Abstract

Background: Prostaglandin E_1 (PGE_1), via cAMP, dilates the ductus arteriosus (DA). For patients with DA-dependent congenital heart diseases (CHDs), PGE_1 is the sole DA dilator that is used until surgery. However, PGE_1 has a short duration of action and frequently induces apnea. Most importantly, PGE_1 increases hyaluronan (HA) production, leading to intimal thickening (IT) and eventually DA stenosis after long-term use. In this study, therefore, we explored potential DA dilators, such as phophodiesterase 3 (PDE3) inhibitors, as alternatives to PGE_1.

Methods and Results: Expression levels of PDE3a and PDE3b mRNAs in rat DA tissues were higher than those in the PA. Intraperitoneal injection of milrinone (10 or 1 mg/kg) or olprinone (5 or 0.5 mg/kg) induced maximal dilatation of the DA lasting for up to 2 hours in rat neonates. In contrast, vasodilation induced by PGE_1 (10 μg/kg) was diminished within 2 hours. No respiratory distress was observed with milrinone or olprinone. Most important, milrinone did not induce HA production, cell migration, or proliferation when applied to cultured rat DA smooth muscle cells. Further, high expression levels of both PDE3a and PDE3b were demonstrated in the human DA tissues of CHD patients.

Conclusions: Because PDE3 inhibitors induced longer-lasting vasodilation without causing apnea or HA-mediated IT, they may be good alternatives to PGE_1 for patients with DA-dependent CHDs.

Word count: 217 words (abstract)

Key words: Ductus arteriosus, Milrinone, Phosphodiesterase, Congenital heart disease

Introduction

The ductus arteriosus (DA), the fetal arterial connection between the pulmonary artery and the descending aorta, is essential to maintain fetal life in utero. The DA closes after birth by two different mechanisms, namely, vasoconstriction and intimal thickening (IT) [1-3]. During the first few hours after birth, acute vasoconstriction occurs as a result of smooth muscle contraction in the DA. This is triggered by increased oxygen tension, due to the initiation of spontaneous breathing, and decreased circulating prostaglandin E_2 (PGE_2), due to disconnection from the placenta [3]. This functional vasoconstriction, however, must be preceded by intimal thickening of the DA, because vascular remodeling, including intimal thickening, is critical for anatomical closure of the DA.

The intimal thickening of DA is a result of many cellular processes, such as an increase in smooth muscle cell (SMC) migration and proliferation, the production of hyaluronan (HA) under the endothelial layer, and decreased elastin fiber assembly [1, 3, 4]. We have previously demonstrated that PGEs promoted HA production via cAMP/protein kinase A and subsequent SMC migration, resulting in intimal thickening of the DA during the late gestational period [1, 4, 5].

In patients with DA-dependent congenital heart diseases (CHDs), such as pulmonary atresia with intact ventricular septum or arch anomalies (coarctation of aorta or interruption of aortic arch), however, patent DA after birth is essential for survival. PGE_1 is widely used to keep the DA open as it increases intracellular cAMP and thus dilates the DA. However, PGE_1 induces hyaluronan (HA)-mediated intimal thickening and thus DA stenosis after prolonged use [6]. The fact that it induces only a very short duration of vasodilation, together with its severe adverse effects, such as apnea, respiratory distress, and hypotension, present additional problems, making it difficult for some patients with CHD to continue the use of PGE_1 until surgery. As such, possible alternatives to PGE_1 need to be explored.

Phosphodiesterases (PDEs), which catalyze the hydrolysis of cAMP/cGMP, constitute a superfamily of at least 11 gene families (PDE1–PDE11) [7]. The two PDE3 subfamilies, PDE3A and PDE3B, are encoded by closely related genes [8], and both hydrolyze cAMP. PDE3 inhibitors have been approved by the U.S. Food and Drug Administration (FDA) for use as vasodilators as well as in heart failure. Two of these are milrinone and olprinone, which are widely used to treat heart failure [9-12] and persistent pulmonary hypertension in neonates [13, 14]. Previous studies have shown that the PDE3 inhibitors milrinone, amrinone, and cilostazol counteract indomethacin-induced DA contraction [15, 16]. Thus, PDE3 inhibitors alone may be sufficient to dilate the DA. Nevertheless, it remains undetermined whether they induce intimal thickening, which is a major problem with PGE_1, via HA production, cell migration, or cell proliferation. In the current study, we investigated the role of PDE3 inhibitors in DA vascular remodeling and vasodilation with a view to their potential use as alternatives to the current PGE therapy.

Materials and Methods

Animals and materials

Timed pregnant Wistar rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan). All animal studies were approved by the institutional animal care and use committees of Yokohama City University. Milrinone, PDGF-BB, MTT, trichloroacetic acid, and 10% buffered formalin were obtained from Wako (Osaka, Japan). Olprinone, cilostazol, rolipram, PGE_1, PGE_2, elastase type II, trypsin inhibitor, bovine serum albumin V, poly-L-lysine, penicillin-streptomycin solution, acetic anhydride, triethylamine, Dulbecco's modified Eagle's medium (DMEM), and Hank's balanced salt solution (HBSS) were purchased from Sigma-Aldrich (St Louis, MO). Collagenase II was purchased from Worthington Biochemical Corp. (Lakewood, NJ). Collagenase/dispase

was purchased from Roche Diagnostics (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from Equitech-Bio (Kerrville, TX).

Primary culture of rat smooth muscle cells (SMCs)

Vascular SMCs in primary culture were obtained from the DA (DASMCs), the aorta (ASMCs), and the pulmonary arteries (PASMCs) of Wistar rats on the 21st day of gestation. Isolation of DASMCs and ASMCs has been described previously [17]. To obtain PASMCs, the branch extralobular pulmonary arteries were dissected, cleaned from adherent tissue, and cut into small pieces. The tissues were transferred to a 1.5-ml centrifuge tube that contained 800 μl of collagenase-dispase enzyme mixture (1.5 mg/ml collagenase-dispase, 0.5 mg/ml of elastase type II-A, 1 mg/ml of trypsin inhibitor type I-S, and 2 mg/ml of bovine serum albumin fraction V in HBSS). Digestion was carried out at 37°C for 15 min. Cell suspensions were then centrifuged, and the medium was changed to a collagenase II enzyme mixture (1 mg/ml collagenase II, 0.3 mg/ml trypsin inhibitor type I-S, and 2 mg/ml bovine serum albumin fraction V in HBSS). After 12 min of incubation at 37°C, cell suspensions were transferred to growth medium in 35-mm poly-L-lysine-coated dishes in a moist tissue culture incubator at 37°C in 5% CO_2-95% ambient mixed air. The growth medium contained DMEM with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. We confirmed that >99% of cells were positive for α-smooth muscle actin and exhibited typical "hill-and-valley" morphology. Expression levels of PDE3, EP4, and prostacyclin (IP) receptor mRNAs in DASMCs, ASMCs, and PASMCs are shown in the Supplemental Fig. S1.

Human tissues from patients with CHDs

We obtained eight neonatal DAs and adjacent aortas during cardiac surgery in children between 0 days and 1 month of age. All excised tissue was fixed in 4% paraformaldehyde within 3 hours. The DA tissues were obtained from the Yokohama City University Hospital and Kanagawa Children's Medical Center. The study was

approved by the human subject committees at both Yokohama City University and Kanagawa Children's Medical Center. Detailed patient information is summarized in Table 1.

RNA isolation and quantitative RT-PCR

Pooled vascular tissues were obtained from Wistar rats on the 21st day of gestation. After excision, tissues were frozen in liquid nitrogen and stored at -80°C. The total RNA was isolated from the tissues using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions and from the cultures using Trizol reagent (Invitrogen, Carlsbad, CA). The primers were designed based on the rat nucleotide sequences of PDE3a (NM_017337) (5'- CGC CTG AGA AGA AGT TTG C -3' and 5'- AGA CAG CAT AGG ACG AAG TGA AG -3'), PDE3b (NM_017229.1) (5'- TCC AAA GCA GAG GTC ATC ATC -3' and 5'- GTA TCA AGA AAT CCT ACG GGT GA -3'), EP4 (NR_032076.3) (5'- CTC GTG GTG CGA GTG TTC AT -3' and 5'- AAG CAA TTC TGA TGG CCT GC -3'), and IP (NM_00177644.1) (5'- GGG CAC GAG AGG ATG AAG -3' and 5'- GGG CAC ACA GAC AAC ACA AC -3'). Reverse transcription polymerase chain reaction (PCR) was performed using a PrimeScript RT reagent Kit (TaKaRa Bio, Tokyo, Japan) and real-time PCR was performed using SYBR Green (Applied Biosystems, Foster City, CA). The abundance of each gene was determined relative to that in 18S ribosomal RNA.

Rapid whole-body freezing method

To study the in situ morphology and inner diameter of the neonatal DA, a rapid whole-body freezing method was used as previously described [2]. Fetuses on the 21st day of gestation were delivered by cesarean section and intraperitoneally injected immediately after birth with milrinone (10 mg/kg, 1 mg/kg, 0.1 mg/kg), olprinone (5 mg/kg, 0.5 mg/kg, 0.05 mg/kg), or PGE_1 (10 μg/kg). The rat pups were frozen in liquid nitrogen at 0, 0.5, 1, 2, 4, 6, 8, and 12 hours after injection. The frozen thoraxes were

then cut on a microtome, and the inner diameter of each DA was measured.

Determination of respiratory rate

Fetuses on the 21st day of gestation were delivered by cesarean section and intraperitoneally injected 0 or 2 hours after birth with milrinone (10 mg/kg, 1 mg/kg), olprinone (5 mg/kg, 0.5 mg/kg), or PGE_1 (10 μg/kg). We measured the respiratory rate by counting the movements of the rat thorax.

Quantitation of hyaluronan (HA)

The amount of HA in the cell culture supernatant was measured according to the latex agglutination method as previously described [1].

SMC migration assay

The migration assay was performed using 24-well transwell culture inserts with polycarbonate membranes (8-μm pores) (Corning Inc., Corning, NY) as previously described [1]. Cells were stimulated with milrinone (10 μM), PGE1 (1 μM), PDEF-BB (10 ng/ml), HA (200 ng/ml), or milrinone+HA for 3 days.

Cell proliferation assay

SMCs were cultured on 24-well plates at 1×10^5 cells per well in DMEM supplemented with 10% FBS. After various treatments over 3 days, 500 μl of 1 mg/ml MTT solution was added to each well and incubated for 2 hours. The supernatants were aspirated, and the formazan crystals in each well were solubilized with 0.05 M HCl (500 μl). Each solution (100 μl) was placed in a 96-well plate. SMC proliferation was measured based on absorbance at 570 nm using a microplate reader.

Immunohistochemistry

Immunohistochemical analysis was performed as previously described [1, 18]. Rabbit polyclonal anti-PDE3A antibody (sc-20792) and goat polyclonal anti-PDE3b antibody (sc-11835) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A color extraction method using BIOREVO bz-9000 (KEYENCE, Osaka, Japan) was performed

to quantify the expression of PDE3s in the DAs and the aortas of case 1, 4, 5, and 8 (Table 1). Eighteen fields in the smooth muscle layer of the DA and the aorta respectively were examined in four cases. Diaminobenzidine (DAB)-stained colors, PDE3a-positive or PDE3b-positive areas, were extracted and counted on the screen.

Cyclic AMP Production Measured by Radioimmunoassay

Measurement of cAMP accumulation in DASMCs was performed as previously described [2, 19]. Briefly, DASMCs grown on 24-well plates were serum-starved for 24 h and assayed for cAMP production after a 10- or 20-min period of incubation with 10 μM of milrinone. Reactions were terminated by aspiration of the media and the addition of 300 μl of ice-cold trichloroacetic acid (7.5%) to each well. Forty microliters of each sample were acetylated and incubated with ^{125}I-cAMP (Perkin Elmer, Waltham, MA) and 50 μl of rabbit anti-cAMP antibody (diluted 1:3000, Millipore, Billerica, MA) overnight at 4°C. Each mixture was then incubated with 50 μl of goat anti-rabbit antibody with magnetic beads (Qiagen, Valencia, CA) for 1 h. Separation of bound antibodies from free antibodies was achieved by filtration, and bound radioactivity was counted. Production of cAMP was normalized to the amount of protein per sample.

Statistics

Data are presented as means ± standard error of the mean (SEM) of independent experiments. Statistical analysis was performed between two groups by unpaired two-tailed Student's *t* test or unpaired *t* test with Welch's correction, and among multiple groups by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. A p value of <0.05 was considered significant.

Results

Messenger RNA of PDE3 isoforms was highly expressed in rat DA

We first examined whether the target molecule of PDE3 inhibitors is highly expressed in the DA. We measured the mRNA expression levels of PDE3s using quantitative RT-PCR in the rat DA, aorta, and pulmonary arteries (PA) on the 21st day of gestation (Figure 1). Expression of PDE3a mRNA was higher in the DA than in the PA. Expression of PDE3b mRNA was higher in the DA than in the aorta or the PA. We also confirmed that EP4 mRNA was more highly expressed in the DA than in the aorta or the PA. Thus, PDE3 isoforms were abundantly expressed in the DA relative to the PA.

Vasodilatory effects of PDE3 inhibitors on rat DA in vivo

PDE3 inhibitors are widely used in neonates and children with low cardiac output following myocarditis and cardiovascular surgery for congenital heart disease [20, 21]. We examined whether milrinone or olprinone dilated the DA using the rapid whole-body freezing method in rat neonates. Neonates were injected with one of these drugs immediately after birth to mimic the vasodilatory treatment currently used in DA-dependent congenital heart diseases.

Intraperitoneal injection of PGE_1 (10 μg/kg, the amount that is intravenously administered daily as a clinical maintenance dose) induced maximal dilatation of the DA for 30 min, but this effect was completely lost within 2 h after injection (Figure 2A). A single intraperitoneal administration of 10 mg/kg of milrinone maintained maximal dilation of the DA for up to 12 h (Figures 2B, 2C). 1 mg/kg of milrinone, the amount that is intravenously administered daily as a clinical maintenance dose, maintained maximal dilatation for 2 h, after which DA closure occurred at 4 h after injection. 0.1 mg/kg of milrinone did not affect DA tone. Both 5 mg/kg and 0.5 mg/kg of olprinone, the latter of which is suitable for daily intravenous administration as a clinical maintenance dose, induced maximal dilatation for 1 h after injection (Figures 2D, 2E). 0.05 mg/kg of olprinone did not dilate the DA. Thus, both milrinone and olprinone produced dose-dependent vasodilatory effects (Figure 2F), but those of milrinone lasted

longer.

PDE3 inhibitors did not induce respiratory distress

Since respiratory distress is a major adverse effect of PGE_1 [22], we examined whether PDE3 inhibitors cause respiratory distress. We counted the respiratory rate of rat neonates administered milrione, olprinone, or PGE_1. When rat neonates were administered each drug immediately after birth, PGE_1 significantly reduced the respiratory rate at 15 or 30 minutes after injection, whereas milrinone (1 and 10 mg/kg) and olprinone (0.5 and 5 mg/kg) did not induce respiratory distress up to 8 h after injection compared to the saline control (Figure 3A). To exclude the possibility that neonates administrated PGE_1 had a congenital respiratory problem, we examined the effect of drugs using a different injection timing. We confirmed that all rat neonates established normal breathing 1 h after birth, and then administrated each drug. PGE_1 significantly reduced the respiratory rate up to 1 h after injection. On the other hand, milrinone (10 mg/kg) and olprinone (5 mg/kg) did not affect the respiratory rate compared to the control (Figure 3B). These data suggest that PDE3 inhibitors did not cause respiratory distress.

Milrinone did not promote HA production or SMC migration and proliferation

Although it was previously suggested that PDE3 inhibitors induced vasodilation of the DA, it remained unknown whether they also induced IT formation, a key process in the anatomical closure of the DA. It is known that PGEs stimulate HA production along with increased DASMC migration through the action of HA as a potent trigger of cell migration. This is the major mechanism underlying the increase in intimal thickening induced by PGEs [1, 2, 5].

We thus examined whether a PDE3 inhibitor, milrinone, regulated HA production or SMC migration. First, we confirmed cAMP production in the presence of milrinone. Milrinone significantly increased cAMP accumulation in DASMCs at a

dosage of 10 μM, which also induced marked dilatation of DA explants [16] (Figure 4A). However, the same dosage of milrinone (10 μM) did not induce HA production in DASMCs (Figure 4B). We also confirmed that the PDE3 inhibitor cilostazol did not induce HA production in DASMCs. Similarly, PGE_1 (1 μM) induced DASMC migration; however, milrinone did not increase DASMC migration, as determined by the Boyden chamber method (Figure 5A). The cells used for these tests were sufficiently stimulated with PGE_1 to induce HA production and with PDGF-BB to induce migration. Next, we examined the effects of a PDE3 inhibitor on SMC proliferation, because SMC proliferation plays a role in IT formation of the DA [23, 24]. Milrinone and PGE_1 did not increase DASMC proliferation, as determined by MTT assays, in the presence of 0 or 10% FBS (Figure 5B). Moreover, we found that milrinone did not enhance HA-mediated migration in DASMCs (Figure 6A). Milrinone also did not affect proliferation in HA-treated DASMCs (Figure 6B). Similarly, in ASMCs and PASMCs, neither milrinone nor PGE_1 increased HA production or cell migration and proliferation (Figures 4B, 5A and 5B). These findings suggest that PDE3 inhibitors do not promote HA production or cell migration or proliferation, although they do produce cAMP and dilate the DA.

PDE3a and PDE3b were highly expressed in the smooth muscle layer in human DA tissues

The expression pattern of PDE3s in the human DA remains unknown. We examined PDE3a and PDE3b protein expression in the DA of eight patients with various CHDs, such as interruption of the aortic arch, complex aortic coarctation, hypoplastic left ventricle, and asplenia. The DA of all patients showed a strong immunoreaction for both PDE3a and PDE3b. Representative images are shown in Figure 7A. It has been demonstrated that PDE3a and PDE3b are abundantly expressed in the rat and human aorta [25, 26]. The expression of PDE3a and PDE3b in the DAs was equivalent to that in the adjacent aortas (Figure 7B). This demonstrates that PDE3s are abundantly

expressed in human patients with CHDs of the type that may require long-term vasodilatotherapy prior to surgery.

Discussion

The present study has demonstrated that the PDE3 inhibitors milrinone and olprinone dilate the DA without causing apnea and have a longer duration of action than PGE_1. These findings are expected to apply to human patients, given that PDE3s are abundantly expressed in the DA tissue of infants with CHD. Importantly, this study has shown for the first time that these PDE3 inhibitors do not promote HA production, cell migration, and cell proliferation in the DASMC, processes which potently induce intimal thickening and thus DA closure [1]. The PDE3 inhibitors are very unlikely to produce these unfavorable effects when used as DA dilators. Furthermore, these PDE3 inhibitors are already used in humans for other purposes [9, 10, 13, 14]. Accordingly, they may serve as useful alternatives to PGE_1, the current means of keeping the DA patent.

PGE_1 increases the production of cAMP by activating G protein and adenylyl cyclase [1, 2, 27]. In contrast, milrinone increases the concentration of cAMP by inhibiting its breakdown [7]. Although both drugs increase cAMP and dilate the DA, PGE_1 induces HA production and subsequent migration in DASMCs while milrinone does not. We do not know the molecular mechanism underlying this difference between PGE_1 and the PDE inhibitors. It can be tentatively speculated, however, that they differ in terms of intracellular localization and thus in terms of coupling with other molecules, as recent studies have suggested [28]. Regardless of the mechanisms involved, it is known that PGE_1 and PGE_2 both increase cAMP production and induce HA production via increased expression of HA synthase 2 [1, 5], and we found that a PDE4 inhibitor, rolipram,

did not induce HA production (Figure 4B). Alternatively, increases in cGMP, which is also induced by milrinone, may play a role. These issues need to be further investigated in future studies.

Previous studies effectively demonstrated the vasodilatory effects of the PDE3 inhibitors milrinone, amrinone and cilostazol on the rat or sheep DA that were contracted by indomethacin [15, 16]. In contrast, we have evaluated the effects of PDE3 inhibitors in the absence of indomethacin to examine the effects of PDE3 inhibitors in more relevant clinical settings. We also found, for the first time, that olprinone, a relatively new PDE3 inhibitor, dilates the DA. Our finding that these PDE3 inhibitors do not increase HA production is also novel, as this question had not been investigated previously.

The present study shows that milrinone does not induce SMC migration and proliferation in the DA (Figures 5, 6). Our findings are, at least in part, consistent with those obtained using vascular SMCs from non-DA vessels. PDE3 inhibitors have elsewhere been shown to reduce proliferation and migration of vascular SMCs and to decrease the accumulation of synthetic/activated vascular SMCs in the intimal layers of damaged blood vessels [7, 29, 30]. Similarly, in peripheral pulmonary arteries, PDE3 and PDE4 inhibition do not promote PASMC migration [31]. Furthermore, PDE3a deficiency caused G0/G1 cell cycle arrest in PDE3a knockout mice [8].

PGE_1 is currently the sole DA dilator, however, PGE_1-induced apnea or respiratory distress was noted in 18% of patients with congenital heart disease [32]. Respiratory depression was particularly common in infants weighing less than 2.0 kg at birth who received PGE_1 therapy (42%) [22]. The present study showed that milrinone and olprinone did not induce respiratory distress in rat neonates (Figure 3). Furthermore, no patient who caused apnea or respiratory distress with PDE3 inhibitors was reported in the previous clinical reports [9, 10, 13, 14]. Therefore, the PDE3 inhibitors are very

unlikely to produce an unfavorable effect on respiration when used as DA dilators. It should be noted that PDE3 inhibitors have adverse effects, such as arrhythmia or hypotension [33]. Milrinone reduces the risk of low cardiac output syndrome for some pediatric patients after congenital heart surgery; however, milrinone use is an independent risk factor for clinically significant tachyarrhythmias [34]. Although it was not feasible to examine arrhythmias and change in blood pressure in rat neonates in this study, careful further study is warranted to examine adverse effects.

It should be emphasized that both the PDE3a protein and the PDE3b protein were abundantly detected in the smooth muscle layer and the IT layer in all human DA samples tested, regardless of the patient's diagnosis or age at the time of operation (Figure 7). A previous study demonstrated that PDE3 inhibitors prevented DA closure in premature infants with persistent pulmonary hypertension [15, 35, 36]. Together with these findings, those of the present study suggest that PDE3 inhibitors can dilate the DA without inducing intimal thickening, and that they may serve as alternatives to PGE_1, the current DA vasodilator used for patients with DA-dependent CHDs.

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Figure Legends

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.

Figure 2. The effects of milrinone and olprinone on vasodilation of the DA as observed by the rapid whole-body freezing method. (A) PGE_1 (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