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An EP4 Receptor Agonist Prevents Indomethacin-Induced Closure of Rat Ductus Arteriosus *In Vivo*

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ABSTRACT

Indomethacin exerts a strong tocolytic effect by suppressing uterine contractions mediated by prostaglandins. However, indomethacin also induces *in utero* closure of fetal ductus arteriosus (DA), leading to serious neonatal consequences. Using rats, we tested the effect of an agonist for a subtype of prostaglandin E2 receptor (EP4), ONO-AE1-437 and its prodrug ONO-4819, as a DA dilator during indomethacin treatment. *In vitro*, ONO-AE1-437 exhibited a potent dilatory effect on DA against O_2- and indomethacin-induced contractions in a concentration-dependent manner. *In vivo*, rat dams were given indomethacin (10 mg/kg, p.o.) alone or with ONO-4819 (0.3 μg/kg/h, s.c.) on d 21 of gestation and pups were delivered 4 h later through cesarean section to evaluate the ratio of diameter of DA to that of pulmonary artery. Pups from dams with no drug had DA/PA ratio of 0.9 ± 0.05, whereas those from dams with indomethacin alone had a decreased ratio of 0.2 ± 0.03. When ONO-4819 was co-administered to the dams, the ratio recovered significantly to 0.7 ± 0.06. The administration of ONO-4819 to the dams did not induce any increase in the uterine activity. These results suggest that administration of an EP4 agonist in addition to indomethacin might prevent adverse reactions of indomethacin on fetal DA without restricting its tocolytic effects. **(*Pediatr Res* 56: 586–590, 2004)**

Abbreviations

COX, cyclooxygenase (EC 1.14.99.1)
DA, ductus arteriosus
NSAID, nonsteroidal antiinflammatory drugs
PA, pulmonary artery
PG, prostaglandin

In the late gestational period, PG play key roles in the physiologic processes leading to labor. In the mother, PG are key players in the initiation and progression of parturition (1,2), whereas in the fetus they participate in the regulation of circulatory change at birth. In this regulation, the closure of the DA is a critical event (3). DA functions *in utero* as a shunt vessel that connects the PA and the systemic circulation, bypassing the unexpanded fetal lungs. After birth, the DA closes rapidly as pulmonary circulation is established. The fetal DA is normally maintained patent by dilators, of which PGE_2 is a major mediator that is supplied mainly by the placenta (3). At birth, the increase in oxygen tension along with the drop in PGE_2 level in neonatal circulation, which follow initiation of pulmonary respiration and placental segregation, respectively, result in postnatal closure of DA (3). Four subtypes of PGE_2 receptor have been identified, designated EP1, EP2, EP3, and EP4 (4). Although the distribution of PG receptors shows variation among species, EP4 has been shown to be a primary receptor subtype of PGE_2 at fetal DA in several mammals (5–7). In the case of humans, data are available only at neonatal DA in which EP4 is also a major receptor subtype of PGE_2 (8). Mice lacking EP4 gene died of patent DA soon after birth (9,10), implying an additional function of EP4 as a sensor of circulating PGE_2 to prepare DA closure after birth.

Indomethacin is an NSAID that suppresses prostanoid synthesis by inhibiting COX. In the context of preterm labor, there is some controversy as to whether NSAID are a useful therapeutic choice, despite their tocolytic effect, because morbid neonatal events such as respiratory distress syndrome, necrotizing enterocolitis, and intraventricular hemorrhage may follow their administration (11). One of the established phenomena during NSAID-induced tocolysis is DA closure *in utero*; this impairs the pulmonary function after birth by forcing a large amount of blood flow through unexpanded fetal lung

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(12–14). In this study, we have tested a selective EP4 agonist as a DA dilator during indomethacin tocolysis using near-term pregnant rats.

METHODS

Animals. We followed the guidelines produced by the ethical committee of our institutes, which conform with the U.S. National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Female Wistar rats, 10–12 wk old, were mated individually under free access to the usual diet and water. The day on which a vaginal plug was found was designated as d 0 of gestation.

In vitro study. Immediately after full-term cesarean 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 excise the heart and major vessels *en bloc* under a dissecting microscope. Then, DA was dissected out to obtain a DA ring using fine tweezers. These operations were done in ice-cold Krebs-Henseleit solution gassed with 5% CO_2/95% N_2. The specimen was carefully mounted between two stainless steel wires (0.1 mm diameter) in an assay chamber of a Micro Easy Magnus UC-5A (UFER Medical Instrument, Kyoto, Japan), basically as described previously (15,16). 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 had 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 added 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 vivo study. At 1600 h on d 20 of gestation, an osmotic pump (ALZET Micro-Osmotic Pump, model 1003 D, DURECT Corp., Cupertino, CA) containing either saline alone or saline plus ONO-4819 was implanted subcutaneously in the dams. At 0900 h on d 21, 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. After pups were obtained by cesarean delivery, they were rinsed quickly in a warm water bath and placed in a humidified chamber at 37°C for 10 min. The living pups were frozen either immediately or 3 h after birth using whole-body freezing method (17). The frozen thorax was sliced in 10-μm thicknesses on a cryomicrotome in the frontal plane, and the inner diameters of the common PA and the DA were measured every 10 slices in 8–15 planes. The narrowest diameter of each vessel was used to determine the DA/PA ratio.

Uterine activity. On d 20 of gestation, a balloon connected to a polyethylene catheter was inserted through a laparotomy into the right uterine horn of the dams after removing one fetus under urethane anesthesia (1.2 g/kg body weight, s.c.). The intrauterine pressure was recorded with a pressure transducer (MPU-0.5A, Nihon Kohden, Tokyo, Japan). After the uterine motility became stabilized, ONO-4819 in saline was administered through a catheter placed in the right internal jugular vein using a syringe pump. The uterine activity in Montevideo units (18) was assessed every 10 min. The values were expressed as percentages, taking the unit value in the 10-min period just before the start of the drug infusion as 100%.

Data analysis. Values were presented as means ± SEM. Statistical significance was tested in a one-way ANOVA and Fisher's exact probability test, and a p value <0.01 was considered to be significant. Concentration-response data for ONO-AE1-437–induced relaxations were analyzed with the Prism computer program (GraphPad Software, San Diego, CA).

Materials. Indomethacin, methyl cellulose, papaverine, urethane, oxytocin, and $PGF_{2\alpha}$ were obtained from Wako Chemicals (Osaka, Japan). Two EP4 agonists, ONO-AE1-437 and ONO-4819 (19), a prodrug of ONO-AE1-437, were provided by ONO Pharmaceutical Company (Osaka, Japan); both were dissolved initially in ethanol then diluted in saline.

RESULTS

DA contraction in vitro and the effect of EP4 agonist. Representative recordings for O_2- and indomethacin-induced DA contraction are shown in Figure 1, *a* and *b*, respectively. The DA constriction by indomethacin is slower and less prominent than that by O_2. This result is in agreement with the previous reports (20,21), supporting the notion that circulating PGE_2 rather than locally released PGE_2 works dominantly as a DA dilator. ONO-AE1-437 reversed both DA contractions in a concentration-dependent manner. The pEC_{50} 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 are in good agreement with the affinity values reported in the binding experiments (19).

DA contraction in vivo and the effect of EP4 agonist. Next, we tested the effect of ONO-4819 *in vivo*, on indomethacin-induced DA contraction *in utero* and on neonatal DA closure after birth.

Representative photographs of DA section are shown in Figure 3 and the results are summarized in Figure 4. Administration of indomethacin (10 mg/kg body weight) induced DA contraction *in utero* (DA/PA ratio was 0.2 ± 0.03 with indomethacin and 0.9 ± 0.05 without), which was reversed significantly by co-administration of ONO-4819 (DA/PA ratio was 0.7 ± 0.06 and 0.8 ± 0.08, for 0.3 and 3 μg/kg body weight/h, respectively). In addition, administration of a large dose of ONO-4819 (3 μg/kg body weight/h) did not affect DA closure after birth.

Uterine activity in vivo and the effect of EP4 agonist. Finally, we tested the effect of ONO-4819 on uterine activity

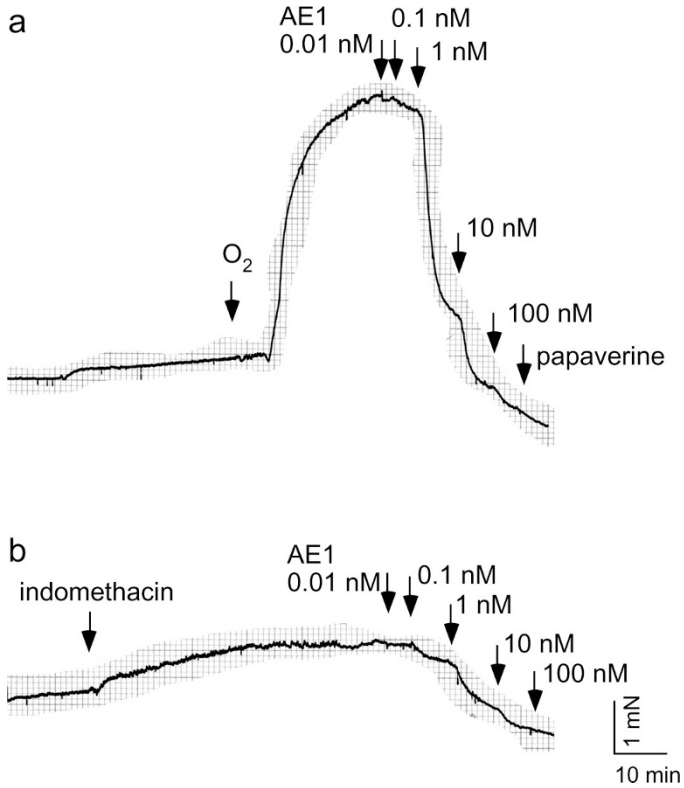

Figure 1. Relaxation effects of ONO-AE1-437 on DA *in vitro*. Representative recordings of ONO-AE1-437–induced relaxation of DA precontracted by O_2 (*a*) or indomethacin 10 μM (*b*) are shown. The bubbling gas was switched from N_2 to O_2 as indicated. After DA contraction became stable, ONO-AE1-437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

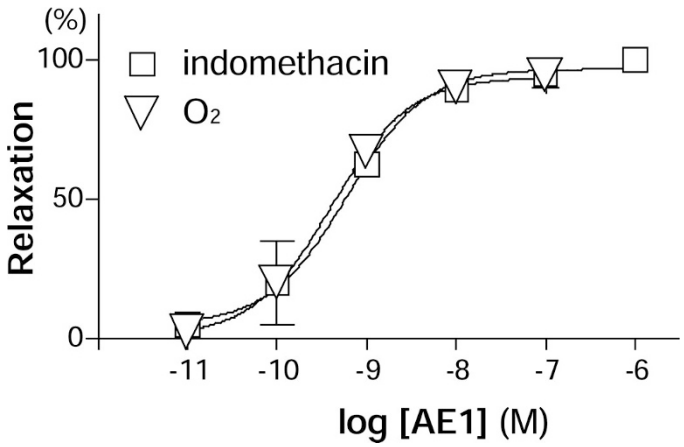

Figure 2. Concentration-dependent relaxation of DA *in vitro* by ONO-AE1-437 administration. Relaxation of DA precontracted by O_2 (*triangle*) or by indomethacin 10 μM (*square*) was studied with cumulative addition of ONO-AE1-437 (0.01–100 nM). In each case, data were obtained from two independent experiments.

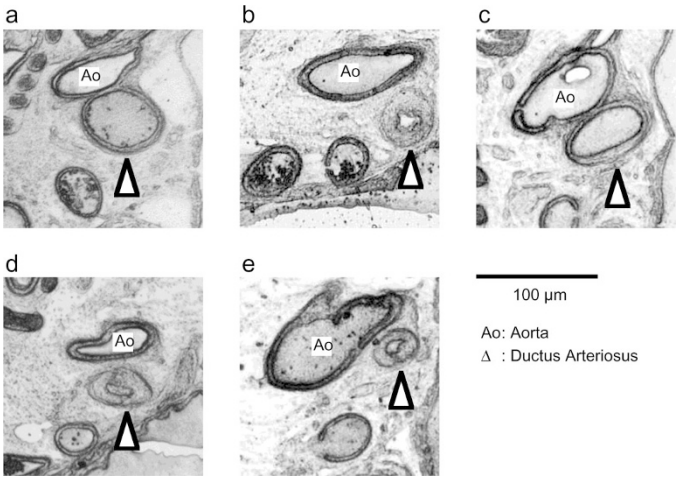

Figure 3. Morphology of DA *in vivo*. Photographs of representative cases are shown. Rat pups were born from dams that were treated with s.c. saline alone (*a, d*), s.c. saline plus p.o. indomethacin 10 mg/kg body weight (*b*), s.c. ONO-4819 0.3 μg/kg body weight/h plus p.o. indomethacin (*c*), or s.c.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

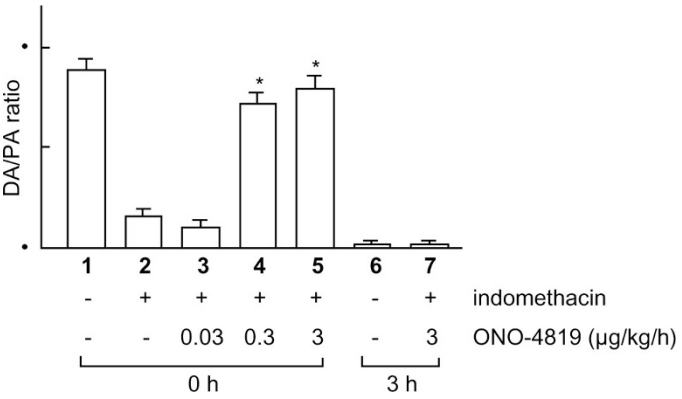

Figure 4. Prevention of indomethacin-induced DA closure at birth *in vivo*. Rat dams were infused with saline or ONO-4819 (0.03, 0.3, or 3 μg/kg body weight/h) using s.c. implanted pumps and were administered indomethacin 10 mg/kg body weight on the morning of d 21 of gestation. Four hours later, pups were delivered through cesarean 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. Data were obtained from three independent experiments. An asterisk indicates significant ($p < 0.01$) increase in DA/PA ratio compared with that of the pups from dams with indomethacin alone at 0 h.

because this is a critical issue in estimating the applicability of EP4 agonists in tocolysis.

No parturition was observed in the dams that received ONO-4819. Moreover, as shown in Table 1, ONO-4819 did not induce any increase in uterine activity, even at a high dose (3 μg/kg/h). These findings are consistent with the observation that EP4 agonists did not induce any contraction of porcine uterine strips *in vitro* (22).

DISCUSSION

In the late gestational period, PG are one of the key players in initiating and completing parturition in mother (1,2) and in fetus (3). During labor, PG that are released from placenta increase the intensity of uterine contraction and, consequently, exogenous PG have been used effectively to induce uterine contraction in inertia uteri. In the fetus, PG, mainly PGE_2, also participate in the regulation of circulatory change at birth, in

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 percentage of Montevideo units, as described in "Methods." The data are means ± SEM from four dams.

* Significant ($p < 0.01$) increase in uterine activity compared with saline.

which the closure of DA is the biggest event (3). DA is kept open during pregnancy by circulating PGE_2, probably *via* a cognate receptor, EP4 (5,7–10), and undergoes rapid closure in response to both a fall in PGE_2 concentration by placental segregation and the action of constrictors induced by an increase in oxygen tension (3). This constriction may involve endothelin secretion and endothelin receptor type A stimulation (15,23,24). In cases of heart anomalies in which survival depends on the patency of DA, PGE_2 analogue has been used as a therapeutic intervention to keep the DA open.

NSAID such as indomethacin that block PG synthesis have been successfully used in the treatment of preterm labor (25). 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 (13,14). As COX-2 has been found to be responsible for PG release from the placenta during labor (26), COX-2 inhibitors were expected to prevent labor and avoid the adverse effects of nonselective COX inhibitors. However, it has been shown that the use of COX-2 inhibitors is not necessarily free of adverse effects on fetuses in animal models (27–31). In this study, we have used indomethacin, a nonselective inhibitor of COX subtypes, as an inducer of DA closure.

We investigated the effect of EP4 selective agonists (19,32) on indomethacin-induced contraction of DA. *In vitro*, ONO-AE1-437 relaxed indomethacin-induced as well as O_2-induced DA contraction in a concentration-dependent manner (Figs. 1 and 2). *In vivo*, ONO-4819 prevented indomethacin-induced DA contraction *in utero* (Figs. 3*c* and 4) but did not affect physiologic closure of DA after birth (Figs. 3*e* and 4). There are two possible explanations for this difference in the DA dilating effects of ONO-4819 before and after birth. The first is the short half-life of ONO-4819 in the body, approximately 10–20 min (32), which prompts the clearance of the agonist from neonates after birth, preventing it from affecting the physiologic process of postnatal closure of DA. Under physiologic conditions, fetal PGE_2 is supplied by the placenta to maintain fetal circulation and, therefore, the concentration of PGE_2 in neonatal blood decreases rapidly after birth to facilitate postnatal DA closure (3). A similar decrease would be seen when the agonist is released from a capsule implanted in rat dams. In fact, an EP4 agonist prevented DA closure when administered directly to the neonate after birth (33). The second explanation is the postnatal rearrangement of EP receptors in DA. It has been reported that numbers of EP4 receptors in DA decrease after birth (6,7). This would result in a postnatal reduction of the dilatory effect of EP4 agonists and is, therefore, consistent with our results.

EP4 plays a role in many physiologic processes (4) and its selective agonist has been shown to have several therapeutic possibilities (19,32,34). The administration of ONO-4819 in rodents induces some acute side effects like diarrhea and hypotension, although they are seen only at higher doses than used in this report (19). COX inhibitor–induced premature closure of DA *in utero* not only causes deleterious pulmonary stress, which may underlie neonatal pulmonary dysfunction (12–14), but it also leads to incomplete but irreversible termination of DA remodeling, possibly resulting in persistent patent DA (35). Administration of EP4 agonist in addition to COX inhibitors might prevent adverse reactions on fetal DA without restricting the tocolytic effects of COX inhibitors.

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