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Involvement of nitric oxide production in the impairment of skin blood flow response to local cooling in diabetic *db*/*db* mice

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ABSTRACT

An enhanced vasoconstrictor activity of cutaneous vessels participates in the reduction of skin blood flow induced by cooling. The present study investigated changes in the local response to cooling in hyperglycemic conditions. Male diabetic *db*/*db* and control C57BL/6J mice, anaesthetized with pentobarbitone, were treated with tetrodotoxin for eliminating the sympathetic nerve tone and artificially ventilated. The plantar skin blood flow (PSBF) was measured by laser Doppler flowmetry. Cooling the air temperature around the foot reduced PSBF in a temperature-dependent manner in control and *db*/*db* mice. The PSBF reduction was significantly smaller in *db*/*db* mice than in control mice. Phentolamine, a non-selective α-antagonist, bunazosin, a selective α_1-antagonist, MK-912, a selective α_{2C}-antagonist, and Y-27632, a Rho-kinase inhibitor, significantly inhibited the PSBF reduction induced by cooling to 15 °C in both mice and the inhibitory effects were comparable between these mice. The cooling-induced PSBF reduction was also significantly inhibited by N^{ω}-nitro-L-arginine, an inhibitor of nitric oxide synthase, in control mice; however, the inhibitory effect of N^{ω}-nitro-L-arginine was not observed in *db*/*db* mice. The reduction of PSBF induced by the intraarterial administration of adrenaline was comparable between control and *db*/*db* mice both before and after the treatment with N^{ω}-nitro-L-arginine. It is thus likely that the reduction of skin blood flow induced by local cooling might be partly mediated by a decrease in endothelium-derived nitric oxide production, and that an impairment of the nitric oxide production might be related to reduced vasoconstrictor response to cooling in *db*/*db* mice.

1. Introduction

The thermoregulation of skin blood flow plays a pivotal role in maintenance of normal body temperatures: cooling causes a reduction of skin blood flow, thereby protecting body from heat loss. This physiological response is mediated not only by a reflex increase in sympathetic output, but also by a locally enhanced contractile activity of cutaneous vessels (Vanhoutte, 1980). As the latter mechanism, the augmentation of α_2-adrenoceptor reactivity during cooling has been suggested in a number of *in vitro* studies using the saphenous vein from dogs (Flavahan et al., 1985; Vanhoutte et al., 1985) and humans (Harker et al., 1990). Moreover, the involvement of α_{2C}-adrenoceptors has been implied by studies showing that the enhanced constrictor response to the α_2-agonist UK-14304 during cooling was inhibited by the α_{2C}-antagonist MK-912 in mouse isolated tail arteries (Chotani et al., 2000) and that the translocation of α_{2C}-adrenoceptors from the Golgi compartment to the plasma membrane was induced by cooling in HEK293 cells transfected with α_{2C}-adrenoceptors (Bailey et al., 2004; Jeyaraj et al., 2001). Several *in vivo* studies in humans also favor the notion 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). However, the analysis of the thermoregulation of skin blood flow *in vivo* is limited, because the neuronal reflex mechanism through the sympathetic efferent, which is well developed in cutaneous circulation, prevents a stable measurement of skin blood flow (Johnson et al., 1986). In animal experiments, the treatment with tetrodotoxin (TTX), a voltage-dependent Na^+ channel blocker, by suppressing sympathetic nervous tone, enables to stabilize the measurement of skin blood flow (Chino et al., 2000; Koganezawa et al., 2006). Using this method, we have demonstrated that local cooling-induced reduction of skin blood flow *in vivo* primarily results from increased reactivity of α_{2C}-adrenoceptors in mice (Honda et al., 2007).

Diabetes is associated with vascular dysfunction, *i.e.*, enhanced vasoconstrictor and impaired vasodilator responses (Ishikawa et al., 2004; Kanie and Kamata, 2000; Matsumoto et al., 2004; Pannirselvam et al., 2002). Microvascular dysfunction is also recognized as important contributors to the morbidity associated with diabetes. However, limited information is available on how microvascular dysfunction in diabetes affects thermoregulatory control mechanisms in cutaneous microcirculation. Although

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cutaneous vasodilator responses to heating stress have been reported to be decreased in patients with type 2 diabetes (Sokolnicki et al., 2009; Wick et al., 2006), the mechanism remains to be fully elucidated. In the case of the cutaneous vasoconstrictor response to cooling, the information is more limited. There is only one report suggesting that preserved reflex cutaneous sympathetic and vasoconstrictor responsiveness during cold exposure in relatively healthy individuals with type 2 diabetes (Strom et al., 2011). In the present study, we investigated if the vasoconstriction of cutaneous vessels induced by local cooling is affected in hyperglycemic conditions. The data show the impairment of cutaneous vasoconstriction induced by local cooling in diabetic *db*/*db* mice, which is suggested to be attributable to endothelial dysfunction.

2. Material and methods

2.1. Animals

A total of 41 male diabetic *db*/*db* mice (C57BLKS/J lar-$^{+}$Lepr^{db}/$^{+}$Lepr^{db}; Japan SLC, Shizuoka, Japan), 12 weeks old and 30–55 g, and 38 male control (non-diabetic) mice (C57BL/6J; Japan SLC), 12 weeks old and 20–30 g, were used in this study. The mice were housed in a 12 h light-dark cycle, with food and water available *ad libitum*. The animal experiments were performed with protocols approved by the Institutional Animal Care and Use Committee of the University of Shizuoka and according to the Guidelines for Animal Experiments established by the Japanese Pharmacological Society.

2.2. Experimental procedures

The plantar skin blood flow (PSBF) in TTX-treated mice was measured as described previously (Honda et al., 2007). Briefly, the mice were anaesthetized with the intraperitoneal administration of pentobarbital sodium (75 mg/kg) 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), the mice were mechanically ventilated with air via a tracheal catheter connected to a rodent ventilator (SN-480-7; Shinano, Tokyo, Japan) at a stroke volume of 0.2 mL per 10 g body weight and a rate of 85 strokes per minute. A laser Doppler flow probe (NS type; Omega Wave, Tokyo, Japan) was set to the position about 5-mm apart from the center of the plantar surface of the left foot to measure PSBF with a non-contact laser Doppler flow meter (ALF 2100; Advance, Tokyo, Japan). The right foot served as the control. The skin temperature of the plantar surface was measured using a thermosensor (AW-601H, Nihon Kohden, Tokyo, Japan), the tip of which was inserted subcutaneously. Data were stored and analyzed 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 intraarterial injection of drugs into the left iliac artery to investigate local effects of drugs. The cooling apparatus composed of a plastic syringe and a rubber tube was constructed in our laboratory as described previously (Koganezawa et al., 2006). The temperature in the apparatus was continuously monitored with a thermosensor (SXB-54; Techno-Seven, Yokohama, Japan), and regulated by changing the temperature of water perfusing the rubber tube. The left foot was placed in the apparatus to apply local cooling. The temperature and humidity of the laboratory were maintained at 24 ± 2 °C and 55 ± 10%, respectively.

2.3. Drugs

The following drugs were used: TTX and clonidine hydrochloride (Wako, Osaka, Japan); phentolamine mesylate (Ciba-Geigi, Hyogo, Japan); Y-27632 ((R)-(+)-*trans*-N-(4-Pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide; Calbiochem, Darmstadt, Germany); Adrenaline bitartrate, *N*ω-nitro-L-arginine (L-NNA), MK-912 ((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), and phenylephrine hydrochloride (Sigma, St Louis, MO, USA). Bunazosin hydrochloride was kindly donated by Eisai (Tokyo, Japan). TTX and bunazosin were dissolved in distilled water. L-NNA was dissolved in 0.1 N HCl. Adrenaline was dissolved in saline containing in 0.01% ascorbic acid. The other drugs were dissolved in saline. L-NNA was intravenously administered as a bolus injection of 30 μL per 10 g body weight and the other drugs were 10 μL per 10 g body weight. Adrenaline was intraarterially administrated as a bolus injection of 1 μL per 10 g body weight. The appropriate vehicle controls showed no apparent effect.

2.4. Statistical analysis

All data are expressed as mean ± S.E.M and *n* shows the number of animals. Statistical significance was evaluated by Student's *t* test for either paired or unpaired observation. When more than two means were compared, statistical significance was evaluated by Williams' or Bonferroni's test for multiple comparison. *P* values less than 0.05 were considered significant.

3. Results

3.1. Cooling-induced reduction of PSBF in db/db mice

When the air temperature in the cooling apparatus was reduced from 25 to 20, 15, 10 and 5 °C, the PSBF of the left foot decreased in a temperature-dependent manner and reached a plateau within 10 min in control and *db*/*db* mice (Fig. 1). 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. The PSBF reduction induced by cooling to 20, 15, and 10 °C was significantly smaller in *db*/*db* mice than in control mice (Fig. 1).

3.2. Possible involvement of α-adrenoceptors

After recording a control response to cooling to 15 °C, phentolamine (10 mg/kg, i.v.), a non-selective α-antagonist, bunazosin (5 mg/kg, i.v.), a selective α_1-antagonist, or MK-912 (30 μg/kg, i.v.), a selective α_{2C}-antagonist, was applied. Our previous study

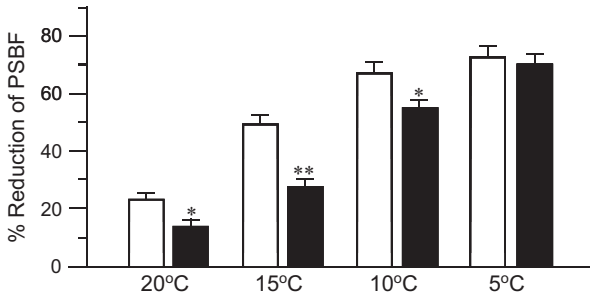

Fig. 1. Reduction of plantar skin blood flow (PSBF) induced by local cooling to 20, 15, 10, and 5 °C in TTX-treated control (open column) and *db*/*db* mice (closed column). The cooling-induced responses are expressed as a percentage of baseline at 25 °C. Data represent mean ± S.E.M. (n=5).