Prostaglandin E2 Receptor EP4 Inhibition Contracts Rat Ductus Arteriosus

Toshiki Sakuma; Toru Akaike, MD, PhD; Susumu Minamisawa, MD, PhD

Background: Patent ductus arteriosus (PDA) is common in premature infants. Cyclooxygenase inhibitors such as indomethacin, which inhibit prostaglandin E2 (PGE2) synthesis, are currently the sole treatments for patients with PDA. Their efficacy are, however, frequently limited, and adverse effects are problematic. Because the PGE2-specific receptor EP4 selectively expresses in rat ductus arteriosus (DA), it is hypothesized that EP4 inhibition would promote DA closure with fewer side-effects.

Methods and Results: A new chemical compound EP4 antagonist, RQ-15986 (renamed from CJ-042794), was used. Whether RQ-15986 selectively contracted the DA was examined by measuring the isometric tension of rat DA ex vivo at embryonic day 19 (e19) and e21. RQ-15986 at a dose of 10−6 mol/L increased the isometric tension of the DA up to 44.8±6.2% and 69.1±12.9% to the maximal KCl-induced tension at e19 and e21 respectively. The effect of RQ-15986 on rat DA in vivo was also tested by using a rapid whole-body freezing method. RQ-15986 inhibited PGE2-induced DA dilatation in neonatal rats. Furthermore, RQ-15986 contracted the DA in a dose-dependent manner, and the constriction was greater at e21 than at e19. Moreover, RQ-15986 did not contract the aorta or the marginal artery of the colon.

Conclusions: EP4 inhibition contracts rat DA with fewer side-effects. EP4 inhibition is a promising alternative strategy to treat patients with PDA.

Key Words: EP4; Indomethacin; Patent ductus arteriosus; PGE2; Vasoconstriction

Prostaglandin E2 (PGE2), one of the prostanoids derived from arachidonic acid, plays a critical role in regulating the vascular tone and anatomical structure of the ductus arteriosus (DA). PGE2 works acutely as the most potent vasodilator for the DA during gestation. We previously demonstrated that PGE2 chronically remolds the DA structure as a muscular-type artery. A rapid decline in PGE2 after birth is one of the most significant factors for DA closure. If the DA fails to close after birth, infants are diagnosed with patent DA (PDA), which is found in 60–70% of premature infants. Since 1973, inhibiting the action of PGE2 has been widely recognized as the primary strategy for DA closure. Cyclooxygenase inhibitors such as indomethacin (IND) and ibuprofen, which inhibit the production of PGE2, are the sole pharmacological treatments for PDA. The vasoconstrictive action of these reagents is not long-lasting, and life-threatening side-effects such as intestinal ischemia, gastrointestinal bleeding, bleeding tendency, and renal impairment are frequent. Therefore, the development of a new drug for the treatment of PDA has been awaited for many years.

One way in which to overcome these issues is to restrictively inhibit the PGE2 pathway in the DA. There are four subtypes of PGE2-specific receptors: EP1, EP2, EP3, and EP4. EP4 is abundantly expressed in the DA, but is not expressed as much in other vessels of all species examined, including humans. Therefore, the selective inhibition of EP4 is expected to selectively contract the DA and have little effect on other organs. Indeed, several studies have already reported that an EP4 antagonist potently contracts the DA. To date, however, no EP4 antagonists have been developed for clinical use to treat PDA. In the present study, we tested whether a new chemical compound of the EP4 antagonist, RQ-15986 (renamed from CJ-042794), which was originally discovered for signs and symptoms of osteoarthritis and rheumatoid arthritis, would promote DA constriction with fewer side-effects.

Methods

Animal Preparation
Timed-pregnant Wistar rats were purchased from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan). The animal experiment protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Jikei University (No. 2015-143), and conformed to the Guidelines for the Proper Conduct of Animal Experiments of the Science Council of Japan.
Reagents
RQ-15986, a selective EP4 antagonist, was provided by AskAt Inc. (Nagoya, Japan). The structure, binding affinity, high selectivity, and pharmacokinetic properties of RQ-15986 have been characterized. Both IND and prostaglandin E1 (PGE1) were purchased from Cayman Chemical (Ann Arbor, MI, USA).

Isometric Tension of the DA and Aorta Vascular Rings
The isometric tension of the vascular rings of the DA and the aorta at embryonic day 19 (e19) and e21 was measured as previously described. Briefly, after maternal rats on the 19th and 21st day of gestation were anesthetized with isoflurane, the fetuses were delivered by cesarean section. Either the vascular ring of the DA or the descending aorta was placed in a tissue bath and kept at 37°C. Two tungsten wires (30 μm in diameter) were threaded into the lumen, and the preparation was mounted in a two-channel myograph (Dual Wire myograph system 410A; Unique Medical, Tokyo, Japan). One tungsten wire was connected to a micro-manipulator, and the other was connected to a force transducer. All of the vascular rings were initially stabilized with a modified Krebs-Henseleit solution (Sigma-Aldrich Co. LLC., St. Louis, MO, USA), and maintained at 37°C by a heated water jacket. Isometric tension was continuously monitored using a PowerLab/8 SP system (ADInstruments, Inc., Colorado Springs, CO, USA). After stabilization, the vascular ring was relaxed. After the resting tension was adjusted to 0.30 ± 0.02 mN, RQ-15986 was added to stimulate vasoconstriction. After the vasoconstriction reached a new steady state, the concentration of RQ-15986 was increased from 10^{-8} to 10^{-4} mol/L. At the end of all experiments, maximal vasoconstriction of the DA and the aorta was induced by potassium-enriched solutions (22 mmol/L NaCl, 120 mmol/L KCl, 1.5 mmol/L CaCl2, 6 mmol/L glucose, 1 mmol/L MgCl2, 5 mmol/L HEPES, pH 7.4). The value of this isometric tension was set to 100% as a reference to compare with the response to RQ-15986.

Rapid Whole-Body Freezing Method
To study the in situ morphology and inner diameter of the DA and other vessels, a rapid whole-body freezing method was used as previously described, with some modifications. (1) For the experiments using neonates, maternal Wistar rats were anesthetized on the 21st day of gestation with isoflurane. Neonates that were delivered by caesarean section were intraperitoneally injected either with RQ-15986 (1 or 10 μg/g), IND (10 μg/g), or phosphate buffered saline (PBS). The neonates were maintained on a warmer bed kept at 37°C with sufficient humidity. After the neonates were subcutaneously injected with either PGE1 (10 μg/g) or dimethyl sulfoxide (DMSO) 60 min after birth, they were frozen in liquid nitrogen 90 min after birth. The frozen neonates were cut on a freezing microtome in the frontal plane, and the inner diameters of the DAs, aortas, and left pulmonary arteries were measured under a microscope. (2) For the experiments using fetuses, maternal Wistar rats on the 19th and 21st day of gestation were anesthetized with isoflurane. After laparotomy, the fetuses at e19 and e21 were intraperitoneally injected with RQ-15986 (1, 10 or 100 μg/g) or IND (10 μg/g) via the uterine wall. For control groups, littermates were injected with the same volume of PBS. After the injection, the maternal rat’s abdomen was immediately sutured and they were maintained under anesthesia. The fetuses were delivered by cesarean section 4 h after the injection and were rapidly frozen in liquid nitrogen. Because this process was applied for both groups, the effect of rapid freezing on autonomic reaction should be considered to be identical. The frozen neonates were cut on a freezing microtome in the frontal plane, and the inner diameters of the DAs, aortas, and left pulmonary arteries were measured under a microscope.

Statistical Analysis
Data are expressed as the mean±standard error of the mean (SEM) of independent experiments. Statistical analysis was performed by using a 2-way repeated measures analysis of variance (ANOVA) followed by Bonferroni correction for post hoc comparisons in the isometric tension study; and by a Stell-Dwass test for multiple comparisons.
Figure 2. RQ-15986, an EP4 antagonist, inhibited prostaglandin E1 (PGE1)-induced vasodilatation in neonatal rat ductus arteriosus (DA) in vivo. (A–H) Representative images of the DA 30 min after the administration of PGE1 or dimethyl sulfoxide (DMSO) by a rapid whole-body freezing method. Either PGE1 or DMSO was injected subcutaneously 60 min after either treatment with RQ-15986 (1 or 10 μg/g), indomethacin (IND; 10 μg/g), or phosphate buffered saline (PBS). The DA was strongly dilated 30 min after the administration of PGE1. However, the DA was rarely dilated 30 min after the administration of RQ-15986 (1 and 10 μg/g). Scale bars=500 μm. (I, J) Quantification of the minimum diameter of the DA (I), and the minimum diameter ratio of the DA to the left pulmonary artery (lt. PA) (J). RQ-15986 (1 and 10 μg/g) significantly inhibited PGE1-induced DA dilatation compared to stimulation with IND and PBS (n=5–11). Ao, aorta. **P<0.01 vs. DMSO in the same treatment.
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RQ-15986 Selectively Contracted Fetal Rat DA Ex Vivo

First, we examined the effect of RQ-15986 on the isometric tension of DA ex vivo at e19 and e21. RQ-15986 at a concentration of $10^{-7}$ mol/L significantly contracted the DA but not the aorta at e21 (Figure 1A). Moreover, RQ-15986 at a concentration of $10^{-6}$ mol/L more selectively contracted the DA compared to the aorta at e21 (DA: 69.1±12.9%, aorta: 13.3±4.3% to the maximal KCl-induced tension). In addition, RQ-15986 at a concentration of $10^{-6}$ mol/L contracted the DA in a dose-dependent manner. RQ-15986 at a concentration of 100 μg/g significantly contracted the DA (n=6–9). *P<0.05 vs. PBS.

**Results**

**Figure 3.** RQ-15986, an EP4 antagonist, contracted the ductus arteriosus (DA) in fetal rat at embryonic day 21 (e21). (A–E) Representative images of the DA 4 h after the administration of RQ-15986, indomethacin (IND) or phosphate buffered saline (PBS) by a rapid whole-body freezing method. RQ-15986 (10 and 100 μg/g) and IND contracted the DA in fetal rat at e21. Scale bars=300 μm. (F–H) Quantification of the minimum diameter of the DA (F), and the aorta (H). RQ-15986 contracted the DA in a dose-dependent manner. RQ-15986 at a concentration of 100 μg/g significantly contracted the DA (n=6–9). (G,I) Quantification of the minimum diameter ratio of the DA (G) to the left pulmonary artery (lt. PA) and the aorta (Ao; I) to the lt. PA. RQ-15986 did not contract the aorta (n=6–9). *P<0.05 vs. PBS.

in the rapid whole-body freezing method study. A P value of less than 0.05 was considered to be statistically significant.
Next, we examined the effect of RQ-15986 on neonatal DA in vivo using a rapid whole-body freezing method. As it is known that rat DA starts to close immediately after birth, the DA was contracted 90 min after birth under basal conditions (Figure 2A). The DA was further contracted significantly contracted the DA, but not the aorta at e19, although its vasoconstrictive response was weaker at e19 (DA: 44.8±6.2%, aorta: 15.6±6.8%) when compared with that at e21 (Figure 1B). These results indicated that RQ-15986 had a selective vasoconstrictive effect on fetal rat DA.

Figure 4. RQ-15986, an EP4 antagonist, tended to contract the ductus arteriosus (DA) in fetal rat at embryonic day 19 (e19). (A–E) Representative images of the DA 4h after the administration of RQ-15986, indomethacin (IND) or phosphate buffered saline (PBS) by a rapid whole-body freezing method. RQ-15986 (1, 10, and 100μg/g) did not contract the DA in the same manner as IND in fetal rat at e19. Scale bars=300μm. (F,H) Quantification of the minimum diameter of the DA (F), and the aorta (H). RQ-15986 did not significantly contract the DA, although RQ-15986 contracted the DA in a dose-dependent manner (n=8–12). (G,I) Quantification of the minimum diameter ratio of the DA (G) to the left pulmonary artery (lt. PA), and the aorta (Ao; I) to the lt. PA. RQ-15986 did not contract the aorta (n=8–12). *P<0.05 vs. PBS.

RQ-15986 Inhibited PGE1-Induced Vasodilatation in Neonatal Rat DA

Next, we examined the effect of RQ-15986 on neonatal DA in vivo using a rapid whole-body freezing method. As it is known that rat DA starts to close immediately after birth, the DA was contracted 90 min after birth under basal conditions (Figure 2A). The DA was further contracted...
in the presence of RQ-15986 (Figure 2B,C) or IND (Figure 2D), although the extent of DA constriction did not reach statistical significance. We then examined the effect of RQ-15986 on PGE₁-induced DA dilatation. PGE₁ strongly dilated the DA 30 min after the administration into neonates (Figure 2E). RQ-15986 inhibited the effect of PGE₁-induced DA dilatation (Figure 2F,G). Indomethacin, however, had no effect on PGE₁-induced DA dilatation (Figure 2H). Statistically, RQ-15986 at concentrations of 1 and 10 μg/g significantly inhibited PGE₁-induced DA dilatation compared to PBS as a negative control (Figure 2I,J). Therefore, these data indicated that RQ-15986 inhibited PGE₁-induced DA dilatation in rat neonates.

**RQ-15986 Contracted Fetal DA**

Next, we assessed the effect of RQ-15986 on fetal DA in vivo at e21. Four hours after intraperitoneal injection, RQ-15986 contracted the DA at e21 in a dose-dependent manner (Figure 3A–D,F,H). RQ-15986 at a concentration of 100 μg/g significantly contracted DA (Figure 3F,H). This vasoconstrictive effect on DA was larger than that caused by IND at a concentration of 10 μg/g (RQ-15986 (100 μg/g): DA 67.1±12.5 μm, IND (10 μg/g): DA 125.6±29.2 μm) (Figure 3D,E respectively). In contrast, RQ-15986 did not contract the aorta of the fetal rat at e21 (Figure 3G,I).

We then investigated the effect of RQ-15986 on fetal DA in vivo at e19 in the same condition of e21. Four hours after an intraperitoneal injection, RQ-15986 also tended to contract the DA at e19 in a dose-dependent manner (Figure 4A–D,F,H), but this did not reach statistical significance. In contrast to e21, the vasoconstrictive effect of RQ-15986 was weaker than that of IND (RQ-15986 (100 μg/g): DA 210.9±50.6 μm, IND (10 μg/g): DA 163.6±40.8 μm) (Figure 4D,E respectively). RQ-15986 did not contract the aorta 4 h after intraperitoneal injection into the fetal rat at e19 (Figure 4G,I). Therefore, these data demonstrated that RQ-15986 selectively contracted fetal rat DA, but the effect was weaker in premature DA.

**RQ-15986 Did Not Contract Other Vessels**

We evaluated whether RQ-15986 contracted other vessels, such as the marginal artery of the colon, in the fetal rat at e21. It is known that IND causes adverse effects, such as intestinal ischemia, gastrointestinal bleeding, bleeding tendency, and renal impairment.²⁰⁻²¹ In our study, RQ-15986 did not contract the marginal artery of the colon at any concentration (1, 10, and 100 μg/g), although IND significantly contracted the marginal artery of the colon compared to PBS (Figure 5). These data indicated that RQ-15986 had less vasoconstrictive effect on small arteries such as the marginal artery of the colon compared with IND.

**Discussion**

This study revealed that RQ-15986, a new chemical compound of an EP4 antagonist, contracted fetal rat DA in both in vivo and ex vivo experiments in a dose-dependent manner. This result is consistent with those of previous studies,¹⁶,¹⁷ although these studies only showed that EP4 antagonists contracted rat DA in term fetuses and neonates. Therefore, this is the first study showing that EP4 inhibition also exhibits a constrictive effect on preterm (e19) rat DA. It should be noted, however, that the DA constrictive effect of RQ-15986 was much stronger in term fetuses than in preterm fetuses. This might reflect the higher expression level of EP4 in term rat DA rather than preterm rat DA.⁶ At present, cyclooxygenase (COX) inhibitors such as IND and ibuprofen are the only pharmacological treatments used for PDA.²³,²⁴ However, the effect of COX inhibitors is sometimes insufficient for premature infants with PDA.²⁵ Therefore, despite the weaker effect of RQ-15986 on preterm fetuses, the result of the present study encourages the introduction of RQ-15986 as a new therapeutic agent
for premature infants with PDA.

More importantly, RQ-15986 had less vasoconstrictive effect on the aorta and the marginal artery of the colon in fetal rats, suggesting DA-dominant vasoconstriction. This result is consistent with that of a previous study using another type of EP4 antagonist, \(^{28}\) IND and ibuprofen are well known to cause adverse effects, such as intestinal ischemia and renal impairment, mainly due to undesirable vasoconstriction.\(^{28,30}\) COX inhibitors prevent all PG synthesis, not only PGE; but also other prostanooids such as PGI; and thromboxane A; in various tissue types. EP4 is known to be predominantly expressed in the DA and bone.\(^{12}\) Therefore, it is reasonable to consider that RQ-15986 works more selectively in the DA with fewer side-effects than COX inhibitors do.

The result of Figure 2, which shows that RQ-15986, but not IND, contracted neonatal DA after the administration of PGE; is also important in a clinical setting, because the result indicates that IND itself does not have an effect on exogenous PGE. PGE; is produced from the placenta and the DA itself.\(^{27}\) Therefore, circulating PGE; is rich in fetuses, and results in keeping the DA open. After birth, the levels of circulating PGE; rapidly decrease due to its degradation in the lungs in addition to no supply from the placenta. Although IND suppresses the synthesis of endogenous PGE; in the DA, it does not inhibit the effect of circulating PGE; or exogenously administered PGE;. In contrast, RQ-15986 can antagonize circulating PGE; or exogenous PGE;. This is why RQ-15986, but not IND, suppressed PGE; induced DA dilatation in neonatal rats. In addition, as Momma et al suggested previously,\(^{16}\) an EP4 antagonist could have a potentially stronger effect than IND, because it can preserve the vasoconstrictive effect of thromboxane A; the production of which is also inhibited by IND.

Although EP4 antagonists theoretically have more advantages over non-selective COX inhibitors, their safety for premature infants should be carefully investigated before clinical use in patients with PDA. For example, Walker et al demonstrated that an EP4 antagonist decreased ileal blood flow in neonatal Sprague-Dawley rats,\(^{29}\) suggesting that EP4 inhibition might increase the risk of necrotizing enterocolitis, which is one of the most severe complications in premature infants. In the present study, we observed that RQ-15986 had less vasoconstrictive effect on the marginal artery of the colon in fetal Wistar rats. We cannot explain this discrepant result well, although the type of drug and rats differ in both studies. This remains to be carefully investigated in future research. In addition, EP4 is highly expressed in the adult heart, and plays a role in protecting against ischemic-reperfusion injury,\(^{28,29}\) whereas mice with the cardiomyocyte-specific deletion of EP4 showed normal cardiac function.\(^{30}\) In 2016, an EP4 antagonist, grapiprant, was launched in the US as a new animal drug intended to control pain and inflammation associated with osteoarthritis in dogs, and Rausch-Derra et al demonstrated that grapiprant at therapeutic dose was safe and well-tolerated for daily oral administration in dogs.\(^{31}\)

A limitation of this study was that rat fetuses were rapidly frozen in liquid nitrogen before they were able to take their first breath. This fixation technique may have some vasoconstrictive effects because it takes some time to solidly freeze the fetuses. However, this rapid whole-body freezing method has been used as an observational method of the inner diameter of vessels in a number of studies.\(^{16,21,22}\) Currently, this method is the best approach to assess the inner diameter of fetal and neonatal vessels. We need to develop an instant fixation technique of vessels or assess the inner diameter of vessels in vivo.

In conclusion, our study proposes that the new EP4 antagonist, RQ-15986, has a selective vasoconstrictive effect on the DA. The effect is more selective than that of IND. Therefore, EP4 antagonists are expected to exhibit fewer side-effects than IND and to be an alternative pharmacological treatment for PDA.

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**Disclosures**

None.

**References**


