Transient Receptor Potential Channel Opening Releases Endogenous Acetylcholine, which Contributes to Endothelium-Dependent Relaxation Induced by Mild Hypothermia in Spontaneously Hypertensive Rat but Not Wistar-Kyoto Rat Arteries

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ABSTRACT

Mild hypothermia causes endothelium-dependent relaxations, which are reduced by the muscarinic receptor antagonist atropine. The present study investigated whether endothelial endogenous acetylcholine contributes to these relaxations. Aortic rings of spontaneously hypertensive rats (SHRs) and normotensive Wistar-Kyoto (WKY) rats were contracted with prostaglandin $F_{2\alpha}$ and exposed to progressive mild hypothermia (from 37 to 31°C). Hypothermia induced endothelium-dependent, $N\omega$-nitro-L-arginine methyl ester–sensitive relaxations, which were reduced by atropine, but not by mecamylamine, in SHR but not in WKY rat aortae. The responses in SHR aortae were also reduced by acetylcholinesterase (the enzyme responsible for acetylcholine degradation), bromoacetylcholine (inhibitor of acetylcholine synthesis), hemicholinium-3 (inhibitor of choline uptake), and vesamicol (inhibitor of acetylcholine release). The mild hypothermia-induced relaxations in both SHR and WKY rat aortae were inhibited by AMTB [N-(3-aminopropyl)-2-[(3-methylphenyl)methoxy]-N-(2-thienylmethyl)-benzamide; the transient receptor potential (TRP) M8 inhibitor]; only those in SHR aortae were inhibited by HC-067047 [2-methyl-1-[3-(4-morpholinyl)propyl]-5-phenyl-N-[3-(trifluoromethyl)phenyl]-1H-pyrrole-3-carboxamide; TRPV4 antagonist] while those in WKY rat aortae were reduced by HC-030031 [2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-isopropylphenyl) acetamide; TRPA1 antagonist]. The endothelial uptake of extracellular choline and release of cyclic guanosine monophosphate was enhanced by mild hypothermia and inhibited by HC-067047 in SHR but not in WKY rat aortae. Compared with WKY rats, the SHR preparations expressed similar levels of acetylcholinesterase and choline acetyltransferase, but a lesser amount of vesicular acetylcholine transporter, located mainly in the endothelium. Thus, mild hypothermia causes nitric oxide–dependent relaxations by opening TRPA1 channels in WKY rat aortae. By contrast, in SHR aortae, TRPV4 channels are opened, resulting in endothelial production of acetylcholine, which, in an autocrine manner, activates muscarinic receptors on neighboring cells to elicit endothelium-dependent relaxations in response to mild hypothermia.

Introduction

The different components of the neuronal cholinergic system, including the choline transporter, choline acetyltransferase, vesicular acetylcholine transporter (VAChT), acetylcholinesterase, as well as muscarinic and nicotinic receptors, are expressed in various non-neuronal cells (Kirkpatrick et al., 2003; Wessler and Kirkpatrick, 2008). Acetyl-CoA and choline

are substrates for acetylcholine synthesis; the former is generated by the metabolism of carbohydrates, and the latter by intracellular degradation of choline-containing phospholipids or by uptake from the extracellular environment via low- or high-affinity choline transporters (Sarter and Parikh, 2005; Michel et al., 2006). In neurons, choline uptake depends mainly on the high affinity choline transporter (CHT1) (Black and Rylett, 2012). CHT1 is also expressed in rat and human endothelial and smooth muscle cells (Lips et al., 2003). The mRNAs of choline acetyltransferase (the enzyme responsible for acetylcholine synthesis) and VAChT (the transporter responsible for loading acetylcholine into secretory vesicles and making acetylcholine available for release) are expressed

ABBREVIATIONS: AMTB, N-(3-aminopropyl)-2-[(3-methylphenyl)methoxy]-N-(2-thienylmethyl)-benzamide; EC, endothelial cell; HC-030031, 2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-isopropylphenyl)acetamide; HC-067047, 2-methyl-1-[3-(4-morpholinyl) propyl]-5-phenyl-N-[3-(trifluoromethyl)phenyl]-1H-pyrrole-3-carboxamide; L-NAME, $N\omega$-nitro-L-arginine methyl ester; NO, nitric oxide; SHR, spontaneously hypertensive rat; TRAM-34, 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole; TRP, transient receptor potential; UCL 1684, 6,12,19,20,25,26-hexahydro-5,27:13,18:21,24-trietheno-11,7-metheno-7H-dibenzo [b,m] [1,5,12,16]tetraazacyclotricosine-5,13-diium; VAChT, vesicular acetylcholine transporter; WKY, Wistar-Kyoto.

in rat pulmonary arteries and in endothelial cells (ECs) isolated from human or porcine pulmonary blood vessels (Haberberger et al., 2002). Choline acetyltransferase protein is present and functional in primary cultures of human umbilical vein ECs (Kirkpatrick et al., 2003). Acetylcholinesterase, which breaks down acetylcholine into choline and acetyl-CoA, is present in gerbil ECs (Lan et al., 1996). Thus, all the components that permit synthesis, release, and degradation of acetylcholine can be expressed or is present in ECs. Thus, it seems reasonable to assume that under certain circumstances they may produce endogenous acetylcholine that, in turn, activates receptors present on neighboring ECs, and consequently helps to regulate local vascular tone. Actually, synthesis and release of endogenous acetylcholine have been suggested in ECs of rat brain capillaries and bovine carotid arteries (Parnavelas et al., 1985; Kawashima et al., 1990).

Activation of muscarinic M3 receptors can initiate the release of nitric oxide (NO) and other vasoactive mediators and cause endothelium-dependent relaxations (Furchgott and Zawadzki 1980; Boulanger et al., 1994). Gradual hypothermia can induce NO-dependent vasodilatation in isolated canine coronary, femoral, and renal arteries, which can be prevented by atropine (Evora et al., 2007). These observations imply not only the involvement of acetylcholine receptors in an intrinsic regulatory loop leading to endothelium-dependent relaxations but also the contribution of a non-neuronal cholinergic system and its end product, acetylcholine. Thus, the present experiments were designed to test the hypothesis that hypothermia induces synthesis and release of endothelial acetylcholine, which in an autocrine fashion stimulates acetylcholine receptors to elicit endothelium-dependent relaxations. The M3 subtype of muscarinic receptor is mainly responsible for endothelium-dependent relaxation to acetylcholine (Furchgott and Zawadzki, 1980; Boulanger et al., 1994). In the aorta of spontaneously hypertensive rats (SHRs) —but not in that of normotensive Wistar-Kyoto (WKY) rats— when muscarinic receptors are inhibited/occupied by atropine, nicotinic receptors contribute to endothelium-dependent relaxations, indicating that hypertension can affect the sensitivity/function of endothelial cholinergic receptors (Zou et al., 2012). Thus, the present study also compared the production and/or function of endogenous acetylcholine in SHR and WKY rat aortae. Finally, because transient receptor potential (TRP) channels can mediate sensation to coldness

(Venkatachalam and Montell, 2007), their involvement in hypothermia-induced relaxations was also investigated.

Materials and Methods

All the animal experimental procedures were approved by the Committee on the Use of Live Animals for Teaching and Research of the University of Hong Kong, and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health (NIH publication no. 85-23, revised 1996).

Animal and Tissue Preparation. Male (36-week-old) SHRs and WKY rats were housed in a room with standardized temperatures ($21 \pm 1°C$) and exposed to a 12-hour dark/light cycle. The rats had free access to a standardized diet (LabDiet 5053; PMI Nutrition, St. Louis, MO) and tap water. They were anesthetized with pentobarbital sodium (70 mg/kg i.p.) before sacrifice. The thoracic aortae or the superior mesenteric arteries were isolated and placed immediately into cold Krebs-Ringer buffer with the following composition (in mM): 118 NaCl, 4.7 KCl, 2.5 CaCl$_2$, 1.2 MgSO$_4$, 1.2 KH$_2$PO$_4$, 25 NaHCO$_3$, and 11.1 glucose (control solution). After removing the adhering fat and connective tissue, the blood vessels were then cut into rings (approximately 3 mm in length). In some rings, the endothelium was removed mechanically by gently rubbing the intimal surface of the rings with a syringe needle (Tang et al., 2005).

Isometric Force Measurement. The rings were positioned in organ chambers that contained 5 ml of control solution aerated with a 95% O$_2$ and 5% CO$_2$ gas mixture at 37°C. Each ring was connected to a force transducer (model MLT0201/D; AD Instruments, Colorado Springs, CO) for isometric tension recording. They were stretched to an optimal tension of 2.5 g (determined in preliminary experiments; data not shown) and allowed to equilibrate for 90 minutes. Then, they were contracted twice with 60 mM KCl to obtain a submaximal reference contraction. The rings were incubated with vehicle or different chemicals for 30 minutes (Table 1). After incubation, they were contracted with prostaglandin F$_{2\alpha}$ (3×10^{-6} M) (Coleman et al., 1994) and then exposed to mild hypothermia. The temperature was decreased gradually from 37 to 31°C by adding ice packs to the heater bath. The temperature within the organ chambers was measured using a thermometer. The changes in tension were recorded continuously. Most experiments were performed in the presence of indomethacin (nonselective cyclooxygenase inhibitor, 10^{-5} M) to prevent the formation of endothelium-derived vasoactive prostanoids (Lüscher and Vanhoutte, 1986).

Choline/Acetylcholine Assay. To study the endothelium dependency of choline uptake and acetylcholine synthesis, aortic rings of SHRs and WKY rats (with or without endothelium) were incubated with vehicle or the substrate choline (10^{-4} M) at 37°C for 30 minutes. After incubation, the aortic rings were homogenized in choline assay

TABLE 1
Chemicals used and their functions and concentrations

Chemical	Function	Concentration	Reference
Acetylcholinesterase	Enzyme responsible for acetylcholine degradation	4 U/ml	Pohanka (2011)
AMTB	TRP channel subfamily M member 8 (TRPM8) inhibitor	10^{-6} M	Lashinger et al. (2008)
Atropine	Muscarinic receptor inhibitor	10^{-5} M	Clark (1926)
Bromoacetylcholine	Choline acetyltransferase inhibitor	10^{-6} M	Tucek (1982)
HC-030031	TRP channel, subfamily A, member 1 (TRPA1) inhibitor	10^{-6} M	Earley et al. (2009)
HC-067047	TRP cation channel subfamily V member 4 (TRPV4) inhibitor	10^{-6} M	Vincent and Duncton (2012)
Hemicholinium-3	High-affinity choline transporter inhibitor	10^{-7} M	Hartmann et al. (2008)
L-NAME	NO synthase inhibitor	10^{-5} M	Rees et al. (1990)
Mecamylamine	Nicotinic receptor inhibitor	10^{-4} M	Bacher et al. (2009); Zou et al. (2012)
TRAM-34	Intermediate-conductance calcium-activated potassium channels inhibitor	5×10^{-7} M	Gluais et al. (2005)
UCL 1684	Small-conductance calcium-activated potassium channels inhibitor	5×10^{-7} M	Gluais et al. (2005)
Vesamicol	VAChT inhibitor	10^{-6} M	Prior et al. (1992)

buffer. Proteins were concentrated by centrifugation and measured by Bradford assay. Following the manufacturer's instructions for the Choline Assay Kit (Abcam, Cambridge, UK), samples were incubated with reaction mixture. Fluorescence was measured at an excitation wavelength/emission wavelength of 535/590 nm in a micro-plate reader (Cary Eclipse Fluorescence Spectrophotometer with Micro-plate Reader System; Agilent Technologies, Santa Clara, CA) for fluorescence assay. The concentration of choline and acetylcholine (pmol) was normalized to the protein concentration (μg). To study the effect of mild hypothermia, aortic rings with endothelium were contracted with prostaglandin $F_{2\alpha}$ in the organ chambers. Then, the rings were removed from the chambers, before or after exposure to mild hypothermia. The concentrations of both choline and acetylcholine in the aortic rings were then quantified. The choline/acetylcholine level in rings treated with vehicle and without exposure to mild hypothermia represents the basal level.

Cyclic GMP Assay. To study the effect of mild hypothermia on cGMP production, aortic rings of SHRs and WKY rats with endothelium were contracted with prostaglandin $F_{2\alpha}$ in the organ chambers. Then, the rings were removed from the chambers, before or after exposure to mild hypothermia, and quickly frozen in liquid nitrogen. The samples (approximately 20 μg of tissue) were homogenized in 200 μl of 6% trichloroacetic acid. The cGMP was separated by centrifugation at 1500g for 15 minutes and then assayed using a cGMP enzyme immunoassay kit (Biomedical Technologies, Stoughton, MA) following the manufacturer's instructions.

Western Blotting. To measure the protein expression of the components of the non-neuronal cholinergic system, aortic rings of SHRs and WKY rats with endothelium were collected for immuno-assay. The rings were homogenized in lysis buffer (0.02 M Tris-HCL, 1% Triton X-100, 0.015 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 1 mM ethylene glycol tetraacetic acid, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, and 1 mM sodium orthovanadate) containing a protease inhibitor cocktail (50 μg/ml leupeptin, 25 μg/ml aprotinin, 10 μg/ml pesptatin, 2 mM dithiothreitol, and 2 mM phenylmethanesulfonylfluoride). The samples were centrifuged (5000 rpm) for 5 minutes at 4°C to obtain the supernatant. The protein concentrations were determined using the Bradford assay. The protein samples (50 μg) were run on a 10% SDS-PAGE. Proteins were transferred electrophoretically onto nitrocellulose membranes. The membranes were incubated overnight at 4°C with primary antibodies (anti-choline acetyltransferase 1:500; anti-acetylcholinesterase, 1:3000; anti-VAChT, 1:1000) and then incubated with horseradish peroxidase–conjugated secondary antibodies (1:3000) at 25°C for 2 hours. The probed membranes were visualized by chemiluminiscence using an ECL Plus Western Detection System (GE Health Life Science, Piscataway, NJ), and subsequently exposed to X-ray film (FujiFilm, Dusseldorf, Germany). The relative expression of target proteins was normalized to the level of β-actin in the same sample.

Immunofluorescent Staining. Cross-sections of SHR and WKY rat aortic rings were incubated with anti-VAChT antibodies (1:300) at 4°C overnight, and then incubated for 2 hours (in the dark and at room temperature) with fluorescein isothiocyanate–conjugated secondary antibody. The sections were also stained with 4′,6-diamidino-2-phenylindole (fluorescence stain for the detection of nuclei). The signals were detected using an Eclipse TE300 fluorescence microscope (Nikon, Tokyo, Japan) and quantified using ImageJ (NIH, Bethesda, MD). The fluorescent signal of the target protein was normalized to the level of 4′,6-diamidino-2-phenylindole in the same sample.

Drugs and Materials. Atropine, acetylcholinesterase, AMTB [N-(3-aminopropyl)-2-[(3-methylphenyl)methoxy]-N-(2-thienylmethyl)-benzamide], bromoacetylcholine, choline, hemicholinium-3, HC-030031 [2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-isopropylphenyl)acetamide], HC-067047 [2-methyl-1-[3-(4-morpholinyl) propyl]-5-phenyl-N-[3-(trifluoromethyl)phenyl]-1H-pyrrole-3-carboxamide], indomethacin, Nω-nitro-L-arginine methyl ester (L-NAME), mecamylamine, TRAM-34 (1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole), UCL 1684 (6,12,19,20,25,26-hexahydro-5,27:13,18:21,24-trietheno-11,7-metheno-7H-

dibenzo [b,m] [1,5,12,16]tetraazacyclotricosine-5,13-diium), vesamicol, and 4′,6-diamidino-2-phenylindole were purchased from Sigma-Aldrich (St. Louis, MO). Pentobarbital sodium was purchased from Ganes Chemicals (Pennsville, NJ). Prostaglandin $F_{2\alpha}$ was purchased from Cayman Chemical (Ann Arbor, MI). A stock solution of indomethacin was prepared in sodium bicarbonate (5×10^{-3} M). Prostaglandin $F_{2\alpha}$ was prepared in ethanol. All other compounds were prepared in deionized water. The concentrations are given in molar in the bath solution. Antibodies were purchased from Abcam. The concentrations of inhibitors used were selected either from preliminary experiments that studied various concentrations of the inhibitors (data not shown) from previous experience in the laboratory (Zou et al., 2012) or from the literature (Table 1).

Calculations and Data Analysis. Relaxations are expressed as the decrease in tension from the maximal contracted level to prostaglandin $F_{2\alpha}$ (3×10^{-6} M) and calculated as the percentage of that contraction (Fig. 1B). The relaxation/temperature curves (Fig. 1C) were analyzed statistically by two-way analysis of variance. The area under the relaxation/temperature curve was calculated and analyzed using Prism 5 (GraphPad Software, San Diego, CA). The bar graph results were analyzed by Student's t test for comparison of the
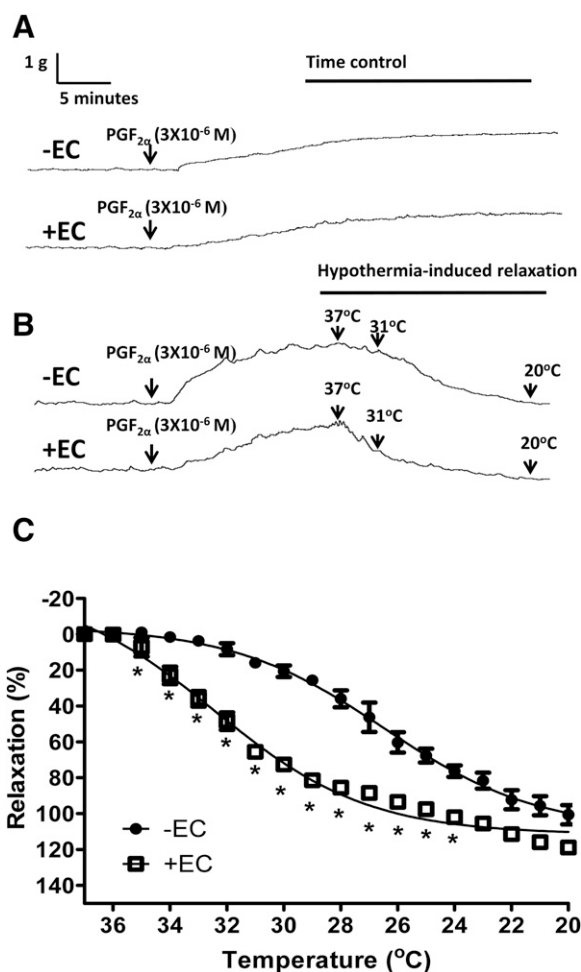
Fig. 1. Effect of hypothermia (37–20°C) in aortic rings with (+EC) or without (−EC) endothelium of 36-week-old male SHRs. Rings were contracted with prostaglandin $F_{2\alpha}$ (PGF$_{2\alpha}$) and then maintained at constant temperature (37°C) or exposed to gradual cooling (from 37 to 20°C). (A) Representative isometric tension recordings without exposure to hypothermia (time control). (B) Representative isometric tension recordings with exposure to hypothermia. (C) Relaxation/temperature curves. Data are expressed as percentage of the contraction to prostaglandin $F_{2\alpha}$ and are shown as mean ± S.E.M.; $n = 4$, *$P < 0.05$ versus −EC.

two groups. Results are presented as mean $\pm$ S.E.M., with n referring to the number of rats used. P values less than 0.05 were considered to indicate statistically significant differences.

Results

Mild Hypothermia Induced Endothelium-Dependent Relaxations. Without exposure to hypothermia, control rings both with and without endothelium sustained prostaglandin $F_{2\alpha}$–induced contraction (3×10^{-6} M) for at least 10 minutes (Fig. 1A). To investigate the effect of progressive cooling on relaxation and its endothelium dependency, preliminary experiments were conducted to measure the effect of deep hypothermia (from 37 to 20°C, in 10 minutes) on prostaglandin $F_{2\alpha}$–contracted SHR aortic rings with and without endothelium (Fig. 1B). Deep hypothermia induced relaxations in both types of preparations. However, with mild hypothermia (from 37 to 31°C, in 3 minutes) the relaxations in rings with endothelium approximated 70% of the contraction to prostaglandin $F_{2\alpha}$, while in rings without endothelium no significant decreases in tension were observed in that temperature range (Fig. 1C). The subsequent experiments focused on the endothelium-dependent relaxation caused by mild hypothermia. Indomethacin (10^{-5} M) was tested in the experiments to investigate the possible involvement of prostacyclin, and the results showed that the inhibitor of cyclooxygenases did not affect the hypothermia-induced endothelium-dependent relaxations in aortae of both WKY rats and SHRs (data not shown). Indomethacin was included in all subsequent experiments.

The mild hypothermia-induced endothelium-dependent relaxations in both SHR and WKY rat aortae were further characterized using pharmacological inhibitors to block the two major pathways leading to endothelium-dependent relaxation, production of NO by endothelial NO synthase, and endothelium-dependent hyperpolarization. Mild hypothermia induced comparable endothelium-dependent relaxations in aortic rings with endothelium of WKY rats and SHRs, which were not affected significantly by incubation with TRAM-34 plus UCL 1684 (5×10^{-7} M each; intermediate-conductance and small-conductance calcium-activated potassium channel inhibitors, respectively) but were reduced significantly by L-NAME (10^{-5} M, NO synthase inhibitor) (Fig. 2A).

In the following experiments, the rings were treated with atropine (10^{-5} M, muscarinic receptor inhibitor), mecamylamine (10^{-4} M, nicotinic receptor inhibitor), acetylcholinesterase (4 U/ml, enzyme responsible for acetylcholine degradation), bromoacetylcholine (10^{-6} M, choline acetyltransferase inhibitor), hemicholinium-3 (10^{-7} M, CHT1 inhibitor), and vesamicol (10^{-6} M, VAChT inhibitor) to determine whether the non-neuronal cholinergic system contributed to the responses. In WKY rat aortae, the mild hypothermia-induced relaxations were not significantly affected by incubation (30 minutes) with atropine, mecamylamine, acetylcholinesterase, bromoacetylcholine, hemicholinium-3, and vesamicol (Fig. 2, B and C). By contrast, in SHR aortae, the relaxations to hypothermia were reduced significantly by atropine but not by mecamylamine (Fig. 2B). The residual response in the presence of atropine approximated 70% of that observed in untreated control preparations and was significantly smaller than the response in WKY rat aortae. The relaxations of SHR aortae were reduced significantly in rings treated with acetylcholinesterase,
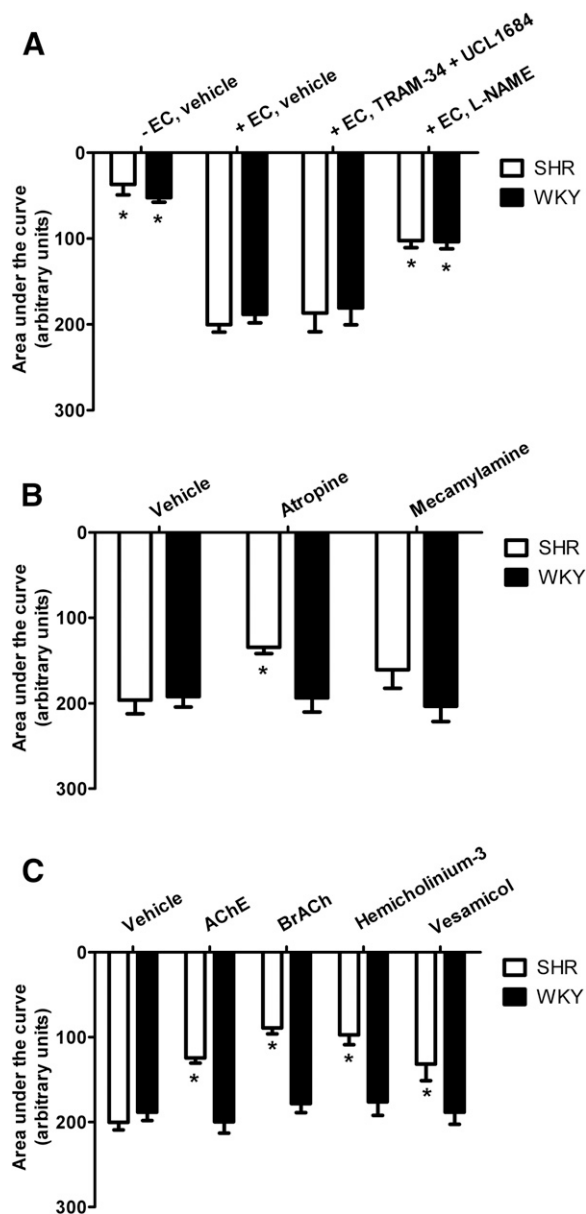
Fig. 2. Characterization of mild hypothermia (37–31°C)-induced endothelium-dependent relaxations in rat aortae. Aortic rings with (+EC) or without (−EC) endothelium of 36-week-old SHRs and WKY rats were incubated (30 minutes) with inhibitors [(A) vehicle, TRAM-34 plus UCL 1684 (5×10^{-7} M each) or L-NAME (10^{-5} M), or with (B) vehicle, atropine (10^{-5} M) or mecamylamine (10^{-4} M), or with (C) vehicle, acetylcholinesterase (AChE) (4 U/ml), bromoacetylcholine (BrACh) (10^{-6} M), hemicholinium-3 (10^{-7} M), or vesamicol (10^{-6} M)], contracted with prostaglandin $F_{2\alpha}$ (3×10^{-6} M) and then exposed to mild hypothermia. Data are expressed as area under the curve derived from relaxation/temperature curves (see Fig. 1) and are shown as mean $\pm$ S.E.M.; $n = 6$–8, *$P < 0.05$ versus (A) +EC, vehicle or (B and C) vehicle.

bromoacetylcholine, hemicholinium-3, and vesamicol compared with those observed in the control group (Fig. 2C). In addition, mild hypothermia also induced endothelium-dependent relaxations in mesenteric arteries of SHRs, which were significantly inhibited by incubation with L-NAME, TRAM-34 plus UCL 1684, and atropine (Fig. 3).

The Non-Neuronal Cholinergic System Was Expressed and Functional in Aortic Tissue. The existence of a non-neuronal cholinergic system in WKY rat and SHR aortae was confirmed by measuring the protein expressions of
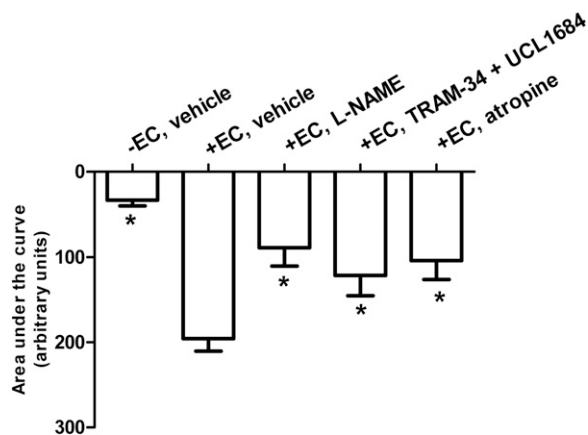
Fig. 3. Characterization of mild hypothermia (37–31°C)-induced endothelium-dependent relaxations in SHR mesenteric arteries. Rings with (+EC) or without (−EC) endothelium of 36-week-old SHRs were incubated with vehicle, L-NAME (10^{-5} M), TRAM-34 plus UCL 1684 (5×10^{-7} M), or atropine (10^{-5} M), contracted with prostaglandin $F_{2\alpha}$ (3×10^{-6} M) and then exposed to mild hypothermia. All rings were studied in the presence of indomethacin (10^{-5} M). Data are expressed as area under the curve derived from relaxation/temperature curves (see Fig. 1) and are shown as mean ± S.E.M.; n = 6–8, *$P < 0.05$ versus +EC, vehicle.

acetylcholinesterase, choline acetyltransferase, and VAChT using Western blotting. Compared with WKY rats, SHRs expressed similar levels of acetylcholinesterase and choline acetyltransferase, while the protein level of VAChT was significantly lower in SHR preparations (Fig. 4).

The distribution of VAChT in the vascular wall was detected by immunostaining (Fig. 5A). The fluorescence detected in the SHR aortae was significantly less than that observed in WKY rat preparations. However, most of the fluorescence in SHR aortae was detected in the endothelium, while it was distributed more evenly in the WKY rat arteries (Fig. 5B).

The ability of aorta to take up choline and to synthesize endogenous acetylcholine was determined by measuring the concentrations of choline and acetylcholine in rings incubated
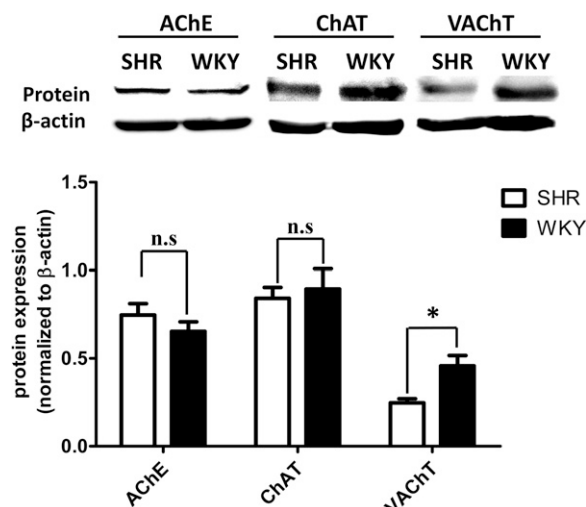

Fig. 4. Protein expressions of acetylcholinesterase (AChE) (70 kDa), choline acetyltransferase (ChAT) (82 kDa), and VAChT (59 kDa) in SHR and WKY rat aortic rings with endothelium. (Top) Representative Western blots. (Bottom) Mean values. The relative expression of target proteins was normalized to the level of β-actin in the same sample. n = 4–6, *$P < 0.05$ versus SHR. n.s., not significant.
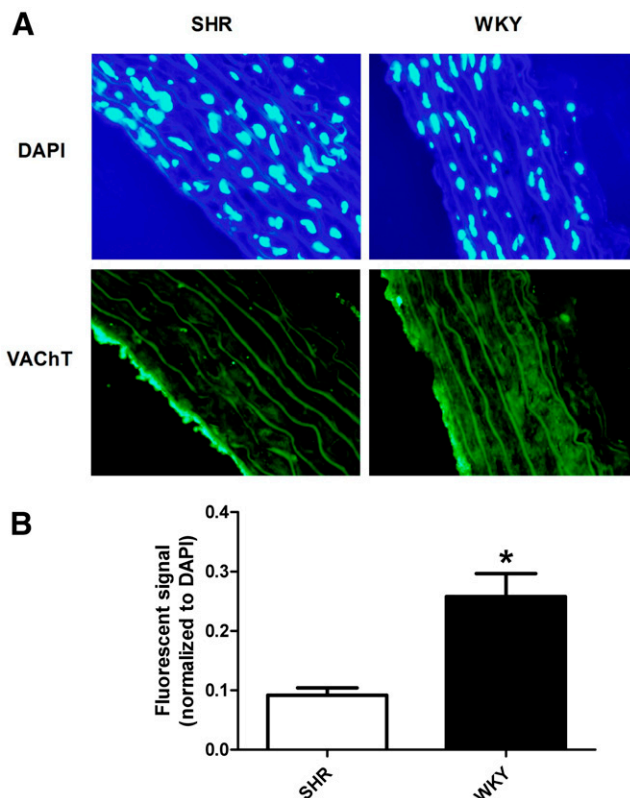
Fig. 5. (A) Representative examples of immunofluorescent demonstrations of the presence of the VAChT and 4′,6-diamidino-2-phenylindole (DAPI) in cross-sections of the aortae of SHRs and WKY rats. VAChT is stained in green; nuclei are stained by DAPI in blue. (B) Mean VAChT fluorescence intensity per positive nuclei; data are shown as mean ± S.E.M. (n = 4), *$P < 0.05$ versus SHR.

with and without exogenous choline (10^{-4} M, substrate for acetylcholine synthesis). The involvement of endothelium in these two processes was examined using aortic rings with and without endothelium. At 37°C, in both WKY rat and SHR aortae (Fig. 6), the choline and the acetylcholine concentrations were significantly higher in rings incubated with exogenous choline than that in preparations incubated with vehicle. The increases were not observed in rings without endothelium.

Effect of Mild Hypothermia on the Non-Neuronal Cholinergic System. The cGMP level and the concentrations of choline and acetylcholine in rings with and without exposure to hypothermia were measured to study whether mild hypothermia activated the NO/cGMP pathway and endogenous acetylcholine production, respectively, to produce relaxation. Without exposure to mild hypothermia, the basal levels of the cGMP level were similar between SHR and WKY rat preparations. Compared with the basal level, the cGMP concentration was significantly increased in both SHR and WKY rat aortae after exposure to mild hypothermia (Fig. 7A). In the presence of endothelium and exogenous choline, the choline concentration was significantly increased by exposure to mild hypothermia in SHR aortae, while no significant changes were observed in WKY rat preparations (Fig. 7B). Likewise, the acetylcholine concentration was increased significantly by exposure to mild hypothermia in SHR aortae, while no significant changes were observed in WKY rat preparations (Fig. 7C).
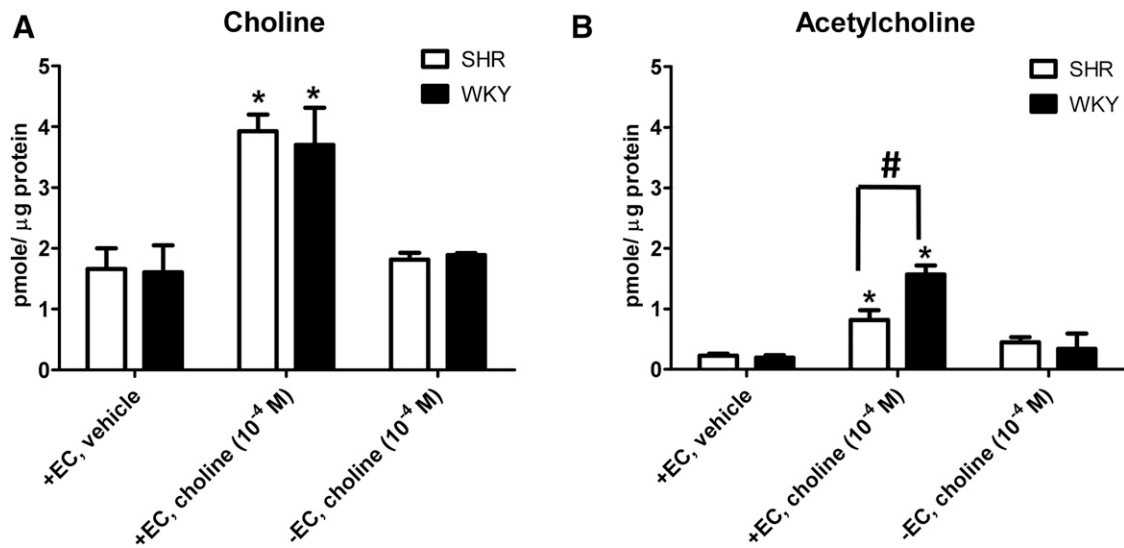
Fig. 6. Concentrations of (A) choline and (B) acetylcholine in SHR and WKY rat aortic rings with (+EC) or without ($-$EC) endothelium incubated with vehicle or choline (10^{-4} M). Data are shown as mean $\pm$ S.E.M.; n = 4–5, *P < 0.05 versus vehicle, +EC; #P < 0.05 versus SHR.

Involvement of TRP Channels in Mild Hypothermia-Induced, Endothelium-Dependent Relaxations. Aortic rings were incubated with AMTB (10^{-6} M, TRPM8 inhibitor), HC-030031 (10^{-6} M, TRPA1 inhibitor), or HC-067047 (10^{-6} M, TRPV1 inhibitor) to study the involvement of TRP channel subtypes in the mild hypothermia-induced endothelium-dependent relaxations. The responses were inhibited significantly by AMTB and HC-067047 in SHR aortae, while they were reduced significantly by AMTB and HC-030031 in WKY rat aortae (Fig. 8). To study the effect of TRP channel inhibitors on hypothermia-induced NO production, the hypothermia-induced increases in cGMP were measured in SHR and WKY rat aortic rings, which were incubated with HC-067047 and HC-030031, respectively. The increase in cGMP was abolished by HC-067047 in SHR aortae and by HC-030031 in WKY rat aortae (Fig. 8). Likewise, the increase in choline induced by mild hypothermia in SHR aortae was inhibited significantly by incubation with HC-067047 (Fig. 9).

Rings of SHR aortae were incubated with vehicle, atropine, HC-067047, or the combination of atropine plus HC-067047 to further investigate whether combined inhibition of muscarinic receptors and TRP channels exerted additive effects on mild hypothermia-induced relaxations. These relaxations were significantly reduced by HC-067067, atropine, and the combination of these two inhibitors. However, the combination of the two inhibitors was not significantly more effective than when each of the HC-067067 or atropine inhibitors were given alone (Fig. 10).

Discussion

The ultimate aim of the present study was to investigate the possible involvement of non-neuronal acetylcholine in the regulation of local vascular tone. Thus, the aorta, which is minimally or not at all innervated (Nilsson et al., 1986), was used in the experiments to exclude the possible involvement of neuronal acetylcholine released from surrounding nerve endings. In rabbit carotid arteries (Mustafa and Thulesius, 2002) or Sprague-Dawley rat pulmonary arteries and aortae

(Mustafa and Thulesius, 2001), cooling induced direct relaxations that were not dependent on the presence of the endothelium and the release of NO or neurotransmitters. By contrast, the present study demonstrates that hypothermia (from 37 to 20°C) induces relaxation of both the SHR and WKY rat aorta, and the comparison of the findings in rings with and without endothelium permits the conclusion that the response is mainly dependent on the endothelium in the temperature range from 37 to 31°C, while it is due to a direct effect on the smooth muscle cells when the temperature decreases below 31°C. In both SHRs and WKY rats, the mild hypothermia-induced endothelium-dependent relaxations are mainly mediated by NO because the responses are reduced by L-NAME (inhibitor of endothelial NO synthase; Rees et al., 1990). The involvement of NO is further confirmed by the finding that mild hypothermia increases the production of cGMP, which is synthesized upon activation of NO-sensitive guanylyl cyclase by NO (Gruetter et al., 1979). Thus, mild hypothermia must activate NO release in the endothelium, which in turn increases cGMP production in the underlying vascular smooth muscle cells leading to relaxation.
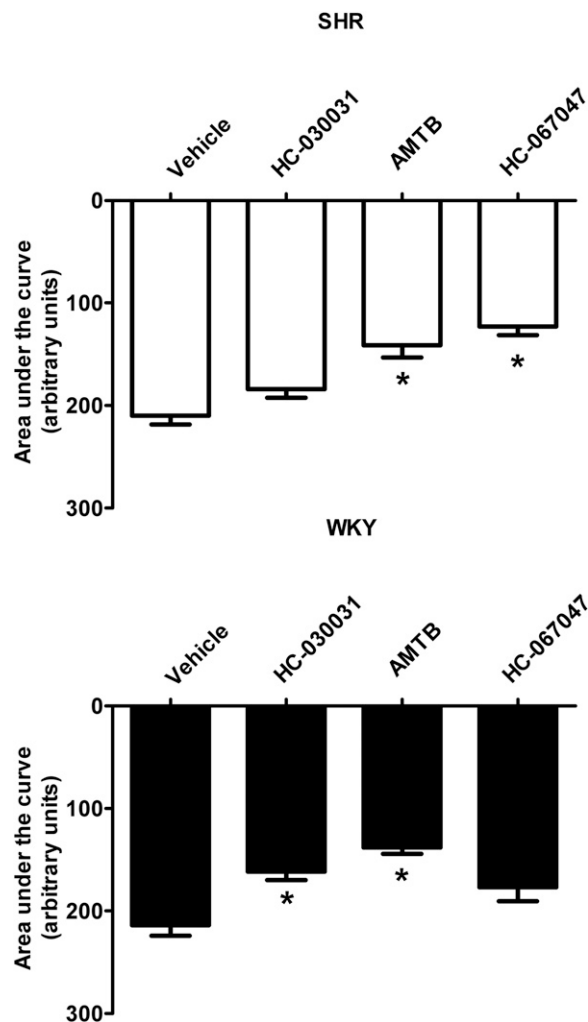
Relaxations induced by mild hypothermia were partly sensitive to atropine, acetylcholinesterase (enzyme responsible for acetylcholine degradation; Pohanka, 2011), bromoacetylcholine (inhibitor of choline acetyltransferase; Tucek, 1982), hemicholinium-3 (inhibitor of choline uptake by CHT1) (Hartmann et al., 2008), and vesamicol (inhibitor of acetylcholine release by VAChT; Prior et al., 1992). However, the inhibitory effect of interfering with the non-neuronal cholinergic system (by accelerating the degradation of acetylcholine, inhibiting its synthesis and release, or inhibiting the acetylcholine receptors) was only observed in preparations of SHRs but not in those of WKY rats, indicating that endogenous acetylcholine is involved in the response to cold in the aorta of the hypertensive animal, whereby the endogenously produced acetylcholine activates endothelial muscarinic receptors in an autocrine manner and elicits endothelium-dependent relaxations. This difference between SHR and WKY rat aortae suggests that hypertension may alter the function of the local non-neuronal cholinergic
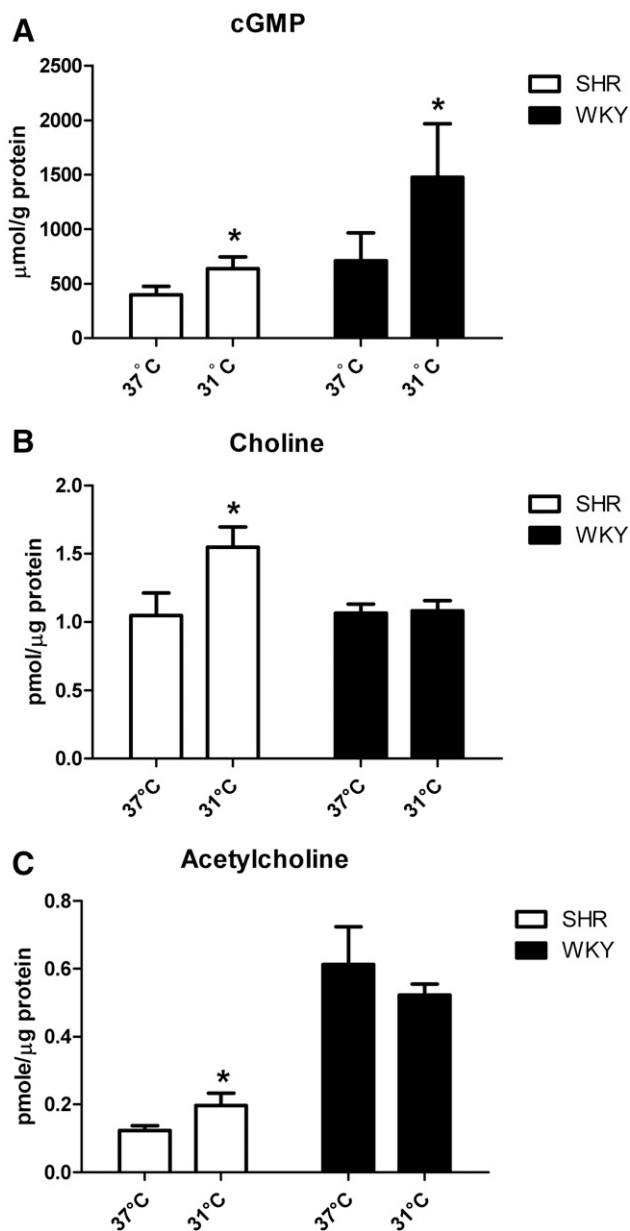
Fig. 7. Concentration of (A) cGMP, (B) choline, and (C) acetylcholine in SHR and WKY rat aortic rings with endothelium with or without exposure to hypothermia. Data are shown as mean ± S.E.M.; n = 4–6, *P < 0.05 versus 37°C.

Fig. 8. Effect of TRP channel inhibitors on mild hypothermia (37–31°C)-induced relaxations in aortic rings with endothelium of 36-week-old SHRs (Top) and WKY rats (Bottom). Rings incubated with vehicle, HC-030031 (10^{-6} M), AMTB (10^{-6} M), or HC-067047 (10^{-6} M) were contracted with prostaglandin $F_{2\alpha}$ (3×10^{-6} M) and then exposed to mild hypothermia. Data are expressed as area under the temperature/relaxation curves (see Fig. 1). All data are shown as mean ± S.E.M.; n = 4–6, *P < 0. 05 versus respective vehicle.
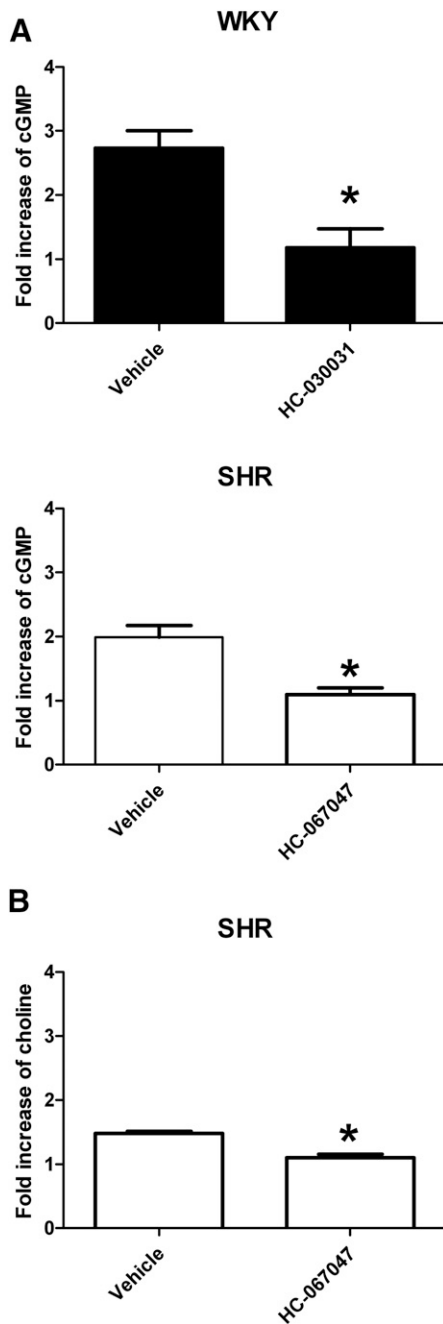
system. A similar conclusion was reached in an earlier study in which nicotinic receptors were shown to contribute to endothelium-dependent relaxations when muscarinic receptors are inhibited in the aorta of hypertensive but not normotensive rats (Zou et al., 2012).

Release of endothelial acetylcholine has been demonstrated in cultured human umbilical vein ECs (Milner et al., 1990) and cultured bovine aortic ECs (Olesen et al., 1988) in response to shear stress and in rat coronary arteries in response to hypoxia (Milner et al., 1989). In bovine aortic ECs (Olesen et al., 1988), the release of acetylcholine in response to shear stress leads to an increase in K^+ inward current. This acetylcholine-induced K^+ inward current was selectively inhibited by atropine. These previous findings suggest a role
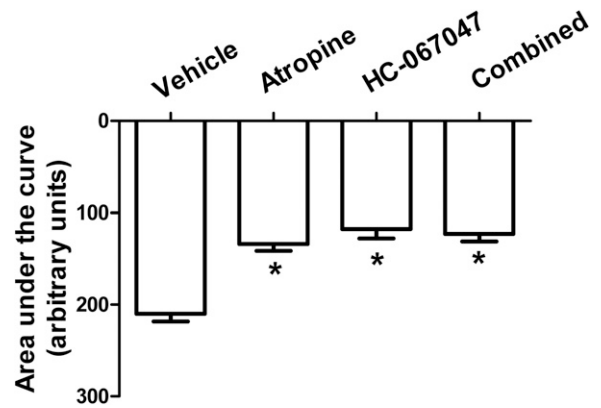
for non-neurogenic acetylcholine in the regulation of vascular tone. The present results show that at 37°C, the endothelium of both SHR and WKY rat aortae can take up choline from the extracellular space and convert it to endogenous acetylcholine. In addition, the measure of the protein expression of the components of the cholinergic system showed that, compared with that of the WKY rat aorta, the SHR aorta contained similar levels of the enzymes choline acetyltransferase (synthesis) and acetylcholinesterase (degradation), but less VAChT (release), a conclusion strengthened by the immunostaining measurements of the latter. Other investigators have reported similar findings showing that the protein expression of VAChT is less in the heart, kidney, and aorta of SHRs than in age-matched WKY rats, indicating reduced storage and release of acetylcholine in the hypertensive animal (Varoqui and Erickson, 1996). However, although there was less total VAChT in the SHR aorta, most of the transporter appeared to be concentrated in the endothelial layer, suggesting a greater propensity to release endogenous endothelium-derived acetylcholine.

Fig. 10. Role of muscarinic receptors and TRPV4 channels on mild hypothermia (37–31°C)-induced endothelium-dependent relaxations in SHR aortae. Aortic rings with endothelium of 36-week-old SHRs were incubated (30 minutes) with vehicle, atropine (10^{-5} M), HC-067047 (10^{-6} M), or a combination of atropine plus HC-067047, contracted with prostaglandin $F_{2\alpha}$ (3×10^{-6} M), and then exposed to mild hypothermia. Data are expressed as area under the curve derived from relaxation/temperature curves and are shown as mean ± S.E.M.; n = 4–6, *P < 0.05 versus vehicle.

Fig. 9. Effect of mild hypothermia (37–31°C) (A, top) on the cGMP concentration in WKY rat aortic rings with endothelium treated with vehicle or HC-030031 (10^{-6} M) and (A, bottom) on cGMP and (B) choline concentrations in SHR aortic rings with endothelium treated with vehicle or HC-067047 (10^{-6} M). Data are expressed as fold increase in concentration. All data are shown as mean ± S.E.M.; n = 4, *P < 0.05 versus vehicle.

The aforementioned findings permit the interpretation that in the endothelium of both SHR and WKY rat aortae the choline taken up by the endothelium can be converted to endogenous acetylcholine, which can consequently be released and possibly act on acetylcholine receptors expressed on neighboring ECs. The finding that the concentrations of choline and acetylcholine were both significantly increased after exposure to mild hypothermia in SHR aortae but not in WKY rat aortae suggests that mild hypothermia augments the activity of the non-neuronal cholinergic system in SHR aortae to further increase choline uptake and acetylcholine synthesis. By contrast, in the WKY rat aortae, the endothelium apparently does not take up the additional choline that would be required to produce a sufficient amount of acetylcholine during cooling to activate endothelial receptors. Thus, when exposed to mild hypothermia, the endothelium of normotensive rat aorta utilizes an acetylcholine-independent pathway to elicit relaxation. This conclusion is in line with the lack of effect of atropine on the response in WKY rat aortae.

TRP receptors, a group of thermosensitive cation channels, are located on the plasma membrane of various human and animal cell types (Venkatachalam and Montell, 2007; Gees et al., 2012). Twenty-eight unique mammalian TRP channel isoforms have been identified, and more than 19 isoforms (TRPA1, all of the TRPC; TRPV1, TRPV2, and TRPV4; all of the TRPM except TRPM5; and TRPP1 and TRPP2) are expressed in ECs (Venkatachalam and Montell, 2007; Gees et al., 2012). Most TRP channels are permeable to calcium ions, thus providing a direct pathway for calcium influx in ECs in response to different stimulations including receptor activation, hypoxia, or temperature changes (Singer and Peach, 1982; Lückhoff and Busse, 1990; Earley, 2011). Activation of TRPM8 (Johnson et al., 2009), TRPA1 (Earley et al., 2009), or TRPV4 (Vincent and Duncton, 2012) leads to endothelium-dependent relaxations (Earley et al., 2009; Johnson et al., 2009). The relaxation caused by TRPA1 activation in rat cerebral arteries is mediated by activation of small and intermediate conductance calcium-activated potassium channels, and hence endothelium-dependent hyperpolarization (Earley et al., 2009; Nilius et al., 2012), while that by TRPV4 activation in mouse small mesenteric and rat carotid arteries involves both NO and endothelium-dependent hyperpolarization (Mendoza et al., 2010). Because these three subtypes of TRP channels can cause increased intracellular Ca^{2+} concentration and generation of endothelium-derived relaxing factors, they are likely candidates for producing endothelium-dependent responses to cooling. In the aorta of the normotensive rats, the inhibitory effect of AMTB (TRPM8 selective antagonist; Lashinger et al., 2008) and HC-030031

(TRPA1 selective antagonist; Earley et al., 2009) suggests that both TRPM8 and TRPA1 play a role in the response to mild hypothermia. However, in the SHR aorta, while the function of TRPM8 apparently remains unaltered, that of TRPA1 must be impaired or lost because HC-030031 does not inhibit the endothelial component of the response to mild hypothermia. To compensate for this loss, TRPV4 appears to take over because HC-067047 (TRPV4 selective antagonist; Vincent and Duncton, 2012) reduces the response but only in preparations of the hypertensive strain. The finding that the mild hypothermia-induced increase in cGMP was abolished by the TRPA1 antagonist in the WKY rat aorta but by the TRPV4 antagonist in the SHR aorta further confirms the interpretation that in the hypertensive strain compensatory TRPV4 activation can make up for the loss of TRPA1-mediated NO production. The observation that in the SHR aorta the mild hypothermia-induced increase in choline uptake is inhibited by the TRPV4 antagonist then implies that the ECs of the hypertensive animal use TRPV4 channels to activate the production of endogenous acetylcholine in response to mild hypothermia. In addition, the finding that the combined inhibition of TRPV4 channels and muscarinic receptors does not have an additive effect on the relaxation responses further confirms the interpretation that TRP channels and muscarinic receptors are working sequentially but not independently in the response to cold. However, the present results do not allow further speculation as to the molecular events connecting TRP channels to the activation of the non-neuronal cholinergic system in the endothelium. As well as in response to hypothermia, TRPV4 activation can preserve vasodilatation under hypoxic conditions and restore NO-mediated relaxations during hypoxic preconditioning (Rath et al., 2012). Thus, TRPV4 channels may play a vascular protective role under pathologic conditions.

The present study provides evidence showing the potential importance of a non-neuronal cholinergic system in the local regulation of vascular tone and suggests that it may play a compensatory role in hypertension. The aorta is a large conduit vessel, which usually remains at constant temperature except during certain therapeutic interventions (Schwarzl et al., 2011). Thus, the results obtained cannot easily be extrapolated to the control of peripheral resistance. However, in the mesenteric artery of the SHR, which is considered to be a resistance vessel and is more likely to be exposed to changes in temperature during digestion, an inhibitory effect of atropine on the response to mild hypothermia is observed in a similar way as in its aorta. These observations suggest that the modulatory role of the non-neuronal cholinergic system on the response to cold may occur in more peripheral blood vessels. The observation that not only L-NAME but also TRAM-34 plus UCL 1684 inhibited the response in the mesenteric arteries implies that in these blood vessels endothelium-dependent hyperpolarization contributes to the response to cold (Earley et al., 2009; Nilius et al., 2012).

In summary, the present findings suggest that in the aorta of normotensive animals mild hypothermia activates TRPA1 and TRPM8 channels, which elicit endothelial NO synthase–dependent, endothelium-dependent, and NO-mediated relaxations in an acetylcholine-independent manner. However, the TRPA1-mediated pathway is impaired/deficient in arteries of spontaneously hypertensive animals, but in compensation for this dysfunction, TRPV4 channels activate the production of endogenous acetylcholine, which induces relaxations by stimulating muscarinic receptors on the ECs leading to an increased endothelial NO synthase–dependent production of NO.

Authorship Contributions

Participated in research design: Zou, Leung, Vanhoutte.
Conducted experiments: Zou.
Performed data analysis: Zou.
Wrote or contributed to the writing of the manuscript: Zou, Leung, Vanhoutte.

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