Differential involvement of α₁-adrenoceptors in vasoconstrictor responses to cooling in mouse plantar arteries in vitro and in vivo

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Abstract

Cooling-induced reduction of skin blood flow results from a reflex increase in sympathetic output and an enhanced vasoconstrictor activity of skin vessels. The latter has been proposed to be mediated by increased reactivity of α₂C-adrenoceptors during cooling in studies with isolated cutaneous vessels in vitro. We have previously shown in studies with tetrodotoxin-treated mice in vivo that reduction of plantar skin blood flow (PSBF) induced by local cooling results primarily from increased reactivity of α₂C-adrenoceptors. In addition, we showed that part of the cooling-induced response was also mediated by α₁-adrenoceptors. However, the mechanisms involved in the cooling-induced responses mediated by α₁-adrenoceptors have not been elucidated. The present study is an investigation seeking to clarify the mechanisms involving α₁-adrenoceptors. Medial plantar arteries were isolated from male ddY mice and changes in vessel diameter were measured in vitro using pressurized arteriography. In vivo measurements of PSBF were performed on artificially ventilated tetrodotoxin treated mice, anaesthetized with pentobarbital sodium, using laser Doppler flowmetry, with the probe positioned above the medial plantar artery. In the in vitro studies with isolated plantar arteries, cooling from 37 to 28°C did not affect the constrictor potency of phenylephrine, an α₁-adrenoceptor agonist, and the threshold concentration to evoke constriction was rather higher at 28°C than it was at 37°C. The cooling also suppressed the constrictor efficacy of UK14,304, an α₂-adrenoceptor agonist. In contrast, cooling the air temperature around the foot from 25 to 10°C in vivo decreased PSBF, which was significantly inhibited by phentolamine, an α-adrenoceptor antagonist, although MK-912, an α₂C-adrenoceptor antagonist, had no effect on it. These results suggest that although α₁-adrenoceptors are involved in cooling-induced reduction of PSBF in mice, the response is unlikely to result from an enhancement of α₁-adrenoceptor-mediated vasoconstriction of plantar arteries during cooling.

Key words: cold-induced contraction, arteriography, tetrodotoxin, laser Doppler flowmetry
Introduction

Cooling causes a reduction of skin blood flow to protect the body from heat loss. This physiological response results from a reflex increase in sympathetic output and a local enhancement of the vasoconstrictor response to noradrenaline in cutaneous vessels (Vanhoutte, 1980). One of the well-established local mechanisms is the augmentation of $\alpha_2$-adrenoceptor reactivity during cooling. Several in vitro studies have shown that moderate cooling enhances vasoconstriction induced by $\alpha_2$-adrenoceptor agonists, but not by $\alpha_1$-adrenoceptor agonists (Flavahan et al., 1985; Vanhoutte et al., 1985; Harker et al., 1990; 1991). Moreover, cooling-induced enhancement of the vasoconstrictor response by the $\alpha_2$-adrenoceptor agonist UK-14304 has been shown to be inhibited by the $\alpha_{2C}$-adrenoceptor antagonist MK-912 in mouse tail arteries (Chotani et al., 2000). In HEK293 cells transfected with $\alpha_{2C}$-adrenoceptors, cooling has been shown to induce the translocation of $\alpha_{2C}$-adrenoceptors from the Golgi compartment to the plasma membrane (Jeyaraj et al., 2001; Bailey et al., 2004). Thus, cooling seems to lead to the translocation of $\alpha_{2C}$-adrenoceptors to the plasma membrane, thereby augmenting vasoconstriction. We have recently demonstrated that this model is also involved in the in vivo cutaneous response to local cooling: MK-912, an $\alpha_{2C}$-adrenoceptor antagonist, inhibited cooling-induced reduction of PSBF in tetrodotoxin-treated mice in vivo (Honda et al., 2007). In addition, we found that a part of the cooling-induced response was also inhibited by bunazosin, an $\alpha_1$-adrenoceptor antagonist (Honda et al., 2007). This is apparently different from the results of earlier in vitro studies. The present study was thus planned to investigate the mechanism for the cooling-induced responses involving $\alpha_1$-adrenoceptors. The results show that the role of $\alpha_1$-adrenoceptors in the responses to cooling in mouse plantar arteries differs between in vitro and in vivo studies.

Methods

Protocols for animal use were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Shizuoka and were in accordance with guidelines approved by the Japanese Pharmacological Society.

Preparations for in vitro studies

Male ddY mice (40–50 g; SLC, Hamamatsu, Japan) were anesthetized with an intraperitoneal (i.p.) injection of pentobarbital sodium (50 mg/kg), and sacrificed by decapitation. The foot was removed and immersed in an ice-cold Tyrode’s solution composed of 158.3 mM NaCl, 4.0 mM KCl, 2.0 mM CaCl$_2$, 1.05 mM MgCl$_2$, 10.0 mM NaHCO$_3$, 0.42 mM NaH$_2$PO$_4$, and 5.5 mM glucose. The second branch of the medial plantar arteries with an outer diameter of between 200–300 $\mu$m was dissected, and incubated at 4°C over night. Changes in arterial diameter were measured by arteriography as previously described (Kashihara et al., 2008). In brief, the isolated arteries were cannulated with two glass pipettes in Tyrode’s solution, and the intraluminal pressure was regulated with an electronic pressure servosystem (Living Systems Instrumentation, Burlington, VT, USA). Those vessels that were not leaking
were used for experimentation. The endothelium was removed by placing an air bubble in the lumen for 1 min. The chamber was superfused with Tyrode’s solution, maintained at 37°C, pH 7.4, and aerated with 97% O₂ and 3% CO₂. The vessel internal diameter was continuously determined using a width analyzer (C3160, Hamamatsu Photonics). At the end of each experiment, the superfusing chamber solution was changed to a Ca²⁺-free Tyrode’s solution containing 2 mM EGTA to obtain the passive diameter of the vessels.

**Experimental protocol**

The cannulated arteries were allowed to equilibrate for 30–40 min at 60 mmHg and exposed to 60 mM K⁺ Tyrode’s solution before commencing experiments. Concentration-response curves to phenylephrine, a selective α₁-adrenoceptor agonist, or UK-14,304, a selective α₂-adrenoceptor agonist, were generated by increasing the concentration of the agonist. When the influence of cooling on α-adrenoceptor responsiveness was investigated, the temperature of the superfusate was decreased from 37 to 28°C for 60 min before commencing another concentration-response curve for each agonist. This provides sufficient time for the effect of cooling on adrenergic reactivity to stabilize (Flavahan et al., 1985; Chotani et al., 2000).

**In vivo experiments**

Male ddY mice (40–50 g; SLC, Hamamatsu, Japan) were anesthetized with pentobarbital sodium (i.p.; 75 mg/kg), and placed on a heating pad in the dorsal position. Changes in PSBF in mice were measured with a non-contact laser Doppler flow meter (ALF 2100; Advance, Tokyo, Japan), as described previously (Honda et al., 2007). In brief, polyethylene tubes were inserted in the right femoral vein to administer drugs and in the right carotid artery to measure the mean arterial blood pressure (MAP) and heart rate (HR). After the intravenous (i.v.) administration of TTX (30 µg/kg), the mice were mechanically ventilated with air via a tracheostomy, using a rodent ventilator (SN-480-7; Shinano, Tokyo, Japan) at a stroke volume of 0.2 ml/10 g body weight and a rate of 85 strokes per minute. A laser Doppler flow probe (NS type; Omega Wave, Tokyo, Japan) was positioned above the medial plantar artery of the left foot. The cooling apparatus for the mouse foot was made in our laboratory, as described previously (Koganezawa et al., 2006). The left foot was placed in the apparatus to apply local cooling. Drugs were intravenously administered as a bolus injection of 0.01 ml/10 g body weight.

**Drugs**

The following drugs were used: TTX (Wako, Osaka, Japan); phentolamine mesylate (Ciba-Geigi, Hyogo, Japan); and MK-912 hydrochloride salt ((2S-trans)-1,3,4,5,6,6',7,12b-Octahydro-1',3'-dimethyl-spiro(2H-benzofuro[2,3-a]quinolizine-2,4'(1'H)-pyrimidin)-2'(3'H)-one), phenylephrine hydrochloride, phentolamine hydrochloride, and UK 14,304 [5-bromo-N-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine] (Sigma, St. Louis, MO, USA).

**Expression of results and statistical analysis**

All data were expressed as the mean ± S.E.M, where n equals the number of experiments. Only one vessel was obtained from each mouse. Vasoconstrictor responses were expressed as
a percentage constriction = 100% × [(D_b – D_d) / D_b], where D is the inner diameter upon stabilization after drug application (d) or baseline (b). The reduction of PSBF is expressed as a percentage of the basal PSBF at 25°C. The statistical significance was evaluated by Student’s t test for paired observations. P values less than 0.05 were considered to be significant.

Results

Influence of cooling on α-adrenoceptor-mediated constriction in isolated plantar arteries

The α₁-adrenoceptor agonist phenylephrine caused concentration-dependent constriction of the plantar arteries (Fig. 1A). The cooling to 28°C did not affect the 50% effective concentration (EC₅₀), and slightly, but significantly, increased the maximum response (Table 1). However, the threshold concentration to evoke constriction was apparently higher at 28°C than at 37°C (Fig. 1A). The α₂-adrenoceptor agonist UK 14,304 also caused concentration-dependent constriction of the plantar arteries (Fig. 1B), although the maximum response was relatively small compared with that of phenylephrine. The cooling to 28°C did not affect the EC₅₀, but significantly decreased the maximum response (Table 1). The cooling to 28°C per se did not affect the basal
diameter of the arteries (data not shown).

Effects of α-adrenoceptor antagonists on cooling-induced reduction of PSBF

Figure 2A shows the HR, MAP, and PSBF during local cooling of the left foot. When the air temperature in the cooling apparatus was changed from 25 to 20, 15, 10 and 5°C, the PSBF of the left foot decreased in a temperature-dependent manner. In contrast, the HR or MAP did not change during the cooling. When the temperature in the apparatus was returned to 25°C, the PSBF of the left foot recovered to the basal level within 10 min.

To elucidate the contribution of α-adrenoceptors to the cooling-induced reduction of PSBF, pharmacological analyses were performed. The non-selective α-adrenoceptor antagonist phentolamine (10 mg/kg) significantly suppressed the reduction of PSBF induced by cooling to 10°C (Fig. 2B), whereas the α<sub>2C</sub>-adrenoceptor antagonist MK-912 (30 µg kg<sup>-1</sup>) did not cause significant changes in it (Fig. 2C). Phentolamine or MK-912 per se caused no remarkable changes in PSBF (data not shown).

Discussion

The in vitro study in isolated mouse plantar arteries showed that the threshold concentration for phenylephrine-induced constriction was shifted to a higher concentration by the cooling, although the maximum response was slightly increased by it. Several in vitro studies have reported that cooling augments the response to exogenously applied noradrenaline, which is however not mediated via α<sub>1</sub>-adrenoceptors, in the canine saphenous vein (Flavahan et al., 1985), rabbit ear artery (Harker et al., 1988), and rat tail artery (Harker et al., 1991). The results of the present study in isolated mouse plantar arteries are essentially consistent with these studies.

In contrast to the in vitro results, local cooling induced the reduction of skin blood flow around the plantar arteries, which was significantly suppressed by the α-adrenoceptor antagonist phentolamine, but not by the α<sub>2C</sub>-adrenoceptor antagonist MK-912. Our previous study in mice in vivo has shown that the cooling-induced reduction of PSBF sensitive to phentolamine is mediated via α<sub>1</sub>- and α<sub>2C</sub>-adrenoceptors (Honda et al., 2007). Thus, α<sub>1</sub>-adrenoceptors were suggested to be involved in the cooling-induced reduction of blood flow in mouse plantar arteries. However, the results in the present in vitro study suggest that the constriction of the medial plantar artery induced by α<sub>1</sub>-adrenoceptor agonists at low

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<th>Phenylephrine</th>
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<td>37°C</td>
<td>28°C</td>
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<tr>
<td>pEC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>7.25 ± 0.06</td>
<td>7.23 ± 0.09</td>
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<td>Max (%)</td>
<td>58.7 ± 0.6</td>
<td>61.8 ± 0.9*</td>
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Values are mean ± SEM of 5 experiments. pEC<sub>50</sub>, the negative log of the 50% effective concentration. Max, the maximum response. *, P<0.05; **, P<0.01 vs. corresponding values at 37°C.
concentrations is inhibited by the cooling. The concentration of noradrenaline should have been low in the present in vivo study, resulting from the inhibition by TTX of the sympathetic nerve. Taken together with the results of the in vitro and in vivo studies, it is suggested that although α₁-adrenoceptors are involved in cooling-induced reduction of PSBF in mice, the response is unlikely to result from an enhancement of α₁-adrenoceptor-mediated vasoconstriction of plantar arteries during cooling.

Several reasons may be proposed for this discrepancy. The first is that the blood flow of the medial plantar artery may be affected by the alterations of the downstream microcirculation.
The constriction of smaller arteries and/or arterioles via $\alpha_1$-adrenoceptors may be increased more by the cooling. The second is that the concentration of noradrenaline at sympathetic nerve terminals may be increased by the cooling \textit{in vivo}. We have previously suggested that cooling induces the release of ATP, which stimulates presynaptic P2 purinoceptors on sympathetic nerve terminals and facilitates the release of noradrenaline, thereby causing constriction of skin blood vessels via the activation of $\alpha_1$- and $\alpha_2$-adrenoceptors in rats (Koganezawa \textit{et al.}, 2006). Although the contribution of ATP to the cooling-induced response in mice was ruled out in our previous study (Honda \textit{et al.}, 2007), other mediators inducing the release of noradrenaline may be involved. Alternatively, the reuptake of noradrenaline into the nerve terminals may also be inhibited by the cooling.

Recent studies in humans have shown that the sustained vasoconstrictor responses during local skin cooling is reduced by an NO synthase inhibitor, suggesting the involvement of NO synthase inhibition by cooling in the response (Hodges \textit{et al.}, 2006; Yamazaki \textit{et al.}, 2006). Since about the half of the cooling-induced reduction of PSBF was not inhibited by phentolamine, other mechanisms also seem to be involved in the response \textit{in vivo}. In addition, the contribution of endothelium-derived substances to the cooling-induced vasoconstriction may also explain the discrepancy between the results of the \textit{in vitro} and \textit{in vivo} studies. The release of NO and other vasodilators from endothelial cells are well known to be enhanced by $\alpha_2$-adrenoceptor stimulation and by sheer stress which could be affected by $\alpha_1$-adrenoceptor stimulation via changes in blood pressure and blood flow (Vanhoutte, 1989). Since the vasoconstrictor responses to $\alpha$-adrenoceptor agonists were investigated in the endothelium-denuded arteries, cooling responses involving endothelial cells were excluded in the \textit{in vitro} study. The possible involvement of endothelial cells in cooling-induced responses will be investigated further.

In isolated mouse plantar arteries, the constriction induced by $\alpha_2$-adrenoceptor stimulation was suppressed by the cooling to 28°C. This is inconsistent with other studies suggesting the enhanced vasoconstrictor response to $\alpha_2$-adrenoceptor stimulation by cooling in cutaneous vessels such as dog saphenous veins (Flavahan \textit{et al.}, 1985; Vanhoutte \textit{et al.}, 1985) and mouse tail arteries (Chotani \textit{et al.}, 2000). However, in the central ear artery of the rabbit, cooling has been shown to have very little effect on adrenergically induced contraction (Harker \textit{et al.}, 1988). Thus, the contribution of $\alpha_2$-adrenoceptors to the responses to cooling might be dependent on the location of the cutaneous vessel being studied.

In the \textit{in vivo} study, the $\alpha_{2C}$-adrenoceptor antagonist MK-912 did not affect the cooling-induced reduction of PSBF in mice, suggesting that $\alpha_{2C}$-adrenoceptors have no contribution to the response to cooling in mouse plantar arteries. This is apparently different from the results of our previous \textit{in vivo} study, in which bunazosin, an $\alpha_1$-adrenoceptor antagonist, RS79948, an $\alpha_2$-adrenoceptor antagonist, and MK-912 all significantly inhibited the cooling-induced reduction of PSBF and the inhibition by bunazosin was relatively small compared with that by RS79948 and MK-912 (Honda \textit{et al.}, 2007). Thus, our previous study concluded that local cooling-induced reduction of PSBF in mice primarily results from increased reactivity of $\alpha_2C$-adrenoceptors (Honda \textit{et al.}, 2007). The difference between the present and previous studies was the position where the laser Doppler probe was set. The probe was set to the position above microvessels
located about 5-mm apart from the center of the plantar surface in the previous study (Honda et al., 2007), whereas it was set to the position above the medial plantar artery in the present study. The arbitrary perfusion units recorded with the same laser Doppler flow meter were drastically different, which were around 30 and 70 PU in the previous (Honda et al., 2007) and present studies, respectively. Thus, the vessels, the blood flow of which we recorded in the previous study, would be considered to be smaller than those in the present study. The contribution of α2C-adrenoceptors to the responses to cooling might be dependent on the vessel diameter. This view is in good agreement with the results in the present in vitro study, where the constriction of the medial plantar artery induced by the α2-adrenoceptor agonist UK 14,304 was not enhanced, rather decreased, by the cooling to 28°C.

In summary, cooling from 37 to 28°C did not affect the constrictor potency of the α1-adrenoceptor agonist phenylephrine in mouse isolated plantar arteries in vitro, whereas a decrease in PSBF induced by cooling the foot from 25 to 10°C was significantly reduced by the α-adrenoceptor antagonist phentolamine in vivo. These results suggest that although α1-adrenoceptors are involved in cooling-induced reduction of the blood flow of plantar arteries in mice, the response is unlikely to result from an enhancement of α1-adrenoceptor-mediated vasoconstriction of plantar arteries during cooling.

References


