

Low-dose thromboxane A₂ receptor stimulation promotes closure of the rat ductus arteriosus with minimal adverse effects

Tomohiro Yokota¹, Takashi Aida¹, Yasuhiro Ichikawa², Takayuki Fujita³, Utako Yokoyama² and Susumu Minamisawa¹

BACKGROUND: Patent ductus arteriosus (PDA) is a common life-threatening complication among premature infants. Although cyclooxygenase inhibitors are frequently used to treat PDA, as they inhibit the synthesis of prostaglandin E₂, the most potent vasodilator in the ductus arteriosus (DA), their efficacy is often limited. As thromboxane A₂ (TXA₂) induces vascular contraction via the TXA₂ receptor (TP), we hypothesized that TP stimulation would promote DA closure.

METHOD: To measure the inner diameter of the vessels, a rapid whole-body freezing method was used.

RESULTS: Injection of the selective TP agonists U46619 and I-BOP constricted the fetal DA at embryonic day 19 (e19) and e21 in a dose-dependent manner. Of note, U46619 also exerted a vasoconstrictive effect on two different types of postnatal PDA models: premature PDA and hypoxia-induced PDA. We also found that U46619 constricted the *ex vivo* DA ring to a greater extent than it constricted the *ex vivo* aorta. Furthermore, we found that U46619 at lower concentrations (up to 0.05 mg/g of body weight) had a minimal vasoconstrictive effect on other vessels and did not induce microthrombosis in the pulmonary capillary arteries.

CONCLUSION: Low-dose TP stimulation constricts the DA with minimal adverse effects at least in rat neonates and our results could point to an alternative potent vasoconstrictor for PDA.

The ductus arteriosus (DA) is an essential vascular shunt connecting the aorta and the pulmonary artery for fetal circulation; ordinarily, it starts to close immediately after birth. In some cases, however, it remains patent after birth; this condition is called patent DA (PDA). PDA occurs frequently in premature infants, and 60–70% of premature infants of <28 wk gestation receive medical or surgical therapy for PDA (1,2). Although cyclooxygenase (COX) inhibitors such as indomethacin and ibuprofen have been widely used for prophylactic or symptomatic treatment of PDA, they fail to close the DA with an incidence of 20–40% in premature infants (3,4). The frequent failure rate of COX inhibitors currently leaves the clinician with the only option being surgical ligation. Although

surgical ligation is considered to be a safe procedure in clinical studies (5), it has been reported that neurosensory impairment, bronchopulmonary dysplasia, and severe retinopathy are more common after surgery (6). Therefore, an alternative pharmacological strategy for PDA treatment is required.

Thromboxane A₂ (TXA₂) is a lipid mediator that exhibits diverse physiological and pathological effects. In term of cardiovascular effects, TXA₂ is known to be a strong vasoconstrictor and to be involved in pathogenesis of vascular diseases including thrombosis, atherogenesis, and neovascularization (7). This lipid mediator is synthesized from arachidonic acid along the COX pathway, via the pivotal intermediate prostaglandin H₂, which, in turn, is converted to TXA₂ by thromboxane synthase (7). TXA₂ receptor (TP) is a G-protein-coupled receptor expressed in many cell and tissue types (7). Previous *ex vivo* experiments using DA explants have yielded conflicting results regarding the effect of TP stimulation on the DA. Smith *et al.* (8) and Reese *et al.* (9) demonstrated that TP stimulation constricted rabbit and mouse DA explants, respectively, whereas Coceani *et al.* (10) demonstrated that it exerted no vasoconstrictive effect on lamb DA. Loftin *et al.* (11), however, have demonstrated through *in vivo* experiments that TP stimulation induces closure of the DA in Cox-1/2 knockout mice with PDA, whereas other Cox-1/2-producing prostanoids did not close the DA of Cox-1/2 knockout mice. Therefore, we undertook to evaluate whether TP stimulation was also effective against other PDA models and to assess its adverse effects.

RESULTS

TP Stimulation Selectively Caused Vasoconstriction in Fetal Rat DA

First, we examined the *in vivo* effect of TP stimulation on the fetal rat DA using two types of TP stimulation: U46619, a prostaglandin H₂ analog and I-BOP, a TXA₂ analog. Both are commonly used as selective TP agonists (7,12). Consistent with the previous *in vivo* study by Loftin *et al.* (11), when U46619 was intraperitoneally injected into the fetal rats at embryonic day 19 (e19) and e21, the DA was significantly constricted in a dose-dependent manner (Figure 1a–g). The constriction of

¹Department of Life Science and Medical Bioscience, Waseda University Graduate School of Advanced Science and Engineering, Tokyo, Japan; ²Cardiovascular Research Institute, Yokohama City University Graduate School of Medicine, Kanagawa, Japan; ³Department of Medical Science and Cardiorenal Medicine, Yokohama City University Graduate School of Medicine, Kanagawa, Japan. Correspondence: Susumu Minamisawa (sminamis@waseda.jp)

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the DA by U46619 was greater at e21 than at e19, even though the levels of circulating prostaglandin E₂ are supposed to be higher during late gestation (Figure 1a–g). Whereas a very low dose of U46619 such as 0.005 μg/g did not show significant constriction of the DA at e19, the effect of U46619 at e21 showed significant constriction even at low dose. I-BOP also constricted the DA (Figure 1h). These results indicated that TP stimulation promoted closure of the DA in the fetal rat.

TP Stimulation Constricted the DA in Two Different PDA Models

Next, we evaluated the effect of U46619 on two PDA models: premature and hypoxia-induced PDA models. Twenty minutes after delivery, we intraperitoneally injected various doses of U46619 (0.0005, 0.05, and 5.0 μg/g) into premature neonates delivered on e20 (PM20). The diameter of the DA was measured 10, 20, and 30 min after injection. U46619 at concentrations of 0.05 and 5.0 μg/g significantly constricted the premature DA as compared with saline injection (Figure 2a).

It should be noted that 75 and 100% of the DA were completely closed 30 min after the injection at concentrations of 0.05 and 5.0 μg/g, respectively (Table 1).

Regarding hypoxic-induced PDA models, PO₂ was lower in rats under hypoxic conditions than under normoxic conditions (19.3 ± 1.5 vs. 56.2 ± 3.8 mm Hg, respectively; *P* < 0.0001, *n* = 4–7). Under normoxic conditions, within 30 min after birth the lumen of the DA shrank by ~91% down from the diameter of the fetal DA on e21. Under hypoxic conditions, on the other hand, DA closure was significantly delayed: the lumen shrank by only ~24% by 30 min after birth. Ten minutes after injection (30 min after birth) we found that U46619 at a concentration of 5.0 μg/g significantly constricted the hypoxic DA as compared with saline injection (Figure 2b). In addition, indomethacin at a concentration of 10 μg/g constricted the hypoxic DA by ~70% of the DA diameter as compared with saline injection. These results indicated that TP stimulation effectively constricted the DA in two different PDA models.

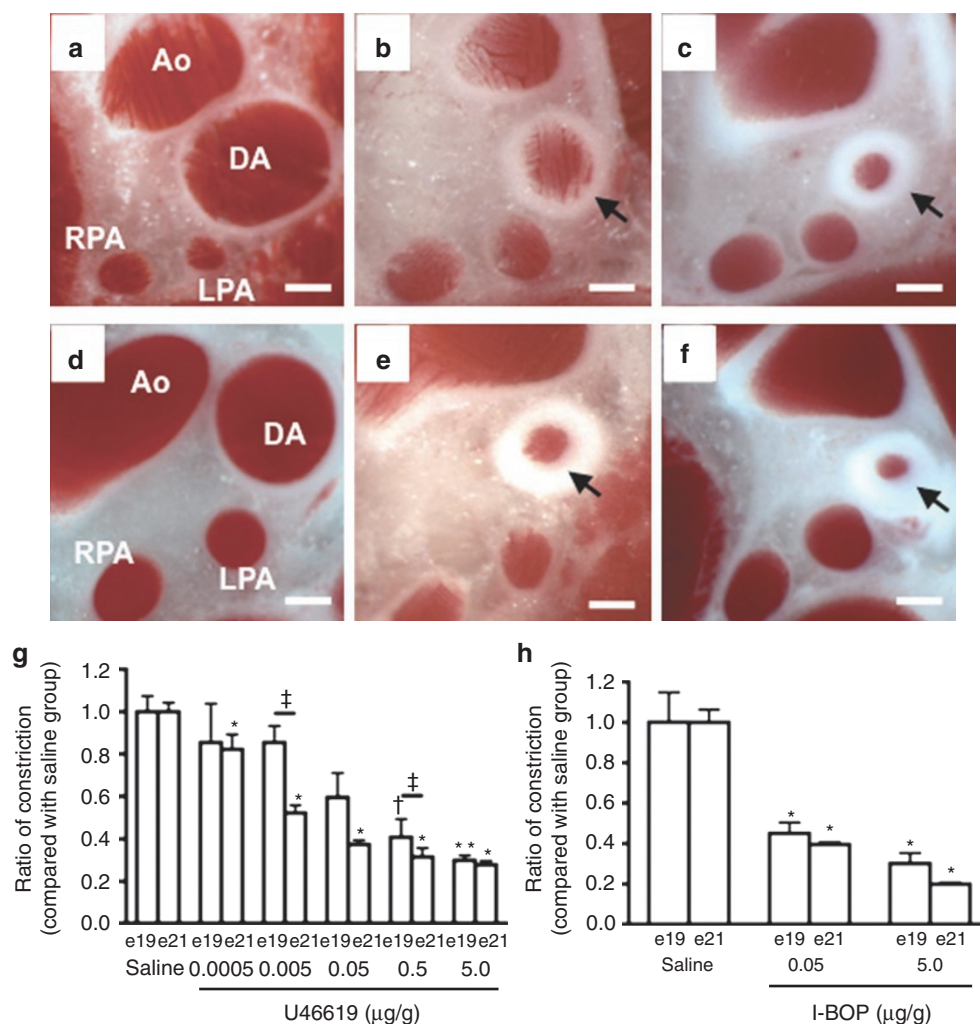


Figure 1. TXA₂ receptor stimulation induced vasoconstriction of the fetal rat ductus arteriosus (DA). (a–f) Effects of U46619 on the fetal rat DA at embryonic day (e)19 (a–c) and at e21 (d–f). Each panel shows representative data from fetuses injected with saline (a and d), U46619 (0.05 μg/g: b and e; 5.0 μg/g: c and f). Arrows show the constricted DA. Scale bar = 0.2 mm. Ao, aorta; LPA, left pulmonary artery; RPA, right pulmonary artery. (g and h) Effects of various doses of (g) U46619 (from 0.0005 to 5.0 μg/g) (*n* = 3–12) and (h) I-BOP (0.05 and 5.0 μg/g) (*n* = 5–10) on the diameter of the fetal rat DA at e19 and e21. *P* value (vs. saline) **P* < 0.05, ***P* < 0.01, ****P* < 0.001. *P* value (e19 vs. e21) †*P* < 0.05. (*n* = 3–12).

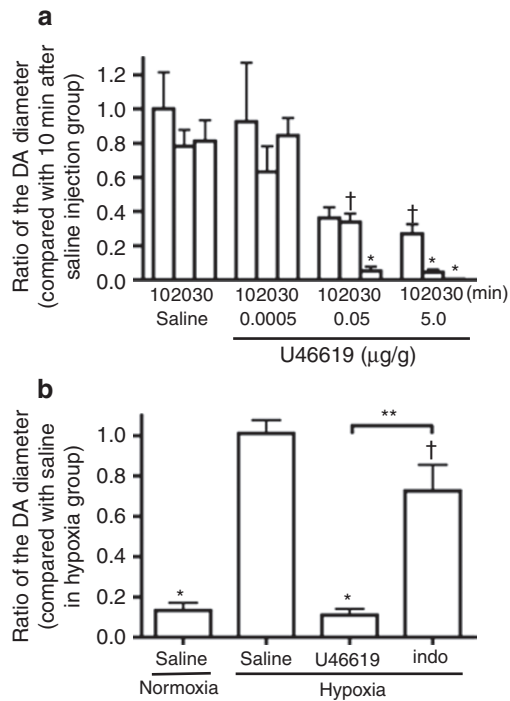


Figure 2. TXA₂ receptor stimulation caused ductus arteriosus (DA) constriction in the premature and hypoxia-induced patent DA (PDA) models. **(a)** The ratios of DA constriction in premature rats injected with U46619 (0.0005, 0.05, or 5.0 µg/g). The diameter of the DA was measured at 10, 20, and 30 min after injection. *P* value (vs. respective saline group) [†]*P* < 0.05, [‡]*P* < 0.001 (*n* = 4–9). **(b)** The ratios of DA constriction in hypoxia-induced PDA model rats injected with U46619 and indomethacin (indo). *P* value (vs. saline on hypoxia) [†]*P* < 0.05, [‡]*P* < 0.001; *P* value (vs. indomethacin on hypoxia) ^{**}*P* < 0.01 (*n* = 3–4).

Table 1. Incidence of complete DA closure

Time after injection (min)	Percentage incidence (number of completely closed DA/number of DA analyzed)			
	Saline (%)	U46619 (µg/g)		
		0.0005 (%)	0.05 (%)	5 (%)
10	0 (0/8)	0 (0/7)	0 (0/8)	11 (1/9)
20	0 (0/8)	0 (0/7)	0 (0/8)	86 (6/7)
30	0 (0/4)	0 (0/4)	75 (6/8)	100 (7/7)

DA, ductus arteriosus.

TP Stimulation Did Not Constrict Other Vessels

We assessed whether U46619 constricts other vessels such as the aorta, the pulmonary artery, the vertebral artery, the renal artery, the portal vein, and the marginal artery of the colon in the mature neonate at day 0 (d0). U46619 at concentrations of up to 0.05 µg/g had no significant vasoconstrictive effect on these vessels (Figure 3a–g). However, U46619 at concentration of 5.0 µg/g significantly constricted the marginal artery of the colon (Supplementary Figure S1 online), but not other vessels at e21. In addition, the aorta and the pulmonary artery at e19 did not respond to U46619 at concentrations of up to 5.0 µg/g (Figure 3a,b). These data suggest that the fetal and neonatal DA had a stronger response to U46619 than did other vessels.

A Low Dose of U46619 Did Not Decrease Peripheral Blood Flow in Neonatal Rats

Although U46619 did not induce vasoconstriction in large arteries, we sought to evaluate whether U46619 constricts a muscular type of arteries or arterioles. Therefore, we measured peripheral blood flow in the tail of d0 rats as an index of microvascular constriction by newly developed methods using a laser speckle measurement technique (13). Up to a concentration of 0.05 µg/g, U46619 did not decrease the peripheral blood flow in the tail (Figure 4a–g). However, when the concentration of U46619 was increased to 5.0 µg/g, the peripheral blood flow was significantly reduced.

U46619-Induced Isometric Tension of the DA Vascular Rings Was Stronger Than That of the Aorta

To consolidate the *in vivo* data demonstrating that the DA responded to U46619 more than did the aorta, we measured the isometric tension induced by U46619 in the DA and aorta vascular rings. U46619 at concentrations of up to 10⁻⁷ mol/l induced isometric tension more strongly in the DA rings than in the aorta rings from both e19 and e21 (Figure 5a,b, respectively). Therefore, it appears that the DA was more sensitive to U46619 than was the aorta.

TP Stimulation Did Not Induce Microthrombosis in the Pulmonary Capillary Arteries

Because one of the most significant adverse effects of TP stimulation is microthrombosis, especially in pulmonary capillary arteries, it is very important to determine whether TP stimulation induces microthrombosis in pulmonary capillary arteries of neonates. U46619 at a concentration of 0.05 µg/g apparently did not induce significant microthrombosis in pulmonary capillary arteries at PM20 (Figure 6b). On the other hand, consistent with previous studies showing significant microthrombosis in the pulmonary capillary arteries (14), arachidonic acid induced significant thrombosis in rat lungs (Figure 6c). When we calculated the ratio of arteries with thrombosis to total capillary arteries, arteries with thrombosis in the lung were 7% in U46619-injected and 4% in saline-injected premature neonates, respectively, whereas those were 42% in arachidonic acid-injected premature neonates (Figure 6d).

TP Inhibition Did Not Dilate the Neonatal DA

To clarify the contribution of endogenous TXA₂ to DA closure, we assessed whether TP inhibition made the closed DA reopen after birth or not. First, to determine the dose of a selective TP antagonist, SQ29548, we pretreated by injecting SQ29548 (1 µg/g) into the fetus at e21 10 min before injecting U46619. Therefore, SQ29548 at a concentration of 1 µg/g is sufficient to inhibit the U46619-mediated DA constriction (Figure 7a). To observe whether or not SQ29548 prevents closure of the DA after birth, SQ29548 was injected 40 min after birth when the DA was still closing. We found that SQ29548 at concentrations of up to 10 µg/g also showed no dilative effect on the DA after birth (Figure 7b,c). On the other hand, prostaglandin E₂ (15 ng/g) significantly dilated the DA until 60 min after injection.

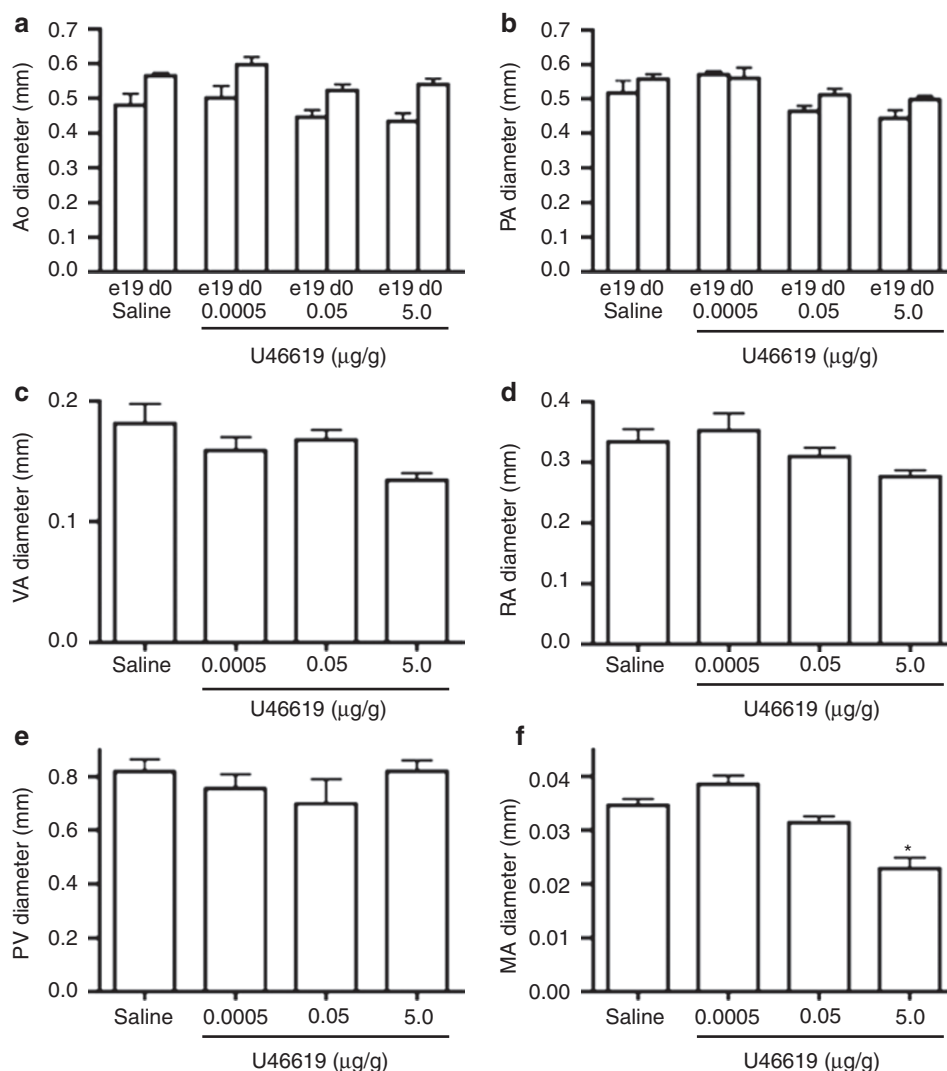


Figure 3. TXA₂ receptor stimulation caused no vasoconstriction of adjunct arteries and veins. **(a and b)** Constrictive effect of U46619 on **(a)** the aorta (Ao) ($n = 3-9$) and **(b)** the pulmonary artery ($n = 4-10$) at e19 and d0. **(c-f)** Constrictive effect of U46619 on **(c)** the vertebral artery (VA) ($n = 4-5$), **(d)** the renal artery (RA) ($n = 4-8$), **(e)** the portal vein (PV) ($n = 4-8$), and **(f)** the marginal artery of the colon (MA) ($n = 3-6$) in mature neonates at day 0. P value (vs. saline) * $P < 0.001$.

DISCUSSION

This study demonstrated that TP stimulation potently constricted the *in vivo* DA in the following subjects: (i) rat fetuses at e19 and e21; (ii) premature rat neonates delivered at e20; and (iii) mature rat neonates under hypoxic conditions (5% O₂). These results are consistent with the previous study by Loftin *et al.* (11) using Cox-1/2 knockout mice with PDA. Previous *ex vivo* studies have shown that TP stimulation produces contraction of ductus smooth muscle through the pathways that control both the concentration of intracellular calcium and the sensitivity of the contractile proteins to changes in intracellular calcium (8). The former is determined by Ca²⁺ influx through L-type Ca²⁺ channels and the latter is regulated by Rho/Rho-kinase activity (15,16).

To apply TP stimulation for patients with PDA as a potential alternative pharmacological therapy, it is important to estimate its possible adverse effects, because TXA₂ is endowed with

powerful systemic vasoconstrictor, cytotoxic, and thrombogenic properties (7). In this regard, we examined the potential adverse effects of TP stimulation on the rat fetuses and neonates. First, systemic vasoconstriction is an important adverse effect of U46619 to be carefully considered. We found that U46619 even at a concentration of 0.05 μg/g, which was sufficient to constrict the DA, did not significantly constrict other vessels including the marginal arteries of the colon and did not decrease blood flow in the tail. In addition, our *ex vivo* data using the rat DA and aorta vascular ring demonstrated that U46619 produced stronger contraction of the DA than that of the aorta. However, U46619 at a concentration of 5.0 μg/g significantly constricted the marginal arteries of the colon and reduced blood flow in the tail (**Supplementary Figure S1** online). Because continuous U46619 infusion is known to decrease cardiac output (17), the reduction in peripheral circulation may be not only due to vascular constriction but also

to a decrease in cardiac output by U46619 at a concentration of 5.0 $\mu\text{g/g}$. Further study will be required to determine whether a decrease in cardiac output is responsible for the U46619-mediated reduction in peripheral circulation.

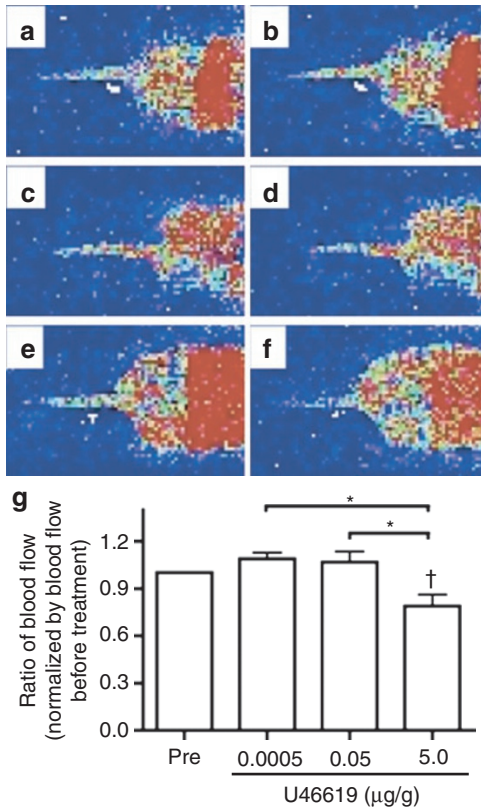


Figure 4. U46619 did not decrease peripheral blood flow in neonatal rats. (a–f) Representative images of blood flow at lower part of the neonates. Left (a, c, and e) and right panels (b, d, and f) indicate relative blood flow before and after U46619 injection, respectively. Upper (b), middle (d), and lower (f) panels indicate U46619-injected group at a dose of 0.0005, 0.05, and 5.0 $\mu\text{g/g}$, respectively. (g) Effect of U46619 on peripheral blood flow in the tails. "Pre" indicates before treatment. P value (vs. pre) $^{\dagger}P < 0.05$; P value (vs. 5.0 $\mu\text{g/g}$) $^*P < 0.01$ ($n = 5$).

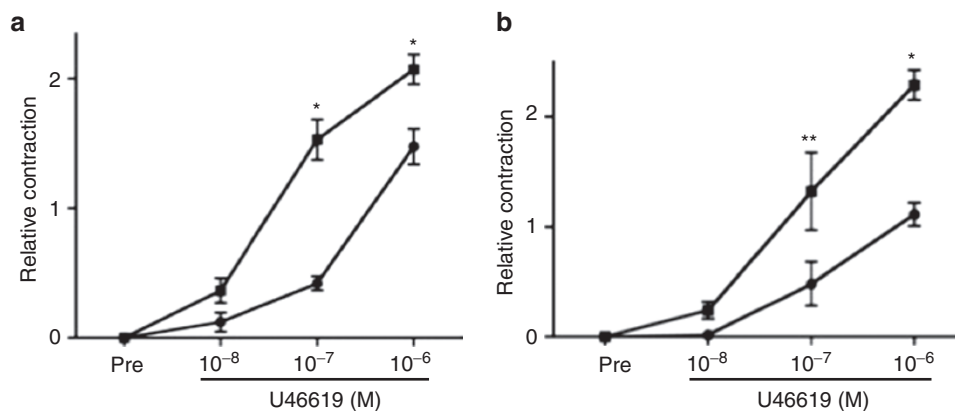


Figure 5. U46619-induced isometric tension of the ductus arteriosus (DA) and aorta vascular rings. (a and b) Isometric tension of the DA and aorta rings at (a) e19 or (b) e21, stimulated by various doses of U46619 (10^{-8} , 10^{-7} , and 10^{-6} mol/l). Squares and circles indicate the DA and aorta rings, respectively. P value (DA vs. aorta) $^{\dagger}P < 0.001$, $^{**}P < 0.01$ ($n = 4-5$).

Next, microthrombosis in the pulmonary capillary arteries is expected to be one of the most severe adverse effects of U46619. We did not find significant microthrombosis in the pulmonary capillary arteries when U46619 at a concentration of 0.05 $\mu\text{g/g}$ was administered to PM20 rats (Figure 6). A number of studies have demonstrated that a relatively high dose of U46619 (e.g., 1.0 mg/kg, i.v.) causes a shock syndrome resulting in sudden death due to systemic platelet aggregation, pulmonary thrombosis, and coronary spasm in adult animals (18–20). However, neonatal platelets are known to be less reactive than adult platelets to U46619, thrombin, and ADP/epinephrine (21). Therefore, thromboembolism may be avoidable when a low dose (up to 0.05 $\mu\text{g/g}$) of U46619 is administered in newborns.

Furthermore, we need to pay careful attention to administering U46619 to newborns, because neonatal pulmonary hypertension is characterized by pulmonary vasoconstriction, due in part to hypoxia-induced TP hyperresponsiveness (22,23). Although pulmonary hypertension is induced by intravenous infusion of U46619 ($\sim 2 \mu\text{g/kg/min}$) (24,25), further investigation is required to examine whether or not a bolus injection of U46619 at a low concentration induces pulmonary hypertension.

Taken together, this study demonstrated that low-dose TP stimulation induced vasoconstriction of the DA with minimal systemic adverse effects when U46619 is administered at a concentration of up to 0.05 $\mu\text{g/g}$. Although COX inhibitors such as indomethacin and ibuprofen are the current unique pharmacological treatment for PDA (1,3), the frequent failure rate of COX inhibitors is clinically problematic. COX inhibitors also share the similar adverse effects with U46619. Therefore, we propose that low-dose TP stimulation can be an alternative pharmacological strategy for PDA treatment when it is problematic to administer COX inhibitors.

The mechanism by which U46619 constricted the DA more than other vessels is the next important question to be clarified, because a considerable number of *ex vivo* experiments have demonstrated that TP agonists constrict a variety of arteries and veins (7,26). We assume that the higher sensitivity

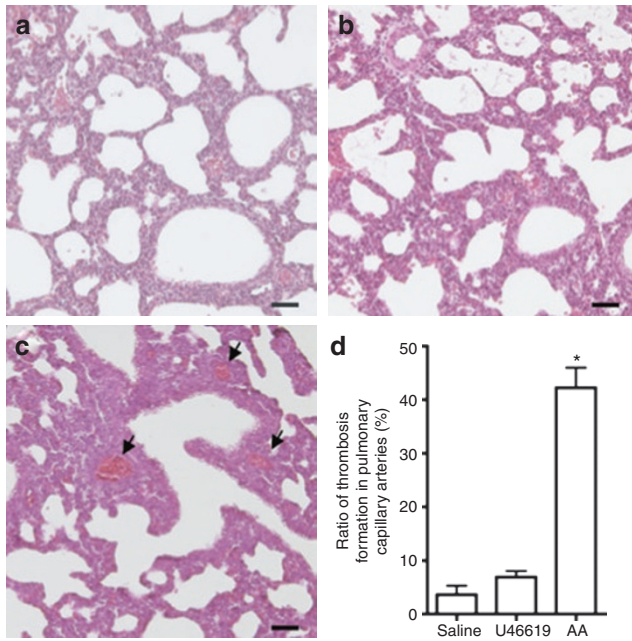


Figure 6. Thrombosis formation in the microvasculature of the rat lung. (a–c) Rat lung sections from premature neonates delivered on embryonic day 20, injected with (a) saline, (b) U46619, and (c) arachidonic acid. Arrows indicate thrombosis formation. Scale bar = 0.1mm. (d): The ratio of thrombosis formation in all pulmonary capillary arteries. *P* value (vs. saline) **P* < 0.001 (*n* = 4).

to U46619 in the DA could be due to its artery type (muscular type), because the structure of the DA is considered to be a muscular type and most of other arteries that we examined belong to an elastic type. U46619 at a concentration of 0.05 μg/g significantly constricted mature fetal DA by ~40% as compared with the control groups, whereas the same dose of U46619 did not reduce the diameter of marginal artery of the colon and blood flow of the rat neonatal tail. Because resistant muscular arteries supply the blood flow in the colon and tail, the arterial type may not be the sole reason for the hypersensitivity to U46619 in the DA.

We also examined the abundance of TP expression between the DA and the aorta during development. Although the expression levels of TP messenger RNA in the DA were higher than those in the aorta in the fetal period, the expression levels of TP protein showed no difference between the DA and the aorta (Supplementary Figure S2 online). Therefore, the abundance of TP expression is not the reason for the hypersensitivity to U46619 in the DA. It is then highly possible that TP in the DA has higher binding affinities to TP agonists than those of the other arteries. Several studies have demonstrated that the affinity state of TP is influenced by interaction with G_{α13} and/or G_{αq} (27,28). Of note, recent studies by Dakshinamurti's group have demonstrated that a change in oxygen tension from normoxia to hypoxia provokes hypersensitivity to TXA₂ in pulmonary arterial myocytes of neonatal piglets (22,23). The authors have indicated that hypoxia promotes the membrane localization of TP and increases its ligand affinity in pulmonary arterial myocytes. The response to oxygen is opposite

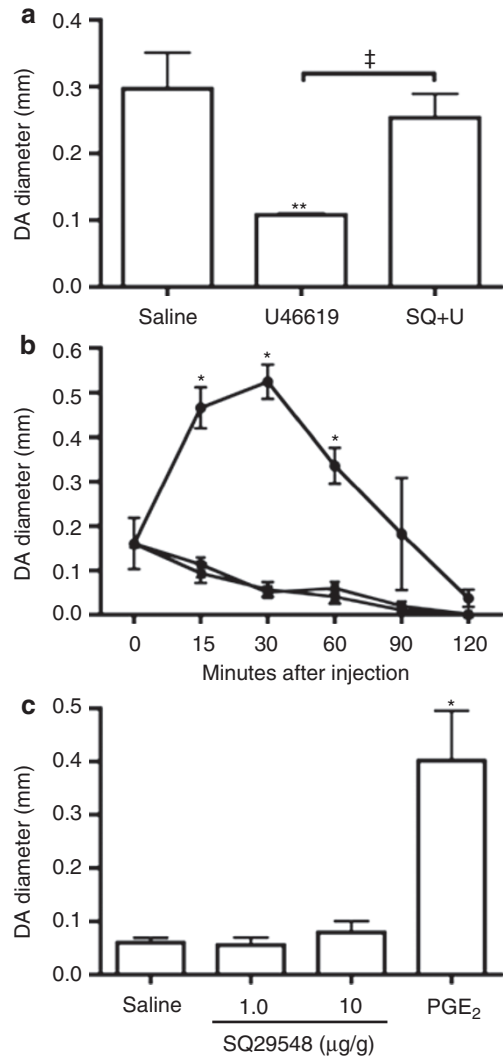


Figure 7. The effect of TXA₂ receptor (TP) inhibition on the neonatal rat ductus arteriosus (DA). (a) The effect of TP antagonist SQ29548 on the U46619-induced DA constriction. SQ + U indicates the group pre-treated with SQ29548 and then injected with U46619. *P* value (vs. saline) ***P* < 0.05; *P* value (vs. SQ + U) †*P* < 0.05 (*n* = 3–4). (b) The effect of the TP antagonist SQ29548 on the DA in rat neonates. Prostaglandin E₂ was injected as a positive control. Circles, squares, and triangles indicate SQ29548, prostaglandin E₂, and saline groups, respectively. *P* value (vs. saline) **P* < 0.001 (*n* = 3–4). (c) Effect of a different dosage of SQ29548 (10 μg/g) on the DA diameter. *P* value (vs. saline) **P* < 0.001 (*n* = 7–8).

between pulmonary arteries and other arteries including the DA. The DA is known to be more sensitive than the adjunct arteries to changes in oxygen tension (29). It should be noted that the response to oxygen is stronger in the mature DA than in the premature DA (30). Our study also demonstrated that the response to U46619 was stronger in the mature DA than in the premature DA (Figure 1). Therefore, this characteristic may be responsible for the DA-specific constriction that results from TP stimulation. Further study is apparently required to understand the mechanism by which the DA is hypersensitive to TP stimulation.

In contrast to exogenous TP stimulation by U46619, our data showed that TP inhibition by the TP antagonist SQ29548

did not have a vasodilatory effect on the neonatal rat DA. Consistent with this observation, a previous study has demonstrated that a native TXA₂ was not synthesized in the DA under physiological conditions (31). In addition, no PDA phenotype has been identified in TP knockout mice to date. Taken together, the evidence suggests that endogenous TXA₂ and TP are likely to play minor roles in the physiological closure of the DA.

In conclusion, our results demonstrate that TP agonists are a selective and potent vasoconstrictor of the fetal and neonatal rat DA with minimal adverse effects when they are administered at a low dose (up to 0.05 µg/g). Although further investigation will be required to clinically use TP agonists for the patient with PDA, we propose that low-dose TP agonists may serve as a possible pharmacological therapeutic strategy for DA closure.

METHODS

Animal Preparation

All animals were cared for in compliance with the guidelines of the American Physiological Society. The experiments were approved by the Ethical Committee on Animal Experiments of Waseda University.

Generation of Premature or Hypoxia-Induced PDA Models

We established two types of PDA animal models: premature and hypoxia-induced PDA models. To establish a premature PDA model, we attempted to use Wistar rat fetuses that were delivered by cesarean section on e19. However, all of them died within 20 min after delivery as a result of respiratory distress. Therefore, we alternatively used Wistar rat fetuses delivered on e20 (PM20). Approximately 85% of PM20 rats could survive for at least 1 h after delivery. They showed a significant delay in closure of the DA 30 min after birth as compared with mature neonatal rats delivered on e21 (d0).

For hypoxia-induced PDA models, Wistar rat fetuses on e21 delivered by cesarean section were promptly placed in a hypoxic chamber with an oxygen concentration of 5% as soon as their respiration was established. All subsequent experiments were performed in the hypoxic chamber. PO₂ was measured with a PO₂ monitor (PO2-150D, Bioresearch Center, Tokyo, Japan) with the probe (polarographic oxygen electrodes: external diameter 0.2 mm) inserted into the subcutaneous tissues. We think that a hypoxia-induced PDA model is valuable because reopening of the DA is often observed in patients with hypoxia as a result of respiratory distress. In addition, a hypoxia-induced PDA model allows us to investigate the effect of TP stimulation on DA constriction in the absence of oxygen because oxygen is a potent vasoconstrictor of the DA. However, it should be noted that a hypoxia-induced PDA model is not clinically relevant to investigate the role of TP stimulation in patients with ductal-dependent cyanotic heart diseases.

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 (32). (i) For the experiments using fetuses, pregnant Wistar rats were anesthetized with isoflurane. Wistar rat fetuses at e19 and e21 were injected with the TP agonists U46619 (Cayman Chemical, Ann Arbor, MI) or I-BOP (Cayman Chemical) via uterine wall at various concentrations (up to 5.0 µg/g i.p.). For control groups, littermates were injected with the same volume of saline. After the injection, the mothers' abdomens were immediately closed and the mothers remained continuously anesthetized. The fetuses were delivered by cesarean section 30 min after the injection and were rapidly frozen in liquid nitrogen. The frozen neonates were cut on a freezing microtome in the frontal plane, and the inner diameters of the DAs, the aortas, and the pulmonary arteries were measured under a microscope. (ii) For the experiments

using neonates including premature and hypoxia-induced PDA models, Wistar rat fetuses were delivered by cesarean section. When the neonates were in a stable respiratory condition, they were intraperitoneally injected with U46619 (0.0005, 0.05, or 5.0 µg/g) or indomethacin (Merck & Co., Whitehouse Station, NJ) (10 µg/g) 20 min after delivery. Especially for premature models, neonates were frozen 10, 20, and 30 min after the injection. (iii) For experiments examining the effect of TP inhibition on rat neonatal DA, the TP antagonist SQ29548 (Cayman Chemical) (1.0 or 10 µg/g) or prostaglandin E₂ (Sigma-Aldrich, St Louis, MO) was injected into rat neonates 20–40 min after delivery; these neonates were then rapidly frozen 15, 30, 60, 90, or 120 min after injection. Neonates were kept at 37°C with sufficient humidity before and after injection.

Isometric Tension of the DA and Aorta Vascular Rings

Isometric tension of the vascular rings of the DA and the aorta at e19 and e21 was measured as previously described (33). After the resting tension was adjusted to 0.30 mN, U46619 was added in the perfusion solution.

Peripheral Blood Flow in Neonatal Rats

The d0 rats were intraperitoneally injected with saline, then with U46619 (0.0005, 0.05, or 5.0 µg/g) 5 min later. Peripheral blood flow in the tail was measured with a laser speckle perfusion imager (MoorFLPI, Moor Instruments, Axminster, UK). During blood flow measurement, each d0 rat was fixed on a hot plate to maintain its body temperature.

Microthrombosis in the Pulmonary Capillary Arteries

Lung tissues from PM20 rats injected with U46619 (0.05 µg/g) or arachidonic acid (Sigma-Aldrich) (100 µg/g) were embedded in paraffin. Paraffin-embedded blocks containing tissues were prepared as previously described (34). These paraffin-embedded blocks were cut into 5-µm-thick sections and placed on glass slides. Slides were stained with hematoxylin and eosin. We counted the total capillary arteries and the arteries with microthrombosis.

Quantification of TP mRNA and Protein

The amounts of TP mRNA and protein were determined using quantitative RT-PCR and western blot, respectively. The procedure is detailed in the **Supplemental Methods** online.

Statistics

Data are presented as mean ± SEM of independent experiments. Statistical analysis was performed among multiple groups by one-way ANOVA followed by Neuman–Keuls multiple-comparison test. A *P* value <0.05 was considered significant.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/pr>

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