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RESEARCH PAPER

Role of α_{2C}-adrenoceptors in the reduction of skin blood flow induced by local cooling in mice

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Background and purpose: The reduction of skin blood flow induced by local cooling results from a reflex increase in sympathetic output and an enhanced vasoconstrictor activity of cutaneous vessels. The present study investigated the latter local response *in vivo* in tetrodotoxin-treated mice, in which the sympathetic nerve tone was abolished.
Experimental approach: Male ddY mice, anaesthetized with pentobarbitone, were treated with tetrodotoxin and artificially ventilated. The plantar skin blood flow (PSBF) was measured by laser Doppler flowmetry.
Key results: Cooling the air temperature around the left foot from 25 to 10°C decreased the PSBF of the left foot. Bunazosin, an α_1-adrenoceptor antagonist, RS79948, an α_2-adrenoceptor antagonist, and MK-912, an α_{2C}-adrenoceptor antagonist, all significantly inhibited the cooling-induced reduction of PSBF; the inhibition by bunazosin was relatively small compared with that by RS79948 and MK-912. The response was not affected by guanethidine or bretylium, but was diminished in adrenalectomized mice. An intra-arterial injection of clonidine, an α_2-adrenoceptor agonist, to the left iliac artery of adrenalectomized mice caused a transient decrease in PSBF, which was significantly augmented at 10°C. MK-912 suppressed only the augmented portion at 10°C. Y-27632, H-1152 and fasudil, Rho kinase inhibitors, also inhibited the cooling-induced reduction of PSBF. RS79948 caused no further reduction of the cooling-induced response after the inhibition by Y-27632.
Conclusions and implications: Local cooling-induced reduction of skin blood flow in mice primarily results from increased reactivity of α_{2C}-adrenoceptors to circulating catecholamines, in which the Rho/Rho kinase pathway is involved.
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Keywords: cold-induced vasoconstriction; tetrodotoxin; thermoregulation; Rho kinase; laser Doppler flowmetry

Abbreviations: ACh, acetylcholine; HR, heart rate; MAP, mean arterial blood pressure; PPADS, pyridoxal phosphate-6-azo(benzene-2, 4-disulphonic acid); PSBF, plantar skin blood flow; PU, perfusion units; ROS, reactive oxygen species; TTX, tetrodotoxin

Introduction

Cooling causes a reduction of the skin blood flow to protect body from heat loss. This physiological response results from a reflex increase in sympathetic output and a local enhancement of vasoconstrictor response to noradrenaline in cutaneous vessels (Vanhoutte, 1980). Several mechanisms have been suggested for the local effect of cooling.

One of the well-accepted local mechanisms is the augmentation of α_2-adrenoceptor reactivity during cooling. *In vitro* studies using the saphenous vein from dogs (Flavahan *et al*., 1985; Vanhoutte *et al*., 1985) and humans (Harker *et al*., 1990) have shown that moderate cooling enhances the constrictor response to α_2-agonists but not α_1-agonists. Recently, evidence has been presented for a novel mechanism of α_2-adrenoceptor-mediated, cooling-induced contraction of skin blood vessels *in vitro*. In the mouse isolated tail artery, cooling-induced enhancement of vasoconstriction to the α_2-agonist UK-14304 was shown to be inhibited by the α_{2C}-antagonist MK-912 (Chotani *et al*., 2000). In human embryonic kidney (HEK) 293 cells transfected with α_{2C}-adrenoceptors, cooling induced the translocation of α_{2C}-adrenoceptors from the Golgi compartment to the plasma membrane (Jeyaraj *et al*., 2001; Bailey *et al*., 2004), and this was prevented by inhibition of Rho kinase, using the Rho kinase inhibitor fasudil or RNA interference (Bailey *et al*., 2004). Moreover, reactive oxygen species (ROS), which are released from smooth muscle mitochondria in response to cooling, have been shown to stimulate Rho/Rho kinase signalling (Bailey *et al*., 2005). Taken together, these findings suggest that cooling leads to the generation of ROS, which stimulates Rho/Rho kinase signalling and the subsequent

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translocation of α_{2C}-adrenoceptors to the plasma membrane, thereby augmenting vasoconstriction. However, whether this model participates in the *in vivo* cutaneous response to local cooling has not been elucidated.

Cutaneous vasoconstriction induced by local cooling has also been demonstrated *in vivo*. Most of the *in vivo* studies were performed in humans and favour the hypothesis that enhanced reactivity of α_2-adrenoceptors is responsible for the cooling-induced reduction of skin blood flow (Ekenvall *et al*., 1988; Freedman *et al*., 1992). Interestingly, patients with Raynaud phenomenon and scleroderma exhibited enhanced constriction of skin blood vessels, mediated by α_2-adrenoceptors, in response to cooling (Cooke and Marshall, 2005). Moreover, a recent study demonstrated that the cooling-induced cutaneous vasoconstriction in human forearms was suppressed by the Rho kinase inhibitor fasudil (Thompson-Torgerson *et al*., 2007). It is thus highly probable that mechanisms similar to those proposed in the *in vitro* studies also function *in vivo*. However, since the protocols of experiments and the drugs available for *in vivo* administration are limited in humans, animal experiments are needed to explore more detailed mechanisms *in vivo*.

The neuronal reflex mechanism through the sympathetic efferent is well developed in cutaneous circulation (Johnson *et al*., 1986), and this prevents a stable measurement of skin blood flow. Recently, we have found that the treatment of rats with tetrodotoxin (TTX), a voltage-dependent Na^+ channel blocker, by suppressing sympathetic nervous tone, stabilizes the measurement of cutaneous blood flow (Chino *et al*., 2000). Using this method, we have proposed a novel mechanism for cooling-induced reduction of skin blood flow in rats; moderate cooling of the skin induces the release of ATP, which stimulates presynaptic P2 purinoceptors on sympathetic nerve terminals and facilitates the release of noradrenaline, thereby causing contractions of skin blood vessels primarily via α_1-adrenoceptor activation (Koganezawa *et al*., 2006).

The purpose of the present study was to elucidate the mechanism for cooling-induced reduction of skin blood flow in mice. As these are better suited to genetic manipulation than other species, this will allow us to investigate in more detail the mechanisms involved. The results show that, although a similar decrease in the plantar skin blood flow (PSBF) is also induced by local cooling in mice, the mechanism involved in mice is apparently different from that in rats; the response in mice is suggested to be independent of ATP and primarily result from enhanced activity of α_{2C}-adrenoceptors.

Methods

A total of 71 male ddY mice weighing 35–45 g (SLC, Hamamatsu, Japan) were used in this study. The mice were housed in a 12 h light–dark cycle, with food and water available *ad libitum*, and treated as approved by the Institutional Animal Care and Use Committee and according to the Guidelines for Animal Experiments established by the Japanese Pharmacological Society.

The mice were anaesthetized with the intraperitoneal (i.p.) administration of pentobarbitone sodium (75 mg kg^{-1}), and placed on a heating pad in the dorsal position. A polyethylene tube was inserted in the right femoral vein to administer drugs and saline. Another one was placed in the right carotid artery and connected to a pressure transducer (TDN-R; Gould, Oxnard, CA, USA) for the measurement of the mean arterial blood pressure (MAP) and heart rate (HR). After the intravenous (i.v.) administration of TTX (30 μg kg^{-1}), the mice were mechanically ventilated, via a tracheotomy, with air, using a rodent ventilator (SN-480-7; Shinano, Tokyo, Japan) at a stroke volume of 0.2 ml 10 g^{-1} body weight and a rate of 85 strokes per minute. After the haemodynamic parameters had stabilized, a laser Doppler flow probe (NS type; Omega Wave, Tokyo, Japan) was set to the position about 5 − mm apart from the centre of the plantar surface of the left foot to measure the PSBF with a non-contact laser Doppler flow meter (ALF 2100; Advance, Tokyo, Japan). The right foot served as the control. The blood flow was expressed as arbitrary perfusion units (PU). The microvessels with the blood flow between 25 and 35 PU were selected for the measurement. The skin temperature of the plantar surface was measured using a thermosensor (AW-601 H, Nihon Kohden, Tokyo, Japan), the tip of which was inserted subcutaneously. Data were stored and analysed on a Macintosh computer with an AD converter (Lab Stack; Keisoku Giken, Tokyo, Japan). In some experiments, a catheter was retrogradely inserted into the right iliac artery for intra-arterial (i.a.) injection of drugs into the left iliac artery to investigate local effects of drugs.

The cooling apparatus for the mouse foot was constructed in our laboratory, as described previously (Koganezawa *et al*., 2006). A rubber tube (a 25 ml plastic syringe) was coiled around the apparatus and water was perfused in the tube by a roller pump (PA-12; Cole Parner Instrument, Chicago, IL, USA). The temperature in the apparatus was continuously monitored with a thermosensor (SXB-54; Techno-Seven, Yokohama, Japan), and regulated by changing the temperature of the perfusing water. The left foot was placed in the apparatus to apply local cooling. The temperature and humidity of the laboratory were at 24 ± 2°C and 55 ± 10%, respectively.

In some experiments, adrenalectomized mice were prepared as described previously (Koganezawa *et al*., 2006). Briefly, 2 days before the experiments, mice were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.) and the bilateral removal of adrenals was achieved via a dorsal approach through two small lateral skin incisions. The adrenals were pulled out through the incision by holding the periadrenal fat and severed with scissors. The adrenalectomized mice were allowed free access to 0.9% saline to maintain their electrolyte balance. The accomplishment of adrenalectomy was confirmed by the measurement of the serum adrenaline concentration; this was done by a company that provides clinical testing services (SRL, Tokyo, Japan).

Drugs

The following drugs were used: TTX and clonidine hydrochloride (Wako, Osaka, Japan); phentolamine mesylate

(Ciba-Geigi, Hyogo, Japan); RS79948 hydrochloride ((8aR, 12aS,13aS)-5,8,8a,9,10,11,12,12a,13,13a-dechydro-3-methoxy-12-(ethylsulphonyl)-6H- isoquino[2,1-g][1,6]naphthyridine hydrochloride; Tocris, Ballwin, MO, USA); H-1152 dihydrochloride ((S)-(+)-2-methyl-1-[(4-methyl-5-isoquinolinyl) sulphonyl]homopiperazine) and Y-27632 ((R)-(+)-trans-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide; Calbiochem, Darmstadt, Germany); and acetylcholine (ACh) chloride, bretylium tosylate, capsaicin, guanethidine monosulphate, 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, pyridoxal phosphate-6-azo(benzene-2, 4-disulphonic acid) (PPADS) and tyramine hydrochloride (Sigma, St Louis, MO, USA). Bunazosin hydrochloride, OPC-28326 hydrochloride monohydrate (4-(*N*-methyl-2-phenylethylamino)-1-(3,5-dimethyl-4-propionylaminobenzoyl) piperidine) and fasudil hydrochloride ((5-isoquinolinesulphonyl)homopiperazine) were kindly donated by Eisai (Tokyo, Japan), Otsuka Pharmaceutical Co (Tokyo, Japan) and Asahikasei Pharma (Tokyo, Japan), respectively.

TTX and bunazosin were dissolved in distilled water. The other drugs were dissolved in physiological salt solution. Drugs were intravenously administered as a bolus injection of 0.01 ml $10g^{-1}$ body weight. In some experiments, clonidine and phenylephrine were intra-arterially administered as a bolus injection of $1\,\mu l\,10\,g^{-1}$ body weight. The appropriate vehicle controls showed no apparent effect.

Statistical analysis

All data are expressed as mean ± s.e.mean, where n equals the number of animals. The statistical significance was evaluated by Student's t-test for either paired or unpaired observations. When more than two means were compared, one-way analysis of variance (ANOVA) was used with Bonferroni or Dunnett's *post hoc* test, and two-way ANOVA was used with Bonferroni *post hoc* test. P-values less than 0.05 were considered significant.

Results

Effect of TTX on ACh-induced tachycardia

We first investigated the reflex tachycardia due to the depressor response to ACh, to confirm that TTX ($30\,\mu g\,kg^{-1}$) could completely inhibit sympathetic nerve activity. ACh induced depressor responses in a dose-dependent manner in both anaesthetized and TTX-treated mice. However, ACh induced tachycardia only in anaesthetized but not in TTX-treated mice (Figure 1). ACh at doses higher than $3\,\mu g\,kg^{-1}$ reduced the HR in TTX-treated mice; this results from the direct negative chronotropic action of ACh on sinus node cells.

Change in PSBF induced by local cooling

In Figure 2a, the changes in the HR, MAP, and PSBF induced by local cooling of the left foot are shown. When the air

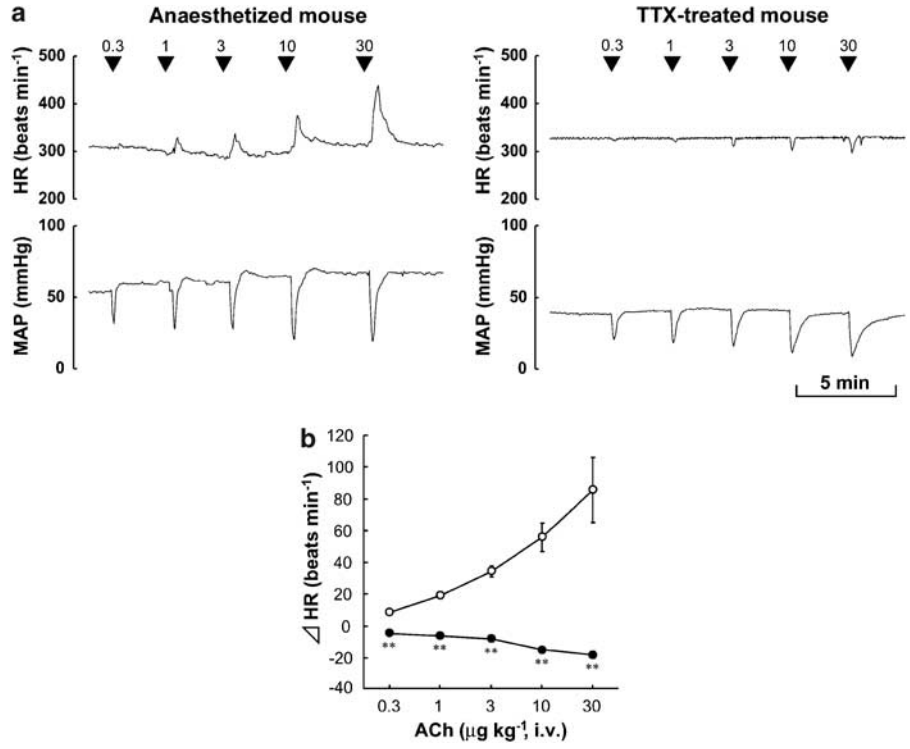

Figure 1 Inhibition by TTX of reflex tachycardia due to depressor response to ACh in anaesthetized mice. (**a**) Typical traces of changes in HR and MAP induced by ACh (0.3–30 $\mu g\,kg^{-1}$, i.v.) in anaesthetized mice (left) and TTX ($30\,\mu g\,kg^{-1}$, i.v.)-treated mice (right). (**b**) Dose–response relationship for ACh-induced changes in HR in anaesthetized (open circles) and TTX ($30\,\mu g\,kg^{-1}$, i.v.)-treated (solid circles) mice. Data represent mean ± s.e.mean ($n=4$). $^{**}P<0.01$ vs corresponding control in anaesthetized mice. ACh, acetylcholine; HR, heart rate; i.v., intravenous; MAP, mean arterial blood pressure; TTX, tetrodotoxin.

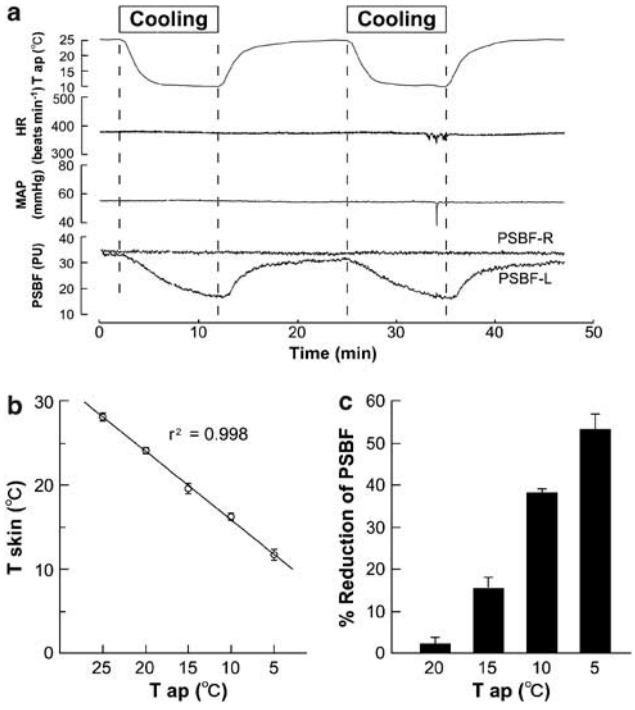

Figure 2 Effect of local cooling on skin blood flow in TTX-treated mice. (**a**) Typical traces of changes in the HR, MAP and plantar skin blood flow of the left (PSBF-L) and right feet (PSBF-R), induced by local cooling of the left foot in TTX-treated mice. T_{ap}, air temperature in the apparatus; PU, arbitrary perfusion units. (**b**) Relationship between T_{ap} and skin temperature of the left plantar surface (T_{skin}). T_{skin} was measured 10 min after the cooling was begun. (**c**) Relationship between changes in PSBF and T_{ap}. Changes in PSBF are expressed as a percentage of the basal PSBF at 25°C. Data represent mean±s.e.mean ($n=4$–6). HR, heart rate; MAP, mean arterial blood pressure; PSBF-L, plantar skin blood flow of the left foot; PSBF-R, plantar skin blood flow of the right foot; PU, perfusion units.

temperature in the apparatus was changed from 25 to 10°C, the PSBF of the left foot decreased and reached a plateau within 10 min. In contrast, the HR, MAP or PSBF of the right foot did not change during cooling. When the temperature in the apparatus was returned to 25°C, the PSBF of the left foot recovered to the basal level within 15 min.

When the temperature in the apparatus was changed from 25 to 20, 15, 10 and 5°C, the skin temperature of the plantar surface was decreased, and reached a plateau within 10 min. There was a linear relationship between the temperature in the apparatus and the skin temperature of the plantar surface(Figure 2b). Cooling the temperature in the apparatus decreased the blood flow in a temperature-dependent manner (Figure 2c). Since the response to cooling to 10°C was very reproducible, the following analyses were performed using this condition.

Role of α-adrenoceptors in the cooling-induced response

To elucidate the mechanism for the cooling-induced reduction of PSBF, pharmacological analyses were performed. Table 1 shows the effects of drugs *per se* on the HR, MAP and PSBF of the left foot in TTX-treated mice. The non-selective α-adrenoceptor antagonist phentolamine (10 mg kg^{-1}) and the α_1-antagonist bunazosin (5 mg kg^{-1}) *per se* caused a sustained decrease in HR, a transient increase in MAP and a transient small decrease in PSBF; MAP and PSBF almost recovered within 5 min. The α_2-antagonist RS79948 (1 mg kg^{-1}) *per se* caused a small increase in MAP and no marked changes in other parameters. Bretylium (10 mg kg^{-1}) and guanethidine (10 mg kg^{-1}), which inhibit noradrenaline release from sympathetic nerve endings, *per se* caused a transient large increase in MAP by 68 and 50%, respectively. The increased MAP was partially recovered and reached a plateau higher than that of the control. These

Table 1 Changes in basal levels of HR, MAP and PSBF after treatment with drugs, in TTX-treated mice

	HR (beats min^{-1})		*MAP (mm Hg)*		*PSBF (PU)*	
	Control	*After*	*Control*	*After*	*Control*	*After*
Vehicle	348±13	349±11	45±5	46±5	29.5±2.5	30.3±3.0
Phentolamine (10 mg kg^{-1})	406±23	332±25*	41±4	46±5	32.7±2.8	33.7±3.0
Bunazosin (5 mg kg^{-1})	395±30	361±22*	44±4	43±3	32.4±3.0	34.9±3.9
RS79948 (1 mg kg^{-1})	401±20	405±19	46±4	48±4*	29.8±1.8	30.4±2.1
Bretylium (10 mg kg^{-1})	424±16	497±2*	43±4	66±6**	35.7±3.3	45.4±8.1
Guanethidine (10 mg kg^{-1})	386±58	495±61**	39±4	50±7	34.2±2.9	39.1±5.0
PPADS (30 mg kg^{-1})	376±20	372±20	45±1	48±2*	34.2±1.6	37.2±2.3*
MK-912 (10 μg kg^{-1})	426±18	428±18	48±3	50±4	29.7±2.5	31.9±2.8
MK-912 (30 μg kg^{-1})	426±18	439±21*	48±3	52±5	29.7±2.5	33.0±3.3
OPC-28326 (100 μg kg^{-1})	467±15	461±15	44±6	44±7	33.0±2.6	34.4±4.1
Y-27632 (10 μg kg^{-1})	387±15	384±14	37±2	38±2	33.8±1.8	33.3±1.6
Y-27632 (100 μg kg^{-1})	387±15	381±14*	37±2	35±2*	33.8±1.8	33.4±2.1
H-1152 (10 μg kg^{-1})	402±9	405±8	48±2	49±2	32.4±0.3	34.6±1.2
H-1152 (30 μg kg^{-1})	402±9	401±12	48±2	48±2	32.4±0.3	36.2±2.1
Fasudil (100 μg kg^{-1})	396±23	406±27	44±3	46±4	28.6±3.2	30.5±2.8

Abbreviations: HR, heart rate; MAP, mean arterial blood pressure; PPADS, pyridoxal phosphate-6-azo(benzene-2, 4-disulphonic acid); PSBF, plantar skin blood flow; PU, perfusion units; TTX, tetrodotoxin.

Values show the stable basal levels of HR, MAP and PSBF before (control) and 5–10 min after (after) administration of drugs in TTX-treated mice; that is, just before the first and second applications of cooling, respectively. Each drug was injected intravenously.

Data represent mean±s.e.mean ($n=4$–6).

*$P<0.05$,**$P<0.01$ vs corresponding control.

drugs *per se* also caused a sustained increase in HR and a small increase in PSBF. When the haemodynamic parameters had stabilized after the treatment with each drug, we applied the cooling stimulation again.

Phentolamine, bunazosin and RS79948 all significantly suppressed the cooling-induced reduction of PSBF (Figure 3), although the inhibitory effect of bunazosin was relatively small compared with those of other antagonists. The specificity of these antagonists was confirmed by using phenylephrine (5 μg kg^{-1}), an α_1-agonist, and clonidine (3 μg kg^{-1}), an α_2-agonist (Table 2). Bunazosin abolished the pressor response to phenylephrine but not clonidine, whereas RS79948 inhibited the pressor response to clonidine but not phenylephrine. Phentolamine abolished both the pressor responses to phenylephrine and clonidine.

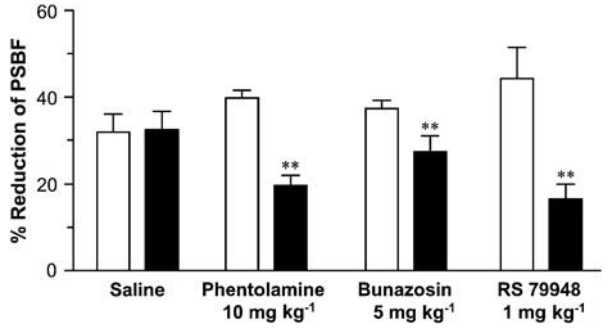

Figure 3 Effects of α-adrenoceptor antagonists on local cooling-induced reduction of PSBF in TTX-treated mice. Reduction of PSBF induced by local cooling to 10°C is expressed as a percentage of the basal PSBF at 25°C before (open columns) and after (solid columns) treatment with phentolamine (10 mg kg^{-1}, i.v.), bunazosin (5 mg kg^{-1}, i.v.) or RS79948 (1 mg kg^{-1}, i.v.). Data represent mean ± s.e.mean (n = 4–6). **P < 0.01 vs corresponding control. i.v., intravenous; PSBF, plantar skin blood flow; TTX, tetrodotoxin.

In the present experiments, the sympathetic tone was totally blocked by the TTX treatment. Nevertheless, the contribution of α-adrenoceptors to the cooling-induced response was suggested. Thus, we investigated the source of the catecholamines in the response. First, the effects of bretylium and guanethidine, compounds that inhibit the release of noradrenaline from the sympathetic nerve, were examined. The cooling-induced reduction of PSBF was not suppressed by bretylium or guanethidine (Figure 4a). We confirmed that these inhibitors abolished the pressor response to tyramine (100 μg kg^{-1}, data not shown). Second, the contribution of catecholamines released from the adrenal gland, to the response to local cooling was examined by using adrenalectomized mice. The serum concentration of adrenaline was less than the detection limit (5 pg ml^{-1}) in two of eight adrenalectomized mice and 23.5 ± 5.5 pg ml^{-1} (n = 6) in the others, whereas it was 292.6 ± 50.0 pg ml^{-1} (n = 8) in the sham-operated mice. After the treatment with

Table 2 Effects of α-adrenoceptor antagonists on an increase in mean arterial pressure induced by phenylephrine or clonidine in TTX-treated mice

	Control	*Phentolamine (10 mg kg^{-1})*	*Bunazosin (5 mg kg^{-1})*	*RS79948 (1 mg kg^{-1})*	*MK-912 (30 μg kg^{-1})*	*OPC-28326 (100 μg kg^{-1})*
Phenylephrine (5 μg kg^{-1})	107.1 ± 11.3	5.0 ± 2.1**	4.3 ± 0.9**	93.1 ± 18.2	104.4 ± 14.4	93.9 ± 9.3
Clonidine (3 μg kg^{-1})	51.7 ± 2.9	7.3 ± 4.7**	44.1 ± 4.5	8.8 ± 2.1**	57.2 ± 7.3	27.1 ± 6.9*

Abbreviation: TTX, tetrodotoxin.
Values show increases in MAP (mm Hg) induced by phenylephrine or clonidine before (control) and after treatment with each α-adrenoceptor antagonist. Each drug was injected intravenously.
Data represent mean ± s.e.mean (n = 4–6).
*P < 0.05, **P < 0.01 vs corresponding control.

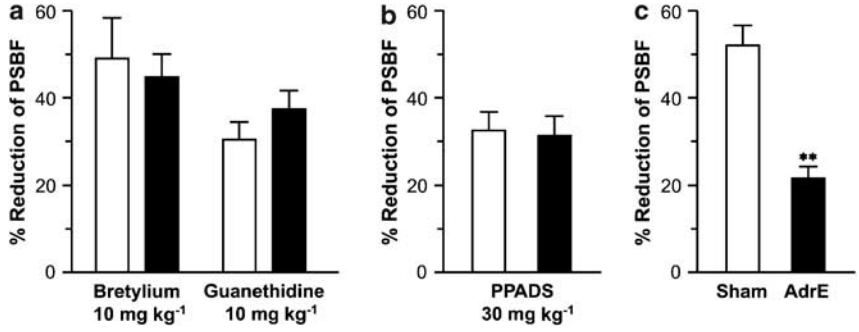

Figure 4 Noradrenaline released from sympathetic nerve terminals does not contribute to the reduction of PSBF in TTX-treated mice. (**a**) Reduction of PSBF induced by local cooling to 10°C is expressed as a percentage of the basal PSBF at 25°C before (open columns) and after (solid columns) treatment with bretylium (10 mg kg^{-1}, i.v.) or guanethidine (10 mg kg^{-1}, i.v.). (**b**) Reduction of PSBF induced by local cooling to 10°C is expressed as a percentage of the basal PSBF at 25°C before (open columns) and after (solid columns) treatment with P2 purinoceptor antagonist pyridoxal-5′-phosphate-6-azophenyl-2′,4′-disulphonate (PPADS; 30 mg kg^{-1}, i.v.). (**c**) Reduction of PSBF induced by local cooling to 10°C in sham-operated (Sham) and adrenalectomized (AdrE) mice is expressed as a percentage of the basal PSBF at 25°C. Data represent mean ± s.e.mean (n = 4–8). **P < 0.01 vs sham-operated mice. i.v., intravenous; PPADS, pyridoxal phosphate-6-azo(benzene-2, 4-disulphonic acid); PSBF, plantar skin blood flow; TTX, tetrodotoxin.

TTX, the HR was not different between the adrenalectomized (387 ± 17 beats min^{-1}, $n=8$) and sham-operated mice (397 ± 21 beats min^{-1}, $n=8$), but the MAP of the adrenalectomized mice (32 ± 3 mm Hg, $n=8$) was significantly lower than that of the sham-operated mice (44 ± 3 mm Hg, $n=8$; $P<0.05$). In the adrenalectomized mice, the cooling-induced reduction of PSBF was significantly smaller than that in the sham-operated mice (Figure 4c). We confirmed that the pressor responses to phenylephrine ($5\,\mu g\,kg^{-1}$) were not different between the adrenalectomized and sham-operated mice (data not shown). These results suggest that the enhanced sensitivity of α-adrenoceptors to circulating catecholamines is responsible for the cooling-induced reduction of PSBF.

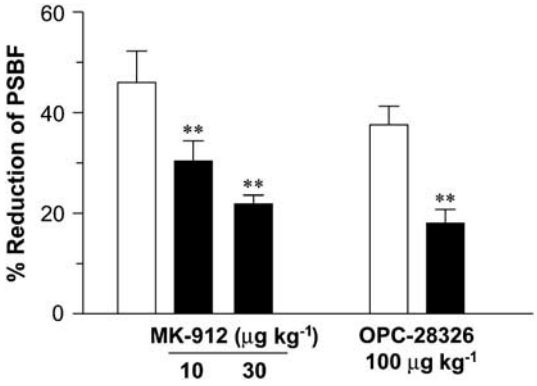

Figure 5 Effects of selective α_{2C}-adreoceptor antagonists on local cooling-induced reduction of PSBF in TTX-treated mice. Reduction of PSBF induced by local cooling to 10°C is expressed as a percentage of the basal PSBF at 25°C before (open columns) and after (solid columns) treatment with MK-912 (10 or $30\,\mu g\,kg^{-1}$, i.v.) or OPC-28326 ($100\,\mu g\,kg^{-1}$, i.v.). Data represent mean ± s.e.mean ($n=6$). **$P<0.01$ vs corresponding control. i.v., intravenous; PSBF, plantar skin blood flow; TTX, tetrodotoxin.

Two different α_{2C}-adrenoceptor antagonists, MK-912 and OPC-28326, were used to clarify whether α_{2C}-adrenoceptors contribute to the cooling-induced response, as has been shown in *in vitro* studies (Chotani *et al*., 2000; Bailey *et al*., 2004, 2005). MK-912 (10 and $30\,\mu g\,kg^{-1}$) *per se* caused a small increase in HR and no remarkable changes in other parameters (Table 1). OPC-28326 ($100\,\mu g\,kg^{-1}$) *per se* did not change any parameters. The reduction of PSBF induced by cooling to 10°C was significantly suppressed by MK-912 in a dose-dependent manner, and by OPC-28326 (Figure 5). The specificity of MK-912 for α_{2C}-adrenoceptors was confirmed by its inability to inhibit the pressor response to phenylephrine or clonidine (Table 2). OPC-28326 was also without any effect on the pressor response to phenylephrine, but only partly suppressed that to clonidine (Table 2).

As shown in Figure 6a, an i.a. injection of clonidine into the left iliac artery caused a transient decrease in PSBF in a dose-dependent manner. The clonidine response was nearly abolished by the α_2-adrenoceptor antagonist RS79948 (Figures 6a and b), suggesting that it was mediated via α_2-adrenoceptors. In the next series of experiments, we used

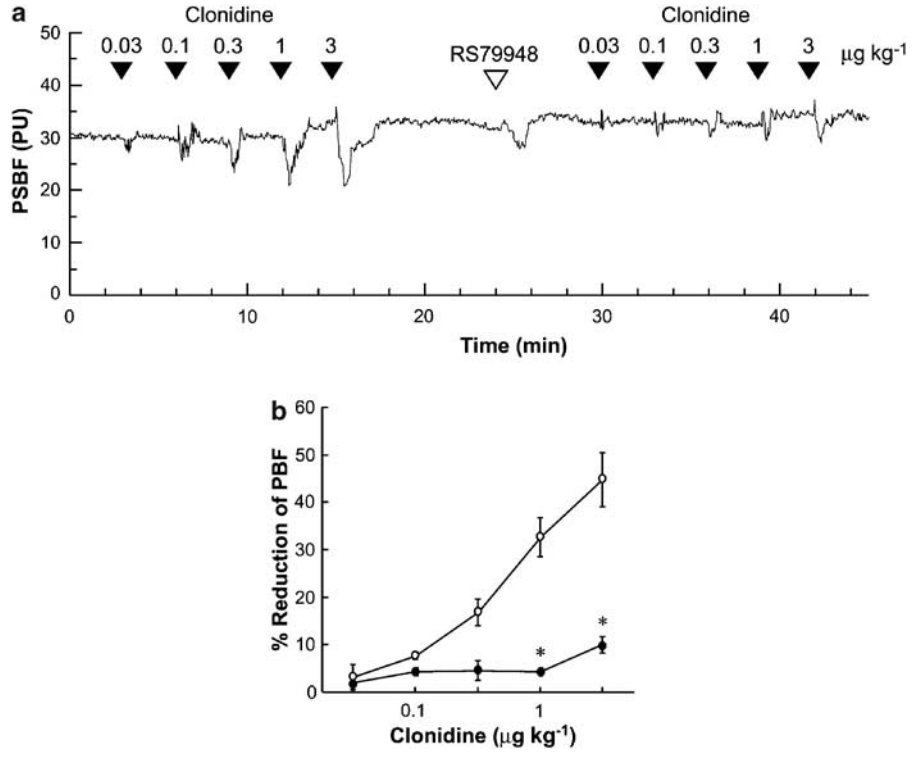

Figure 6 Effect of an α_2-adrenoceptor agonist on PSBF in TTX-treated mice. (**a**) Typical trace of changes in PSBF induced by clonidine (0.03–$3\,\mu g\,kg^{-1}$, i.a.) at 25°C. The decrease in PSBF was largely suppressed by RS79948 ($1\,mg\,kg^{-1}$, i.v.). (**b**) Dose–response relationship for clonidine-induced reduction of PSBF. The reduction of PSBF induced by clonidine (i.a.) is expressed as a percentage of the basal PSBF before (open circles) and after (solid circles) treatment with RS79948 ($1\,mg\,kg^{-1}$, i.v.). Data represent mean ± s.e.mean ($n=4$). *$P<0.05$ vs corresponding control. i.a., intra-arterial; i.v., intravenous; PSBF, plantar skin blood flow; TTX, tetrodotoxin.

adrenalectomized mice, in which cooling *per se* induced a relatively small change in PSBF, as shown in Figure 4c. The clonidine-induced decrease in PSBF was much larger at 10°C than at 25°C (Figures 7a, c and e). The α_{2C}-adrenoceptor antagonist MK-912 selectively inhibited only the clonidine response enhanced at 10°C (Figure 7d). In contrast, the α_2-antagonist RS79948 nearly abolished both the responses at 25 and 10°C (Figure 7f). These results suggest that the cooling-induced augmentation of the clonidine constrictor response is mediated by α_{2C}-adrenoceptors. Local administration of phenylephrine (0.3 μg kg^{-1}, i.a.) also caused a transient decrease in PSBF; however, it was not affected by cooling (Figures 7g and h).

We further investigated whether P2X purinoceptors contribute to the cooling-induced response, because our previous study in rats has shown that ATP released by cooling facilitates release of noradrenaline (Koganezawa *et al*., 2006). The P2X purinoceptor antagonist PPADS (30 mg kg^{-1}, i.v.)

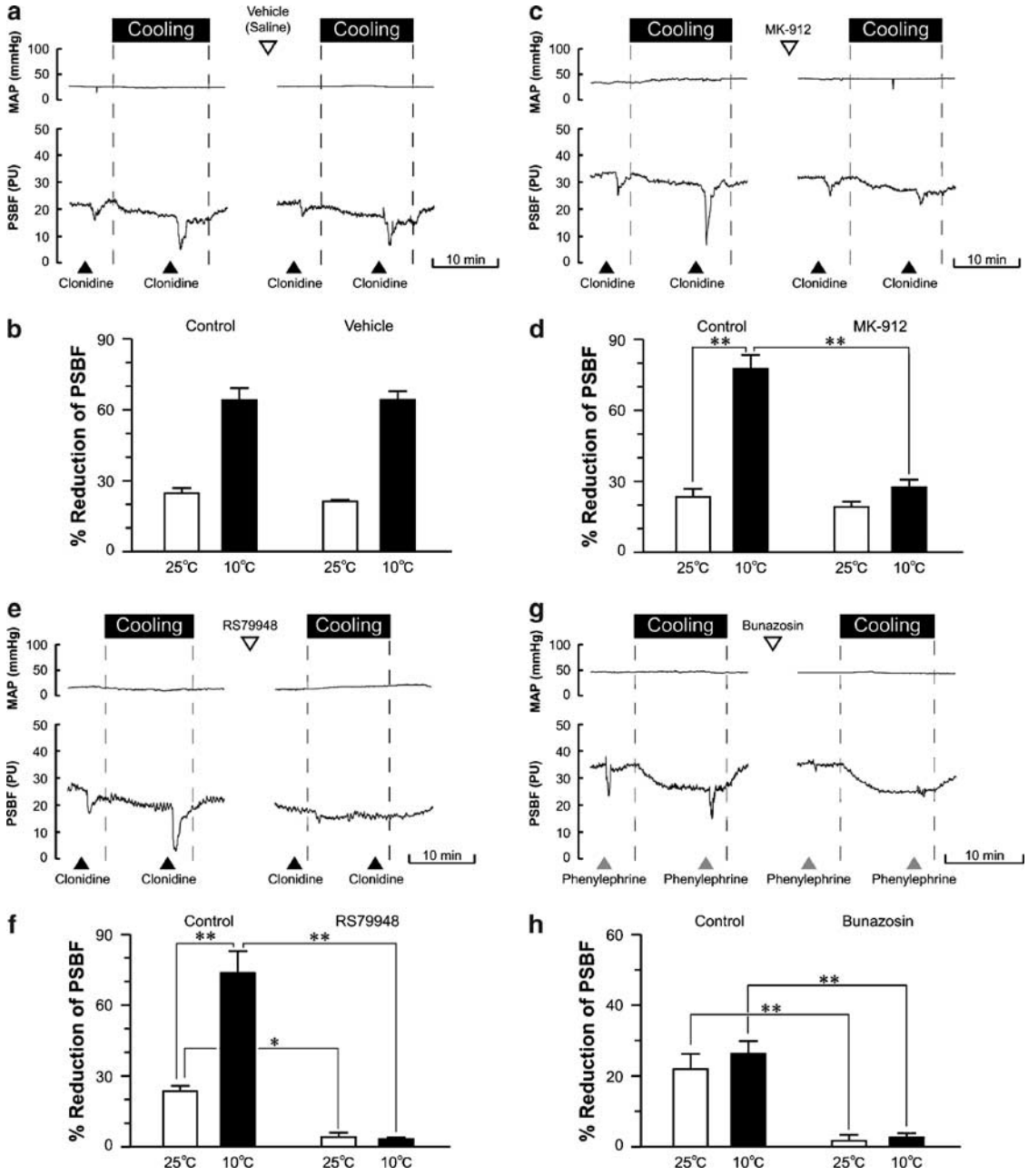

Figure 7 Influence of cooling on α_2- or α_1-adrenoceptor agonist-induced reduction of PSBF in adrenalectomized, TTX-treated mice. Typical traces of changes in PSBF induced by clonidine (1 μg kg^{-1}, i.a.; **a**, **c**, **e**) or phenylephrine (0.3 μg kg^{-1}, i.a.; **g**) before (left) and after (right) treatment with saline (**a**), MK-912 (30 μg kg^{-1}, i.v.; **c**), RS79948 (1 mg kg^{-1}, i.v.; **e**) or bunazosin (5 mg kg^{-1}, i.v.; **g**), at 25 and 10°C. Effects of saline (**b**), MK-912 (30 μg kg^{-1}, i.v.; **d**), RS79948 (1 mg kg^{-1}, i.v.; **f**) and bunazosin (5 mg kg^{-1}, i.v.; **h**) on clonidine (1 μg kg^{-1}, i.a.; **b**, **d**, **f**)- or phenylephrine (0.3 μg kg^{-1}, i.a.; **h**)-induced reduction of PSBF at 25 (open columns) and 10°C (solid columns). The reduction of PSBF is expressed as a percentage of the basal PSBF at 25 and 10°C. Data represent mean ± s.e.mean ($n = 4$). *$P < 0.05$, **$P < 0.01$. i.a., intra-arterial; i.v., intravenous; PSBF, plantar skin blood flow; TTX, tetrodotoxin.

per se caused increases in MAP and PSBF, but it did not affect the response to cooling (Figure 4b).

Involvement of Rho kinase in the response to cooling

Recent *in vitro* studies have proposed that the contraction of isolated cutaneous arteries induced by cooling is mediated by the Rho/Rho kinase pathway; this causes a translocation of α_{2C}-adrenoceptors to the plasma membrane (Bailey *et al*., 2004, 2005). We therefore investigated the involvement of Rho kinase in the cooling-induced reduction of PSBF with three different Rho kinase inhibitors, Y-27632, H-1152 and fasudil. Y-27632 (10 μg kg^{-1}), H-1152 (10 and 30 μg kg^{-1}) and fasudil (100 μg kg^{-1}) *per se* had no marked effects on the HR, MAP and PSBF in the TTX-treated mice. However, Y-27632 at 100 μg kg^{-1} caused small decreases in the HR and MAP (Table 1). The Rho kinase inhibitors all suppressed the cooling-induced reduction of PSBF (Figure 8a). After the treatment with Y-27632 (100 μg kg^{-1}), the α_2-adrenoceptor antagonist RS79948 (1 mg kg^{-1}) did not cause an additional decrease in the response induced by cooling, whereas the α_1-antagonist bunazosin (5 mg kg^{-1}) reduced it further (Figures 8b and c). Although the Rho kinase inhibitors were shown to inhibit other kinases (Davies *et al*., 2000; Sasaki *et al*., 2002), the inhibitors at doses used in the present study did not affect the pressor response to phenylephrine (5 μg kg^{-1}; data not shown).

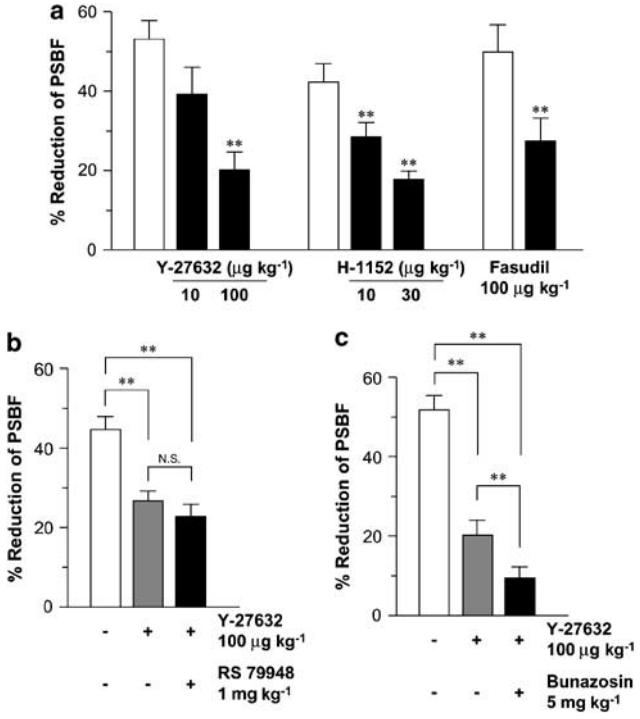

Figure 8 Effects of Rho kinase inhibitors on local cooling-induced reduction of PSBF in TTX-treated mice. (**a**) Reduction of PSBF induced by local cooling to 10°C is expressed as a percentage of the basal PSBF at 25°C before (open columns) and after (solid columns) treatment with Y-27632 (10 or 100 μg kg^{-1}, i.v.), H-1152 (10 or 30 μg kg^{-1}, i.v.) or fasudil (100 μg kg^{-1}, i.v.). (**b**, **c**) Reduction of PSBF induced by local cooling to 10°C is expressed as a percentage of the basal PSBF at 25°C before (open columns) and after treatment with Y-27632 (100 μg kg^{-1}, i.v.) and with Y-27632 plus RS79948 (1 mg kg^{-1}, i.v.) or bunazosin (5 mg kg^{-1}, i.v.). Data represent mean$\pm$s.e.mean ($n=4$–6). *$P<0.05$, **$P<0.01$ vs corresponding control. i.v., intravenous; N.S., not significant; PSBF, plantar skin blood flow; TTX, tetrodotoxin.

Discussion

The present *in vivo* study in TTX-treated mice demonstrates that cutaneous microcirculation is regulated locally by a direct action of cooling on the skin. The cooling-induced reduction of skin blood flow seems to result primarily from increased reactivity of α_{2C}-adrenoceptors to circulating catecholamines. We further provide pharmacological evidence that the Rho/Rho kinase pathway is involved in the response mediated by α_{2C}-adrenoceptors.

As in rats (Koganezawa *et al*., 2006), both α_1- and α_2-adrenoceptors were concluded to mediate cooling-induced reduction of PBSF in mice. However, apparent differences were observed between rats and mice. First, the cooling-induced vasoconstriction was primarily mediated via α_1-adrenoceptors in rats, but via α_2-adrenoceptors in mice. Second, the cooling-induced response was inhibited by guanethidine and bretylium in rats, but was insensitive to them in mice. Finally, adrenalectomy did not affect the cooling-induced response in rats, but reduced the same in mice. These findings imply that the cooling-induced response in rats is mediated by noradrenaline released from sympathetic nerve endings, which primarily stimulates α_1-adrenoceptors, whereas that in mice is mediated by circulating adrenaline and noradrenaline released from the adrenal glands, which primarily stimulates α_2-adrenoceptors. In rats, it has been suggested that the cooling-induced release of noradrenaline is mediated via the activation of presynaptic P2X purinoceptors on sympathetic nerve endings (Koganezawa *et al*., 2006). However, the cooling-induced response in mice was insensitive to a P2 purinoceptor antagonist, which is also in line with the view that the response in mice is not dependent on the release of noradrenaline from sympathetic nerve endings. The difference between rats and mice may be attributable to species differences. However, it should be noted that the results in the present study were obtained in TTX-treated mice; noradrenaline release from sympathetic nerves may be related to the cooling-induced response under physiological conditions.

In the present study, cooling augmented α_2-adrenoceptor reactivity in skin blood vessels in mice. This result accords with those from a number of earlier studies in isolated cutaneous blood vessels (Flavahan *et al*., 1985; Vanhoutte *et al*., 1985; Harker *et al*., 1990) and in human forearms and fingers (Ekenvall *et al*., 1988; Freedman *et al*., 1992). Recently, several *in vitro* studies have further suggested the contribution of α_{2C}-adrenoceptors to the cutaneous vascular response (Chotani *et al*., 2000; Bailey *et al*., 2004, 2005). In the mouse isolated tail artery, the cooling-induced augmentation of vasoconstriction to the α_2-adrenoceptor agonist UK-14304 was inhibited by the α_{2C}-adrenoceptor antagonist MK-912 (Chotani *et al*., 2000). In the HEK293 cells transfected with α_{2C}-adrenoceptors, cooling induced the translocation of α_{2C}-adrenoceptors to the plasma membrane

(Jeyaraj *et al*., 2001; Bailey *et al*., 2004). Thus, α_{2C}-adrenoceptors, which are not functional and localized to the Golgi compartment at 37°C, are suggested to be translocated to the plasma membrane by cooling, thereby augmenting vascular contraction. The present study is, to our knowledge, the first to demonstrate the contribution of α_{2C}-adrenoceptors to the cooling-induced response *in vivo*. In α_{2B}-adrenoceptor knockout mice, the pressor response to α_2-adrenoceptor agonists is absent (Link *et al*., 1996), indicating that postsynaptic α_{2B}-adrenoceptors mediate the vasoconstriction to α_2-agonists in mice at normal temperature. Since MK-912 did not suppress the pressor response to clonidine, MK-912 at the doses used in the present study is likely to selectively inhibit α_{2C}-adrenoceptors.

It has been suggested that the Rho/Rho kinase pathway mediates the cooling-induced α_{2C}-adrenoceptor translocation to the plasma membrane, thereby leading to enhanced vasoconstriction induced by cooling (Bailey *et al*., 2004, 2005). In accordance with the results from these studies, the cooling-induced response sensitive to α_2-adrenoceptor antagonists was inhibited by Rho kinase inhibitors. However, it has not yet been clarified how the Rho/Rho kinase pathway participates in the cooling-induced response *in vivo*. ROS generated in smooth muscle mitochondria in response to cooling may mediate the activation of the Rho/Rho kinase pathway and the subsequent translocation of α_{2C}-adrenoceptors to the plasma membrane, as has been suggested in an *in vitro* study (Bailey *et al*., 2005). Alternatively, the Rho/Rho kinase pathway may directly cause the α_{2C}-adrenoceptor-mediated vasoconstriction through inhibition of myosin phosphatase, as has been shown in rat-isolated aorta and porcine palmer lateral veins (Carter *et al*., 2002; Roberts, 2004).

Previously, we found that the treatment of rats with TTX allows the measurement of skin blood flow to be stabilized (Chino *et al*., 2000; Koganezawa *et al*., 2006). This method was applied to mice in the present study. The i.v. bolus injection of TTX was shown to abolish the pressor response induced by electrical stimulation of the spinal cord in rats (Chino *et al*., 2000). In the present study, the inhibition by TTX of sympathetic nervous tone was confirmed by depression of the reflex tachycardia due to the depressor response to Ach, and by failure of α-adrenoceptor antagonists to lower blood pressure in TTX-treated mice. However, no qualitative changes in the vasoconstrictor responses to α_1- and α_2-agonists were observed and cooling caused the reproducible reduction of PSBF in TTX-treated mice. It is likely, therefore, that the TTX treatment is a useful tool to investigate the local regulation of cutaneous microcirculation, at least vasoconstrictor responses, *in vivo*.

In rats, a large part of the cooling-induced reduction of PSBF was inhibited by phentolamine (Koganezawa *et al*., 2006). Unlike in rats, about half of the cooling-induced response in mice was insensitive to phentolamine. Several mediators other than catecholamines and ATP, such as NO (Hodges *et al*., 2006; Yamazaki *et al*., 2006), vasopressin (García-Villalón *et al*., 1999), endothelin-1 (García-Villalón *et al*., 1997), neuropeptide Y (García-Villalón *et al*., 2000), 5-hydroxytryptamine and angiotensin II (Furspan *et al*., 2004), have also been suggested to participate in cooling-induced vasoconstriction. Further investigations are needed to elucidate the mechanism for the cooling-induced response that is not affected by α-adrenoceptor antagonists in mice.

In summary, a reduction in skin blood flow could be induced by local cooling in mice. However, the mechanism in mice is apparently different from that in rats; the ATP-mediated novel mechanism for the cooling-induced response that we proposed in the rat study (Koganezawa *et al*., 2006), is unlikely to function in mice. The mechanism in mice seems to be rather similar to that in humans; α_2-adrenoceptors and Rho kinase contribute to the cooling-induced response in mice. Our results also indicate that the response is mediated by enhanced activity of α_{2C}-adrenoceptors, which supports the results from *in vitro* studies in isolated cutaneous arteries (Bailey *et al*., 2004, 2005). Patients with Raynaud phenomenon and scleroderma exhibit enhanced cutaneous vasoconstriction in response to cooling, which is mediated via α_2-adrenoceptors (Cooke and Marshall, 2005). Thus, the analysis of cooling-induced reduction of skin blood flow in mice will be of benefit in assessing the mechanisms underlying these diseases and in developing novel therapeutic strategies for them.

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Conflict of interest

The authors state no conflict of interest.

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