freezing method (arrow). (D) Vasodilatory effect of milrinone on rat DA. Rat neonates were intraperitoneally injected with olprinone (n = 4–6). (E) Representative images of rat DAs treated with 5 mg/kg of olprinone or control for 2 h using the whole-body freezing method (arrow). (F) Milrinone or olprinone dilated DA in a dose-dependent manner. Vasodilatory effects of PDE3 inhibitors were examined 2 h after injection (n = 4–6). *** $p < 0.001$ and NS vs. control. NS indicates not significant.

Figure 3. Effects of PDE3 inhibitors and PGE_1 on respiratory distress. (A) Respiratory rate of rat neonates administered each drug immediately after birth, the same as in Figure 2 (n = 6–9). (B) Respiratory rate of rat neonates administered each drug 2 h after birth (n = 4). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. control. No mark indicates not significant vs. control.

Figure 4. Milrinone increased cAMP production, however, it did not induce HA production. (A) Milrinone (10 μM) significantly increased cAMP accumulation in DASMCs (n = 4). (B) HA production in SMCs treated with milrinone (10 μM), cilostazol (10 μM), rolipram (10 μM), PGE_1 (1 μM), or PGE_2 (1 μM) (n = 4–6). Cilostazol: PDE3 inhibitor. Rolipram: PDE4 inhibitor. **$p < 0.01$ and ***$p < 0.001$ vs. control. No mark

indicates not significant vs. control.

Figure 5. Milrinone did not promote migration and proliferation in SMCs. (A) Migration of SMCs treated with milrinone (10 μM), PGE_1 (1 μM), or PDGF-BB (10 ng/ml) using the Boyden chamber method (n = 4–5). (B) Proliferation of SMCs treated with milrinone (10 μM) or PGE_1 (1 μM) in the presence of 0 or 10% FBS by an MTT assay (n = 5–9). $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. NS indicates not significant.

Figure 6. Effect of co-treatment of HA with milrinone on migration and proliferation in DASMCs. (A) Migration of SMCs with co-treatment of HA (200 ng/ml) and milrinone (10 μM) using the Boyden chamber method (n = 4–5). (B) Proliferation of SMCs with co-treatment of HA (200 ng/ml) and milrinone (10 μM) in the presence of 0 or 10% FBS by an MTT assay (n = 8). $**p < 0.01$, NS indicates not significant.

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Fig. 1

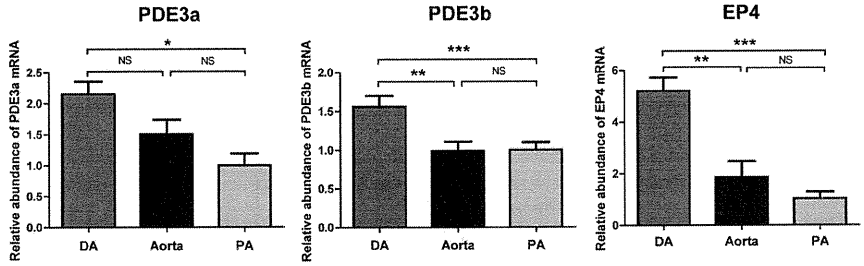

Figure 1. Quantitative RT-PCR analyses of PDE3a, PDE3b, and EP4 in rat e21 DA, aorta, and pulmonary artery (PA) tissue. n = 4–5, *p < 0.05, **p < 0.01, ***p < 0.001, NS indicates not significant.
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Fig. 2

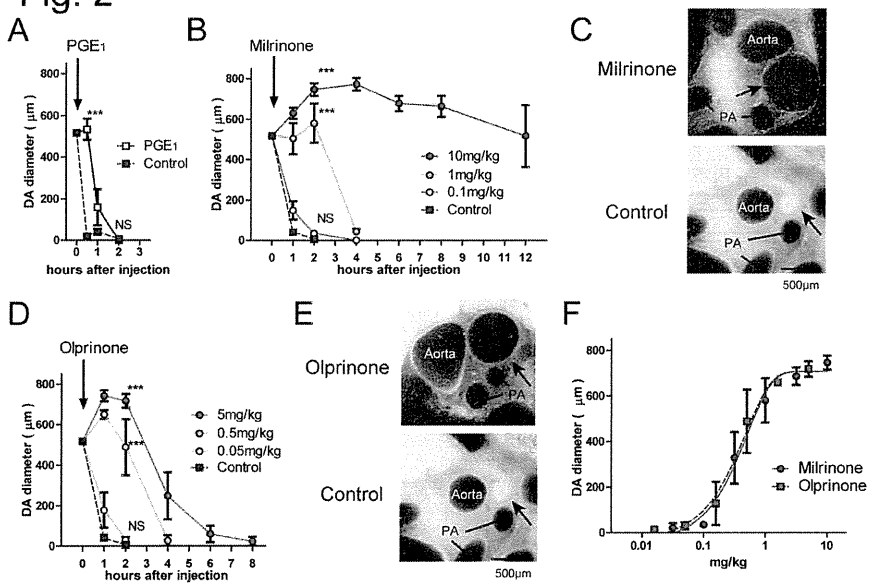
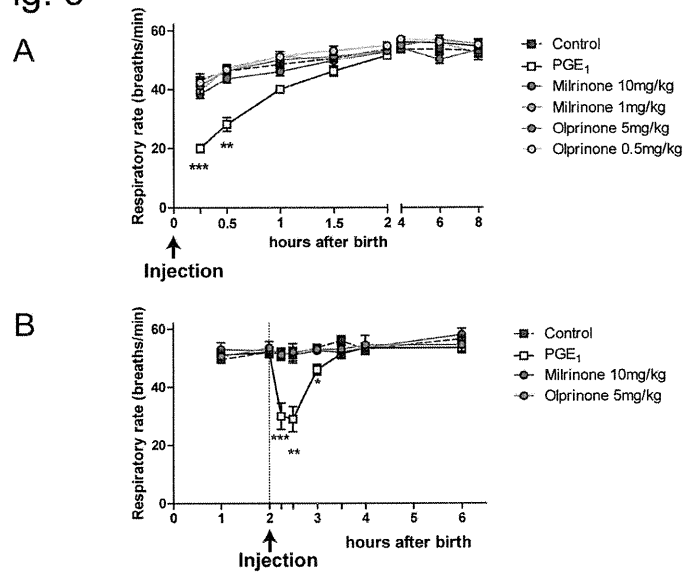


Figure 2. The effects of milrinone and olprinone on vasodilation of the DA as observed by the rapid whole-body freezing method. (A) PGE1 (10 μg/kg)-induced dilation of rat DA (n = 4–6). (B) Vasodilatory effect of milrinone on rat DA. Rat neonates were intraperitoneally injected with milrinone (n = 4–6). (C) Representative images of rat DAs treated with 10 mg/kg of milrinone or saline (control) for 2 h using the whole-body freezing method (arrow). (D) Vasodilatory effect of milrinone on rat DA. Rat neonates were intraperitoneally injected with olprinone (n = 4–6). (E) Representative images of rat DAs treated with 5 mg/kg of olprinone or control for 2 h using the whole-body freezing method (arrow). (F) Milrinone or olprinone dilated DA in a dose-dependent manner. Vasodilatory effects of PDE3 inhibitors were examined 2 h after injection (n = 4–6). *** $p < 0.001$ and NS vs. control. NS indicates not significant.
303x216mm (150 x 150 DPI)

Fig. 3



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303x216mm (150 x 150 DPI)

Fig. 4

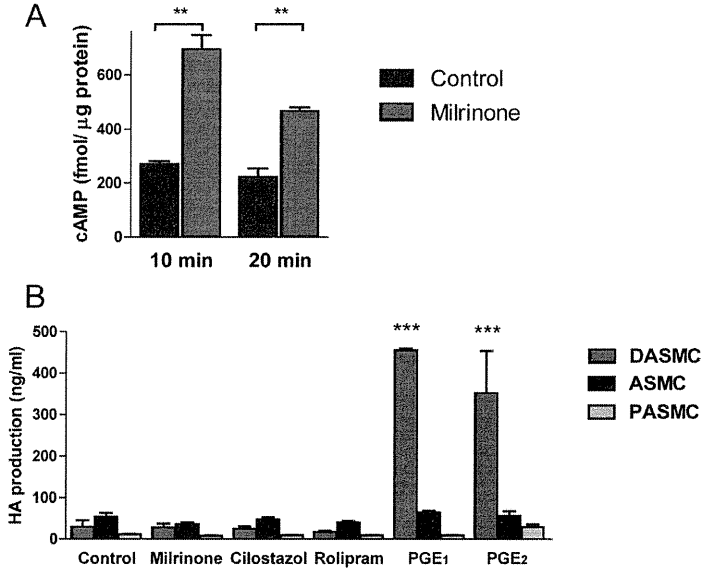

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Fig. 5

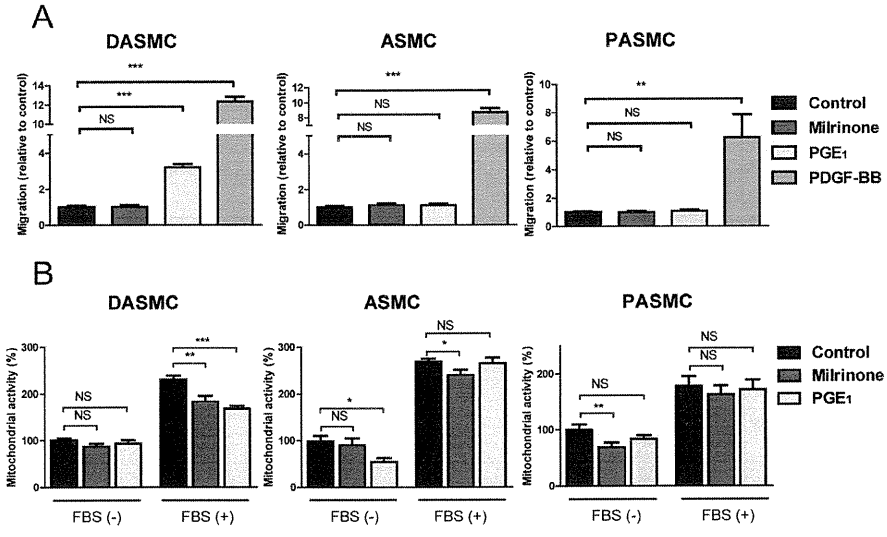

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Fig. 6

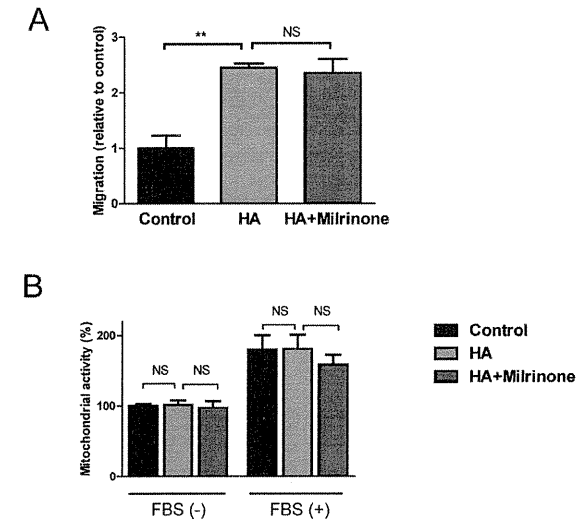

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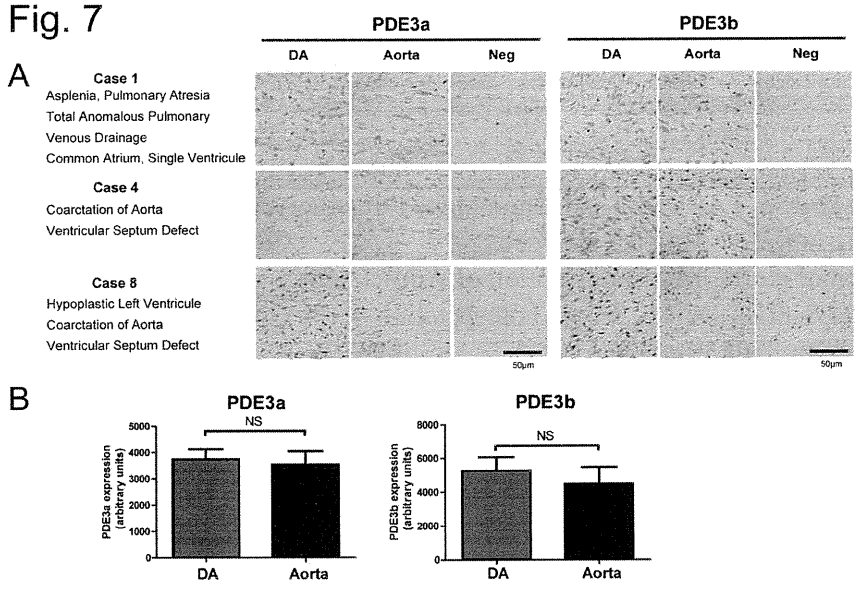
Fig. 7



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303x216mm (150 x 150 DPI)

Table 1.

Summary of patient characteristics

Case No.	Age at Operation	Diagnosis
1	0 days	Asplenia, PA, TAPVD, CA, SV
2	1 day	Asplenia, CoA, CA, SV
3	2 days	IAA, Aorticopulmonary window
4	2 days	CoA, VSD
5	3 days	TGA, CoA
6	4 days	CoA, VSD
7	13 days	CoA, VSD
8	1 month	hypoLV, CoA, VSD

PA: Pulmonary Atresia, TAPVD: Total Anomalous Pulmonary Venous Drainage,
CA: Common Atrium, SV: Single Ventricule,
CoA: Coarctation of Aorta, IAA: Interruption of Aortic Arch,
VSD: Ventricular Septum Defect, TGA: Transposition of the Great Arteries,
hypoLV: Hypoplastic Left Ventricule

Low-dose Thromboxane A_2 Receptor Stimulation Promotes Closure of the Rat Ductus Arteriosus with Minimal Adverse Effects

Running title: **TXA_2 Receptor in Ductus Arteriosus**

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Category of study: 1) basic science

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Abstract

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 E_2, the most potent vasodilator in the ductus arteriosus (DA), their efficacy is often limited. Since Thromboxane A_2 (TXA_2) induces vascular contraction via TXA_2 receptor (TP), we hypothesized that TP stimulation promoted DA closure. **Methods** and **Results** The selective TP agonists U46619 and I-BOP constricted the fetal DA at embryonic day 19 (e19) and e21 in a dose-dependent manner. Importantly, 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 *exo vivo* DA ring stronger than it constricted the *exo vivo* aorta. Furthermore, we found that U46619 at lower concentrations (up to 0.05 μg/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 be a motive for an alternative potent vasoconstrictor for PDA.

Introduction

The ductus arteriosus (DA) is an essential vascular shunt connecting the aorta and the pulmonary artery (PA) 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 weeks 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 20-40% in premature infants[1, 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 as a safe procedure in clinical studies[5], it has been reported that neurosensory impairment, bronchpulmonary dysplasia, and severe retinopathy are more common after surgery[6]. Therefore, an alternative pharmacological strategy for PDA treatment is required.

Thromboxane A_2 (TXA_2) is a lipid mediator that exhibits diverse physiological and pathological effects. In term of cardiovascular effects, TXA_2 is known to be a strong vasoconstrictor and 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 H_2 (PGH_2), which, in turn, is converted to TXA_2 by thromboxane synthase[7]. TXA_2 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. and Reese et al. demonstrated that TP stimulation constricted rabbit and mouse DA explants, respectively[8, 9], whereas Coceani et al. demonstrated that it exerted no vasoconstrictive effect on lamb DA[10]. Loftin et al., 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[11]. Therefore, we undertook to evaluate whether TP stimulation was also effective against other PDA models and to assess its adverse effects.

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 embryonic day 19 (e19). However, all of them died within 20 minutes after delivery due to respiratory distress. Therefore, we alternatively used Wistar rat fetuses delivered on e20 (premature neonate: PM20). Approximately 85% of PM20 could survive for at least one hour after delivery. They showed a significant delay in closure of the DA 30min after birth when compared with mature neonatal rats delivered on e21 (mature neonate at day 0: d0).

For hypoxic-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 has been established. All subsequent experiments were performed in the hypoxic chamber.

P_{O2} was measured with a P_{O2} monitor (PO2-150D, Bioresearch Center, Tokyo) with the probe (polarographic oxygen electrodes: external diameter 0.2mm) 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 due to 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[12]. 1) For the experiments using fetuses, pregnant Wistar rats were anesthetized with isoflurane. Wistar rat fetuses at e19 and e21 were intraperitoneally injected with the TP agonists U46619 (Cayman Chemical, Ann Arbor, MI) or I-BOP (Cayman Chemical) via uterine wall at various concentrations (up to 5.0μg/g). 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 30min 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. 2) For the experiments using neonates including premature and hypoxia-induced PDA models, Wistar rat fetuses were delivered by cesarean section. When neonates were in a stable respiratory condition, they were intraperitoneally injected with U46619 (0.0005, 0.05, or 5.0μg/g) or indomethacin (Merck & Co., Inc., Whitehouse Station, NJ) (10μg/g) 20min after delivery. Especially for premature models, neonates were frozen 10, 20, and 30min after the injection. 3) For experiments examining the effect of TP inhibition on rat neonatal DA, the TP antagonist SQ29548 (Cayman Chemical) (1.0 or 10μg/g) or PGE_2 (Sigma-Aldrich, St. Louis, MO) was injected into rat neonates 20-40min after delivery; these neonates were then rapidly frozen 15, 30, 60, 90, or 120min 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[13]. After the resting tension was adjusted to 0.30mN, U46619 was added in the perfusion solution.