Fig. 5

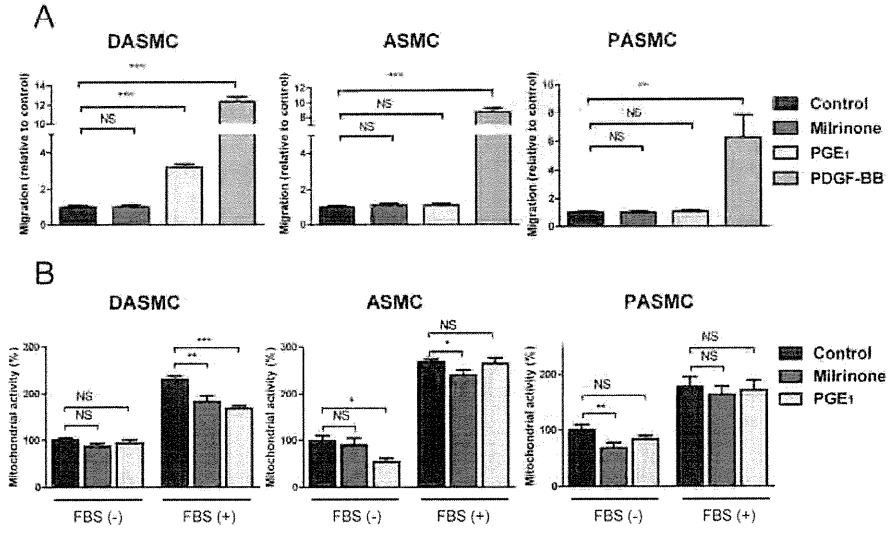

Figure 5. Milrinone did not promote migration and proliferation in SMCs. (A) Migration of SMCs treated with milrinone (10 μM), PGE1 (1 μM), or PDGF-BB (10 ng/ml) using the Boyden chamber method (n = 4–5). (B) Proliferation of SMCs treated with milrinone (10 μM) or PGE1 (1 μM) in the presence of 0 or 10% FBS by an MTT assay (n = 5–9). *p < 0.05, **p < 0.01 and ***p < 0.001. NS indicates not significant.
303x216mm (150 x 150 DPI)

Fig. 6

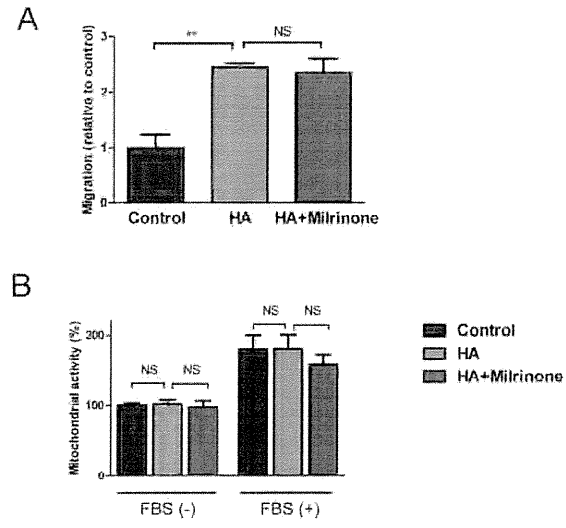

Figure 6. Effect of co-treatment of HA with milrinone on migration and proliferation in DASMCs. (A) Migration of SMCs with co-treatment of HA (200 ng/ml) and milrinone (10 μM) using the Boyden chamber method (n = 4–5). (B) Proliferation of SMCs with co-treatment of HA (200 ng/ml) and milrinone (10 μM) in the presence of 0 or 10% FBS by an MTT assay (n = 8). **p < 0.01, NS indicates not significant.
303x216mm (150 x 150 DPI)

Fig. 7

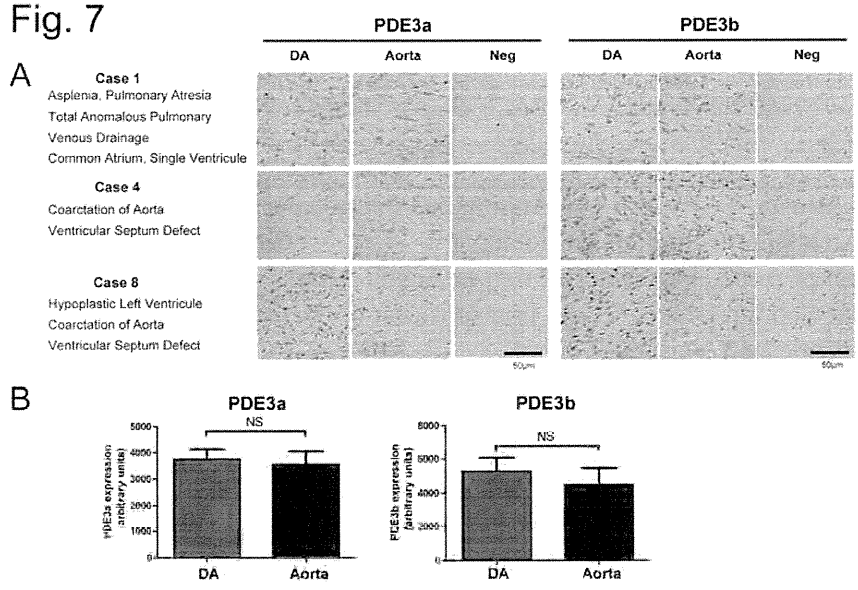

Figure 7. (A) Representative images of immunoreaction to PDE3a and PDE3b in the human DA and aortic smooth muscle layers from various CHDs. No immunoreaction was detected when omitting the primary antibody as in PDE3a Neg and PDE3b Neg. (B) Quantification of PDE3a and PDE3b in the DA and the aorta by a color extraction method (n = 4). NS indicates not significant.
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**

Tomohiro Yokota[1)], Takashi Aida[1)], Yasuhiro Ichikawa[2)], Takayuki Fujita[3)], Utako Yokoyama[2)], Susumu Minamisawa[1)]

1) Department of Life Science and Medical Bioscience, Waseda University Graduate School of Advanced Science and Engineering, Tokyo 162-8480, Japan

2) Cardiovascular Research Institute, Yokohama City University Graduate School of Medicine, Kanagawa 236-0004, Japan

3) Department of Medical Science and Cardiorenal Medicine, Yokohama City University Graduate School of Medicine, Kanagawa 236-0004, Japan

Corresponding author: Susumu Minamisawa[1)] M.D./Ph.D.
TWIns C224, 2-2 Wakamatsu-cho, Shinjuku-ku, Tokyo 162-8480, Japan
Fax: +81-3-5369-7022
Telephone: +81-3-5369-7322
sminamis@waseda.jp

Statement of financial support: This work was supported by grants from the Ministry of Education,

Culture, Sports, Science and Technology of Japan (U.Y., S.M.), Foundation for Growth Science (S.M.), the Yokohama Foundation for Advanced Medical Science (U.Y., S.M.), the "High-Tech Research Center" Project for Private Universities: MEXT (S.M.), the Vehicle Racing Commemorative Foundation (S.M.), Miyata Cardiology Research Promotion Founds (U.Y., S.M.), Takeda Science Foundation (U.Y., S.M.), the Japan Heart Foundation Research Grant (U.Y.), the Kowa Life Science Foundation (U.Y.), the Sumitomo Foundation (U.Y), Japan Cardiovascular Research Foundation (S.M.), and the Uehara Memorial Foundation (U.Y.) .

Category of study: 1) basic science

Word count of abstract: 190 words

Word count of manuscript: 4991 words

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.

Peripheral blood flow in neonatal rats

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

Microthrombosis in the pulmonary capillary arteries

Lung tissues from PM20 injected with U46619 (0.05μg/g) or arachidonic acid (AA) (Sigma-Aldrich) (100μg/g) were embedded in Paraffin. Paraffin-embedded blocks containing tissues were prepared as previously described[14]. 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.

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 of less than 0.05 was considered significant.

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 PGH_2 analogue and I-BOP, a TXA_2 analogue. Both are commonly used as selective TP agonists[7, 15]. Consistent with the previous *in vivo* study by Loftin et al.[11], when U46619 was intraperitoneally injected into the fetal rats at e19 and e21, the DA was significantly constricted in a dose-dependent manner (**Figure 1A-G**). The constriction of the DA by U46619 was greater at e21 than at e19, even though the levels of circulating PGE_2 are supposed to be higher during late gestation (**Figure 1A-G**). Whereas very low-dose of U46619 such as 0.005μg/mg did not showed significant constriction of the DA at e19, effect of U46619 at e21 showed significant constriction even in 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 hypoxic-induced PDA models. Twenty minutes after delivery, we intraperitoneally injected various doses of U46619 (0.0005, 0.05, and 5.0μg/g) into PM20. The diameter of the DA was measured 10, 20,

and 30min after injection. U46619 at concentrations of 0.05 and 5.0μg/g significantly constricted the premature DA when compared with saline-injection (**Figure 2A**). It should be noted that 75% and 100% of the DA were completely closed 30min after the injection at concentrations of 0.05 and 5.0μg/g, respectively (**Table**).

Regarding hypoxic-induced PDA models, P_{O2} was lower in rats under a hypoxic condition than under a normoxic condition (19.3 ± 1.5 versus 56.2 ± 3.8mmHg, respectively. $p<0.0001$, n=4-7). Under normoxic conditions, within 30min 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 30min after birth. Ten minutes after injection (30min after birth) we found that U46619 at a concentration of 5.0μg/g significantly constricted the hypoxic DA when compared with saline injection (**Figure 2B**). In addition, indomethacin at a concentration of 10μg/g constricted the hypoxic DA by ~70% of the DA diameter compared with saline-injection. These results indicated that TP stimulation effectively constricted the DA in two different PDA models.

TP stimulation did not constrict other vessels

We assessed whether U46619 constrict other vessels such as the aorta, the PA, the vertebral

artery, the renal artery, the portal vein, and the marginal artery of the colon (MA) at d0. U46619 at concentrations of up to 0.05μg/g had no significant vasoconstrictive effect on these vessels (**Figure 3A-G**). However, U46619 at concentration of 5.0μg/g significantly constricted the MA (**Supplemental figure 1**), but not other vessels at e21. In addition the aorta and the PA in e19 did not responded to U46619 at concentrations of up to 5.0μg/g (**Figure 3A, B**). These data suggested that the fetal and neonatal DA responded much to U46619 rather than other vessels.

Low-dose of U46619 did not decrease peripheral blood flow in neonatal rats

Though U46619 did not induce vasoconstriction in large arteries, it should be evaluated 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[16]. Up to a concentration of 0.05μg/g, U46619 did not decrease the peripheral blood flow in the tail (**Figure 4A-G**). However, if the concentration of U46619 was increased to 5.0μg/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 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}M developed isometric tension stronger 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 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 0.05μg/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[17], AA induced significant thrombosis in rat lungs (**Figure 6C**), When we counted 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 AA-injected (**Figure 6D**).

TP inhibition did not dilate the neonatal DA

To clarify the contribution of endogeneous TXA_2 to DA closure, we assessed whether TP inhibition made the closed DA reopened after birth or not. First, to determine the dose of a selective TP

antagonist, SQ29548, we pretreated with SQ29548 (1μg/g) into fetus at e21 10min before injecting U46619. Therefore, SQ29548 at concentration of 1μg/g is sufficient to inhibit the U46619-mediated DA constriction (**Figure 7A**). To observe whether SQ29548 prevents closure of the DA nor not after birth, SQ29548 was injected 40min after birth when the DA is still closing. We found that SQ29548 at concentrations of up to 10μg/g also showed no dilative effect on the DA after birth (**Figure 7B, C**). On the other hand, PGE_2 (15ng/g) significantly dilated the DA until 60min after injection.

Discussion

The present study demonstrated that TP stimulation potently constricted the *in vivo* DA in the following subjects: 1) rat fetuses at e19 and e21; 2) premature rat neonates delivered at e20; 3) mature rat neonates under hypoxic conditions (5%O_2). These results are consistent with the previous study by Loftin et al. using Cox-1/-2 knockout mice with PDA[11]. Previous *ex vivo* studies have identified 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 Ca^{2+} influx through L-type Ca^{2+} channels and the latter is regulated by Rho/Rho-kinase activity[18, 19].

To apply TP stimulation for patients with PDA as a potential alternative pharmacological therapy, it is important to estimate its possible adverse effects, because TXA_2 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 0.05μg/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 5.0μg/g significantly constricted the marginal arteries of the colon and reduced blood flow in the tail (**Supplemental figure 1**). Because continuous U46619 infusion is known to decrease cardiac output[20], the reduction in peripheral circulation may be not only due to vascular constriction but also a decrease in cardiac out by U46619 at a concentration of 5.0μg/g. Further study will be required whether a decrease in cardiac out is responsible for the U46619-mediated reduction in peripheral circulation.

Next, microthrombosis in the pulmonary capillary arteries is expected to be one of the worst adverse effects of U46619. We did not find significant microthrombosis in the pulmonary capillary arteries when U46619 at a concentration of 0.05μg/g was administered into PM20 rats (**Figure 6**). A number of studies have demonstrated that a relatively high dose of U46619 (e.g. 1.0mg/kg, i.v.) causes a shock syndrome resulting in sudden death due to systemic platelet aggregation, pulmonary thrombosis, and coronary spasm in adult animals[21-23]. However, neonatal platelets are known to be less reactive than adult platelets to U46619, thrombin, and ADP/epinephrine[24]. Therefore, thromboembolism may be avoidable when a low dose (up to 0.05μg/g) of U46619 is administered in