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An EP4 receptor agonist prevents indomethacin-induced closure of rat ductus arteriosus in vivo

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EP4 agonist prevents indomethacin-induced closure of ductus arteriosus *in vivo*

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Abstract

Indomethacin is known to have a strong tocolytic effect by suppressing uterine contraction which is mediated by prostaglandins and their cognate receptors. However, indomethacin also induces in utero closure of fetal ductus arteriosus (DA) when administered at late pregnancy, leading to serious neonatal consequences. The patency of fetal DA is maintained via prostaglandin E/EP4 which is overcome after birth by constrictor mechanism to allow rapid DA closure in response to an increase in oxygen tension in circulating blood. Using rats, we tested the effect of an EP4 agonist, ONO-AE1-437, as a relaxant of DA in vitro and the effect of ONO-4819, that is a pro drug of ONO-AE1-437, as a complimental DA dilator during indomethacin treatment in vivo.

In vitro, ONO-AE1-437 showed a potent dilatory effect against O2- and indomethacin-induced DA contractions (EC50 was less than 1 nM in both cases). In vivo, the effect of ONO-4819 on indomethacin-induced in utero closure of the rat DA was evaluated measuring a ratio of the diameters of DA and pulmonary artery (PA). Rat dams at 21st day of gestation were given indomethacin (10 mg/kg, p.o.) and/or ONO-4819 (0.3 μg/kg/h, s.c.) and 4 hours later pups were delivered through Caesarean section to evaluate the DA/PA ratio. The pups without indomethacin had DA/PA ratio 0.9 ± 0.05, while the pups with indomethacin had the ratio 0.2 ± 0.03 at birth. When ONO-4819 was co-administered, the ratio recovered significantly to 0.7 ± 0.06.

These results suggest that ONO-4819 as a pro drug of EP4 agonist may be a useful therapeutic aid in indomethacin therapy for tocolysis.

Introduction

In the late gestational period, prostaglandins (PGs) play key roles not only for mothers but also for babies. In the former, PGs trigger uterine contraction to complete parturition (Sugimoto et al., 1997) . In the latter, PGs participate in the transitional regulation of neonatal gas exchange and circulation from placental to terrestrial in which the closure of ductus arteriosus (DA) is a critical event (Smith, 1998) . DA functions in utero as a shunt vessel which connects the pulmonary artery and systemic circulation, bypassing the unexpanded lungs. After birth, the DA undergoes rapid closure to terminate fetal circulation as the pulmonary circulation is established. The patency of DA in utero is maintained by dilators, in which PGE2 is a major mediator probably via a cognate receptor EP4 (Bouayad et al., 2001; Leonhardt et al., 2003; Nguyen et al., 1997; Segi et al., 1998; Smith et al., 1994) . Indomethacin is a nonsteroidal anti-inflammatory drug which inhibits cyclooxygenase (COX) to suppress PG synthesis. In the setting of preterm labor, there is a controversy whether indomethacin is a useful therapeutic choice because of neonatal morbid events like respiratory distress syndrome, necrotizing enterocolitis and intraventricular hemorrhage, in spite of its tocolytic effect (Macones et al., 2001) . One of the established phenomena during indomethacin tocolysis is DA closure in utero which would obviously impair neonatal pulmonary function by forcing a large amount of blood flow to pass through unexpanded fetal lung (Moise et al., 1988; Norton et al., 1993) . In this study, we have tested a specific EP4 agonist as a compliment of DA dilator during indomethacin tocolysis in a near-term-pregnant rat model.

Methods

Animals----- We followed the guidelines produced by the Ethical Committee of our Institutes, which conform with the American National Institutes of Health's guide for the Care and Use of Laboratory animals. Female Wistar rats, 10 to 12 weeks at the time of mating, were housed individually in breeding cages with free access to the usual diet and water. The day on which a vaginal plug was found was designated as day 0 of gestation. On the evening of the day 20 of gestation, an osmotic pump (Micro-Osmotic Pump, model 1003 D, ALZET) containing either saline alone or saline plus ONO-4819 was implanted subcutaneously. On the morning of the day 21 of gestation, either 2 ml of 1 % methyl cellulose alone or 2 ml of 1 % methyl cellulose plus indomethacin (10 mg/kg body weight) was administered through an orogastric tube. The pups were obtained by Caesarean delivery from the dams killed by decapitation.

In vitro study ----- Immediately after delivery, pups were decapitated and soaked in ice-cold Krebs-Henseleit solution (composition in mM: NaCl 112, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2, $NaHCO_3$ 25, NaH_2PO_4 1.2, and glucose 11.5) gassed with 5 % CO_2/95 % N_2. The thorax was opened to remove the heart and major vessels en bloc under a dissecting microscope. Then, DA was dissected out to obtain a DA ring. These operations were done in ice-cold Krebs-Henseleit solution gassed with 5 % CO_2/95 % N_2. The specimen was mounted in an assay chamber of a Micro Easy Magnus UC-5A (UFER Medical Instrument, Kyoto, Japan). The chamber was filled with Krebs-Henseleit solution which was continuously gassed with 5 % CO_2/95 % N_2 at 37 °C. A resting tension of 1 mN was initially applied and the responses were recorded isometrically through force displacement transducers (T7-8-240, Orientec, Tokyo, Japan). In preliminary experiments, we confirmed that the resting tension is optimal because 60 mM KCl produced a maximum contraction (data not shown). All preparations were equilibrated for 60 min, followed by a test contraction with 60 mM KCl. Samples were then exposed either to 5 % CO_2/95 % O_2 or to 10 μM indomethacin under an atmosphere of 5 % CO_2/95 % N_2.

When the contraction of DA reached a plateau, ONO-AE1-437 was applied in the chamber in a cumulative manner. At the end of each experiment, samples were dilated by the addition of 0.1 mM papaverine to obtain the zero tension value, in relation to which all contractile responses were normalized.

In vivo study ----- After delivery, pups were rinsed quickly in a warm water bath and were placed in a humidified chamber at 37 °C for 10 min. The living pups were either fixed using whole-body freezing method (Hornblad et al., 1967) or maintained in the chamber for 3 h after birth, then fixed using whole-body freezing method for evaluation of the DA closure.

The thorax of the frozen pup was trimmed and sectioned in a frontal plane on a cryomicrotome. The inner diameters of DA and of common PA were measured with a calibrated microscope. The ratio of the DA diameter at its narrowest part to the PA diameter was employed for evaluation.

Data analysis ----- Data are presented as means ± s. e. mean. Concentration-response data for ONO-AE1-437-induced relaxations were analyzed with a program, Prism. Statistical significance was tested in one-way ANOVA, Fisher's exact probability test or Student's t test.

Materials ----- The following chemicals were used: indomethacin and methyl cellulose were obtained from Wako Chemicals (Osaka, Japan). ONO-AE1-437 and ONO-4819 (a cellulose dextran conjugate of a pro drug of ONO-AE1-437) were provided by ONO Pharmaceutical Company (Osaka, Japan) and both of them were dissolved initially in ethanol then diluted into saline.

Results

DA contraction in vitro and the effect of EP4 agonist ----- Representative recordings for O_2- and indomethacin-induced DA contraction is shown in Fig. 1A and B,

respectively. ONO-AE1-437 reversed the DA contractions in a concentration-dependent manner. The pEC50 values of the relaxation responses of the DA are 9.4 ± 0.2 and 9.2 ± 0.1 for O_2- and indomethacin-induced DA contractions, respectively (Fig. 2). These values are in good agreement with the pKd values reported in the binding experiments (Yoshida et al., 2002) .

DA contraction in vivo and the effect of EP4 agonist ----- Next, we tested the effect of administration of ONO-4819, a pro drug of ONO-AE1-437 in vivo, to the dams against indomethacin-induced DA contraction in utero as well as physiological DA contraction after birth in the neonate.

No parturition was observed in the dams which received ONO-4819. This is consistent with the observation that ONO-AE1-437 did not induce any contraction of uterine strips of rat (data not shown) or pig (Cao et al., 2002) in vitro. Representative photographs of DA section were shown in Fig. 3 and the results were summarized in Fig. 4. Administration of indomethacin (10 mg/kg body weight) induced DA contraction in utero (Figs. 3B and 4) which was reversed by co-administration of ONO-4819 (0.3 μg/kg body weight/h) as shown in Figs. 3C and 4. In addition, administration of large dose of ONO-4819 (3 μg/kg body weight/h) did not affect DA closure after birth (Figs. 3E and 4).

Discussion

In the late gestational period, PGs are one of the key players to initiate and complete parturition (Sugimoto et al., 1997) and to regulate circulatory switching from a fetus to a neonate (Smith, 1998) . During labor, PGs released from fetal membranes increase the intensity of uterine contraction and consequently, exogenous PGs have been used effectively to induce uterine contraction in termination of pregnancy. On the other hand, PG, mainly PGE2, participates also in the control of fetus-to-neonate circulatory transition along with the initiation of pulmonary respiration, in which the closure of DA is the biggest event (Smith, 1998) . DA is kept open during pregnancy by circulating PGE2 probably via a cognate

receptor, EP4 (Bouayad et al., 2001; Leonhardt et al., 2003; Nguyen et al., 1997; Segi et al., 1998; Smith et al., 1994) , and undergoes a rapid closure in response to both a fall of PGE2 concentration by a placental segregation (Smith, 1998) and an action of constrictors induced by an increase in oxygen tension which may involve endothelin secretion and ETA stimulation (Coceani et al., 1992; Momma et al., 2003; Taniguchi et al., 2001) . In the cases of heart anomaly which depends on the patency of DA, PGE2 has been used to keep DA open and a EP4 agonist has been shown to prevent physiological DA closure after birth (Loftin et al., 2002) .

Cyclooxygenase (COX) inhibitors that block PG synthesis have been successfully used in the treatment of preterm labor (Niebyl et al., 1986) . However, the use of indomethacin has been limited because of concern about adverse effects on the fetus including constriction of the fetal DA in utero which may lead to serious neonatal complications (Moise et al., 1988; Norton et al., 1993) . As COX-2 has been found to be responsible for PG release from the fetal membranes during labor (Slater et al., 1995) , COX-2 inhibitors were predicted to prevent labor and avoid the adverse effects of nonselective COX inhibitors. However, it has been shown that the use of COX-2 inhibitors are not necessarily free of adverse effects in animal models (Clyman et al., 1999; Coceani et al., 2001; Kajino et al., 2002; Sakai et al., 2001; Takahashi et al., 2001) .

We investigated the effect of a EP4 specific agonist, ONO-AE1-437, and its prodrug, ONO-4819, against indomethacin-induced contraction of DA in vitro and vivo, respectively. As shown in Figs. 1 and 2, ONO-AE1-437 relaxed indomethacin- as well as O2-induced DA contraction in a concentration-dependent manner. ONO-4819, when co-administrated with indomethacin to dams, prevented indomethacin-induced DA contraction in utero (Figs. 3c and 4) but did not affect physiological closure of DA after birth (Figs. 3e and 4). There are two possibilities to explain this discrepancy of DA dilating effects of ONO-4819 before and after birth. One is a short half life of ONO-4819 in the body (approximately 10 - 20 min, (Kabashima et al., 2001) and data not shown) which prompts the clearance of the agonist from

neonates after birth, preventing the agonist to affect the physiological process of post natal closure of DA. In the physiological condition, fetal PGE2 is supplied by placenta to maintain fetal circulation and therefore the concentration of PGE2 in neonatal blood decreases rapidly after birth to facilitate postnatal DA closure (Smith, 1998) . The situation of the agonist which is in a capsule implanted in dams is similar. Another is a postnatal rearrangement of EPs in DA. It is reported that EP subtypes in DA undergo postnatal rearrangement in which EP4 becomes minor population after birth (Bhattacharya et al., 1999; Bouayad et al., 2001) , supporting our results of postnatal incompetence of ONO-4819.

EP4 functions in many physiological process (Narumiya et al., 1999) and its specific agonist has been shown to have several therapeutic potentials (Kabashima et al., 2001; Kasai et al., 2001; Yoshida et al., 2002) . Although one of the predicted systemic adverse effects is hypotension (Narumiya et al., 1999) , blood pressure in the rat was not affected at all in the doses of ONO-4819 employed in this report (data not shown). COX inhibitor-induced premature closure of DA in utero not only causes deleterious pulmonary stress which may underlie neonatal pulmonary dysfunction (Moise et al., 1988; Norton et al., 1993) but also lead to incomplete but irreversible termination of DA remodelling which may result in persistent patent DA (Clyman et al., 2001) . Administration of EP4 agonist in addition to COX inhibitor would prevent adverse reactions on fetal DA without encumbering tocolytic effects of COX inhibitor.

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Legends to the figures

Figure 1 Relaxation effects of ONO-AE1-437 on DA in vitro. Representative recordings of ONO-AE1-437-induced relaxation of DA which is pre-contracted by O_2 (a) or indomethacin 10 μM (b) are shown. The bubbling gas was switched from N_2 to O_2 as indicated. After DA contraction becomes stable, ONO-AE1-437 is added in a chamber cumulatively from 0.01 nM to 100 nM. At the end of experiments, relaxation by 0.1 mM papaverine were tested. Scales are same in all charts as shown at bottom right.

Figure 2 Concentration-dependent relaxation of DA in vitro by ONO-AE1-437 administration. Relaxation of DA which was pre-contracted by O_2 (triangle) or indomethacin 10 μM (square) was studied with cumulative addition of ONO-AE1-437 (0.01 to 100 nM). Data were obtained from 2 independent experiments.

Figure 3 Morphology of DA in vivo. Photographs of representative cases are shown. Rat pups born from dams treated with s.c. saline alone (a, d) or s.c. saline plus p.o. indomethacin 10 mg/kg body weight (b) and s.c. ONO-4819 0.3 μg/kg body weight/h plus p.o. indomethacin (c) or s.c. ONO-4819 3 μg/kg body weight/h plus p.o. indomethacin (e) were examined for DA closure at 0 h (a, b, c) or 3 h after birth as described in Methods. Ascending aorta (Ao) and DA (arrow heads) are indicated.

Figure 4 Prevention of indomethacin-induced DA closure at birth in vivo. Rat dams were infused with saline or ONO-4819 (0.03, 0.3 or 3 μg/kg body weight/h) using subcutaneously implanted pumps and were administered indomethacin 10 mg/kg body weight at the morning of the day 21 of gestation. Four hour later, pups were delivered through Caesarean section to examine DA closure (DA/PA ratio) just after birth or 3 h after birth as described in Methods. The administration

of indomethacin resulted in DA closure (reduction of DA/PA ratio) and the treatment with ONO-4819 prevented the indomethacin-induced DA closure at birth but did not affect DA closure at 3 h after birth. Both an asterisk and double asterisks show statistical significance ($p < 0.01$). Data were obtained from 3 independent experiments.

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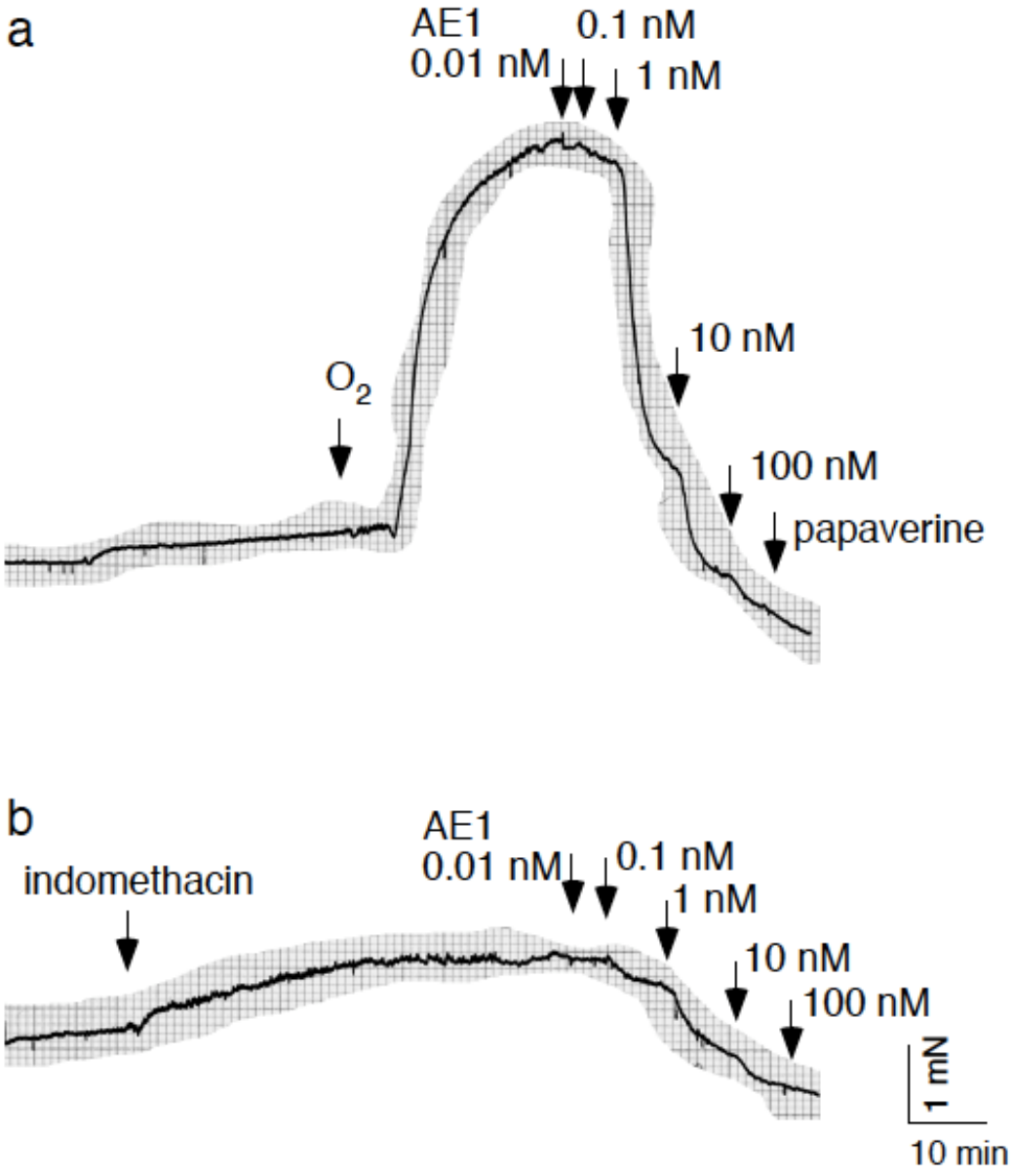
Table 1. EP4 agonist induced no increase in rat uterine activity in vivo.

	-10 – 0 min	20 – 30 min	50 – 60 min
saline	100	96.8 ± 25.3	103.2 ± 6.9
ONO-4819			
0.3 μg/kg/h (5 ng/kg/min)	100	90.2 ± 14.4	104.4 ± 13.9
3 μg/kg/h (50 ng/kg/min)	100	90.1 ± 12.2	100.6 ± 13.1
oxytocin (20 mU/kg/min)	100	184.6 ± 14.5 *	208.8 ± 27.8 *
$PGF_{2\alpha}$ (20 μg/kg/min)	100	168.4 ± 14.8 *	179.1 ± 14.2 *

Uterine activity was monitored with a balloon catheter placed in the right uterine horn and was calculated and expressed as % of Montevideo units, as described in Methods. The data are means ± s. e. mean from four dams. An asterisk indicates significant ($p < 0.01$) increase in uterine activity compared to saline.

Fig. 1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O2
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Fig. 2

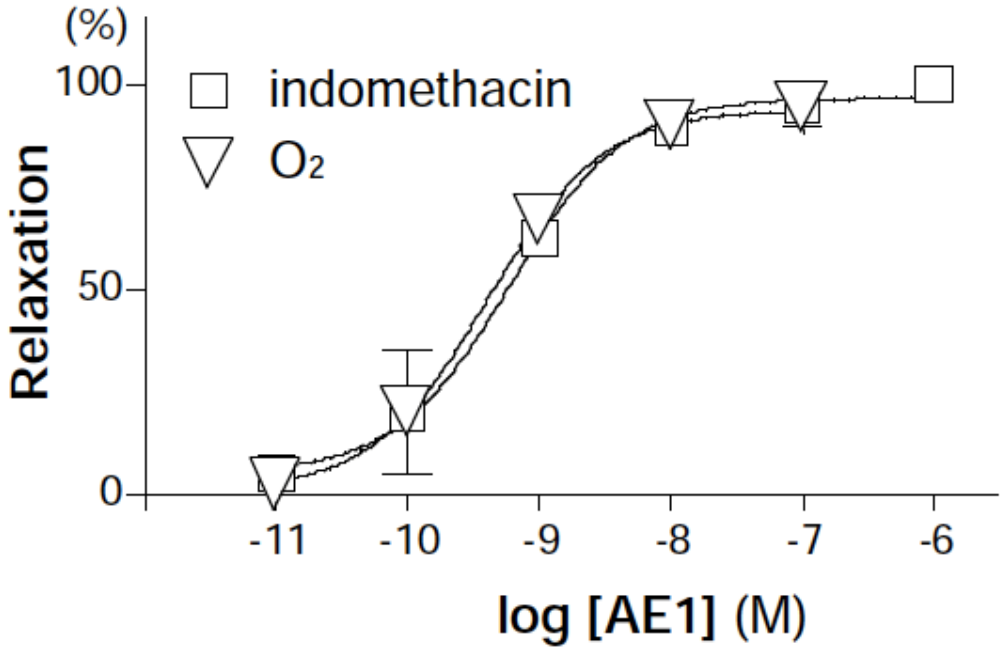

Fig. 3

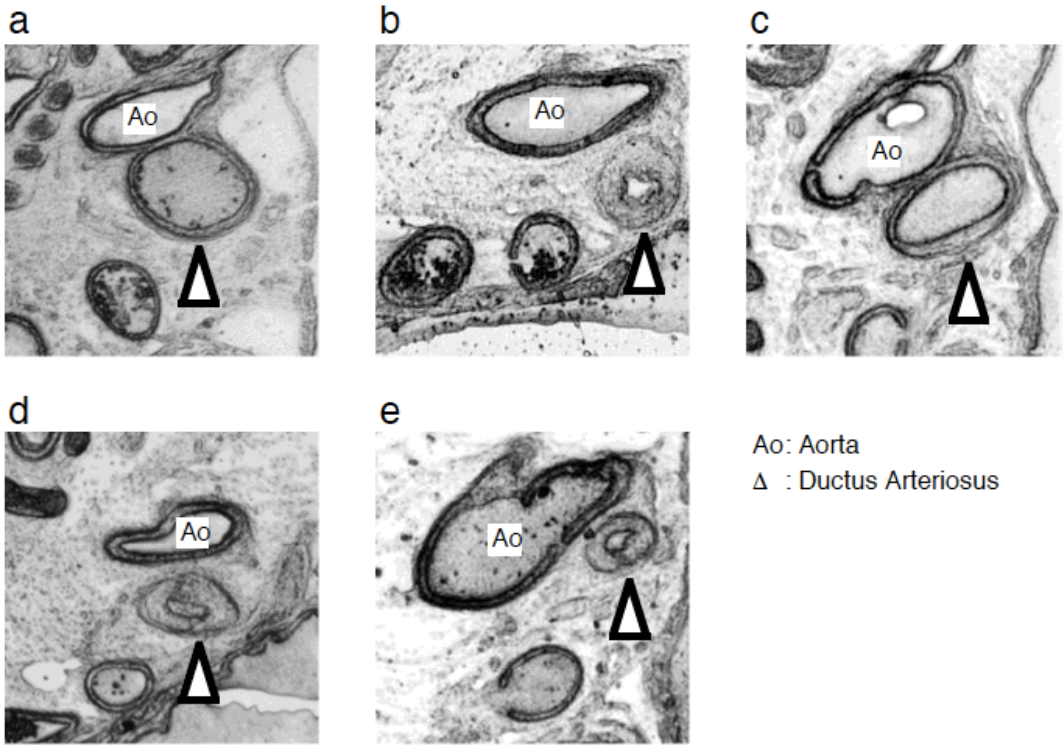

Fig. 4