lumen (2, 3, 43). Only 21% of DA from the preterm baboons, with PaO2 in the normoxic range, had evidence of endothelial cell accumulation in the DA lumen (3 or more layers of luminal endothelial cells) (Fig. 9B); the rest was lined by a single layer of luminal endothelial cells (Fig. 7C). In contrast, all of the DA from the baboons ventilated with 100% oxygen had neointimal mounds filled with endothelial cells (Figs. 7D and 9B) (P < 0.001). The DA from the baboons ventilated with 100% oxygen also had a significantly thicker neointima than the DA from the normoxic baboons (Figs. 7, A, B, C, and D, and 9A) (P < 0.01).

In the fetal DA, vasa vasorum are restricted to the adventitia and rarely penetrate the outer muscle media (Fig. 7G) (3). After birth, vasa vasorum invade the muscle media, even in the preterm newborn (Fig. 7H) (3). The baboons ventilated with 100% oxygen had a much greater ingrowth of vasa vasorum than those kept in the normoxic range (P < 0.001; Fig. 9D).

In the full-term DA, cells are lost from the center of the EF5-stained hypoxic zone due to cell death (2). Similarly, condensed pyknotic nuclei could be seen throughout the hypoxic zone of DA obtained from preterm newborns ventilated with 100% oxygen (Fig. 7E). This was associated with loss of cellular elements from the intimal-inner media region (Fig. 7, D and F). DA from premature newborns ventilated with 100% oxygen had more extensive regions of cell loss than those from normoxic animals (P < 0.001; Fig. 9C).
DISCUSSION

Hypoxia of the DA wall (or events that lead to the development of hypoxia) seems to be the required stimulus for irreversible closure. Anatomic remodeling occurs only in the presence of moderate/intense hypoxia (2). Oxygen reaches the muscle media of most blood vessels through either the vessel’s lumen or its intramural vasa vasorum (derived from small adventitial arteries) (45). The preterm baboon DA can meet its oxygen needs from luminal flow alone, because it has no vasa vasorum in its muscle media (Fig. 7G) (3). Therefore, when the preterm DA wall fails to become hypoxic after its initial postnatal constriction, the most likely cause is a small persistent patent lumen (2, 34).

In the current study, we found that despite the absence of Doppler-detectable luminal blood flow, a small residual patent lumen was still present in most of the preterm baboons, when their arterial PaO2 was maintained in the normoxic range (Fig. 5, B and C). Therefore, when the preterm DA wall fails to become hypoxic after its initial postnatal constriction, the most likely cause is a small persistent patent lumen (2, 34).

In the current study, we observed that the oxygen-induced constriction in the preterm DA was less than that observed near term (Fig. 2A). This is similar to what has previously been reported (8, 30, 36, 37). However, we found that in the presence of inhibitors of PG and NO production, not only was the preterm DA more sensitive to oxygen, it responded to oxygen with a greater increment in contractile force than it did near term (Fig. 4). Therefore, the excessive inhibitory effects of endogenous PG and NO on DA tone make it appear as if the preterm DA has a smaller increment in tension, in response to oxygen, than it does near term. The increased inhibitory effects of PG and NO in the preterm DA are not due to increased production of the vasodilators (Fig. 3). The increased inhibitory effects of PG and NO are probably due to the increased sensitivity of the preterm DA to the two vasodilators (5, 7, 10).

Although oxygen is an important regulator of DA tone, the exact mechanisms that mediate the DA response to oxygen are still unknown. Oxygen causes membrane depolarization in vascular smooth muscle, which, in turn, is associated with a rise in smooth muscle intracellular calcium (33). Oxygen inhibits K+ channels and releases endothelin-1 in DA smooth muscle (11, 33, 44); however, the importance of K+ channels and endothelin-1 in mediating oxygen-induced contraction is still unclear (R. I. Clyman, unpublished data) (17).

High oxygen concentrations also decrease PGE2 production by the DA (Fig. 3); their effects on PGI2 (6-keto-PGF1α) production are less apparent. This suggests an effect predominantly on PG synthase rather than cyclooxygenase. PGE2 synthesis is glutathione dependent (12), and hyperoxia depletes reduced glutathione, especially in tissues of immature animals (41). These events may explain the reduction in PGE2 that occurs in 95% oxygen (Fig. 3).

In the current study, we observed that the oxygen-induced constriction in the preterm DA was less than that observed near term (Fig. 2A). This is similar to what has previously been reported (8, 30, 36, 37). However, we found that in the presence of inhibitors of PG and NO production, not only was the preterm DA more sensitive to oxygen, it responded to oxygen with a greater increment in contractile force than it did near term (Fig. 4). Therefore, the excessive inhibitory effects of endogenous PG and NO on DA tone make it appear as if the preterm DA has a smaller increment in tension, in response to oxygen, than it does near term. The increased inhibitory effects of PG and NO in the preterm DA are not due to increased production of the vasodilators (Fig. 3). The increased inhibitory effects of PG and NO are probably due to the increased sensitivity of the preterm DA to the two vasodilators (5, 7, 10).

In addition to the oxygen-induced tension, there is an intrinsic vasoconstrictor tone in the DA that is independent of ambient oxygen concentration (Figs. 2A and 4). Whether this sustained intrinsic tone is due to vasoconstrictors that are released by the endothelial or SMC of the DA wall or to the increased calcium sensitivity of the DA contractile proteins is currently unknown (14, 20, 26, 27). In the late-gestation DA, the intrinsic tone is equivalent to 64 ± 7% of the maximal...
tone that can be developed by the DA. On the other hand, in the preterm DA, the intrinsic tone is only 34 ± 10% of the maximal tone (Fig. 4). The factors that regulate the intrinsic tone and its increase during late gestation are currently unknown.

Therefore, despite the increase in arterial PaO₂ after birth, the preterm DA has an ineffective postnatal constriction due to both its weak intrinsic tone and its exaggerated response to PG- and NO-mediated vasodilation. These findings offer an explanation for a previously contradictory observation: indomethacin appears to have greater inhibitory effects on DA contraction early in gestation (5, 7, 10). Our findings suggest that early in gestation, the effects of indomethacin may not be apparent in vivo, due to the poor intrinsic tone in the immature DA and its exaggerated response to NO. Indomethacin may appear to have a greater effect in vivo, late in gestation, because the increase in intrinsic tone and decreased responsiveness to NO may make the absolute change in DA tension more noticeable.

On the basis of our in vitro studies (Fig. 2A), we predicted (and subsequently found) that preterm newborns kept at a higher arterial PaO₂ would have increased DA tone and would be more likely to obliterate their lumen (Fig. 5C). We found that even though DA
constriction was due to elevated PaO₂, obliteration of the lumen produced profound hypoxia in the center of the constricted vessel (Fig. 6B). In contrast with the animals whose PaO₂ was maintained in the normoxic range, those ventilated with 100% oxygen developed both a similar degree of hypoxia and the same features of anatomic remodeling as observed in the full-term newborn (Figs. 6, 7, 8, and 9; see Ref. 2). Although other explanations could account for the individual appearances of VEGF and cell death in the wall of the DA (15, 18, 22, 28, 29, 38, 42), their combined appearance and their geographic distribution can be completely accounted for by the intensity and distribution of hypoxia as measured by EF5.

**Perspectives**

We used markedly elevated PaO₂ to produce permanent DA closure. We are not recommending this as a clinical approach to be used in preterm infants. Even short periods of elevated PaO₂ have been associated with retinopathy of prematurity. Rather, these studies lend support to the concept that functional obliteration of the DA lumen is both necessary and sufficient to initiate the remodeling process (even in the preterm infant). We hypothesize that other more appropriate treatments that cause sustained closure of the DA (e.g., indomethacin or a combination of indomethacin plus NO inhibition) should also produce DA remodeling (9).

An additional speculation can be made from our findings. The profound hypoxia that occurs during postnatal contraction in the full-term DA inhibits the production of PG and NO (Fig. 2, B and C) (24). These two vasodilators oppose the high intrinsic tone of the full-term DA; their decreased production more than compensates for the loss of oxygen-induced tension and enables the DA to remain constricted as it remodels. On the other hand, even at similar degrees of profound hypoxia, the preterm DA may not be able to maintain its constriction during the remodeling process due to its low intrinsic tone (Fig. 2A). In addition, the preterm infant rarely develops the same degree of profound DA hypoxia as found at term (Fig. 6A) (2). At mild-to-moderate degrees of hypoxia, PG and NO

**Fig. 8.** Relationship between VEGF expression and constriction of the DA lumen in normoxic (n = 6) or hyperoxic (n = 6) preterm newborn baboons. Units of VEGF (0–2) refer to intensity of immunostaining. Diameter of lumen: narrowest measurement of luminal diameter at the time of necropsy (see Fig. 5C).

**Fig. 9.** Neointima formation, luminal endothelial accumulation, cell loss, and vasa vasorum ingrowth are increased in the DA wall of preterm baboons ventilated with 100% oxygen (hyperoxia). A: neointimal thickness (in μm): means ± SD. B: luminal endothelium. The eNOS- and VWF-positive cells that lined the DA lumen formed either single or multiple (≥3) layers. C: cell loss was considered minimal if the area of absent hematoxylin-stained nuclei was >3,000 and <15,000 μm²; moderate if >20,000 μm². D: vasa vasorum ingrowth. Four separate, random sections from the middle of the DA were stained for VWF and eNOS and photographed. The muscle media was partitioned into concentric regions based on the percentage of muscle media thickness between the intima and adventitia. Vessel ingrowth was categorized based on the innermost region of the wall that contained at least 1 vasa vasorum.
production is no longer inhibited (Fig. 3); by contrast, mild-to-moderate hypoxia does inhibit oxygen-induced tone (Fig. 2A). These findings may explain why extremely immature infants still have a high rate of reopening, despite the elimination of luminal blood flow during DA constriction (34).

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