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ORIGINAL ARTICLE

Colforsin-induced vasodilation in chronic hypoxic pulmonary hypertension in rats

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Abstract
Purpose Colforsin, a water-soluble forskolin derivative, directly activates adenylate cyclase and thereby increases the 3′,5′-cyclic adenosine monophosphate (cAMP) level in vascular smooth muscle cells. In this study, we investigated the vasodilatory action of colforsin on structurally remodeled pulmonary arteries from rats with pulmonary hypertension (PH).
Methods A total of 32 rats were subjected to hypobaric hypoxia (380 mmHg, 10% oxygen) for 10 days to induce chronic hypoxic PH, while 39 rats were kept in room air. Changes in isometric force were recorded in endothelium-intact (+E) and -denuded (−E) pulmonary arteries from the PH and control (non-PH) rats.
Results Colforsin-induced vasodilation was impaired in both +E and −E arteries from PH rats compared with their respective controls. Endothelial removal did not influence colforsin-induced vasodilation in the arteries from control rats, but attenuated it in arteries from PH rats. The inhibition of nitric oxide (NO) synthase did not influence colforsin-induced vasodilation in +E arteries from controls, but attenuated it in +E arteries from PH rats, shifting its concentration–response curve closer to that of −E arteries from PH rats. Vasodilation induced by 8-bromo-cAMP (a cell-permeable cAMP analog) was also impaired in −E arteries from PH rats, but not in +E arteries from PH rats, compared with their respective controls.
Conclusions cAMP-mediated vasodilatory responses without β-adrenergic receptor activation are impaired in structurally remodeled pulmonary arteries from PH rats. In these arteries, endothelial cells presumably play a compensatory role against the impaired cAMP-mediated vasodilatory response by releasing NO (and thereby attenuating the impairment). The results suggest that colforsin could be effective in the treatment of PH.

Keywords Pulmonary hypertension · Hypoxia · Colforsin · Nitric oxide · Endothelium

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Introduction

3′,5′-Cyclic adenosine monophosphate (cAMP) has been implicated in the control of pulmonary vascular tone [1, 2]. Patients and animal models with chronic pulmonary hypertension (PH) develop pulmonary vascular changes, such as endothelial injury, medial hypertrophy of muscular arteries, and new muscularization of normally nonmuscular peripheral arteries [3–7]. These structurally remodeled pulmonary arteries may respond to cAMP-mediated vasodilatory drugs differently than normal pulmonary arteries.

Indeed, the vasodilator response to β-adrenoceptor agonists (i.e., isoproterenol), which is believed to be mediated mainly by an increase in the level of cAMP in vascular smooth muscle (VSM) cells, was found to be impaired in the isolated pulmonary arteries of chronic hypoxic PH rats [4, 5]. However, the vasodilator response to dibutyryl cyclic AMP (DBcAMP), a membrane-permeable cAMP analog, was found not to be impaired in pulmonary arteries isolated from chronically hypoxic PH rats [6]. Thus, the impairment [4, 5] might be attributable, at least in part, to decreases in adenylate cyclase activity and the number of β-adrenoceptors, both of which were previously identified in VSM cells subjected to prolonged hypoxia [8, 9]. However, it might also be attributable to impaired endothelial function, because besides the increase in cAMP level in VSM cells, nitric oxide (NO) released from vascular endothelial cells may be involved in β-adrenoceptor-mediated vasodilation [10–13]. However, less is known about the role of endothelium in impaired β-adrenoceptor- or cAMP-mediated vasodilation. In addition, less is known about the impact of chronic hypoxic PH on cAMP-mediated vasodilation without β-adrenoceptor activation.

Colforsin, a water-soluble forskolin derivative, directly activates adenylate cyclase (without activating the β-adrenoceptors), and thereby increases the cAMP level in cardiac and VSM cells. The increases in cAMP level in cardiac and VSM cells lead to an increase in cardiac contractility and a decrease in vascular tone, respectively. Thus, colforsin is expected to increase cardiac output by increasing ventricular contractility, as well as by decreasing both venous and arterial tones (i.e., preload and afterload). Indeed, colforsin is considered for the treatment of acute heart failure in a clinical setting [14–16]. However, although colforsin was shown to attenuate PH in previous animal studies [17], its clinical usefulness in the treatment of PH is yet to be established.

Colforsin has been shown to cause vasodilation in various vascular beds, including coronary [14], cerebral [18, 19], internal mammary [20], cortical renal [21], and pulmonary [17] vasculatures. However, less information is available regarding its direct action on pulmonary arteries that have been structurally remodeled by exposure to chronic hypoxia and hence associated with the development of PH. Thus, this study was designed to investigate the direct action of colforsin on structurally remodeled pulmonary arteries from chronic hypoxic PH rats, focusing on the role of endothelium.

Materials and methods

Induction of hypoxic PH

Rats with hypoxia-induced PH were prepared in accordance with previously described methods [4, 22]. Seventy-one male Wistar rats (SLC, Shizuoka, Japan) weighing 180–210 g were randomly assigned to be kept in a hypobaric hypoxic chamber (air at 380 mmHg) ($n = 32$) for 10 days. Age-matched control rats ($n = 39$) were housed in room air at normal atmospheric pressure for 10 days.

Tissue preparation

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). The lungs and heart were removed en bloc and placed in a modified Krebs–Henseleit solution (room temperature) of the following composition (in mM): NaCl 115, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, and dextrose 10. In randomly selected rats, the right ventricle of the heart was dissected with and then separated from the left ventricle plus septum, and these cardiac portions were weighed separately. Extrapulmonary arteries (1.4–1.6 mm in external diameter) were isolated, gently cleaned of fat and connective tissue, and cut into rings (2 mm in length). For experiments without endothelium, the endothelium was removed by gently rubbing the luminal surface with a small stainless steel needle.

Tension studies

Rings were suspended vertically between stainless steel hooks in organ baths (20 ml) containing the modified Krebs–Henseleit solution to record tension with an isometric force transducer. The changes in isometric tension were measured with a force–displacement transducer (TB612; Nihon Kohden, Tokyo, Japan) connected to a carrier amplifier (AP600G; Nihon Kohden) and recorded with a pen recorder (MC6622; Watanabe, Tokyo, Japan). In accordance with a previous study, we applied an optimal resting tension of 0.75 g to rings from control rats and 1.0 g to those from PH rats [11]. The bath medium was maintained at 37°C and bubbled continuously with 95% air and 5% CO_2. Arterial rings were washed and allowed to equilibrate for 60 min, and 70 mM KCl contraction curves were recorded twice to confirm that the vascular reactivity had reached a steady state. The response shown by the second curve was taken as the KCl (70 mM)-induced contraction. The rings were then washed with fresh Krebs–Henseleit solution until a stable baseline tension was achieved. Rings were allowed to equilibrate for a further 15 min in the presence of 1 μM indomethacin, after which removal of the endothelial cells was confirmed by the inability of 10 μM acetylcholine to induce vasodilation (less than 20% relaxation of 10 μM $PGF_{2\alpha}$-induced precontraction). The rings were then washed again and allowed to equilibrate for a further 45 min. Following another 15 min equilibration period in the presence of

1 μM indomethacin to exclude the influence of prostacyclin, rings were precontracted with $PGF_{2\alpha}$ (10 μM). Colforsin (10^{-8} to 10^{-5} M) was added in a cumulative fashion after the $PGF_{2\alpha}$-induced contraction reached a stable plateau. Finally, 100 μM papaverine was added. Vasodilation induced by 100 μM papaverine was taken as 100%.

In another series of experiments with endothelium-intact (+E) and endothelium-denuded (−E) rings from control or PH rats, in order to examine the possible involvement of NO in the vasodilator response to colforsin, the relaxation response to colforsin was determined with or without a NO synthase inhibitor {*N*-nitro-L-arginine (LNNA), 100 μM [4, 6]} in the presence of 1 μM indomethacin. In these experiments, each ring was precontracted with $PGF_{2\alpha}$ to obtain 50–70% of the KCl (70 mM)-induced contraction. This method of standardizing the precontraction strength does not completely guarantee the homogeneity of the precontraction of the blood vessels; the need to consider the influences of neurotransmitters, endothelial NO, and so on remains. However, comparison of the relaxation responses of the blood vessels in various states simultaneously is one of the established classic methods [4, 6, 22]. In control rats, the logarithmically ($\log_{10}$) transformed concentrations of $PGF_{2\alpha}$ (M) in LNNA-untreated +E rings ($n = 7$), LNNA-treated +E rings ($n = 6$), and LNNA-untreated −E rings ($n = 19$) were -5.14 ± 0.12, -5.67 ± 0.24, and -5.74 ± 0.30, respectively. The values of $PGF_{2\alpha}$-induced precontractions in untreated +E rings, LNNA-treated +E rings, and untreated −E rings were 58.0 ± 2.7, 57.2 ± 3.6, and 56.6 ± 3.3% of KCl (70 mM)-induced contractions, respectively; there were no significant differences ($P = 0.88$). In PH rats, the logarithmically transformed concentrations of $PGF_{2\alpha}$ in untreated +E rings ($n = 11$), LNNA-treated +E rings ($n = 7$), and untreated −E rings ($n = 12$) were -5.41 ± 0.17, -6.49 ± 0.38, and -6.50 ± 0.39, respectively. The values of $PGF_{2\alpha}$-induced precontractions in untreated +E rings, LNNA-treated +E rings, and untreated −E rings were 57.2 ± 3.3, 57.2 ± 3.2, and 56.5 ± 2.7% of KCl (70 mM)-induced contractions, respectively; there were no significant differences ($P = 0.57$).

In the final series of experiments with +E and −E rings from control and PH rats, in order to investigate the mechanisms behind the impaired vasodilator response to colforsin observed in the above series of experiments, we compared the vasodilator response to 8-bromo-cAMP (a cell-permeable cAMP analog that causes vasodilation without adenylate cyclase activation) in the presence of 1 μM indomethacin. Again, in these experiments, each ring was precontracted with $PGF_{2\alpha}$ to obtain 50–70% of the KCl (70 mM)-induced contraction. The logarithmically transformed concentrations of $PGF_{2\alpha}$ in +E rings ($n = 7$) from control rats, −E rings ($n = 7$) from control rats, +E rings ($n = 6$) from PH rats, and −E rings ($n = 6$) from PH rats were -5.14 ± 0.12, -5.76 ± 0.29, -5.41 ± 0.18, and -6.52 ± 0.42, respectively. The values of $PGF_{2\alpha}$-induced precontractions in +E rings from control rats, −E rings from control rats, +E rings from PH rats, and −E rings from PH rats were 58.1 ± 2.8, 56.0 ± 5.0, 58.0 ± 3.1, and 55.5 ± 6.5% of KCl (70 mM)-induced contractions, respectively; there were no significant differences ($P = 0.64$).

For each ring, one situation (+E, −E, or LNNA-treated +E) and one vasodilatory drug (colforsin or 8-bromo cAMP) was examined.

Monitoring of Fura-PE3 fluorescence

Fura-PE3/AM [23] solution [25 mM, 100% dimethyl sulfoxide (DMSO)] was diluted in the modified Krebs–Henseleit solution to a final concentration of 25 μM, which was mixed vigorously by applying ultrasonic waves. During this time, pluronic F-127 (0.02%) was added to increase the solubility of fura-PE3/AM. Helically cut, −E strips (1 mm in width, 5 mm in length) were treated with the Fura-PE3/AM solution for 5 h at 30°C. The strips then were suspended horizontally in a 5 ml organ bath under 0.35 g tension in modified Krebs–Henseleit solution containing 1 μM indomethacin. The bath medium was maintained at 37°C and bubbled with 95% air and 5% CO_2. Following a 100 min equilibration period, the contractile force and fura-PE3 fluorescence of the strips were monitored simultaneously. The fluorescence intensities (500 nm) of the Fura-PE3–Ca^{2+} complex were monitored using a spectrofluorometer specifically designed for Fura-PE3 fluorometry (CAF110; JASCO Co., Tokyo, Japan) [24]. Background fluorescence was measured by applying 4 mM $MnCl_2$, and was determined to be less than 25% of the total fluorescence. The ratio (R340/380) of Fura-PE3 fluorescence intensities excited by 340 nm (F340) to those excited by 380 nm (F380) was calculated after subtracting the background fluorescence and used as an index of $[Ca^{2+}]i$ [25]. The effects of colforsin (0.1 μM) on $PGF_{2\alpha}$ (0.5 μM)-induced increases in R340/380 and muscle tension were measured simultaneously. The percentage inhibitions of R340/380 and muscle tension were calculated, with the 0.5 μM $PGF_{2\alpha}$-induced increases in R340/380 and muscle tension taken as 100%. The percentage inhibition of tension was calculated as follows: (tension with $PGF_{2\alpha}$ − tension after colforsin) × 100/tension with $PGF_{2\alpha}$. The same calculation was performed for R340/380. Absolute concentrations of Ca^{2+} were not obtained because the dissociation constant of Fura-PE3/AM for Ca^{2+} in smooth muscle cytoplasm may be different from that obtained in vitro [24]. In all of the Ca^{2+} measurements, changes in F340 and F380 were consistently in opposite directions,

suggesting that the observed changes in F340 and F380 reflected changes in $[Ca^{2+}]i$ and were not motion artifacts. In addition, none of the agents used during the Ca^{2+} measurements influenced the fluorescence signals.

Reagents

The following drugs were used: Fura-PE3/AM (Calbiochem-Novabiochem Corp., La Jolla, CA, USA); indomethacin, 8-bromo-cAMP (Sigma Chemical Co.); pluronic F-127 (Molecular Probes, Inc., Eugene, OR, USA); colforsin (Nippon Kayaku Co., Ltd., Tokyo, Japan); and $PGF_{2\alpha}$ (Ono Pharmaceutical, Osaka, Japan). The solvent solution was 100% DMSO for Fura-PE3/AM and pluronic F127. The final concentration of DMSO in the bath was never greater than 0.1%, at which the concentration had no significant effect on the tension.

Statistical analysis

Values are expressed as mean ± SD. The normality of the data was analyzed using the Kolmogorov–Smirnov test. Differences between two means were analyzed using either the unpaired t test or Welch's t test, according to the homogeneity of the variances, which was confirmed by F test. Differences between multiple means were analyzed using one-way analysis of variance (ANOVA). The homogeneity of the variances between multiple means was confirmed by the Bartlett test. Concentration–response curves were compared using repeated-measure ANOVA. If the ANOVA result was significant, differences between individual means were estimated using one-way ANOVA. Post hoc multiple comparisons were performed by Tukey–Kramer's method. The concentration–response dependency was evaluated using contrasts. Differences were considered significant at $P < 0.05$.

Results

Right ventricular hypertrophy

The right ventricle to left ventricle plus septum ratio was significantly increased to 0.35 ± 0.05 ($n = 21$) by 10 days of hypoxia compared with a control value of 0.22 ± 0.05 ($n = 23$) ($P < 0.05$), indicating the occurrence of right ventricular hypertrophy.

Response to KCL

The absolute tension levels of KCl (70 mM)-induced contraction in +E rings from control rats, +E rings from PH rats, −E rings from control rats, and −E rings from PH rats were 235 ± 18 mg ($n = 27$), 236 ± 27 mg ($n = 29$), 233 ± 29 mg ($n = 33$), and 217 ± 30 mg ($n = 22$), respectively. No significant differences were observed in these values among the four groups.

Effects of colforsin on contractile response to $PGF_{2\alpha}$

In both +E and −E rings from control rats, colforsin (+E, 3×10^{-8} to 10^{-5} M; −E, 10^{-8} to 10^{-5} M) inhibited the contractile response to $PGF_{2\alpha}$ (10 μM) in a concentration-dependent manner ($P < 0.05$) (Figs. 1A, B, 2). The concentration–response curve was almost identical ($P \geq 0.05$) between +E and −E rings (Fig. 2). In addition, no significant difference was observed in the logarithmically transformed IC50 value ($\log_{10}$IC50 [M]) between +E rings (−6.746 ± 0.062, $n = 7$) and −E rings (−6.749 ± 0.057, $n = 7$) ($P \geq 0.05$).

In both +E and −E rings from PH rats, colforsin (+E, 3×10^{-8} to 10^{-5} M; −E, 10^{-7} to 10^{-5} M) also inhibited the contractile response to $PGF_{2\alpha}$ (10 μM) in a concentration-dependent manner ($P < 0.05$) (Figs. 1C, D, 2). However, unlike in the experiments with rings from control rats, the concentration–response curve was shifted ($P < 0.05$) to the right for −E rings compared with that for +E rings (Fig. 2); the $\log_{10}$IC50 for −E rings (−5.739 ± 0.183, $n = 4$) was significantly higher than that for +E rings (−6.268 ± 0.120, $n = 5$) ($P < 0.05$).

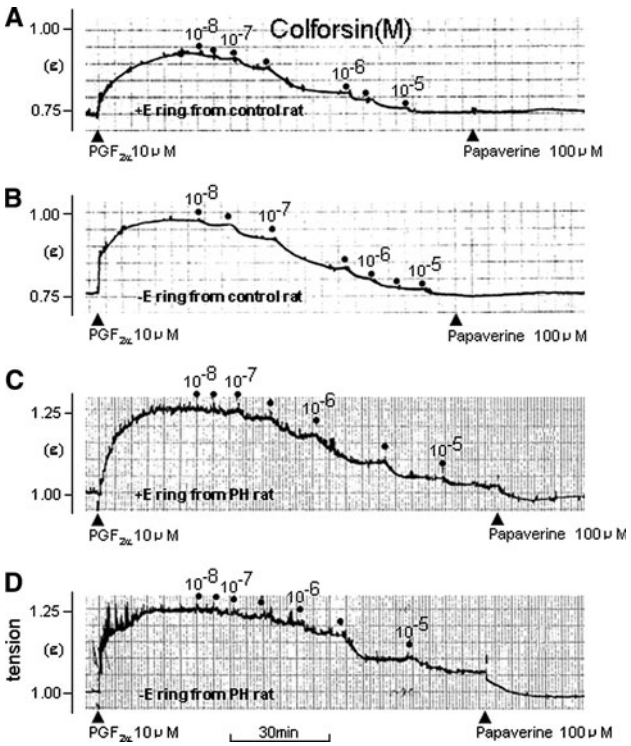

Fig. 1 Typical inhibitory effects of colforsin on $PGF_{2\alpha}$ (10 μM)-induced contractions

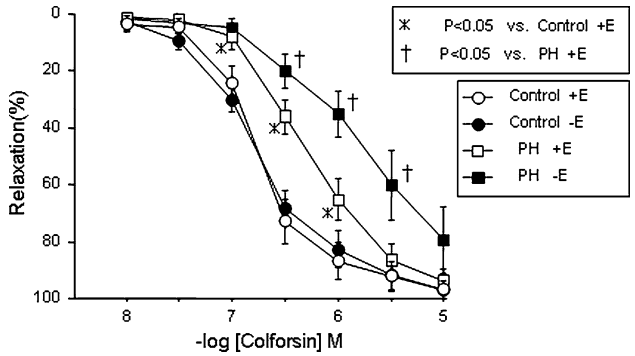

Fig. 2 Effects of colforsin on contractile response to $PGF_{2\alpha}$ (10 μM). Vasodilation induced by 100 μM papaverine was taken as 100%. *Open circles* for +E rings ($n = 7$) from control rats; the $\log_{10}$IC50 was -6.746 ± 0.062. *Closed circles* for −E rings ($n = 7$) from control rats; the $\log_{10}$IC50 was -6.749 ± 0.057. *Open squares* for +E rings ($n = 5$) from PH rats; the $\log_{10}$IC50 was -6.268 ± 0.120. *Closed squares* for −E rings ($n = 4$) from PH rats; the $\log_{10}$IC50 was -5.739 ± 0.183. Values are expressed as mean ± SD; (n) number of rings from 7 control and 5 PH rats. $^{*}P < 0.05$, compared with +E rings from control rats. $^{\dagger}P < 0.05$, compared with +E rings from PH rats

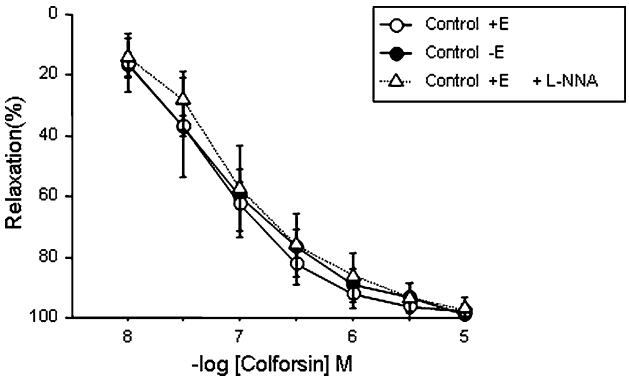

Fig. 3 Effects of LNNA on colforsin-induced vasodilation in rings from control rats. Each ring was precontracted with $PGF_{2\alpha}$ to obtain 50–70% of the KCl (70 mM)-induced contraction. Vasodilation induced by 100 μM papaverine was taken as 100%. *Open circles* for untreated +E rings ($n = 7$); the $\log_{10}$IC50 was -7.258 ± 0.265. *Closed circles* for LNNA-treated +E rings ($n = 6$); the $\log_{10}$IC50 was -7.137 ± 0.224. *Open triangles* for untreated −E rings ($n = 19$); the $\log_{10}$IC50 was -7.238 ± 0.084. Values are expressed as mean ± SD; (n) number of rings from 19 control rats

Regarding comparisons of the vasodilatory action of colforsin between control and PH rats, the concentration–response curve was shifted ($P < 0.05$) to the right for +E rings from PH rats compared with that for +E rings from control rats (Fig. 2); the $\log_{10}$IC50 was significantly higher for +E rings from PH rats (-6.268 ± 0.120, $n = 5$) compared with that for +E rings from control rats (-6.746 ± 0.062, $n = 7$) ($P < 0.05$). The concentration–response curve was also shifted ($P < 0.05$) to the right for −E rings from PH rats compared with that for −E rings from control rats (Fig. 2); the $\log_{10}$IC50 was significantly higher for −E rings from PH rats (-5.739 ± 0.183, $n = 4$) compared with that for −E rings from control rats (-6.749 ± 0.057, $n = 7$) ($P < 0.05$).

Effects of LNNA on colforsin-induced vasodilation

In rings from control rats, colforsin (untreated +E rings, 10^{-8} to 10^{-5} M; LNNA-treated +E rings, 10^{-8} to 10^{-5} M; untreated −E rings, 10^{-8} to 10^{-5} M) inhibited the contractile response to $PGF_{2\alpha}$ in a concentration-dependent manner ($P < 0.05$) (Fig. 3). The concentration–response curves for LNNA-treated +E rings, untreated +E rings, and untreated −E rings were almost identical ($P \geq 0.05$) (Fig. 3). In addition, no significant difference was observed in the $\log_{10}$IC50 between LNNA-treated +E rings (-7.137 ± 0.224, $n = 6$), nontreated +E rings (-7.285 ± 0.265, $n = 7$), and nontreated −E rings (-7.238 ± 0.084, $n = 19$) ($P \geq 0.05$).

In rings from PH rats, colforsin (untreated +E rings, 3×10^{-8} to 10^{-5} M; LNNA-treated +E rings, 10^{-8} to 10^{-5} M; untreated −E rings, 3×10^{-8} to 10^{-5} M) also inhibited the contractile response to $PGF_{2\alpha}$ in a concentration-dependent manner ($P < 0.05$) (Fig. 4). Unlike in the experiments with rings from control rats, the concentration–response curve for LNNA-treated +E rings was shifted ($P < 0.05$) to the left compared with that for untreated +E rings (Fig. 4); however, it was still to the right of that for untreated −E rings ($P < 0.05$, Fig. 4). The $\log_{10}$IC50 for LNNA-treated +E rings (-6.502 ± 0.168, $n = 7$) was significantly higher than that for untreated +E rings (-6.833 ± 0.239, $n = 11$) ($P < 0.05$). No significant difference was observed in the $\log_{10}$IC50 between LNNA-treated +E rings (-6.502 ± 0.168, $n = 7$) and untreated −E rings (-6.425 ± 0.204, $n = 12$) ($P \geq 0.05$).

Effects of 8-bromo-cAMP on contractile response to $PGF_{2\alpha}$

For both +E and −E rings from control rats, 8-bromo-cAMP (+E, 3×10^{-6} to 3×10^{-4} M; −E, 3×10^{-6} to 3×10^{-4} M) inhibited the contractile response to $PGF_{2\alpha}$ in a concentration-dependent manner ($P < 0.05$) (Fig. 5). The concentration–response curves for 8-bromo-cAMP for +E and −E rings were almost identical ($P \geq 0.05$) (Fig. 5). In addition, no significant difference was observed in the $\log_{10}$IC25 between +E rings (-4.350 ± 0.258, $n = 7$) and −E rings (-4.268 ± 0.253, $n = 7$) ($P \geq 0.05$).

In both +E and −E rings from PH rats, 8-bromo-cAMP (+E, 3×10^{-6} to 3×10^{-4} M; −E, 3×10^{-6} to 3×10^{-4} M) inhibited the contractile response to $PGF_{2\alpha}$ in a concentration-dependent manner ($P < 0.05$) (Fig. 5). However, unlike in the experiments with rings from control

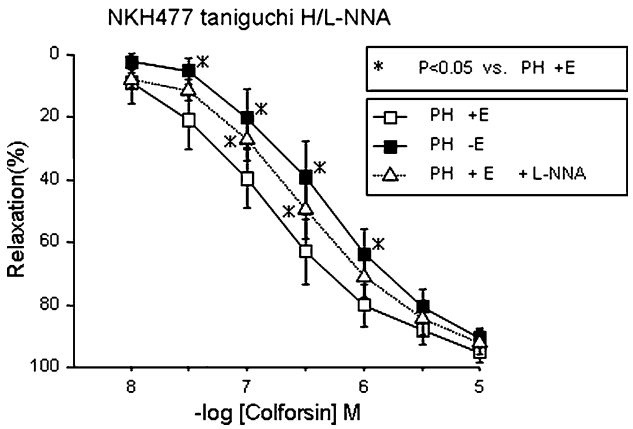

Fig. 4 Effects of LNNA on colforsin-induced vasodilation in rings from PH rats. Each ring was precontracted with $PGF_{2\alpha}$ to obtain 50–70% of the KCl (70 mM)-induced contraction. Vasodilation induced by 100 μM papaverine was taken as 100%. *Open circles* for untreated +E rings ($n = 11$); the $\log_{10}$IC50 was -6.833 ± 0.239. *Closed circles* for LNNA-treated +E rings ($n = 7$); the $\log_{10}$IC50 was -6.502 ± 0.168. *Open triangles* for untreated −E rings ($n = 12$); the $\log_{10}$IC50 was -6.425 ± 0.204. Values are expressed as mean ± SD; (n) number of rings from 15 PH rats. $^{*}P < 0.05$, compared with nontreated +E rings from PH rats

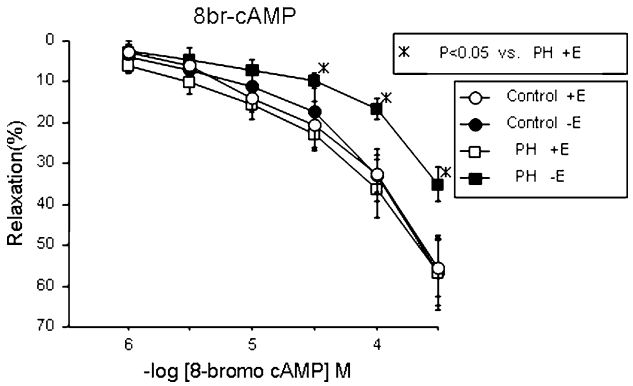

Fig. 5 Effects of 8-bromo-cAMP on contractile response to $PGF_{2\alpha}$. Each ring was precontracted with $PGF_{2\alpha}$ to obtain 50–70% of the KCl (70 mM)-induced contraction. Vasodilation induced by 100 μM papaverine was taken as 100%. *Open circles* for +E rings ($n = 7$) from control rats; the $\log_{10}$IC25 was -4.350 ± 0.258. *Closed circles* for −E rings ($n = 7$) from control rats; the $\log_{10}$IC25 was -4.268 ± 0.253. *Open squares* for +E rings ($n = 6$) from PH rats; the $\log_{10}$IC25 was -4.439 ± 0.154. *Closed squares* for −E rings ($n = 6$) from PH rats; the $\log_{10}$IC25 was -3.781 ± 0.070. Values are expressed as mean ± SD; (n) number of rings from 7 control and 6 PH rats. $^{*}P < 0.05$, compared with +E rings from control rats. $^{\dagger}P < 0.05$, compared with +E rings from PH rats

rats, the concentration–response curve for 8-bromo-cAMP was shifted ($P < 0.05$) to the right for −E rings compared with that for +E rings (Fig. 5); the $\log_{10}$IC25 for −E rings (-3.781 ± 0.070, $n = 6$) was higher than that for +E rings (-4.439 ± 0.154, $n = 6$) ($P < 0.05$).

Regarding comparisons of the inhibitory action of 8-bromo-cAMP on $PGF_{2\alpha}$-induced contraction between control and PH rats, the concentration–response curves for +E rings for control and PH rats were almost identical ($P \geq 0.05$) (Fig. 5); no significant difference was found in the $\log_{10}$IC25 between control rats (-4.350 ± 0.258, $n = 7$) and PH rats (-4.439 ± 0.154, $n = 6$) ($P \geq 0.05$). On the other hand, the concentration–response curve was shifted ($P < 0.05$) to the right for −E rings from PH rats compared with that for −E rings from control rats (Fig. 5); the $\log_{10}$IC25 for −E rings from PH rats (-3.781 ± 0.070, $n = 6$) was significantly higher than that for −E rings from control rats (-4.268 ± 0.253, $n = 7$) ($P < 0.05$).

Effects of colforsin on $PGF_{2\alpha}$-induced increases in tension and R340/380

Colforsin (0.1 μM) inhibited ($P < 0.05$) the $PGF_{2\alpha}$ (0.5 μM)-induced increases in both tension and R340/380 in the Fura-PE3-loaded −E strips from both control and PH rats (Fig. 6A, B). The observed inhibitions of the $PGF_{2\alpha}$-induced increase in R340/380 for control and PH rats were identical ($P = 0.79$), while the observed inhibition of the $PGF_{2\alpha}$-induced increase in tension was significantly smaller ($P < 0.05$) for the PH rats compared with the control rats (Fig. 6B).

Discussion

The observed differences in the vasodilatory action of colforsin (an adenylate cyclase activator) and 8-bromo-cAMP (a cell-permeable cAMP analog) between rings from control rats and rings from PH rats suggest that the cAMP-mediated vasodilatory response is impaired in structurally remodeled pulmonary arteries from rats with hypoxic PH, and that the impairment occurs at least at a level distal to the activation of adenylate cyclase in VSM cells.

The inability of endothelial removal to influence the vasodilatory action of colforsin or 8-bromo-cAMP in rings from control rats suggests that the cAMP-mediated vasodilatory response is independent of endothelium in pulmonary arteries from control (i.e., non-PH) rats. In contrast, the observed difference in the vasodilatory action of colforsin or 8-bromo-cAMP between +E and −E rings from PH rats suggests that the cAMP-mediated vasodilatory response is partly dependent on the presence of endothelium in structurally remodeled pulmonary arteries from rats with hypoxic PH. The observed ability of LNNA to attenuate colforsin-induced vasodilation in +E rings from PH rats further suggests that the cAMP-mediated vasodilatory response is, at least in part, dependent on the endothelium-derived NO. Although we did not examine the 8-bromo-cAMP-induced vasodilation responses in the

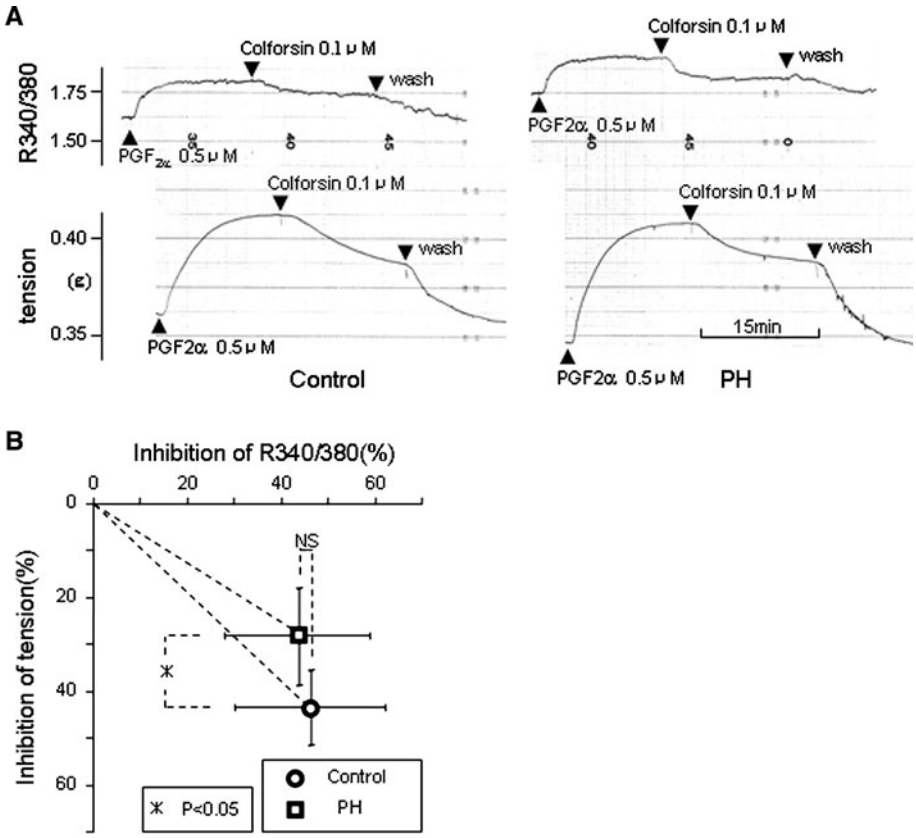

Fig. 6 **a** Typical responses to colforsin (0.1 μM) of R340/380 (*upper trace*) and muscle tension (*lower trace*) in Fura-PE3-loaded −E strips from control and PH rats. **b** Summarized results of the inhibitory effect of colforsin (0.1 μM) on $PGF_{2\alpha}$ (0.5 μM)-induced increases in R340/380 and muscle tension. *Open circles*, ($n = 6$) Fura-PE3-loaded −E strips from control rats (inhibition of R340/380, 46.2 ± 16.0%; inhibition of tension, 43.4 ± 8.2%). *Open squares*, ($n = 6$) Fura-PE3-loaded −E strips from PH rats (inhibition of R340/380, 43.7 ± 15.5%; inhibition of tension, 28.3 ± 10.4%). Values are expressed as mean ± SD; (n) number of rings from 6 control and 6 PH rats. The percentage inhibitions of R340/380 and muscle tension were calculated with 0.5 μM $PGF_{2\alpha}$-induced increases in R340/380 and muscle tension taken as 100%. $^*P < 0.05$

presence of LNNA, we speculate that the endothelium-derived NO might also be involved in the vasodilator response to 8-bromo-cAMP in the +E rings from PH rats.

Several mechanisms may underlie our results. First, in the pulmonary arteries of PH rats, the crosstalk between the 3′,5′-cyclic guanosine monophosphate (cGMP) and cAMP pathways in VSM cells may be transformed [26–31]. This might be one of the causes of the change in the effect of colforsin, for example, the decrease in vasodilatory response in −E rings from PH rats and the partial endothelium dependency of vasodilatory response in +E rings from PH rats. Second, colforsin might only increase the level of endothelium-derived NO in rings from PH rats, not in rings from control rats, which could explain the partial endothelium dependency of vasodilatory response in +E rings from PH rats. To examine these possibilities, further study is necessary.

In our study, in rings from control rats, neither endothelial removal nor treatment with LNNA (in the +E rings) attenuated the vasodilator response to colforsin. Thus, Toyoshima et al. [26] reported that endothelium-dependent components of vasodilatory responses to DBcAMP and colforsin were not detected in non-PH rat pulmonary arteries, consistent with the present results. In this way, normal rat pulmonary artery differs from other vascular beds such as rat thoracic aorta [10, 11, 26], rat mesenteric vascular bed [32], human umbilical vein [12], and coronary blood vessel [13], in which endothelial removal attenuates cAMP-induced vasodilation.

In our study, in +E rings from hypoxic PH rats, 8-bromo-cAMP-induced vasodilation was not attenuated. Thus, Rodman [33] reported no reduction in DBcAMP-induced vasodilation in pulmonary arteries isolated from hypoxic PH rats, which is consistent with the present results for +E rings from hypoxic PH rats. Apparently, vasodilator response to these cell-permeable cAMP analogs is not impaired in +E rings from PH rats. However, unlike in the experiments with rings from control rats, endothelial removal attenuated 8-bromo-cAMP-induced vasodilation in our study. This suggests that the cAMP-mediated relaxation response of VSM cells is impaired, and the endothelium plays a compensatory role against the impaired cAMP-mediated relaxation response.

In our study, colforsin-induced vasodilation was attenuated in +E rings from hypoxic PH rats, and it was further attenuated by endothelial removal. To some degree, this attenuation by endothelial removal was reproduced by LNNA (a NO synthase inhibitor) in +E rings from PH rats. In +E rings from PH rats, the colforsin-induced relaxation response of VSM cells is suggested to be impaired. Moreover, this suggests that colforsin enhances endothelial NO action on VSM cells in +E rings from PH rats, or

suggests that colforsin promotes endothelial NO release in +E rings from PH rats.

Previous studies showed that chronic hypoxia upregulates pulmonary arterial endothelial NO synthase [34, 35], and that NO synthase plays a role in preventing hypoxic PH [36]. Thus, the effect of colforsin on endothelial NO-dependent vasodilation might benefit the pulmonary vasculature in chronic hypoxic PH.

Agents that increase cAMP level are believed to induce vasodilation with a decrease in Ca^{2+} level or a decrease in Ca^{2+} sensitivity of the contractile apparatus [1, 37]. R340/380 was used as a relative indicator of cytosolic free Ca^{2+}, changes in which show the relative changes in cytosolic free Ca^{2+} [23, 24]. In our study, the observed results were different between the strips from PH rats and the strips from control rats, although colforsin of the same amount had been added with $PGF_{2\alpha}$ of the same concentration. Thus, it is suggested that the Ca^{2+} sensitivity of the contractile apparatus or the intracellular Ca^{2+} concentration in VSM cells differs between strips from PH rats and strips from control rats, which might explain the decrease in colforsin-induced relaxation in −E rings from PH rats. To examine this possibility, further study is necessary.

In summary, in chronic hypoxic PH, colforsin might have an endothelium-dependent vasodilation effect as well as an endothelium-independent vasodilation effect. This endothelium-dependent effect of colforsin might benefit the relaxation of pulmonary artery in chronic hypoxic PH. Further research is necessary to clarify the mechanism involved.

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