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Factors that increase the contractile tone of the ductus arteriosus also regulate its anatomic remodeling
H. Kajino, Y.-Q. Chen, S. R. Seidner, N. Waleh, F. Mauray, C. Roman, S. Chemtob, C. J. Koch and R. I. Clyman
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Tissue hypoxia inhibits prostaglandin and nitric oxide production and prevents ductus arteriosus reopening

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Kajino, Hiroki, Yao-Qi Chen, Sylvain Chemtob, Nahid Waleh, Cameron J. Koch, and Ronald I. Clyman. Tissue hypoxia inhibits prostaglandin and nitric oxide production and prevents ductus arteriosus reopening. *Am J Physiol Regulatory Integrative Comp Physiol* 279: R278–R286, 2000.—Regulation of ductus arteriosus (DA) tension depends on a balance between oxygen-induced constriction and PG and nitric oxide (NO)-mediated relaxation. After birth, increasing Pa_{O_2} produces DA constriction. However, as the full-term ductus constricts, it develops severe tissue hypoxia in its inner vessel wall (oxygen concentration <0.2%). We used isolated rings of fetal lamb DA to determine why the constricted ductus does not relax and reopen as it becomes hypoxic. We used a modification of the 2-(2-nitro-1*H*-imidazol-1-yl)-*N*-(2,2,3,3,3-pentafluoropropyl) acetamide (EF5) technique (Clyman RI, Chan CY, Mauray F, Chen YQ, Cox W, Seidner SR, Lord EM, Weiss H, Wale N, Evan SM, and Koch CJ. *Pediatr Res* 45: 19–29, 1999) to determine mean tissue oxygen concentration. A decrease in the ductus' mean tissue oxygen concentration from 1.4 to 0.1% lowers the isometric tone of the ductus by 15 ± 10% of its maximal active tension (the maximal tension that can be produced by the ductus). Although decreases in oxygen concentration diminish ductus tension, most of the vasoconstrictor tone in the ductus is independent of ambient oxygen concentration. This oxygen-independent tone is equivalent to 64 ± 10% of the maximal active tension. At mean tissue oxygen concentrations >0.2%, endogenous PGs and NO inhibit more than 40% of the active tension developed by the ductus. However, when tissue oxygen concentrations drop below 0.2%, the constitutive relaxation of the ductus by endogenous PGs and NO is lost. In the absence of PG and NO production, tension increases to a level normally observed only after treatment of the ductus with indomethacin and nitro-L-arginine methyl ester (inhibitors of PG and NO production). Therefore, under conditions of severe hypoxia (tissue oxygen concentration <0.2% oxygen), the loss of PG- and NO-mediated relaxation more than compensates for the loss of oxygen-induced tension. We hypothesize that this increased ductus tone enables the vessel to remain closed as it undergoes tissue remodeling.

prostaglandin E_2; prostacyclin; oxygen; vascular smooth muscle

REGULATION OF VASCULAR TONE in the ductus arteriosus depends on a balance between oxygen-induced contraction (7) versus PG (10, 12)- and nitric oxide (NO)-mediated relaxation (9, 13). In full-term newborns, an increase in arterial Pa_{O_2} after birth shifts this balance toward contraction. This initial, oxygen-induced constriction leads to functional occlusion of the ductus lumen; following this, extensive anatomic remodeling results in permanent obliteration of the lumen.

The initial muscular constriction of the ductus arteriosus appears to set the stage for its subsequent anatomic remodeling. As the full-term ductus constricts in vivo, the oxygen concentration within the muscle media decreases from 4% to <0.2% (2). Hypoxia of the ductus wall seems to be a required stimulus for anatomic remodeling, because permanent anatomic closure will not occur unless hypoxia is present (2). Because an increase in tissue P_{O_2} is necessary for the initial ductus constriction, we wondered why the ductus remained constricted and did not reopen its lumen during this stage of tissue hypoxia. We performed the following experiments to examine how progressive hypoxia alters the balance between oxygen-induced constriction and PG- and NO-induced relaxation. We hypothesized that profound degrees of hypoxia, similar to those found in the constricted newborn ductus, would inhibit PG and NO production within the vessel wall. This would enable the ductus to remain constricted during anatomic remodeling, despite the loss of oxygen-mediated contraction.

METHODS

Thirty fetal lambs (mixed Western breed, between 125 and 137 days of a 145-day term gestation) were delivered by cesarean section. The ewe was anesthetized with a constant intravenous infusion of ketamine HCl and diazepam throughout the procedure. The fetus was given ketamine HCl (30 mg/kg im) before rapid exsanguination. These procedures were approved by the Committee on Animal Research at the University of California, San Francisco.

The ductus arteriosus was dissected free of loose adventitial tissue and divided into 1-mm-thick rings that were placed in separate 10-ml organ baths and kept in a dark room, as we have described previously (8). Throughout the experiment, the rings were suspended between two stainless steel hooks at 38°C in a modified Krebs solution (in mM): 118 NaCl, 4.7 KCl, 2.5 CaCl$_2$, 0.9 MgSO$_4$, 1 KH$_2$PO$_4$, 11.1 glucose, and 23 NaHCO$_3$, pH 7.4. The bath solution was bubbled with several different gas mixtures. An oxygen electrode (YSI model 53 Biological Oxygen Monitor, Yellow Springs, Ohio) placed in the organ bath measured the oxygen concentration of the bath solution. The oxygen concentrations in the bath solution were 2%, 4%, 6%, 16%, and 30% after equilibration with the following gas mixtures: 95% N$_2$, 2.5% O$_2$, 5% O$_2$, 15% O$_2$, and 30% O$_2$ (each gas mixture containing 5% CO$_2$ and balance N$_2$), respectively. The bath solution was changed every 30 min. Isometric responses of circumferential tension were measured by Grass FT03C force transducers (Grass Instruments, Quincy, MA).

Each of the rings was stretched to an initial length, resulting in a maximal contractile response to increases in oxygen tension (7). The rings were stretched during a 15-min interval in medium equilibrated with 6% oxygen (starting tension). Once they achieved a steady-state tension, they 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 after a drug addition before another experimental agent was added to the bath.

Protocol 1: effects of hypoxia, PG, and NO on ductus arteriosus tension. Rings were equilibrated with a solution containing one of the following oxygen concentrations: 2%, 4%, 6%, 16%, or 30% until the tension reached a new plateau (Fig. 1). Indomethacin (5.6×10^{-6} M) then was added to the bath solution. We have previously shown that this concentration of indomethacin inhibits PGE$_2$ and PGI$_2$ production in the ductus (3, 4, 8). The rings were exposed to indomethacin for the remainder of the study protocol. After the rings reached a new steady-state tension, an antagonist of NO synthesis, nitro-L-arginine methyl ester (L-NAME, 10^{-4} M), was added to the bath solution. After the addition of indomethacin and L-NAME, K$^+$-Krebs solution (containing 100 mM KCl substituted for an equimolar amount of NaCl) equil-
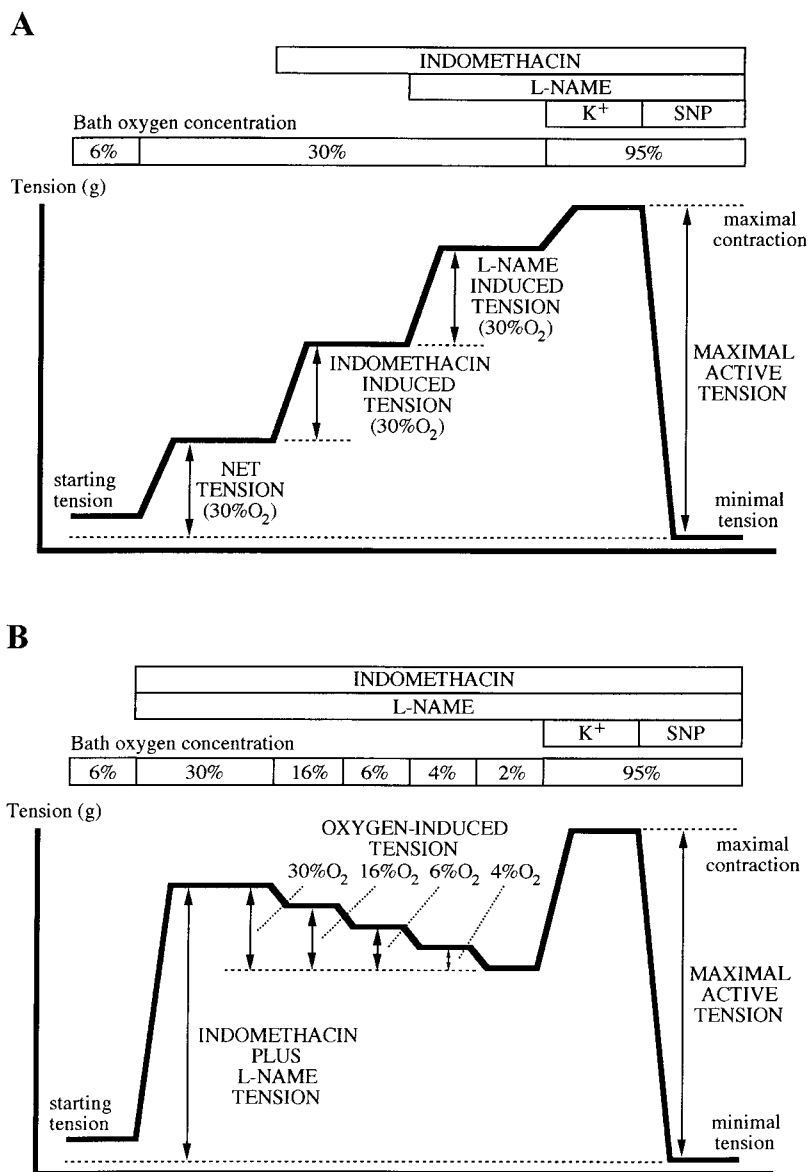

Fig. 1. Definition of tensions measured in *protocols 1* (*A*) and *2* (*B*) for a representative ring exposed first to 30% oxygen; see METHODS for details. K$^+$, potassium Krebs solution; L-NAME, nitro-L-arginine methyl ester; SNP, sodium nitroprusside.

ibrated with 95% O_2-5% CO_2 was used to measure the maximal contraction, and sodium nitroprusside (SNP, 10^{-4} M) was used to determine the minimal tension that could be developed by the ductus.

The difference in tensions between the maximal contraction (with K^+-Krebs) and the minimal tension (with SNP) was considered the maximal active tension developed by the ring. The difference in tensions between the steady-state tension achieved at a particular oxygen concentration and the SNP-induced minimal tension was considered the net tension. The difference in tensions between the steady-state tension achieved with indomethacin and the steady-state tension with oxygen 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.

Protocol 2: effects of hypoxia on ductus arteriosus pretreated with indomethacin and L-NAME. Rings of ductus arteriosus were equilibrated for 4.5 h with solution containing indomethacin (5.6×10^{-6} M) and L-NAME (10^{-4} M) and one of the following oxygen concentrations: 4%, 6%, 16%, or 30%. Next, the rings were exposed sequentially to 30%, 16%, 6%, 4%, and 2% oxygen. They were allowed to reach a new steady-state tension after each change in oxygen concentration (25–30 min) before another change was made. After being exposed to the different oxygen concentrations, the rings were incubated in K^+-Krebs solution (equilibrated with 95% O_2/5% CO_2) followed by SNP to determine the maximal and minimal tensions. The difference in tensions between the steady-state tension at a particular oxygen concentration (plus indomethacin and L-NAME) and the minimal tension (after SNP) was considered the indomethacin plus L-NAME tension at that oxygen concentration. The difference in indomethacin plus L-NAME tensions between 2% oxygen and either 4%, 6%, 16%, or 30% oxygen was considered the oxygen-induced tension due to that particular oxygen concentration.

Changes in tension for each experimental condition were expressed as a percentage of maximal active tension. The initial starting tension, at the beginning of the experiment, was always greater than the tension after SNP relaxation by $11 \pm 5\%$ ($P < 0.001$) of maximal active tension; this indicates that the ductus rings were actively contracting even at the time of their initial mounting in the organ bath.

PGE_2 and $6ketoPGF_{1\alpha}$ (PGI_2 metabolite) determination. Rings of ductus arteriosus were equilibrated with a solution containing one of the following oxygen concentrations: 2%, 4%, 6%, or 30%, for 5 h while tension was monitored. The bath solution was changed every 30 min. The last change in bath solution was collected for PGE_2 or $6ketoPGF_{1\alpha}$ analysis. Next, the rings were equilibrated with solution containing 30% oxygen for 1.5 h. The third change in bath solution was collected for PGE_2 and $6ketoPGF_{1\alpha}$ analysis. We compared the amount of PG released at a particular oxygen concentration (2, 4, 6, or 30%) with the amount released at 30% oxygen from the same ring. After the experiment, the rings were removed from the baths, blotted dry, and their wet weights were determined. Samples of bath solution were acidified to pH 3 with glacial acetic acid and applied to octadecylsilyl silica columns. The columns were washed with 15% aqueous ethanol followed by petroleum ether and then eluted with methyl formate. Methyl formate was dried (Speed Vac), and PGs were reconstituted in PBS for radioimmunoassay (21, 35). The recovery of radioactive PGE_2 and $6ketoPGF_{1\alpha}$ was >96%, and the interassay variability of the radioimmunoassay was <5%.

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 ductus arteriosus suspended in the organ baths, we used a modification of the 2-(2-nitro-1*H*-imidazol-1-yl)-*N*-(2,2,3,3,3-pentafluoropropyl) acetamide (EF5) detection system that we have previously reported (2, 16, 27). The pentafluorinated derivative of etanidazole, EF5, binds to cysteine residues of intracellular proteins after it has been acted on by hypoxia-dependent nitroreductases (36, 41). 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 (16, 27). We have previously shown that EF5 binding can be assessed quantitatively using immunofluorescent techniques (27). We developed a dot immunoblot modification of these techniques for the current experiments so that we could make EF5 measurements in a large number of tissue samples.

Rings of ductus arteriosus were suspended in the organ baths and incubated in Krebs solution containing 100 μM EF5. The bath solution was preequilibrated with the desired oxygen concentrations. The bath solution, with EF5, was exchanged every hour. After a 3-h incubation, unbound EF5 was rinsed out of the baths. The rings were either frozen with liquid N_2 and stored at $-80°C$ until assayed or embedded in Tissuetek (Miles, Elkhart, IN) and frozen for immunohistochemical detection of EF5 (2).

In preliminary immunohistochemical studies (for techniques, see Ref. 2), we found that EF5 was not uniformly distributed throughout the wall of the ductus ring. EF5 binding at the luminal and adventitial surfaces of the ring was less than in the center of the muscle media (Fig. 2). This pattern of EF5 binding in the ductus is similar to the distribution of tissue P_{O_2} measured in other vessels with oxygen microelectrodes (23, 34). The immunoblot technique (described below) measures the net EF5 binding in the tissue, thereby giving us an average value of oxygen concentration in the ring.

We used vascular smooth muscle cells (SMC) that were isolated from medial explants of 138-day gestation fetal
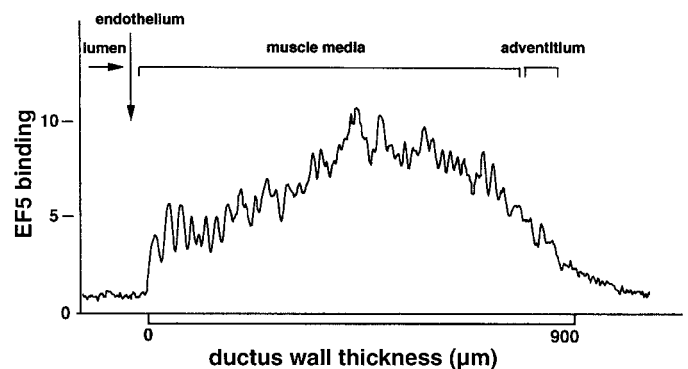

Fig. 2. Distribution of 2-(2-nitro-1*H*-imidazol-1-yl)-*N*-(2,2,3,3,3-pentafluoropropyl) acetamide (EF5) binding in a ring of ductus arteriosus incubated in 2% oxygen. The ductus ring was incubated for 3 h in a solution containing 100 μm EF5, before embedding in Tissuetek (see METHODS). Frozen sections (14 μm) were processed for immunohistochemistry with the monoclonal antibody ELK 3–51, photographed, and analyzed with the National Institutes of Health "Image" software and Adobe Photoshop as previously described (2). The vertical axis represents the amount of EF5 binding at various points across the ductus wall (from lumen to adventitia) compared with background binding. Binding is highest in the center of the muscle media.

lambs (39) to characterize the immunoblot detection technique. The SMC were grown in monolayer culture in Eagle's minimal essential medium, 10% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were passaged with trypsin-EDTA and were used between *passages 4* and *9*. On the day preceding the experiment, cells were trypsinized, and ~250,000 cells were plated onto glass Petri dishes (50-mm diameter) and incubated overnight. The next morning, the dishes were removed from the incubator, cooled to 4°C, and their medium replaced with 1 ml fresh medium containing 100 µM EF5. Dishes were placed in leak-proof aluminum chambers, which were connected to a manifold allowing the gas phase of the chambers to be exchanged for the desired oxygen concentration (26, 27). The chambers then 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, lysed with lysis buffer [150 mM NaCl, 50 mM Tris · HCl, pH 7.5, 1% Nonidet P-40 (NP-40), 0.1% SDS, 2 mM aprotinin, 2 mM leupeptin, and 2 mM phenylmethylsulfonylfluoride (PMSF)], and stored at −80°C after adjusting the protein concentration to 1.0 mg/ml.

To perform the immunoblot assay, 2 µl (2 µg protein) of the cell lysate were added to Nitrocellulose Hybond enhanced chemiluminescence (ECL) membranes (Amersham, Arlington Heights, IL) and air-dried for 30 min. The membrane was washed three times with D-PBS and blocked with 5% nonfat dry milk plus 0.05% Tween 20 in D-PBS for 1 h. This was followed by incubation with the mouse monoclonal IgG primary antibody ELK 3–51 (5 µg/ml) overnight at 4°C. ELK 3–51 is highly specific for EF5 tissue adducts and was used to detect the presence of bound EF5 in the tissue (16, 27, 32). The next morning, the membrane was washed three times before incubation with goat-antimouse IgG antibody coupled to horseradish peroxidase (0.13 µg/ml; Jackson Laboratories, Westgrove, PA) for 30 min. After the membranes were washed three times, the protein dots were visualized by ECL using the ECL kit (Amersham). Densitometric analysis was performed with the National Institute of Health "Image" software and Adobe Photoshop.

SMCs incubated in 0.01% oxygen have maximal binding (100%) of EF5. Cells incubated in severe and moderate hypoxia (0.1% and 0.3% oxygen) have $47 \pm 4\%$ and $12 \pm 2\%$ of the maximal EF5 binding, respectively. At milder degrees of hypoxia, 1% and 3% oxygen, binding is only $6 \pm 2\%$ and $4 \pm 1\%$ of maximum, respectively, and at 10% and 21% oxygen, binding is negligible ($1.2 \pm 0.5\%$ and $0.5 \pm 0.5\%$, respectively). This technique is most accurate between 0.05 and 4% oxygen.

We performed immunoblot analyses of EF5 binding in ductus rings and compared them to measurements made in SMCs that were incubated in known oxygen concentrations (0.01, 0.1, 0.3, 1, 3, 10, and 21% O_2). The frozen rings were pulverized in a stainless steel mortar and pestle kept on dry ice. Frozen ground tissue (~30 mg) was homogenized with 200 µl lysis buffer and centrifuged at 900 rpm for 5 min at 4°C. The supernatant was collected and centrifuged at 4,900 rpm for 10 min, followed by 10,000 rpm for 2 min. The protein concentration of the supernatant was determined by the dye-binding method (1), with bovine serum albumin as the standard, and adjusted to a final protein concentration of 1.0 mg/ml. Lysates from both the tissue samples and the SMC standard curve were blotted on the same membrane and processed together. Although the oxygen dependency of EF5 binding is independent of cell type, it is linearly related to drug exposure (16, 27). Therefore, the relative binding of EF5 to a tissue lysate was corrected for its drug exposure. The in vitro drug exposure to EF5 was 300 µM × h in both the tissue

samples and the SMC standard curve. For in vivo experiments (see below), exposure to EF5 was calculated from the area under the curve of EF5 serum concentrations (2). All values for both the standard curve and the tissue samples were expressed as a ratio (EF5 binding ratio) of the value obtained from SMCs equilibrated with 0.01% oxygen (maximal binding). The oxygen concentration in the tissue was determined by comparing the EF5 binding ratio of the tissue with the EF5 binding ratios in the SMC standard curve lysates. Two types of control tissues were used in the ductus 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 ELK 3–51 binding to either control tissue (data not shown).

To compare measurements of oxygen concentration in our in vitro ductus studies with those found in vivo, we operated on one fetal lamb (128 days gestation) and one 1-day-old full-term newborn lamb. The fetal surgical preparation has been described in detail previously (18). Briefly, under intravenous anesthesia with ketamine hydrochloride and diazepam, a midline laparotomy was performed on the ewe. Catheters were advanced into the fetal descending aorta and inferior vena cava from the femoral artery and vein, respectively. The incisions were closed, and the ewe was returned to her cage for recovery. One day after surgery, the fetus was given EF5 (100 µmol/kg) over 10 min followed by 100 µmol · $kg^{-1} \cdot h^{-1}$ for 3 h. At 10 and 30 min and 1, 2, 3, 4, 6, 10, and 24 h after starting the infusion, blood samples were prepared for EF5 determination. The samples were precipitated with 10% trichloroacetic acid. Unmetabolized drug was measured in serum by acid extraction followed by high-performance liquid chromatography with ultraviolet detection of the nitrochromophore (30).

Under intramuscular ketamine hydrochloride and local lidocaine anesthesia, femoral arterial and venous catheters were placed in the 1-day-old newborn lamb. One day after surgery, the newborn lamb was given EF5 (100 µmol/kg) over 10 min, and blood samples were collected for EF5 determinations during the next 24 h.

The arterial P_{O_2} in the fetus and newborn during the 24-h EF5 exposure was 19 ± 1 and 77 ± 5 Torr, respectively. In both the fetal and newborn study, necropsies were performed at 24 h after EF5 administration. The ductus arteriosus was divided in two: one-half was frozen in liquid nitrogen for immunoblot analysis of EF5 and the other embedded in Tissuetek (Miles, Elkhart, IN) and frozen for immunohistochemical detection of EF5 (2).

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 Wilcoxon signed-rank test. Values are expressed as means ± SD. Drug doses refer to their final molar concentration in the bath.

Chemicals. The following compounds were used: indomethacin, L-NAME, SNP, pepstatin, EDTA, aprotinin, PMSF, trichloroacetic acid, Tween 20, and all cell culture reagents (Sigma, St. Louis, MO); leupeptin (Boehringer Manheim, Indianapolis, IN); NP-40 (Calbiochem, La Jolla, CA); protein determination assay (Bio-Rad, Richmond, CA); radioimmunoassay kit for PGE_2 and $6ketoPGF_{1\alpha}$ (Advanced Magnetics, Boston, MA); [^{3}H]PGE_2 (183 Ci/mmol) (Amersham); and EF5 (synthesized by Dr. M. Tracy, SRI International, Palo Alto, CA). Indomethacin was prepared in ethanol (15 mg/ml). The final concentration of ethanol did not affect tissue contractility.

RESULTS

We measured the in vivo tissue oxygen concentration of the ductus arteriosus in one late-gestation fetus and one 2-day-old newborn lamb using the EF5 immunoblot technique. We found that the tissue oxygen concentration of the fetal ductus, in vivo, was ~2% throughout its muscle media; in contrast, the inner half of the 2-day-old newborn ductus had a tissue oxygen concentration of <0.1%. To examine how hypoxia affects ductus contractility, we used isolated rings of ductus arteriosus and exposed them to tissue oxygen concentrations that approximated the values found in vivo (between 2% and 0.1%). We used five specific bath-solution oxygen concentrations and measured the corresponding average tissue oxygen concentrations by the EF5 immunoblot technique. As the bath solution oxygen concentration dropped from 30% to 2%, the tissue oxygen concentration decreased from 1.4% to 0.1% (Table 1).

In *protocol 1*, rings of ductus arteriosus developed varying degrees of tension depending on the oxygen concentration to which they were exposed (Fig. 3). The rings contracted slowly, taking 60–90 min to reach their maximal net tension at mean tissue oxygen concentrations between 0.2% and 1.4%, and 180 min when the mean tissue oxygen concentration was 0.1% (Fig. 3*A*). As the mean tissue oxygen concentration in the ductus muscle media decreased from 1.4% to 0.2%, the ductus relaxed and developed less tension. However, when the mean oxygen concentration dropped to 0.1%, there was a profound increase in tension in the ductus (Fig. 3*A*). Rings of ductus arteriosus that were severely hypoxic (tissue oxygen concentration 0.1%) contracted to a tension that was greater than that achieved when the rings had a mean tissue oxygen concentration of 1.4% (Fig. 3*A*).

We used indomethacin to examine the role played by endogenous PG production in opposing the contractile state of the ductus. Indomethacin caused an increase in tension when the mean tissue oxygen concentration of the rings was ≥0.2% (Fig. 3*B*). In contrast, indomethacin had no contractile effect on ductus rings that were severely hypoxic (tissue oxygen concentration 0.1%). The lack of effect of indomethacin at severe hypoxia was not due to loss of sensitivity of the tissue to PGE_2; exogenous PGE_2 had the same relaxant effect
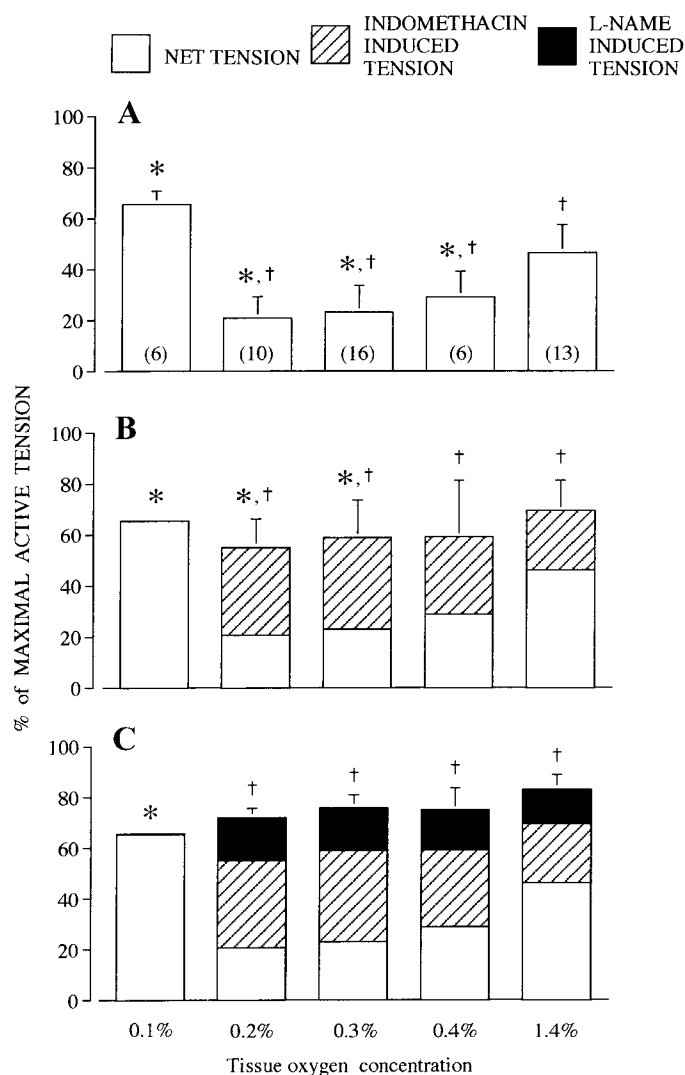

Fig. 3. Severe hypoxia produces increased tension in the ductus arteriosus by inhibiting PG and nitric oxide-mediated relaxation. *A*: ductus rings were incubated at 1 of the following mean tissue oxygen concentrations: 0.1% ($n = 6$), 0.2% ($n = 10$), 0.3% ($n = 16$), 0.4% ($n = 6$), or 1.4% ($n = 13$). Height of column represents mean ($\pm$SD) net tension developed by the rings at each oxygen concentration. Number of ductus rings in parentheses. *B*: height of column represents the steady-state tension after indomethacin (5.6×10^{-6} M) was added to the bath ($\pm$SD of indomethacin-induced tension). *C*: height of column represents the steady-state tension after indomethacin and L-NAME (10^{-4}) were added to the bath ($\pm$SD of L-NAME-induced tension). Tensions are expressed as percent maximal active tension. Starting tension: 0.1% oxygen = 6 ± 2 g; 0.2% = 5 ± 1 g; 0.3% = 5 ± 1 g; 0.4% = 6 ± 1 g; 1.4% = 5 ± 2 g. Maximal active tension: 0.1% oxygen = 19 ± 5 g; 0.2% = 22 ± 5 g; 0.3% = 21 ± 4 g; 0.4% = 24 ± 2 g; 1.4% = 23 ± 4 g. Tissue weight: 0.1% oxygen = 64 ± 24 mg; 0.2% = 49 ± 19 mg; 0.3% = 43 ± 16 mg; 0.4% = 61 ± 23 mg; 1.4% = 55 ± 20 mg. *$P < 0.05$ compared with net tension, indomethacin-induced tension, or L-NAME-induced tension at 1.4% tissue oxygen concentration. †$P < 0.001$ compared with net tension, indomethacin-induced tension, or L-NAME-induced tension at 0.1% tissue oxygen concentration.

Table 1. *Comparison of oxygen concentration in the organ bath solution with mean tissue oxygen concentration in the ductus arteriosus rings*

Bath solution	Oxygen, %				
	2	4	6	16	30
Tissue	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.4 ± 0.1	1.4 ± 0.4
n	11	8	8	6	11

Values are mean $\pm$ SD. n, no. of ductus rings. Organ bath solution oxygen concentration measured by oxygen electrode. Tissue oxygen concentration measured by 2-(2-nitro-1H-imadazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl) acetamide (EF5) technique (see METHODS). Tissue wet weight = 63 ± 21 ($n = 44$).

on indomethacin-exposed rings at all tissue oxygen concentrations tested (Fig. 4). Rather, the lack of effect of indomethacin was due to the marked inhibition of endogenous PG production that occurs at this low tissue oxygen concentration (Fig. 5)
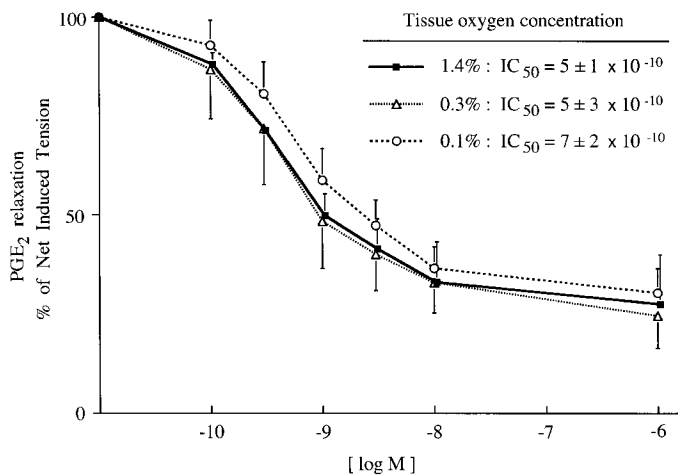
Fig. 4. Tissue oxygen concentration does not affect the ability of PGE_2 to relax the ductus. Ductus arteriosus from 3 fetuses was precontracted with indomethacin and L-NAME at either 0.1, 0.3, or 1.4% mean tissue oxygen concentration. Net induced tension is the difference between the tension after precontraction (with indomethacin and L-NAME) and the minimal tension produced after SNP. Net induced tension: 1.4% tissue oxygen concentration $= 16 \pm 1$ g; 0.3% $= 12 \pm 2$ g; 0.1% $= 13 \pm 4$ g. Relaxation is expressed as a percent change in net induced tension. Tissue weight: 1.4% oxygen $= 56 \pm 27$ mg; 0.3% oxygen $= 40 \pm 13$ mg; 0.1% oxygen $= 62 \pm 44$ mg.

We used L-NAME, an antagonist of NO synthesis, to examine the role played by endogenous NO production in opposing the contractile state of the ductus (Fig. 3C). L-NAME caused an increase in tension in the indomethacin-contracted rings when the mean tissue oxygen concentration was $\geq 0.2\%$ (Fig. 3C). L-Arginine $(10^{-3}$ M) completely reversed the L-NAME-induced contraction (data not shown). L-NAME had no effect on the contractile tone of ductus rings that were severely hypoxic (tissue oxygen concentration 0.1%). The lack of effect of L-NAME at severe hypoxia was not due to loss of sensitivity of the tissue to NO because the NO donor SNP had the same relaxant effect on L-NAME plus indomethacin-exposed rings at all tissue oxygen concentrations (2).

In *protocol 2*, ductus rings were pretreated with indomethacin and L-NAME before oxygen concentrations in the rings were varied. In the absence of endogenous PG and NO production, there was a significant $(P < 0.05)$ decrease in ductus tension as the mean tissue oxygen concentration decreased from 1.4% to 0.1% (Fig. 6). The final tension achieved by rings that were severely hypoxic (oxygen concentration 0.1%), in the presence of both indomethacin and L-NAME, was significantly less than that achieved by rings exposed
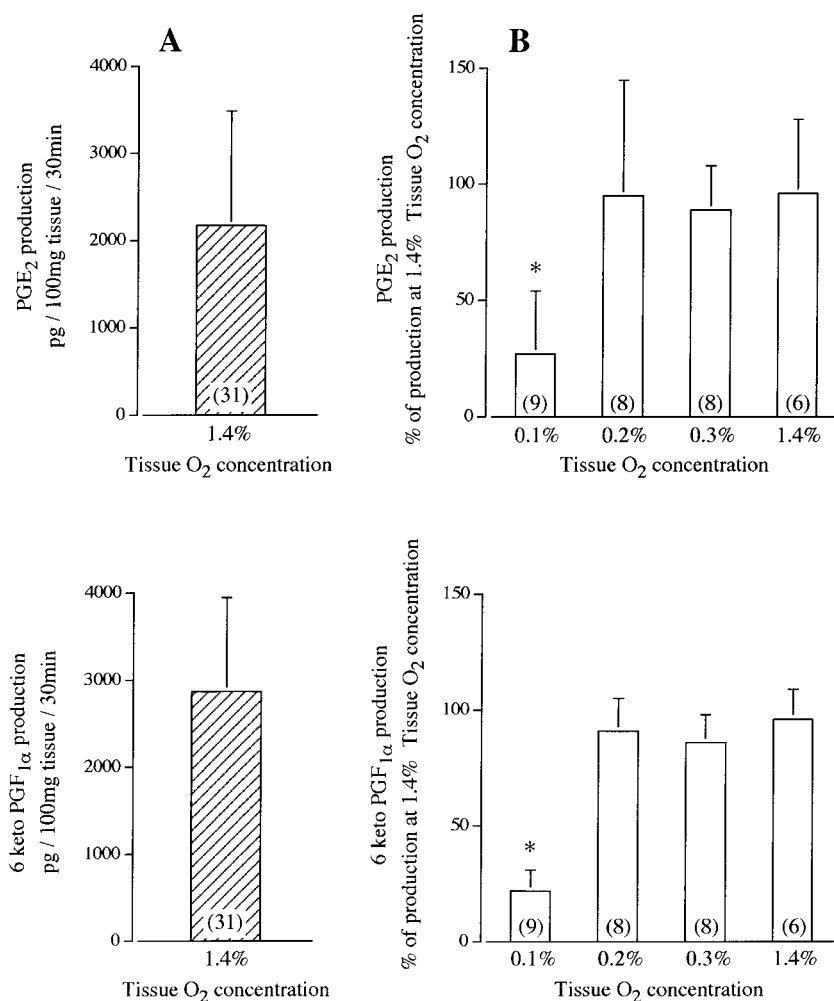

Fig. 5. Severe hypoxia inhibits PGE_2 and 6ketoPGF$_{1\alpha}$ production by rings of ductus arteriosus. *A*: height of column represents mean $\pm$ SD immunoreactive PGE_2 or 6ketoPGF$_{1\alpha}$ released from ductus rings when their mean tissue oxygen concentration was 1.4%. PG release was measured during a 30-min collection period and expressed per 100 mg tissue wet wt. *B*: column height represents the mean percent of PGE_2 or 6ketoPGF$_{1\alpha}$ released from rings at 0.1%, 0.2%, 0.3%, or 1.4% tissue oxygen concentration compared with the amount released from the same rings after the bath solution was changed and the rings were incubated at 1.4% tissue oxygen concentration (see METHODS). *$P < 0.01$ vs. amount released at 1.4% oxygen. Number of ductus rings in parentheses.
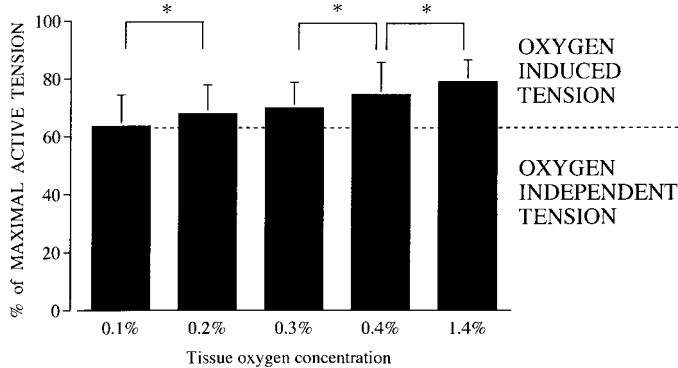
Fig. 6. After inhibition of PG and nitric oxide production, decreasing oxygen concentrations lead to ductus relaxation. Rings of ductus arteriosus (from 13 fetuses) were first equilibrated with indomethacin (5.6×10^{-6} M) and L-NAME (10^{-4} M); next, they were exposed sequentially to the following mean tissue oxygen concentrations: 1.4%, 0.4%, 0.3%, 0.2%, and 0.1%. Height of column represents mean ($\pm$SD) indomethacin plus L-NAME tension at a particular oxygen concentration. The difference in tension between 0.1% tissue oxygen concentration and either 0.2%, 0.3%, 0.4%, or 1.4% oxygen concentration is considered the oxygen-induced tension. Tensions are expressed as percent maximal active tension. Starting tension = 5 ± 1 g; maximal active tension = 23 ± 3 g; tissue weight = 56 ± 17 ($n = 13$). *$P < 0.05$ tension at 0.1% oxygen vs. tension at 0.2%; tension at 0.3% vs. tension at 0.4%; tension at 0.4% vs. tension at 1.4%

to higher oxygen concentrations (Fig. 6). However, even at negligible tissue oxygen concentrations (0.1%), the ductus had an intrinsic tone that was $64 \pm 10\%$ of its maximal active tension.

DISCUSSION

We have previously shown that when the full-term newborn ductus constricts after birth, it cuts off its luminal blood supply and develops a region of profound hypoxia comprising the inner half of the muscle media (2). In full-term primates, the tissue oxygen concentration drops to <0.2% within 24 h birth (2). With the use of a modification of the EF5 technique previously used in primates (2), we observed a similar drop in tissue oxygen concentration in the newborn sheep ductus in vivo. Although the animal's arterial Pa_{O_2} was 79 Torr, the tissue oxygen concentration within the inner half of the ductus muscle media was <0.1%. To study the effects of different degrees of tissue hypoxia on ductus contractility, we used isolated rings of sheep ductus arteriosus in which we produced similar tissue oxygen concentrations to those found in vivo.

Regulation of ductus contractility depends on a balance between vasoconstricting and vasodilating influences. Prior studies have suggested that the increase in postnatal oxygen tension leads not only to ductus constriction (11, 24, 33, 40), but also to the production of vasodilators like PGs (4, 12) and NO (9, 13) that oppose this constriction. In the presence of indomethacin and L-NAME (inhibitors of PG and NO production, respectively), we observed a direct relationship between changes in tissue oxygen concentration and ductus tone (Fig. 6). We found that oxygen could alter the contractile tone of the ductus by as much as $15 \pm 10\%$ of its maximal active tension as we varied mean tissue

oxygen concentrations from 0.1% to 1.4% (Fig. 6). Increasing the environmental (bath solution) oxygen concentration to >90% increased the range of oxygen-induced tension to $29 \pm 10\%$ of the maximal active tension (data not shown). Conversely, there was a consistent, stepwise relaxation of the ductus as its mean tissue oxygen concentration dropped to 0.1% (Fig. 6). The exact mechanisms that mediate this response are still unknown (14, 17). Oxygen causes membrane depolarization, which, in turn, is associated with a rise in smooth muscle intracellular calcium (33). Oxygen inhibits K^+ channels and releases endothelin-1 in ductus smooth muscle (11, 33, 40); however, the importance of K^+ channels and endothelin-1 in mediating oxygen-induced contraction is still unclear (R. I. Clyman, unpublished results, Ref. 18).

In addition to the oxygen-induced tension, we observed an intrinsic vasoconstrictor tone in the ductus that was independent of ambient oxygen concentration (Fig. 6). This oxygen-independent tone was equivalent to $64 \pm 10\%$ of the maximal tension that could be produced by the ductus. Although changes in oxygen concentration produce a change in ductus tension, it seems that most of the tension developed by the ductus is independent of oxygen concentration. Whether this sustained intrinsic tone is due to vasoconstrictors that are released by the endothelial or SMCs of the ductus wall or to the increased calcium sensitivity of ductus contractile proteins is currently unknown (15, 20, 28, 31).

The ductus also produces several known vasodilators (e.g., PGs and NO) that inhibit both the oxygen-induced and -independent contractions. We used indomethacin and L-NAME to uncover the role of endogenous PGs and NO in opposing the contractile state of the ductus. At mean tissue oxygen concentrations between 0.2% and 0.4%, endogenous PGs and NO play a significant role in regulating ductus tone because they inhibit >60% of the active tension developed by the vessel (Fig. 3C). As tissue oxygen concentration increases above 0.4%, the inhibitory effects of PGs and NO are outweighed by the vasoconstrictor effects of oxygen (Fig. 3C).

Oxygen is also an essential substrate for both PG and NO formation (29, 43). The formation of PGs by cyclooxygenase can be regulated by oxygen, but only at very low oxygen concentrations. The apparent Michaelis constant (K_m) for oxygen in PG biosynthesis is ~5 μM (0.5% oxygen concentration) (29). Our results are consistent with this value because PG production was not inhibited until ductus tissue oxygen concentration dropped below 0.2% (Fig. 5). Similarly, indomethacin had no effect on the ductus at tissue oxygen concentrations <0.2%, whereas it caused significant ductus constriction at tissue oxygen concentrations above this range (Fig. 3B).

Prior in vitro studies have suggested that PG production can be inhibited by extracellular oxygen concentrations in the range of 2–3% ($Po_2 = 14$–21 Torr) (4, 12) . This has prompted some investigators to hypothesize that, in vivo, the fetal ductus may have a limited

ability to make PGs because the P_{O_2} of fetal arterial blood falls within this range (4). The current studies point out a problem with this hypothesis: whereas the fetus may be hypoxemic in vivo, the ductal tissue oxygen concentration is really ~2% [which should be sufficient for PG production (see Fig. 5)]; in contrast, when the ductus is studied in vitro, even if its bath solution is kept at a similar "fetal" P_{O_2}, the oxygen concentration in the tissue drops to <0.2% [Table 1; a tissue oxygen concentration that inhibits PG production (see Fig. 5)]. Presumably, this is due to the loss of vasa vasorum perfusion in vitro (2, 9). Our findings suggest that whereas low extracellular oxygen concentrations can inhibit PG production, they do so only when the actual tissue oxygen concentrations fall below 0.2% (Fig. 5).

NO production also has been reported to be inhibited at low oxygen concentrations (19, 22, 25, 38, 43). The K_m for oxygen appears to be ~9 μM (0.9% oxygen) in isolated endothelial NO synthase preparations (37). Unfortunately, we were unable to obtain any direct measurements of hypoxia's inhibitory effects on NO production. We used a Sievers model 280 NO analyzer (Boulder, CO) to measure NO release by measuring its nitrate and nitrate reaction products in the solution surrounding the ductus. In our experiments, at all oxygen concentrations tested, the rate of release of NO was below the limits of detection of the assay system (data not shown). On the other hand, our contraction studies were consistent with oxygen having a regulatory role in NO production; however, this was seen only under conditions of severe hypoxia: for example, whereas L-NAME produced significant ductus constriction at all oxygen concentrations $\geq$0.2% (Fig. 3C), it had no effect on the ductus at oxygen concentrations <0.2%.

It is unlikely that the inability of ductus rings to contract with indomethacin or L-NAME under conditions of severe hypoxia was due to tissue deterioration during the hypoxic exposure: ductus rings incubated at severe hypoxia (0.1% tissue oxygen concentration) for >4 h develop similar maximal active tensions (see Fig. 3) and have similar relaxant responses to PGE_2 (Fig. 4) and NO (9) as those incubated at higher oxygen concentrations; similarly, although ductus rings incubated in severe hypoxia do not produce PGs or contract when exposed to indomethacin, they can produce significant amounts of PGs (see Fig. 5) (4) and will contract with indomethacin if they are subsequently incubated in elevated P_{O_2} (4). Our findings suggest that whereas hypoxic conditions can regulate PG and NO production in the ductus, they do so only when tissue oxygen concentrations fall below 0.2% (Figs. 3, B and C, and 5). When tissue oxygen concentration falls into this severely hypoxic range (<0.2%), the relaxant effects of endogenous PGs and NO are lost and the tissue tension rises to a level normally observed only after treatment with indomethacin and L-NAME (compare Fig. 3A with 3C or 6).

After birth, factors that normally oppose ductus constriction in the fetus (e.g., elevated pulmonary blood pressure and circulating plasma PGs) (5, 6) decrease rapidly in the newborn with the onset of spontaneous ventilation. The combination of these events, with the increase in postnatal arterial P_{O_2}, leads to ductus constriction and loss of luminal blood flow. If we can extrapolate our in vitro results to the situation in vivo, we would conclude that the profound tissue hypoxia that develops during postnatal contraction in the full-term ductus inhibits PG and NO production. We hypothesize that in vivo, the loss of these two vasodilators more than compensates for the loss of oxygen-induced tension. This would enable the ductus to remain constricted, despite the development of tissue hypoxia, as the vessel undergoes anatomic remodeling.

Perspectives

In contrast with the full-term ductus, the preterm ductus develops only mild to moderate hypoxia (2.0–0.7% tissue oxygen concentration) after postnatal constriction in vivo (2). We have previously shown that without a drop in tissue oxygen concentration to <0.4%, ductus remodeling does not occur (2). We would suggest that these mild to moderate degrees of hypoxia in the preterm do not inhibit PG and NO production, whereas they do lead to loss of oxygen-induced tension (Fig. 3). This would lead to ductus relaxation and may explain the high incidence of ductus reopening in the preterm infant (42).

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