ARTICLES

The Effect of Vitamin A on Contraction of the Ductus Arteriosus in Fetal Rat

GUI-RONG WU, SHEN JING, KAZUO MOMMA, AND TOSHIO NAKANISHI

Department of Pediatric Cardiology, The Heart Institute of Japan, Tokyo Women's Medical University,
Tokyo, 162 Japan

ABSTRACT

Endogenous retinoic acid may play a role in inducing smooth muscle differentiation in the fetal ductus arteriosus. Maternal administration of retinoic acid may accelerate the process. This study was designed to investigate the effect of vitamin A on developmental changes in the contractile system of the ductus. Vitamin A was injected into pregnant rats and the ductus was isolated from the fetus at 19, 20, or 21 d of gestation. The fetus at 19 d of gestation served as a model of the preterm fetus. The force of contraction and $[Ca]_i$ were measured. Membrane depolarization caused by high KCl induced ductal contraction in all age groups studied. In the 19-d fetus, O_2 did not cause significant contraction or changes in $[Ca]_i$ in the control group, but it did induce a significant contraction and increases in $[Ca]_i$ in the vitamin A-treated group. In the 20- and 21-d fetuses, 5% O_2 -induced contraction in the vitamin A-treated group was significantly greater than in the control group. In the 19-d fetus, noradrenaline-induced contraction and increases in $[Ca]_i$, indica-

tors of the size of the intracellular Ca pool, were observed and they were similar in the control group and in the vitamin A-treated group. These data suggest that 1) in the preterm fetus, the contractile system, including membrane depolarization, $[Ca]_i$ increase, and its activation of contractile proteins, is already functioning, but the O_2 -sensing mechanism is underdeveloped, 2) vitamin A accelerates the development of the O_2 -sensing mechanism of the ductus arteriosus. (*Pediatr Res* 49: 747–754, 2001)

Abbreviations:

DA, ductus arteriosus
Ca, calcium
K, potassium
 $[Ca]_i$, intracellular Ca concentration
fura-2/AM, fura-2/acetoxymethyl ester

The ductus arteriosus (DA) normally closes after birth. Increases in arterial PO_2 , decreases in endogenous dilator prostaglandins and nitric oxide, and/or production of endothelin-1 may be responsible for the ductal closure (1–4).

Failure of the DA to close after birth is more frequent in premature than in mature infants. In full-term neonates, closure of the DA occurs in two phases after birth: 1) initial muscular constriction, and 2) neointimal thickening after mechanical closure. Both muscular constriction and neointimal thickening may be inadequate in premature neonates (5). Indomethacin, a prostaglandin synthetase inhibitor, has been used clinically to induce ductal closure, but its effect is minimal before 24 wk of

gestation in humans (6, 7). In an *in situ* morphometric analysis in rats, Momma *et al.* (8–10) showed that maternal administration of indomethacin caused only mild DA constriction at 19 d of gestation and strong constriction in the near-term fetus at 21 d of gestation. The precise mechanisms by which the DA in premature infants or animals is resistant to vasoconstrictive stimulation remain unclear.

Kim *et al.* (11) have shown in near-term fetal rabbits that the expression of the adult type isoform of smooth muscle myosin heavy chain in the DA was more prominent than in other arteries. They suggested that vascular smooth muscle cells might be more differentiated in the DA than in other arteries. Colbert *et al.* (12) showed that endogenous retinoic acid signaling colocalized with advanced expression of the adult type of smooth muscle myosin heavy chain isoform during development of the DA. These authors speculated that retinoic acid might play a role in inducing and maintaining smooth muscle differentiation. Momma *et al.* (9) hypothesized that exogenously administered retinoic acid might accelerate maturation

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Correspondence: Toshio Nakanishi, MD, Department of Pediatric Cardiology, The Heart Institute of Japan, Tokyo Women's Medical University, 8-1 Kawadacho, Shinjuku, Tokyo, 162, Japan; e-mail: pnakanis@hij.twmu.ac.jp

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of the fetal DA, and they, indeed, showed that maternally administered vitamin A enhanced indomethacin-induced DA constriction in *in situ* morphometric analysis. The mechanisms underlying acceleration of ductal constriction by vitamin A, however, remained unclear.

The purpose of the present study was to clarify 1) developmental changes in the contractile system of the DA and 2) the effect of vitamin A treatment on the contractile system of the DA using a ductal preparation isolated from the fetal rat.

Previous studies have suggested that O₂-induced contraction of the DA is, at least partly, induced by membrane depolarization, which then results in increases in [Ca]_i (13, 14). Since extracellular Ca was required for the increase in [Ca]_i (13), an increase in Ca influx across the sarcolemma is probably the main mechanism for the increase in [Ca]_i, but Ca-induced Ca release from intracellular store sites may also occur. In the present study, the effect of O₂ on DA contraction and [Ca]_i was studied in mature as well as premature fetuses. To induce membrane depolarization, high K was used and the effect of high KCl on ductal contraction and [Ca]_i was measured. The size of the intracellular Ca pool was examined using norepinephrine, which releases Ca from intracellular store sites (15–17).

MATERIALS AND METHODS

Animals. Wistar virgin rats were mated overnight from 1700 to 900 h. The presence of sperm in vaginal smears was confirmed as day 0 of pregnancy (pregnancy period 21.5 d). All rats were fed commercial solid food (Labo RUM Breeder, Nippon-Nousan Co., Ltd., Yokohama, Japan) and water. The food contained 1200 IU of vitamin A per 100 g, as specified by the manufacturer. All animals were cared for in compliance with the guiding principles of the American Physiologic Society. The experiments were approved by the Ethical Committee of Animal Experiments of our Institute.

Vitamin A Administration. Retinyl palmitate (Eisai Co. Ltd, Tokyo, Japan), 33 mg, which is equivalent to 18 mg of retinol (vitamin A, 50,000 IU), was dissolved in safflower seed oil (Nissinseiyu Co. Ltd., Tokyo, Japan) to a concentration of 1.8 mg of retinol/mL. The vitamin A solution was injected intramuscularly into the dorsum of pregnant rats at 900 h on the 17th, 18th, 19th, or 20th days of gestation at a dose of 1 mg (3000 IU)/kg of body weight. The dose of vitamin A used in the present study was chosen to be within the therapeutic range for latent deficiency, as described previously (9). Experiments were performed using the ductus arteriosus isolated from the fetal rat at 19, 20, or 21 d of gestation. When vitamin A was administered once, it was injected 1 day before the experiment. When vitamin A was administered twice, it was injected 1 and 2 days before the experiment. In the control group, safflower seed oil only was injected into pregnant rats in a similar fashion described above.

Experimental Preparation. After the doe was anesthetized with pentobarbital sodium (50 mg/kg), the fetuses were delivered by cesarean section. The ductal tissue was excised from the chest cavity using a dissecting microscope (model SMA, Nikon, Tokyo, Japan). The isolated ductus arteriosus was

placed in a HEPES-buffered solution equilibrated with room air. The surrounding tissues were removed under a dissecting microscope. The ductus preparation was placed in a tissue bath and kept at 37°C. Two tungsten wires (25 μ m in diameter, Thermonic Products Company, North Plainfield, NJ, U.S.A.) were threaded into the lumen and the preparation was placed in a myograph (Living Systems, Burlington, VT, U.S.A.) (16, 18–20). The tungsten wires were secured by stainless screws to specially designed holders. One holder was connected to a micro-manipulator and the other was connected to a force transducer (Kulite Semiconductor, Leonia, NJ, U.S.A.). Iso-metric tension was monitored using the force transducer and a recorder (San-ei Sokki, Tokyo, Japan). When set up, the preparation was superfused with solutions at 20 mL/min. For [Ca]_i determination, the ductus preparation was placed in a tissue bath that was mounted on the stage of an inverted microscope (model TMD, Nikon). All ductus rings were initially stabilized for 30 min with hypoxic Krebs-Henseleit solution at 37°C equilibrated with 5% CO₂ and 95% N₂.

Axial length, outer diameter, and inner diameter of the preparation were measured before it was stretched, and the muscle volume (V), (mm³), was calculated assuming that the shape of the preparation was cylindrical. Axial vessel length (L), the distance between the wires (D), and force (F) were measured while the vessel was stretched gradually. The wall thickness (H) at the time of force measurement was calculated as: $H = V/2LD$. The wall tension, expressed as mN/mm (16, 21, 22), was calculated as: wall tension = $F/2L$. The contractile force, normalized by the cross-sectional area of the preparation, was also calculated as: wall stress (mN/mm²) = $F/2LH$. The resting tension was adjusted to 0.35–0.5 mN/mm (mean 0.40 ± 0.05) in the fetus at 19 d of gestation, 0.4–0.7 mN/mm (mean 0.55 ± 0.08) at 20 d of gestation, and 0.6–0.8 mN/mm (mean 0.72 ± 0.09) at 21 d of gestation. In a preliminary study, the resting tension/developed tension relation was examined and at these values of resting tension, developed tension (total tension/resting tension) induced by 50 mM KCl was 80% of the maximal level. These values of resting tension were chosen to avoid any over-stretching of the vessel.

Solution. The control Krebs-Henseleit solution contained (in mM): NaCl, 118; KCl, 5; CaCl₂, 1.5; glucose, 6; MgCl₂, 1; NaHCO₃, 24; and NaH₂PO₄, 0.436. The solution was equilibrated with one of the following gasses: 95% N₂ - 5% CO₂ (P_{O2} = 22–25 mm Hg, solution #1), 5% O₂ - 90% N₂ - 5% CO₂ (P_{O2} = 57–60 mm Hg, solution #2), 30% O₂ - 65% N₂ - 5% CO₂ (P_{O2} = 218–232 mm Hg, solution #3), or 95% O₂ - 5% CO₂ (P_{O2} = 660–690 mm Hg, solution #4). The final pH was 7.38–7.42 and P_{CO2} was 35–42 mm Hg in all solutions.

The HEPES solution contained (in mM): NaCl, 142; KCl, 5; CaCl₂, 1.5; glucose, 6; MgCl₂, 1; HEPES, 5; pH 7.4 with 1 M NaOH. The HEPES solution was equilibrated with either 100% N₂ or 100% O₂ (P_{O2} = 22–25 or 790–810 mm Hg, respectively). In potassium-enriched solutions (50 or 120 mM), the NaCl was replaced, mol by mol, with KCl. The Ca- and Na-free solution contained in mM: N-methylglucamine, 142; KCl, 4; K₂-EGTA, 1; glucose, 6; MgCl₂, 1; HEPES, 5; pH 7.4 with 1 M HCl.

Ductal Contraction. Ductal contraction was induced by four methods: O₂, indomethacin, noradrenaline, and 50 mM KCl. In experiments in which ductal contraction was induced by O₂, hypoxic Krebs-Henseleit solution #1 was changed to solution #2 (5% O₂), solution #3 (30% O₂), and then to solution #4 (95% O₂). After the force reached a new steady state, the solution was again returned to the hypoxic solution. In all experiments, ductal contraction was finally induced by 50 mM KCl under hypoxic conditions. The 50 mM KCl was used to induce moderate membrane depolarization. In some experiments, however, ductal contraction was induced by 120 mM KCl + 10 mM CaCl₂ to examine the degree of maximal contraction.

In additional experiments, the role of the endothelium in an age-related difference in the effect of O₂ was studied. The endothelium was removed by gently rubbing the internal wall of the vessel with a polyethylene tube (PE-50), which had been tapered to 50–100 μ m in diameter. At the end of the experiments, all the ductal preparations were fixed in 10% formalin and the absence of the endothelium was confirmed histologically.

In experiments in which ductal contraction was induced by indomethacin, hypoxic Krebs-Henseleit solution #1 (0% O₂), was changed to solution #2 (5% O₂), to partially activate the O₂-sensitive mechanisms of ductal contraction. The solution was then changed to solution #2 containing 10⁻⁶ M of indomethacin. This concentration of indomethacin inhibits cyclooxygenase activity completely (23, 24). In some experiments, the indomethacin concentration was increased to 3 $\times$ 10⁻⁶ M.

The size of the intracellular Ca pool was examined using norepinephrine. Norepinephrine increases Ca influx *via* Ca channels by stimulating beta-receptors, and releases Ca from intracellular store sites by stimulating alpha-receptors (15–17). To eliminate the Ca influx *via* the Ca channel and Ca efflux *via* Na-Ca exchange, the effect of norepinephrine was examined in the absence of extracellular Na and Ca (16). Initially, the vessel was superfused with a control HEPES solution equilibrated with 100% N₂. The vessel was then superfused with a HEPES solution that did not contain Ca and Na. Ten minutes after superfusion with the Ca-and Na-free solution, the vessel was superfused with the Ca-and Na-free solution containing 10⁻⁵ M noradrenaline. After the developed tension reached its peak value, the vessel was superfused again with a control HEPES solution. After the mechanical function returned to control, vascular contraction was induced by a solution containing 50

mM KCl. The degree of noradrenaline-induced contraction was normalized by the degree of KCl-induced contraction. Since norepinephrine may alter the sensitivity of contractile proteins to Ca, the effect of norepinephrine on intracellular Ca concentrations was also measured.

Fluorescence Measurements. [Ca]_i of the ductal tissue was measured using fura-2, as described previously (13, 25, 26). In brief, a mixture was made of 60 μ L of 1 mM fura-2/AM in DMSO, 20 μ L of 25% cremophor and 240 μ L of FCS. The mixture was added to 5 mL HEPES solution and mixed well. The ductus preparation was then superfused with the solution equilibrated with 100% N₂ for 1–2 h at room temperature (22–24°C). After the dye loading, the ductus preparation was superfused for 30 min with dye-free solutions to wash out extracellular fluorescent dye. Excitation lights of 340 and 380 nm wavelengths were obtained alternatively using a xenon lamp (450-W), two monochrometers, and a chopper (Spex, Edison, NJ, U.S.A.). The emitted light passed through a 505nm band-pass filter and its intensity was measured by a photomultiplier (Hamamatsu Photonics R928, Hamamatsu, Japan). The intensity of fura-2 fluorescence at 505 nm during excitation at each wavelength was observed for 1 s at 5-s intervals. The interval between acquisition of the 340 nm excitation and that of the 380 nm excitation was 1 s. The fura-2 fluorescence ratio was then calculated as follows: ratio = (fluorescence at 340 nm excitation-autofluorescence at 340 nm excitation)/(fluorescence at 380 nm excitation-autofluorescence at 380 nm excitation).

Fura-2 and fura-2/AM were purchased from Dojin Chemical Co. (Kumamoto, Japan). DMSO was purchased from Eastman-Kodak. Fura-2 and fura-2/AM were dissolved in DMSO to 1 mM. Cremophor EL (Sigma Chemical Co.) was diluted to 25% with DMSO.

Statistical Analysis. Results were expressed as mean $\pm$ SE. One-way analysis of variance was used for multiple comparisons. Statistical significance of difference between group means was determined using Dunnett *t* test. Percent changes were compared using the nonparametric method (Wilcoxon's rank sum test) (27). The statistical significance of response to vasoconstrictive stimulations was analyzed using a paired *t* test and repeated measurements of analysis of variance. A difference was considered statistically significant when *P* was less than 0.05.

Table 1. Body weight of fetuses and size of ductus arteriosus

Age groups	Pre-treatment	<i>n</i>	Body weight (g)	Ductal length (mm)	Inner diameter (mm)	Outer diameter (mm)	Wall thickness (mm)
1. 19-day fetus	Control	24	2.1 $\pm$ 0.1	0.42 $\pm$ 0.04	0.13 $\pm$ 0.01	0.38 $\pm$ 0.01	0.25 $\pm$ 0.01
2. 19-day fetus	Vitamin A (once)	9	2.0 $\pm$ 0.2	0.47 $\pm$ 0.14	0.12 $\pm$ 0.02	0.39 $\pm$ 0.02	0.27 $\pm$ 0.01
3. 19-day fetus	Vitamin A (twice)	26	2.1 $\pm$ 0.1	0.48 $\pm$ 0.07	0.13 $\pm$ 0.01	0.38 $\pm$ 0.02	0.25 $\pm$ 0.01
4. 20-day fetus	Control	10	2.8 $\pm$ 0.2	0.49 $\pm$ 0.06	0.12 $\pm$ 0.02	0.42 $\pm$ 0.01	0.30 $\pm$ 0.01
5. 20-day fetus	Vitamin A (once)	6	2.8 $\pm$ 0.3	0.51 $\pm$ 0.03	0.12 $\pm$ 0.01	0.43 $\pm$ 0.01	0.31 $\pm$ 0.01
6. 20-day fetus	Vitamin A (twice)	11	2.9 $\pm$ 0.2	0.52 $\pm$ 0.02	0.12 $\pm$ 0.01	0.41 $\pm$ 0.01	0.29 $\pm$ 0.01
7. 21-day fetus	Control	5	4.2 $\pm$ 0.3	0.59 $\pm$ 0.05	0.18 $\pm$ 0.01	0.50 $\pm$ 0.06	0.32 $\pm$ 0.02
8. 21-day fetus	Vitamin A (once)	5	4.3 $\pm$ 0.6	0.60 $\pm$ 0.02	0.18 $\pm$ 0.03	0.51 $\pm$ 0.05	0.33 $\pm$ 0.02
9. 21-day fetus	Vitamin A (twice)	5	4.3 $\pm$ 0.7	0.60 $\pm$ 0.02	0.19 $\pm$ 0.01	0.53 $\pm$ 0.04	0.34 $\pm$ 0.01

Table 2. Force of contraction induced by 50 mM KCl

Age groups	Pre-treatment	n	Contractile force (mN/mm)	Contractile force (mN/mm ²)
1. 19-day fetus	Control	24	0.76 ± 0.06	15.7 ± 1.3
2. 19-day fetus	Vitamin A (once)	9	0.88 ± 0.06	17.1 ± 1.3
3. 19-day fetus	Vitamin A (twice)	26	0.84 ± 0.07	15.8 ± 1.6
4. 20-day fetus	Control	10	1.12 ± 0.08*	14.7 ± 1.0
5. 20-day fetus	Vitamin A (once)	6	1.27 ± 0.08*	16.4 ± 1.2
6. 20-day fetus	Vitamin A (twice)	11	1.15 ± 0.08*	15.4 ± 1.0
7. 21-day fetus	Control	5	1.47 ± 0.13†	16.0 ± 1.4
8. 21-day fetus	Vitamin A (once)	5	1.40 ± 0.12	14.7 ± 1.2
9. 21-day fetus	Vitamin A (twice)	5	1.61 ± 0.14†	17.2 ± 1.6

* Significantly different from the value in the 19-day fetus.

† Significantly different from the value in the 20-day fetus.

RESULTS

Body weight in the fetuses with vitamin A treatment was not significantly different from that in the control fetuses. Length, outer diameter, and inner diameter of the ductus isolated from the fetuses with vitamin A treatment were similar to those in the control fetuses (Table 1).

KCl-Induced Contraction. Membrane depolarization caused by high KCl (50 mM) induced ductal contraction in all age groups studied. The developed tension expressed as mN/mm increased with age (Table 2). The contractile force normalized by the cross-sectional area, however, did not change with age. In each age group, the KCl-induced contraction in the fetuses treated with vitamin A was not significantly different from that in the control group.

Maximal contraction was also induced by 120 mM KCl + 10 mM CaCl₂ in the fetuses at 19 and 21 d of gestation. Ductal contraction induced by 50 mM KCl was 83% to 85% of the maximal contraction induced by 120 mM KCl + 10 mM CaCl₂

in all age groups studied, and in each age group, the maximal contraction in the fetus treated with vitamin A twice was not significantly different from that in the control fetus.

O₂-Induced Contraction. The degree of O₂-induced contraction was expressed as a relative value of 50 mM KCl-induced contraction (Fig. 1). In the control fetus at 19 d of gestation, O₂ did not cause significant contraction. In the control fetus at 20 d of gestation, 30% and 95% O₂, induced significant contraction. In the control fetus at 21 d of gestation, 5% to 95% O₂ caused significant contraction.

In the fetus at 19 d of gestation, which was treated with vitamin A once or twice, 5% to 95% O₂ induced a small but significant contraction (Fig. 2).

In the fetus at 20 d of gestation, 30% and 95% O₂ caused significant contraction in both the control fetus and the vitamin A-treated fetus (Fig. 3). O₂ (5%) caused significant contraction only in the fetus treated with vitamin A twice.

In the fetus at 21 d of gestation, 5% to 95% O₂ caused significant contraction in both the control and vitamin A-treated groups (Fig. 4). In the fetus treated with vitamin A twice, contraction induced by 5% O₂ was significantly greater than in the control group.

Indomethacin-Induced Contraction. The effect of indomethacin was studied in the fetus at 19 d of gestation. Indo-

Figure 1. Graph showing the effect of O₂ on ductal contraction, expressed as a relative value of 50 mM KCl-induced contraction, in the control fetuses. In the fetus at 19 d of gestation, O₂ did not cause significant contraction. In the control fetus at 20 d of gestation, 30% and 95% O₂, but not 5% O₂, induced significant contraction. In the control fetus at 21 d of gestation, 5% to 95% O₂ caused significant contraction. *Significant contraction.

Figure 2. Graph showing the effect of ductal contraction in the fetus at 19 d of gestation. In the group treated with vitamin A once or twice, 5% to 95% O₂ induced a small but significant contraction. *Significant contraction.

Figure 3. Graph showing the effect of O_2 on the ductus in the fetus at 20 d of gestation. Thirty percent and 95% O_2 caused significant contraction in both the control fetus and the vitamin A-treated fetus, while 5% O_2 caused significant contraction only in the fetus treated with vitamin A twice. *Significantly different from the value in the control group.

Figure 4. Graph showing the effect of O_2 on ductal contraction in the fetus at 21 d of gestation. In all groups, 5% to 95% O_2 caused significant contraction. In the fetus treated with vitamin A twice, contraction induced by 5% O_2 was significantly greater than in the control group. *Significantly different from the value in the control group.

methacin did not cause significant contraction in the control fetus and the fetus treated with vitamin A once (Fig. 5). In the fetuses treated with vitamin A twice, indomethacin did cause significant contraction.

Noradrenaline-Induced Contraction. The effect of vitamin A treatment on the size of the intracellular Ca pool was estimated using noradrenaline in the fetuses at 19 and 20 d of gestation. Noradrenaline-induced contraction in the group

Figure 5. Graph showing the effect of indomethacin on ductal contraction in the fetus at 19 d of gestation. Indomethacin caused significant contraction only in the fetuses treated with vitamin A twice. *Significant contraction. Number in parenthesis indicates number of the experiments performed.

Figure 6. Graph showing the effect of noradrenaline on ductal contraction in the fetus at 19 and 20 d of gestation. Noradrenaline (10^{-5} M)-induced contraction was examined in Ca- and Na-free solutions. Noradrenaline-induced contraction in the group treated with vitamin A twice was similar to that in the control group. Number in parenthesis indicates number of the experiments performed.

treated with vitamin A twice was similar to that in the control group (Fig. 6).

[Ca]_i Measurements. The effect of O_2 on $[Ca]_i$ in the ductal tissue was studied in the fetus at 19 d of gestation. In the control fetus, 30% and 95% O_2 did not cause a significant increase in $[Ca]_i$. In the fetus treated with vitamin A twice, 30% and 95% O_2 caused significant increases in $[Ca]_i$ (Table 3).

The effect of noradrenaline on $[Ca]_i$ was studied in the fetus at 19 d of gestation (Table 4). The noradrenaline-induced increases in $[Ca]_i$ in the control fetus was similar to that in the fetus treated with vitamin A twice. High KCl (50 mM) increased $[Ca]_i$ similarly in the control fetus and in the fetus treated with vitamin A (Table 4).

The Role of the Endothelium. The role of the endothelium in the O_2 -induced contraction was studied in the ductus arteriosus from which the endothelium was removed in the fetuses at 19 and 20 d of gestation. In the control fetuses at 19 and 20 d

Table 3. Effect of O₂ on [Ca]_i

Groups	Pre-treatment	n	Baseline [Ca] _i (nM)	[Ca] _i at 30% O ₂ (nM)	Net increase by 30% O ₂ (nM)	[Ca] _i at 95% O ₂ (nM)Net	Net increase by 95% O ₂ (nM)	[Ca] _i at 50 nM KCl (nM)	Net increase by KCl (nM)
1. 19-day fetus	Control	5	45 ± 5	45 ± 5	0	45 ± 5	0	68 ± 4*	23 ± 2
2. 19-day fetus	Vitamin A (twice)	6	46 ± 12	55 ± 13*	9 ± 2†	59 ± 13*	13 ± 1†	68 ± 12*	22 ± 2

* Significant increase from baseline.

† Significantly different from the control value.

Table 4. Effect of noradrenaline on [Ca]_i

Groups	Pre-treatment	n	Baseline [Ca] _i (nM)	[Ca] _i at noradrenaline (nM)	Net increase (nM)	[Ca] _i at 50 mM KCl (nM)	Net increase (nM)
1. 19-day fetus	Control	4	45 ± 9	57 ± 10*	12 ± 1	73 ± 7*	28 ± 4
2. 19-day fetus	Vitamin A (twice)	4	42 ± 9	58 ± 8*	16 ± 3	73 ± 8*	28 ± 4

* Significant increase from baseline value.

Figure 7. Graph showing the role of the endothelium in the O₂-induced contraction in the fetus at 19 and 20 d of gestation. In the control fetuses at 19 and 20 d of gestation, removal of the endothelium did not alter the O₂-induced contraction. In the fetuses at 19 and 20 d of gestation treated with vitamin A twice, the O₂-induced contraction in the ductus from which the endothelium was removed was not different from that in the ductus in which the endothelium was intact.

of gestation, removal of the endothelium did not alter the O₂-induced contraction. In the fetuses at 19 and 20 d of gestation treated with vitamin A twice, the O₂-induced contraction in the ductus from which the endothelium was removed was not different from that in the ductus in which the endothelium was intact (Fig. 7).

DISCUSSION

The O₂-sensing mechanisms of the ductus arteriosus remain undetermined. Cocci *et al.* (1, 28–30) suggested that cytochrome P450 is an O₂ sensor and interaction of P450 with oxygen might increase endothelin production by the ductal smooth muscle cells, resulting in ductal contraction. Nakanishi *et al.* (13) hypothesized that O₂ might close the ATP-sensitive K channel. Tristani-Firouzi *et al.* (14), however, suggested that the voltage-dependent K channel was responsible for the O₂-induced membrane depolarization. O₂ may, either directly or

mediated by endothelin, cause membrane depolarization of the smooth muscle cells, increase Ca influx, induce Ca release from intracellular store sites, increase [Ca]_i, and finally cause ductal contraction.

In the control group in the present study, membrane depolarization, induced by high KCl, increased [Ca]_i and resulted in ductal contraction even in the preterm fetus at 19 d of gestation, in which no significant O₂-induced contraction was observed. Furthermore, noradrenaline caused significant increases in [Ca]_i and ductal contraction in this age group, suggesting that the intracellular Ca pool is present even in the preterm fetus at 19 d of gestation. These data suggest that the contractile system of the DA, from membrane depolarization to increases in [Ca]_i and muscle contraction, is functioning even in the preterm fetus and only O₂-sensing mechanisms are underdeveloped. The data in Fig. 1–4 suggest that the sensitivity to O₂ increases with development and vitamin A treatment may accelerate the development of O₂-sensitivity.

Kim *et al.* (11) have shown in near-term fetal rabbits that the expression of the adult-type isoform of smooth muscle myosin heavy chain in the DA was more prominent than in other arteries. They suggested that vascular smooth muscle cells in the DA might be more differentiated than in the other arteries. Colbert *et al.* (12) showed that endogenous retinoic acid signaling colocalized with advanced expression of the adult type of smooth muscle myosin heavy chain isoform during development of the DA. In the present study, vitamin A treatment did not change the contractile force obtained with 50 mM KCl in each age group (Table 2). The maximal contractile force obtained with 120 mM KCl and 10 mM CaCl₂ was also similar in the control fetus and the fetus treated with vitamin A. Although changes in myosin isozymes may alter myosin ATPase activity and the rate of actin-myosin cross-bridge cycling (31), the maximal contractile force is determined by the amount of contractile proteins per unit of ductal tissue. Therefore, it is unlikely that vitamin A induced quantitative changes in contractile proteins. In the present study, the effect of vitamin A treatment on myosin isozymes was not evaluated and it remained possible that vitamin A induced qualitative changes in contractile proteins. Although the developed tension normalized by the length of the vessel increased with age,

absolute values of contractile force, normalized by the cross-sectional area of the preparation, did not change with age (Table 2). This suggests that the amount of contractile proteins per unit cross-sectional area in the preterm fetus is similar to that in the near-term fetus.

One may argue that vitamin A treatment has altered the sensitivity of contractile proteins to Ca and increased O₂-induced contraction in the fetus treated with vitamin A, even though changes in [Ca]_i were similar in the control fetus and in the fetus treated with vitamin A. In the fetus at 19 d of gestation, however, ductal contraction was always associated with increases in [Ca]_i and both O₂-induced ductal contraction and increases in [Ca]_i were observed only in the fetus treated with vitamin A. Furthermore, the degree of contraction obtained by high KCl and increases in [Ca]_i were similar in the control fetus and in the fetus treated with vitamin A. Therefore, although the sensitivity of contractile proteins to Ca was not directly measured in the present study, it is unlikely that the increased sensitivity to O₂ in the fetus treated with vitamin A was due to qualitative changes in contractile proteins induced by vitamin A treatment.

In the fetuses at 20 and 21 d of gestation, treatment with vitamin A did not further alter the degree of contraction induced by 30% and 95% O₂, most likely because the degree of contraction induced by 30% to 95% O₂ was close to the maximal contraction that can be achieved by the ductal smooth muscle. In support of this hypothesis, the addition of high KCl (120 mM) and high Ca (10 mM) to the oxygenated solution did not further increase the degree of contraction induced by 95% O₂ in these age groups. In the fetus at 19 d of gestation, which was treated with vitamin A once or twice, 5% O₂ induced a small but significant contraction (Fig. 3). The reason why ductal contraction did not further increase with the increase in O₂ concentration (to 30% to 95%) is not clear, but higher O₂ concentration might not have further activated processes inducing membrane depolarization. Measurement of the intracellular Ca pool using norepinephrine was performed to determine whether vitamin A treatment alters its size. It must be noted that norepinephrine stimulates not only alpha-adrenergic receptors, but also beta-receptors and increases Ca influx *via* Ca channels (15–17). To eliminate the Ca influx *via* the Ca channel, the effect of norepinephrine was examined in the absence of extracellular Ca (16). The increase in [Ca]_i caused by norepinephrine in the fetus treated with vitamin A was similar to that in the control fetus, suggesting that the size of the Ca pool was similar in the two groups.

In the present study, pretreatment with vitamin A also augmented indomethacin-induced ductal contraction (Fig. 7). This finding is in agreement with the *in situ* morphometric data of Momma *et al.* (9). It is likely that in the control fetuses at 19 d of gestation, the vasoconstrictive effect of 5% O₂ was not significant and, therefore, inhibition of prostaglandin E₂ production by indomethacin did not cause any ductal contraction. In the fetuses treated with vitamin A, however, 5% O₂ had significant vasoconstrictive effect. Thus, the augmented indomethacin-induced contraction in the fetus treated with vitamin A may be, at least in part, due to increased sensitivity to O₂ in this group. However, since prostaglandins were not measured

directly in the present study, the effect of vitamin A treatment on prostaglandin pathways in the DA remains unclear. Since the location of the O₂-sensing mechanism is unknown (4), we performed additional experiments in the endothelium-denuded preparation. The fact that O₂-induced contraction was observed in the absence of the endothelium in the control fetus at 20 d of gestation suggests that an O₂-sensing mechanism does not exist in the endothelium. In the fetuses at 19 and 20 d of gestation treated twice with vitamin A, the O₂-induced contraction in the ductus from which the endothelium was removed was not different from that in the ductus in which the endothelium was intact. These data may suggest that vitamin A treatment enhances development of an O₂-sensing mechanism, which is present in the vascular smooth muscle cells.

Although precise mechanisms whereby the ductus senses O₂ remain unknown, the cytochrome P450-endothelin pathway (30) and K channels (13, 14) have been implicated. O₂ may alter the redox status of the cell and K channels may be redox-modulated (32, 33). The regulation of K channels by the redox state may differ at different stages of development. Furthermore, many enzymes and ion channels may be involved in an O₂-sensing mechanism. It has been shown that retinoic acid, a derivative of vitamin A, can induce a number of genes involved in regulation of smooth muscle differentiation (34, 35). Several investigators have reported that vitamin A or retinoic acid accelerated lung development in the fetal and neonatal rat (36, 37). Likewise, it is possible that vitamin A accelerates development of an O₂-sensing mechanism in the DA. The effect of vitamin A on the development of putative O₂ sensors and effectors, such as the cytochrome P450-endothelin pathway, and/or K channels in the fetal DA has not yet been studied.

Direct extrapolation of the present data to the clinical situation is not easy. Firstly, the degree of involvement of retinoic acid in the maturation of the human DA is not clear. Secondly, the relative importance of O₂ sensors and effectors for ductal closure in the rat may be different from that in the human. Nevertheless, we think that administration of vitamin A to the pregnant woman may accelerate the maturation of the fetal DA and its closure after birth even in the premature baby.

In conclusions, the present study suggests that 1) in the premature fetus, the contractile system, including membrane depolarization, [Ca]_i increases and Ca activation of contractile proteins, is already functioning, but an O₂-sensing mechanism is underdeveloped, 2) administration of vitamin A accelerates development of the O₂-sensing mechanism of the ductus arteriosus.

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