RhoA/ROCK Signaling

Activation of Cold-Sensing Transient Receptor Potential Melastatin Subtype 8 Antagonizes Vasoconstriction and Hypertension Through Attenuating RhoA/Rho Kinase Pathway

Jing Sun,* Tao Yang,* Peijian Wang, Shuangtao Ma, Zhenyu Zhu, Yunfei Pu, Li Li, Yu Zhao, Shiqiang Xiong, Daoyan Liu, Zhiming Zhu

Abstract—Environmental cold is a nonmodifiable hypertension risk factor. Transient receptor potential melastatin subtype 8 (TRPM8) is a cold-sensing cation channel that can be activated by menthol, a compound with a naturally cold sensation in mint. Little is known about the effect of TRPM8 activation on vascular function and blood pressure. Here, we report that TRPM8 is abundantly expressed in the vasculature. TRPM8 activation by menthol attenuated vasoconstriction via RhoA/Rho kinase pathway inhibition in wild-type mice, but the effect was absent in TRPM8−/− mice. Chronic dietary menthol blunted mesenteric arterial constriction and lowered blood pressure in genetic hypertensive rats via inhibition of RhoA/Rho kinase expression and activity in the vivo study. TRPM8 effect was associated with inhibition of intracellular calcium release from the sarcoplasmic reticulum, RhoA/Rho kinase activity, and sustained arterial contraction in the vitro study. Importantly, 8-week chronic menthol capsule treatment moderately lowered systolic blood pressure and diastolic blood pressure in prehypertensive individuals compared with the placebo group. Furthermore, chronic menthol capsule administration also improved flow-mediated dilatation in prehypertensive individuals, but not in the placebo group. In conclusion, our study demonstrates that TRPM8 activation by menthol benefits vascular function and blood pressure by inhibiting calcium signaling–mediated RhoA/Rho kinase activation in the vasculature. These findings add to the evidence that long-term dietary menthol treatment had favorable effects on hypertension treatment. (Hypertension. 2014;63:1354-1363.) ● Online Data Supplement

Key Words: hypertension ■ menthol ■ muscle, smooth, vascular ■ rho-associated kinases ■ TRPM8 protein, human ■ vasoconstriction

Blood pressure levels are affected by cold stress. Cold exposure results in high blood pressure through several mechanisms, such as sympathetic nervous system activation, endothelial function impairment, renal sodium load enhancement, blood viscosity changes, platelet activation, and increased inflammation. Recent studies demonstrate that several transient receptor potential channels, such as transient receptor potential potential canonical 3 (TRPC3) and transient receptor potential melastatin 7 (TRPM7), are involved in hypertension pathogenesis. TRPM8 is a main molecular entity that is responsible for detecting cold stimulation. TRPM8 is a calcium-permeable nonselective cation channel that is activated by cold temperatures (≤26°C) and pharmacological agents that mimic the psychophysical sensation of cold such as menthol. TRPM8 is mainly localized in the sensory neurons, but its distribution in the fundus and adipose tissue has also been reported. Johnson et al demonstrated that menthol-induced TRPM8 activation regulates vascular tone. Mustafa et al reported that administration of menthol, a TRPM8 agonist, caused rat fundus contraction that was associated with the Rho kinase (ROCK) pathway. ROCK and its substrate myosin phosphatase targeting subunit 1 (MYPT-1) are responsible for vascular contraction by increasing calcium sensitivity. Abnormal RhoA/ROCK activity is associated with human and experimental hypertension. Fasudil, a selective ROCK inhibitor, can lower high blood pressure in hypertension. U46619 (9,11-deoxy-9a, 11a-methanoepoxy Prostaglandin F2a), which is a thromboxane A2 analogue, is commonly used to contract pulmonary vascular smooth muscle cells and induce pulmonary arterial hypertension. Although numerous hypertensive drugs play important roles in hypertension treatment, diets such as the Dietary Approaches to Stop Hypertension (DASH) diet have emerged as effective antihypertensive strategies. Our recent study demonstrated that TRPM8 activation by chronic dietary menthol can prevent high-fat diet–induced obesity in mice via Uncoupling Protein-1–mediated thermogenesis upregulation.
in brown fat beyond sympathetic nervous system activation.7 Currently, little is known about the effect of TRPM8 activation on vascular function and blood pressure. In this study, we hypothesized that dietary menthol contributes to vascular benefits and high blood pressure reduction via TRPM8 activation. Here, we demonstrate that menthol-induced TRPM8 activation attenuated vascular tone by inhibiting calcium signaling–mediated RhoA/ROCK activation.

Materials and Methods

Animal Treatment
We obtained male spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY) from Charles Rivers Laboratories (Malvern, PA) and obtained TRPM8−/− mice from the laboratory of Dr Patapoutian.8 To maintain an isogenic strain, heterozygous knockout mice and their wild type (WT) littersmates were maintained and used for experiments, as previously described9 (online-only Data Supplement).

Cell Culture
Vascular smooth muscle cells (VSMCs) were obtained from the thoracic aortas of mice and cultured by the tissue explant method, as previously described10 (online-only Data Supplement).

Intracellular Calcium Measurements
Fluorescence measurements were performed at 510 nm emission, with excitation wavelengths of 340 and 380 nm (Fluroskan Ascent Fluorometer; Thermo Helsinki, Finland; online-only Data Supplement).

Blood Pressure Measurement
Animals were implanted surgically with telemetric transmitters (Data Sciences International, MN), and 24-hour ambulatory systolic and diastolic pressures were measured by telemetry in conscious unrestrained animals8 (online-only Data Supplement).

Prehypertensive Participant Characteristics
Prehypertensive participants aged between 45 to 65 years were included in this study. The prehypertension diagnosis was based on systolic blood pressure (SBP) of 120 to 139 mm Hg or diastolic blood pressure of 80 to 89 mm Hg (online-only Data Supplement).

Flow-Mediated Vasodilation
Flow-mediated vasodilation was measured by a single trained sonographer with high-resolution ultrasonography using a 7.5-MHz linear transducer on an HY 6000 system (Wixi Haiying Electronic Medical System Co. Ltd, Jiangsu, China) in accordance with international guidelines (online-only Data Supplement).

Western Blot and Immunofluorescence Analysis
Immunoblots of TRPM8, RhoA, ROCK-2, p-MYPT-1, Rho-GTP, and GAPDH were detected as previously described7 (online-only Data Supplement).

RNA Isolation and Polymerase Chain Reaction Analysis
Total RNA was isolated from the VSMCs of C57BL/6j mice (WT) and the aorta of WT and TRPM8−/− mice using the TRIzol reagent (Invitrogen, USA). RNA was used to synthesize the first-strand cDNA using the Reverse Transcription System (Promega A3500, USA; online-only Data Supplement).

Measurement of Vascular Reactivity
Vascular reactivity of freshly isolated mesenteric arteries was studied by wire myograph (Danish Myo Technology, Denmark), as described.11,12

Statistical Analyses
All data represent mean±SEM from different rodents. Statistical significance was determined by 2-tailed Student t test or 1-way ANOVA followed by the Bonferroni post hoc test as appropriate. P values <0.05 indicate statistically significant difference. The clinical data were expressed as mean±SD. Statistical significance was determined by Student t test for the placebo and menthol capsule–treated groups. The paired t test was used to compare with their baseline and treatment.

Results
Characterization of TRPM8 in the Vasculature
To characterize TRPM8 in the mouse vasculature, primary cultured VSMCs and mesenteric arteries were obtained from WT and TRPM8−/− mice. TRPM8 expression was detected clearly by immunofluorescence both in the cultured VSMCs and in the mesenteric arterial medial layers from WT but not TRPM8−/− mice (Figure 1A and 1B). TRPM8 mRNA and protein expression were also detected by reverse transcriptase-polymerase chain reaction (PCR) or immunoblotting in both cultured VSMCs and freshly isolated aortas from WT mice and TRPM8−/− mice (Figure 1C and 1D). These data suggest that TRPM8 is abundantly localized in the vasculature.

TRPM8 Activation Attenuates Hypertension
Next, we examined whether TRPM8 activation modulated vascular function and what mechanisms were involved. The mesenteric arteries were precontracted with 60 mmol/L KCl. The administration of the RhoA agonist U46619 caused dose-dependent vasoconstriction, which was attenuated by a TRPM8 activator, menthol or icilin, or ROCK inhibitor Y27632 pretreatment in WT mice, but the effects were absent in TRPM8−/− mice, whereas ROCK inhibitor (Y27632) treatment still inhibited U46619-induced vasoconstriction in TRPM8−/− mice (Figure 2A). TRPM8 antagonist, [N-(3-aminopropyl)-2-[(3-methylphenyl) methyl]oxy]-N-(2-thienylmethyl) benzamide hydrochloride salt], AMTB, significantly inhibited menthol-induced vasodilation from mesenteric arteries in concentration-dependent manner (Figure S1A in the online-only Data Supplement). We evaluated the effect of menthol on basal tone in mesenteric arteries. In basal force vessel (1.8 mN), menthol caused a weak, but transient and dose-dependent vasoconstriction (Figure S1B). In addition, endothelium-denuded reduced menthol-induced vasodilation (Figure S1C). Furthermore, it showed that menthol reduced both tonic and phasic contractile induced by U46619, this effect was more dominated in the presence of calcium channel blocker, nimodipine (100 mmol/L; Figure S2). Treatment with U46619 significantly upregulated RhoA and ROCK-2 protein expression as well as ROCK activity, as assessed by Rho-GTP and MYPT-1 phosphorylation protein expression in the cultured VSMCs from WT mice. Furthermore, these effects were inhibited by pretreatment with 100 μmol/L menthol, 10 μmol/L icilin, or 1 μmol/L ROCK inhibitor Y27632 (Figure 2B) and absent in TRPM8−/− mice (Figure 2C). These results indicate that TRPM8 activation attenuates vasoconstriction via RhoA/ROCK pathway inhibition.
TRPM8 Activation–Mediated Vasoconstriction Inhibition Is Associated With Blunted RhoA/ROCK-Related Calcium Signaling

We further examined the mechanisms by which menthol-induced TRPM8 activation can antagonize U46619-induced vasoconstriction. U46619 treatment only triggered a weak vasoconstriction in the absence of external calcium, but adding 2.5 mmol/L Ca\(^{2+}\) caused a significant phasic and tonic constriction. Menthol dose dependently inhibited both U46619-induced phasic and tonic contraction and completely abolished the tonic component at high menthol concentrations (Figure 3A). Administration of thapsigargin to deplete internal Ca\(^{2+}\) stores abolished U46619-induced tonic constriction but had no effect on phasic contraction. The effect of menthol on tonic constriction mimicked the actions of thapsigargin. Furthermore, administration of ROCK inhibitor Y27632 significantly reduced the tonic constriction in mesenteric arteries (Figure 3B). We further evaluated whether menthol inhibits U46619-induced Ca\(^{2+}\) influx. In calcium-free medium, thapsigargin (1 \(\mu\)mol/L) was used to deplete the intracellular Ca\(^{2+}\) store from sarcoplasmic reticulum (SR). After calcium store depleted, the menthol completely inhibited U46619-induced calcium influx in VSMCs from WT, but not in TRPM8\(^{-/-}\) mice (Figure 3C). It indicated that the inhibitory effect of menthol on mesenteric artery constriction was associated with menthol reducing U46619-induced Ca\(^{2+}\) influx in TRPM8-dependent manner.

**Figure 1.** Transient receptor potential melastatin subtype 8 (TRPM8) characterization in the vasculature. A and B, Representative immunofluorescence images of TRPM8 in vascular smooth muscle cells (VSMCs) and mesenteric arteries from wild-type (WT) mice or TRPM8\(^{-/-}\) mice. α-Actin (red) coexpression with TRPM8 (green) is shown. C and D, TRPM8 mRNA (C) or protein (D) was detected in the primary cultured VSMCs and thoracic aortas from WT mice or TRPM8\(^{-/-}\) mice. Polymerase chain reaction performed in samples containing RNA with reverse transcriptase (RT), RNA without RT (–RT) and water (no RNA with RT, –RNA) for TRPM8 and GAPDH. DAPI indicates 4′,6-diamidino-2-phenylindole; H&E, hematoxylin–eosin; and M, marker.
Chronic Dietary Menthol Lowers Blood Pressure by Inhibiting the RhoA/ROCK Pathway in a TRPM8-Dependent Manner

To further study whether menthol administration has an effect on blood pressure, TRPM8−/− and WT mice were treated with 0.5% dietary menthol for 28 weeks. Chronic dietary menthol treatment significantly reduced U46619-induced vasoconstriction (Figure 4A and 4B) and lowered 24-hour ambulatory arterial pressure (Figure 4C–4F) in WT mice but not in TRPM8−/− mice. Chronic dietary menthol also inhibited RhoA, ROCK-2, and p-MYPT-1 expression in mesenteric arteries from WT mice but not in TRPM8−/− mice (Figure 4G and 4H). These data demonstrate that chronic dietary menthol can attenuate vasoconstriction by inhibiting the RhoA/ROCK pathway in a TRPM8-dependent manner.

Chronic Dietary Menthol Antagonizes High Blood Pressure in Spontaneous Hypertensive Rats

To examine whether chronic dietary menthol has an antihypertensive effect, spontaneous hypertensive rats (SHRs) were fed with...
Figure 3. Menthol reduced U46619-induced vasoconstriction and calcium influx in a transient receptor potential melastatin subtype 8 (TRPM8)–dependent manner. A, U46619 treatment induced a small contraction in Ca²⁺-free media and 2.5 mmol/L CaCl₂–induced contractions demonstrating phasic and tonic components (Control). Menthol pretreatment for 10 minutes dose dependently inhibited both the phasic and tonic components. The tonic components were measured 20 minutes after CaCl₂ was added. Data are expressed as the mean±SEM, n=6. *P<0.05 compared with control. B, Pretreatment with menthol (1 mmol/L), thapsigargin (1 μmol/L) or Y27632 (10 μmol/L) for 10 minutes significantly inhibited the U46619-induced tonic component. Tonic components were measured 20 minutes after CaCl₂ was added. *P<0.05 compared with control, **P<0.01 compared with control. Data are expressed as the mean±SEM, n=5. C, After calcium store of vascular smooth muscle cells (VSMCs) was depleted by thapsigargin (1 μmol/L) in calcium-free medium, menthol (1 mmol/L) reduced U46619 (100 nmol/L)–induced calcium influx in cultured VSMCs from wild-type (WT) but not in TRPM8⁻/⁻ mice. Data are expressed as the mean±SEM, n=8. *P<0.05 compared with control.
Figure 4. Transient receptor potential melastatin subtype 8 (TRPM8) activation by dietary menthol decreases vascular contraction and blood pressure in mice through RhoA/Rho-kinase pathway inhibition. A and B, Twenty-eight weeks of 0.5% dietary menthol (Ment) decreased the RhoA agonist, U46619 (10^{-9.5} to 10^{-6} mol/L)-induced mesenteric artery contraction in wild-type (WT) mice but not in TRPM8^{−/−} mice. *P<0.05 compared with a normal diet (Cont), n=6. C to F, A total of 0.5% dietary Ment for 28 weeks lowered systolic blood pressure (SBP) and diastolic blood pressure (DBP) in mice in a TRPM8-dependent manner. *P<0.05 compared with a normal diet (Cont), n=4. G and H, RhoA, Rho kinase 2 (ROCK-2), and phosphorylation of myosin phosphatase targeting subunit 1 (p-MYPT-1) protein expression in mesenteric arteries (MA) of WT or TRPM8^{−/−} mice that were fed with a normal diet (ND) or a ND plus 0.5% menthol (NM) for 28 weeks. *P<0.05; or **P<0.01 compared with ND, n=3. The data are expressed as the means±SEM and were analyzed with Student unpaired t test.
or without 0.5% dietary menthol for 28 weeks. SHR’s blood pressure was measured by tail cuff every week. It showed that dietary menthol significantly reduced SBP after 4 weeks and this effect sustained in SHR (Figure 5A). Chronic dietary menthol significantly reduced U46619-induced vasoconstriction in mesenteric arteries from SHR (Figure 5B). The 24-hour blood pressure was significantly lower in menthol-treated SHR compared with untreated SHR (Figure 5C and 5D). Furthermore, chronic dietary menthol also significantly reduced RhoA, ROCK-2 Rho-GTP, and p-MYPT-1 protein expression in mesenteric arteries from SHR (Figure 5E and 5F). These results suggest that dietary menthol attenuates vasoconstriction and lowers high blood pressure in genetic hypertensive rats via RhoA/ROCK pathway inhibition.

**Menthol Capsules Lower Blood Pressure and Improve Vasodilation in Prehypertensive Participants**

As proof of principle, we evaluated whether continuous menthol-induced TRPM8 channel activation can lower blood pressure in prehypertensive individuals. Continuous dietary menthol significantly reduced SBP after 4 weeks and this effect sustained in SHR (Figure 5A). Chronic dietary menthol significantly reduced U46619-induced vasoconstriction in mesenteric arteries from SHR (Figure 5B). The 24-hour blood pressure was significantly lower in menthol-treated SHR compared with untreated SHR (Figure 5C and 5D). Furthermore, chronic dietary menthol also significantly reduced RhoA, ROCK-2 Rho-GTP, and p-MYPT-1 protein expression in mesenteric arteries from SHR (Figure 5E and 5F). These results suggest that dietary menthol attenuates vasoconstriction and lowers high blood pressure in genetic hypertensive rats via RhoA/ROCK pathway inhibition.
pressure and improve vasodilation in human prehypertension. A total of 36 eligible prehypertensive participants underwent randomization, in which 18 participants were given menthol capsules and 18 participants were in the placebo group for 8 weeks. Ambulatory blood pressure monitoring and brachial artery vasodilation measurements were performed before and after treatment. There were no significant differences in baseline characteristics between the 2 groups (Table). The data demonstrated a significant reduction in blood pressure in the menthol capsule group compared with their baseline (SBP, 129±5 versus 123±7 mm Hg in menthol treatment group, P<0.05; SBP, 129±5 versus 127±9 mm Hg in placebo group, P>0.05; diastolic blood pressure, 81±5 versus 76±6 mm Hg in menthol treatment group, P<0.01; diastolic blood pressure, 78±7 versus 77±8 mm Hg in placebo group, P>0.05; Figure 6A and 6B). The changes in 24-hour mean diastolic blood pressure from baseline in the menthol capsule group was greater than in the placebo group (P=0.015; Figure 6C). Furthermore, flow-mediated vasodilation was significantly improved in the menthol capsule group compared with that in the placebo group (P=0.004; Figure 6D). These data indicate that menthol capsules administration favorably lowered blood pressure and improved vasodilation in human prehypertension.

### Discussion

The present study provides experimental evidences for the beneficial effect of dietary menthol in reducing vascular reactivity and high blood pressure. Such benefit is related to a stimulatory action of menthol on the vascular TRPM8 channels, a conclusion supported by both in vitro and in vivo data obtained in TRPM8−/− mice. It showed that chronic dietary menthol inhibited RhoA, ROCK-2, and phosphorylation of MYPT-1 in the vasculatures, attenuated vasoconstriction, as well as reduced blood pressure in genetically hypertensive rats. In human prehypertension, menthol capsule treatment for 8 weeks significantly reduced blood pressure and improved vasodilation. Thus, vascular TRPM8 activation can be considered as a potential strategy for the management of hypertension.

Several TRP channels participate in hypertension pathogenesis. TRPC3 dysfunction contributes to hypertension pathogenesis.21 TRPC6-null mice exhibit increased TRPC3 expression, elevated blood pressure, and vasoconstriction.20 A significant increase in TRPC6 expression and receptor-stimulated current was found in VSMCs of doxycorticosterone acetate-salt hypertensive rats.23 Reduced TRPV1 expression has been reported in Dahl salt-sensitive rats, which renders them sensitive to salt load in blood pressure regulation.22 Capsaicin-induced TRPV1 activation enhances endothelium-dependent relaxation and lowers blood pressure in genetic hypertensive rats.16 TRPV4 activation decreases blood pressure in rats on a normal salt diet, and TRPV4 inhibition increased blood pressure in rats on a high-salt diet.21 Reduced TRPM7 expression is associated with reduced cytosolic magnesium concentrations in VSMCs from SHR and may facilitate vasoconstriction.24 In addition, increased catecholamine release from TRPM4 knockout chromaffin cells may contribute to increased sympathetic tone and hypertension.25

TRPM8 is a well-established cold sensor that can be activated by pharmacological agents that mimic the psychophysical sensation of cold, such as menthol. TRPM8 is mainly located on sensory neurons26 and in several vascular beds in rats.4-27 TRPM8-deficient mice exhibit strikingly reduced avoidance of cold temperatures and lack behavioral response to cold-inducing icilin application.8 TRPM8 channels are also expressed and functional in blood vessels. In situ menthol produced Ca2+ transients that consisted of an initial phasic component, followed by a sustained component.28-29 Johnson et al demonstrated that TRPM8 channel activation in the rat artery causes vasoconstriction or vasodilation depending on previous vasomotor tone.

However, our results suggest that menthol ultimately reduced the vascular reactivity by inhibiting calcium signaling–mediated RhoA/ROCK pathway inhibition based on the following reasons: (1) arteries pretreated with menthol showed significant lower reactivity to vasoconstrictors; (2) in the absence of extracellular calcium, agonists only caused weak vasoconstriction, but adding calcium evoked significant vasoconstriction, which was dose dependently inhibited by menthol, indicating that menthol inhibited Ca2+ influx; (3) the tonic contraction that represents SR Ca2+ release–mediated RhoA/ROCK activation can be inhibited by menthol; (4) in TRPM8−/− mice, menthol failed to inhibit U46619-induced vasoconstriction and the related vascular calcium signaling. Fernández-Tenorio et al showed that agonist-induced isometric contractions involve 2 components: the phasic component representing Ca2+ influx and the tonic component representing SR Ca2+ release–mediated RhoA/ROCK activation. Our previous study demonstrated that menthol inhibits contraction in rat aorta, mesenteric and coronary arteries primarily through inhibiting Ca2+ influx via nifedipine-sensitive Ca2+ channels.30 Calmodulin-dependent myosin light chain kinase catalyzes myosin light chain phosphorylation to cause vasoconstriction through either L-type calcium channel–mediated calcium

### Table. Characteristics of Prehypertensive Participants

<table>
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<tr>
<th>Characteristic</th>
<th>Placebo Group (n=18)</th>
<th>Menthol Group (n=18)</th>
</tr>
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<tbody>
<tr>
<td>Age, y</td>
<td>57.0±5.7</td>
<td>56.8±5.0</td>
</tr>
<tr>
<td>Sex (men/women)</td>
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<td>8/10</td>
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<tr>
<td>Systolic blood pressure, mm Hg</td>
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<td>128±6.5</td>
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<td>Diastolic blood pressure, mm Hg</td>
<td>78±7.1</td>
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<tr>
<td>Weight, kg</td>
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<td>62.8±9.9</td>
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<tr>
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<td>25.8±3.2</td>
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<td>Hemoglobin, g/L</td>
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<td>139.8±11.9</td>
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<td>Leukocytes, 10³/L</td>
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<td>Serum sodium, mmol/L</td>
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<td>Serum potassium, mmol/L</td>
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<td>Glucose, mmol/L</td>
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<tr>
<td>Creatinine, mmol/L</td>
<td>78.9±14.0</td>
<td>80.1±9.6</td>
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Data are mean±SD.
influx or the intracellular SR store–induced calcium release. In addition to this classical pathway, alterations in myofilament Ca\textsuperscript{2+} sensitivity can also affect vasoconstriction. For example, ROCK directly phosphorylates MYPT-1, a myosin targeting subunit of myosin light chain phosphatase, which consequently inhibits myosin light chain phosphatase activity and thereby maintains vascular contraction via a calcium-independent mechanism. Recent studies suggest that SR Ca\textsuperscript{2+} release–mediated RhoA/ROCK activation plays an important role in maintaining sustained vascular contraction and refilling of the SR stores by a residual influx of extracellular calcium through L-type calcium channels participates in depolarization-evoked RhoA/ROCK activity and sustains arterial tonic constriction.

Increased RhoA/ROCK activity has been demonstrated in human and experimental hypertension. Antagonizing ROCK using fasudil lowers blood pressure in hypertensive patients. Mustafa et al reported that TRPM8-induced gastric fundus contraction is associated with the RhoA/ROCK pathway. Although menthol can inhibit the vasoconstriction by blocking L-type calcium channel–induced calcium influx at several vessel beds, it remains to be determined whether menthol has an effect on calcium release from SR and its underlying mechanism.

The present study demonstrated that chronic menthol administration not only reduced vasoconstriction but also inhibited vascular RhoA/ROCK pathway in a TRPM8-dependent manner. Furthermore, we demonstrated that menthol inhibited both L-type calcium channel and SR calcium release, which reduced sustained vasoconstriction by inhibiting of RhoA/ROCK activation.

Importantly, a favorable effect of menthol on blood pressure and vascular function was also proved in genetically hypertensive rats and in human prehypertension. We conclude that TRPM8 activation by dietary menthol improves vasodilation function, which may represent a promising therapeutic intervention of hypertension.

Perspectives

This study is the first to demonstrate that menthol treatment benefits vascular function and blood pressure via TRPM8-mediated RhoA/ROCK pathway activation. Taken together, dietary menthol-mediated TRPM8 activation may be a promising lifestyle intervention for prehypertension prevention in populations that have high cardiometabolic risk factors.

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Disclosures

None.

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Running title: TRPM8 activation attenuates hypertension
Supplementary Materials and Methods

Animal Treatment

Eight-week-old male spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY) were obtained from Charles Rivers Laboratories. Rats and mice were housed under controlled temperatures (21-23 °C) with a 12/12 hours light-dark cycle with free access to food and water. Animals were given the normal chow diet (Control group) or normal chow diet plus 0.5% menthol (Menthol group) for 28 weeks. All of the experimental procedures were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee.

Blood pressure measurement

Animals were surgically implanted with telemetric transmitters (Data Sciences International, MN, USA) after receiving a normal diet or a normal diet plus 0.5% menthol for 28 weeks. The implant catheter was placed into the descending carotid artery (mice) or the abdominal aorta (rats). The animals were allowed to recover from the surgery for 10 days, and then 24-hour ambulatory systolic and diastolic pressures were measured by telemetry in conscious unrestrained animals. We collected data for 10 seconds every 30 minutes and used the mean values of 24 hours for the analysis.1

Cell culture

VSMCs were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (HyClone) containing 100 mg/mL penicillin and 100 mg/mL streptomycin (GIBCO, USA). Cultured VSMCs were plated and grown at 37 °C in a humidified atmosphere of 95% air/5% CO₂. VSMCs between passages 2 and 6 were used. Quiescent VSMCs were obtained by incubating the cells in serum free medium for 12 h before all of the in vitro experimental procedures were performed.

Intracellular Calcium Measurements

Cultured VSMCs were incubated with 2 μmol/L Fura 2-AM and 0.025% Pluronic F-127 for 60 minutes at room temperature in physiological saline solution (PSS, containing in mmol/L, NaCl 135, KCl 5, CaCl₂ 1.8, MgCl₂ 1, D-glucose 11, and HEPES 10, pH 7.4) and then washed three times with PSS to remove the extracellular Fura 2-AM. Cells were resuspended in one tube, and 100 μL of cells were added to each well, and each well contained the same amount of cells. Serial dilutions of cells showed that the resting-fluorescence F340 nm: F380 nm ratio remained constant, indicating that within the range of cells used, the fluorescence F340 nm: F380 nm ratio was not markedly influenced by cell number.

Prehypertensive participants characteristics

36 eligible prehypertensive participants were randomly assigned to receive an 8-week treatment with menthol capsules (144 mg/day) or placebo. No participants were taking medication and major medical illnesses were excluded. All of the participants
gave written informed consent, and the study was approved by the institute ethics committee. The office blood pressure was measured in a sitting position with a suitably sized cuff after a rest of 10 minutes by conventional sphygmomanometric methods. Ambulatory blood pressure monitoring (ABPM) was recorded using Spacelabs 90217 (Spacelabs, Medical Inc., Redmond, Washington, USA). Automatic BP readings were taken every 15 minutes throughout the day (08:00-20:00) and every 30 minutes during the night (20:00-08:00).

**Flow-mediated vasodilation (FMD)**
Baseline brachial artery diameter was determined, which was approximately 15 to 50 mm above the antecubital fossa in supine participants. Blood occlusion was then created using a sphygmomanometer cuff, which was increased to 220 mmHg for 5 minutes. The brachial artery diameter was recorded 60 seconds after cuff deflation. FMD was calculated as the percent increase in mean diastolic diameter compared with baseline diameter. FMD was measured by a single trained sonographer with high-resolution ultrasonography using a 7.5 MHz linear transducer on an HY 6000 system (Wuxi Haiying Electronic Medical System Co. Ltd., JIANGSU, CHN) in accordance with international guidelines.

**Western blot and Immunofluorescence analysis**
The protein expression was normalized to the internal control GAPDH. To verify the antibodies specificity we included the tissues from TRPM8-/- mice in the western blots. Immunofluorescence for TRPM8 and α-actin in the VSMCs and the mesenteric arteries of the mice was performed as previously described. α-actin was used to identify vascular smooth muscle cells and the vascular medial layer. All of the primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). After incubation with secondary antibodies (ZSGB-BIO, China) at room temperature for 2 hours, the proteins were detected with enhanced chemiluminescence and quantified using a Gel Doc 2000 Imager (Bio-Rad, USA).

**RNA isolation and PCR analysis**
Total mRNA (2.5 μg) was reverse-transcribed with a reverse transcription mixture consisting of oligo dT and 15 U AMV reverse transcriptase at 42°C for 60 minutes, followed by heating to 95°C for 5 minutes. PCR was performed in samples containing RNA with reverse transcriptase, RNA without reverse transcriptase and water (no RNA with reverse transcriptase) for TRPM8 and GAPDH. Control PCR was performed in samples containing RNA, without RNA, RNA without reverse transcriptase, and in samples containing no cDNA. Three micro litter of a 1:3 diluted single-stranded cDNA was added to a 25 μL PCR mix and amplified using a 2×Taq plus PCR MasterMix (Tiangen Biotech CO, China). The PCR was started with a denaturation at 95°C for 5 minutes, and then 44 cycles were performed under the following conditions: denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 40 seconds. The sense and antisense primers for the coding regions of the TRPM8 or GAPDH genes were the following: TRPM8 (Ref
Measurement of Vascular Reactivity

After the mice or rats were anesthetized with pentobarbital sodium, the mesenteric vascular bed was removed and placed in a cold (4 °C) Krebs solution containing (mM) 118 NaCl, 25 NaHCO₃, 11 D-glucose, 4.7 KCl, 1.2 KH₂PO₄, 1.17 MgSO₄, and 2.5 CaCl₂. For the Ca²⁺-free Krebs, CaCl₂ was removed and 1 mM EGTA was added. The first branches (of mice) or second branches (of rats) for the mesenteric arteries were dissected out and cleaned off of the connective tissue. The arterial segments (2-2.5 mm in length) were mounted in a myograph. According to our previous study, each ring was bathed in Krebs solution aerated with 95%O₂ and 5%CO₂ at 37 °C (pH 7.4) and stretched to the optimum baseline tension: 1.8 mN for mice and 2.5 mN for rat the mesenteric arteries. The rings were equilibrated for 60 min before each experiment started. In some rings, endothelium was removed mechanically by scrubbing the luminal side of the ring using stainless steel wire. High K⁺ (60 mM)-containing Krebs solution was added to test the contractility. After several wash outs, each ring was contracted by U46619 to test for the integrity of endothelium. Isometric contractions were recorded using a computerized data acquisition system (PowerLab/8SP; ADInstruments Pty Ltd., Castle Hill, Australia).

References

Supplementary Figures
Figure S1

**A**
Menthol (0.01-1) mM

**B**
100 uM Menthol
300 uM Menthol
1 mM Menthol

**C**
Endothelium-intact
10^6 M Ach
10^6 M Ach

Endothelium-denude
10^6 M Ach 10^6 M Ach

Figure S1: A, AMTB, a TRPM8 antagonist, inhibited menthol-mediated vasodilation. *p<0.05 versus Control. Means ± SEM, n=3. B, In basal force vessel (1.8mN), menthol caused small, transient and dose-dependent vasoconstriction. Means ± SEM, n=3. C, Endothelium-denuded reduced menthol-induced vasodilation. Segments relaxing >80% by 10^-6 M Ach were considered being endothelium intact, while those
relaxing <5% were defined as being endothelium-denuded. Means ± SEM, n=4.

Figure S2

**Figure S2:** The effect of menthol on U46619-induced contractile in the presence of calcium channel blocker, nimodipine. All vascular rings were incubated in Ca\(^{2+}\) free Krebs solution. Menthol or nimodipine were added 10 minutes before U46619 were
added. * $p<0.05$ versus Control. Means ± SEM, n=3.