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

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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

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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 PGE1. 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 PGE1, the current means of keeping the DA patent.

PGE1 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, PGE1 induces HA production and subsequent migration in DASMCs while milrinone does not. We do not know the molecular mechanism underlying this difference between PGE1 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 PGE1 and PGE2 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,

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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

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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) 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

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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

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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.

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

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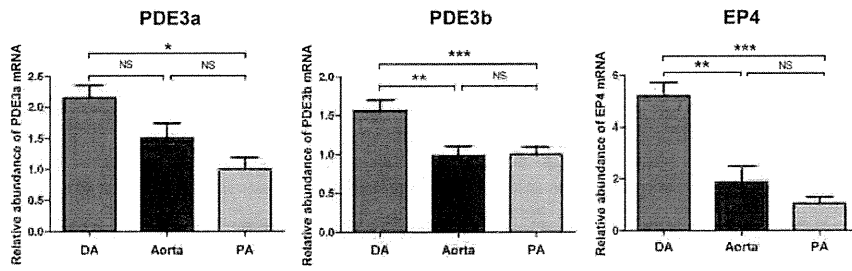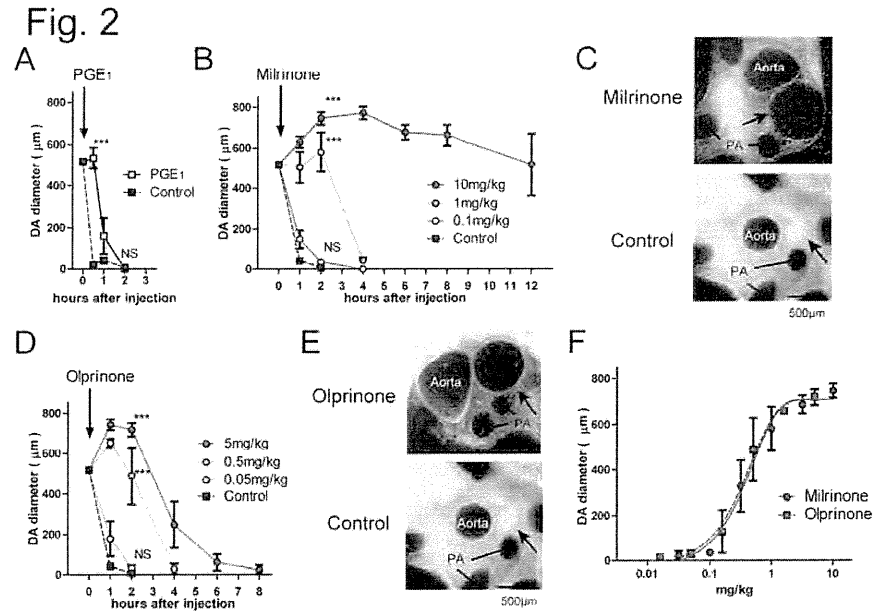49
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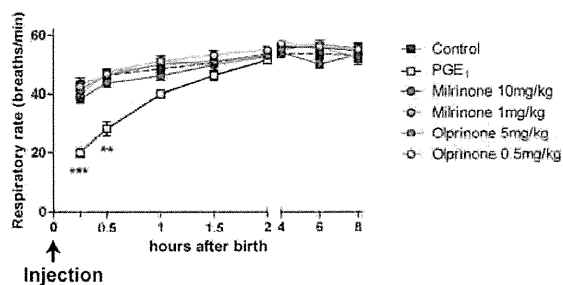56
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Fig. 1

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.
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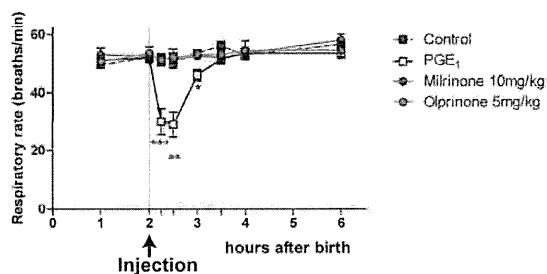46
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Fig. 3

Figure 3. Effects of PDE3 inhibitors and PGE1 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.
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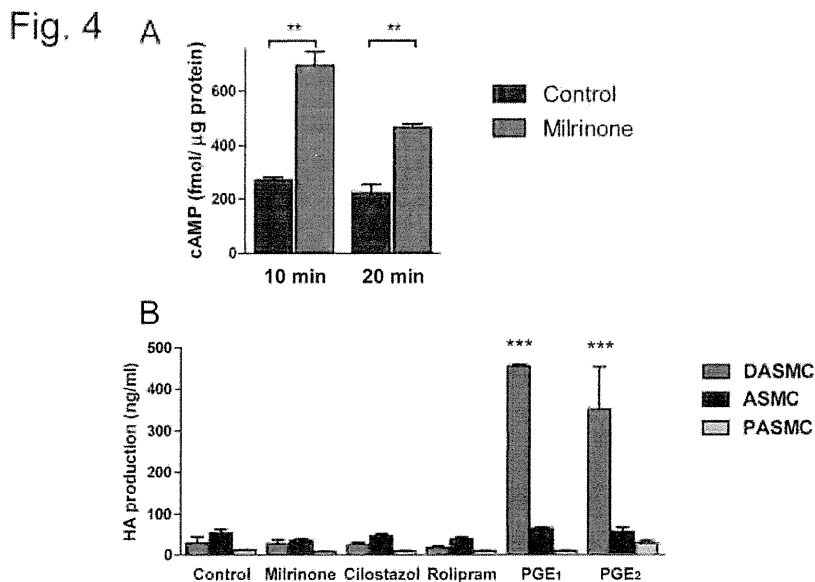51
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Figure 4. Milrinone increased cAMP production, however, it did not induce HA production. (A) Milrinone (10 μM) significantly increased cAMP accumulation in DASMCs (n = 4). (B) HA production in SMCs treated with milrinone (10 μM), cilostazol (10 μM), rolipram (10 μM), PGE1 (1 μM), or PGE2 (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.
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