TRPM8 downregulation by angiotensin II in vascular smooth muscle cells is involved in hypertension

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Abstract. Angiotensin II (Ang II)-induced injury of vascular smooth muscle cells (VSMCs) serves an important role in hypertension and other cardiovascular disorders. Transient receptor potential melastatin 8 (TRPM8) is a thermally-regulated Ca^{2+}-permeable channel that is activated by reduced body temperature. Although several recent studies have revealed the regulatory effect of TRPM8 in vascular tone and hypertension, the precise role of TRPM8 in dysfunction of vascular smooth muscle cells (VSMCs) induced by Ang II remains elusive. In the present study, the possible function of TRPM8 in Ang II-induced VSMCs malfunction *in vivo* and *in vitro* was investigated. In the aortae from rats that had undergone a two-kidney one-clip operation, which is a widely-used renovascular hypertension model, the mRNA and protein levels of TRPM8 were reduced. In addition, exogenous Ang II treatment decreased TRPM8 mRNA and protein expression levels in primary cultures of rat VSMCs. TRPM8 activation by menthol, a pharmacological agonist, in VSMCs, significantly attenuated the Ang II-induced increase in reactive oxygen species and H_2O_2 production. In addition, TRPM8 activation reduced the Ang II-induced upregulation of NADPH oxidase (NOX) 1 and NOX4 in VSMCs. Furthermore, TRPM8 activation relieved the Ang II-induced activation of ras homolog gene family, member A-rho associated protein kinase 2 and janus kinase 2 signaling pathways in VSMCs. In conclusion, the results presented in the current study indicated that TRPM8 downregulation by Ang II in VSMCs may be involved in hypertension.

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Introduction

Hypertension, a disorder associated with structural and functional vascular alterations, has been recognized as a major risk to cardiovascular health (1). As hypertension is correlated with coronary artery disease, left ventricular hypertrophy, congestive heart failure, obesity, hyperlipidemia, diabetes and cerebrovascular complications, it represents the biggest single contributor to the global burden of disease and to global mortality (1-4). Recent studies have demonstrated that some gene variants may be involved in hypertension (2,3). Dysfunction of the renin-angiotensin-aldosterone system (RAAS), and over-activation of angiotensin II (Ang II), have been revealed as most important factors in the onset and progression of hypertension (5), cardiac remodeling (6), renal injury (7) and aortic aneurysm (8-11). Consequently, inhibiting the action of Ang II using angiotensin-converting enzyme inhibitors (ACEI) and Ang II type 1 receptor inhibitors have been widely accepted as a cornerstone in the treatment of hypertension (12,13). Although the biological functions and molecular mechanisms of Ang II in cardiovascular system have not yet been not fully elucidated, the injury of vascular smooth muscle cells (VSMCs) induced by hypertension, as well as the accompanying high blood Ang II concentration, are considered to be primary causes for hypertension-associated vascular injury, including remodeling (14), thrombi transformation (15), neointimal hyperplasia (16) and calcification (17).

Transient receptor potential melastatin 8 (TRPM8) is a thermally-regulated Ca^{2+}-permeable channel that is activated by cold sensations (18). As a universal cold receptor in the thermoregulation system, TRPM8 regulates body temperature (18,19). Mice and rats experience a transient reduction in core body temperature following administration of TRPM8 antagonists (20). By contrast, activation of TRPM8 channels by selective agonists, such as menthol and icilin, demonstrates the opposite physiological hyperthermic effect by acting on peripheral neurons rather than the central nervous system (21,22). In addition to this thermoregulatory effect, TRPM8 is involved in carcinogenesis (23), pain (24), inflammation (25), obesity (26), testosterone sensing (27) and eye-blinking regulation (28). Although previous studies have demonstrated that TRPM8 functions as a regulator of vascular tone that may be a promising therapeutic target for hypertension (29,30), the role of TRPM8 in vascular biology is not completely understood.

In the current study, it was hypothesized that TRPM8 serves an important role in VSMC biology under hypertensive conditions. Therefore, the expression of TRPM8 was investigated in the aorta media from rats subject to a two-kidney one-clip operation (2K1C), which is a widely-used renovascular hypertension model. In addition, the effect of benazepril, a commonly used ACEI, on the protein expression levels of TRPM8 was studied. Furthermore, the effects of exogenous Ang II treatment on TRPM8 expression in cultured VSMCs were explored. Furthermore, the potential influence of TRPM8 activation by a pharmacological agonist on oxidative stress, ras homolog gene family, member A (RhoA)-rho-associated protein kinase 2 (Rock2) signaling activation and janus kinase 2 (JAK2) signaling activation induced by Ang II treatment in VSMCs were investigated.

Materials and methods

Animals. A total of 24 male Sprague-Dawley rats (age, 8 weeks; weight, 220-260 g) were obtained from Sino-British SIPPR/BK Lab Animal Co., Ltd. (Shanghai, China) and housed under conditions involving a 12 h/12 h light/dark cycle and a temperature of 23-25°C with free access to food and water. All animals used in this work received humane care in compliance with the institutional animal care guidelines and the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health (Bethesda, MA, USA). The animal experiments were approved by the Animal Care and Use Committee of Tongji University (Shanghai, China).

2K1C hypertension model. The 2K1C hypertension model was performed as described previously (31). Rats were anesthetized with a combination of ketamine (40 mg/kg i.p.; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) /xylazine (13 mg/kg i.p.; Sigma-Aldrich; Merck Millipore), the right renal artery was isolated through a flank incision, and a silver clip (0.2 mm internal gap) was placed on the renal artery. Sham-operated rats underwent the same surgical procedure except for the placement of the renal artery clip, and served as controls. The rats were returned to the cage after waking from the operation, and were maintained for 4 weeks. Blood pressure and heart rate (HR) measurements were recorded at 4 weeks post-2K1C surgery in conscious rats as described in a previous study by the authors (31). For ACEI benazepril treatment, benazepril (1.5 mg/kg/day; Beijing Novartis Pharma Co., Ltd., Beijing, China) was administrated via the chow for an additional 4 weeks in 2K1C rats.

Primary VSMC culture. Rat VSMCs were isolated using a standard enzymatic digestion technique as described previously (32,33). Primary VSMCs were cultured in Dulbecco's modified Eagle's medium (DMEM; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in 95% O_2 and 5% CO_2. Experiments were performed using cells between passages 3 and 8 (34).

Cell study. The VSMCs ($1x10^6$) were seeded in wells of a 6-wells plate. Cells were grown to 60% confluence, and switched to serum-starved medium (0.1% FBS) for 12 h prior to further treatments to achieve synchronization. At the second day, the cells were switched to normal medium (DMEM + 10% FBS) and treated with Ang II (10^{-7} M; Sigma-Aldrich; Merck Millipore) or menthol (100 mM; Sigma-Aldrich; Merck Millipore) for 5 days at 37°C. Cells were then washed with PBS three times. Subsequently, cells were lysed with RIPA solution (Beyotime Institute of Biotechnology, Haimen, China) for determination of mRNA and protein levels. In some experiments, cells were induced by incubation of H_2O_2 (150 mM) for 12 h and then lysed for determination of the effect of ROS on TRPM8 expression.

Oxidative stress. The levels of reactive oxygen species (ROS) were measured using a flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA) with a 2',7'-dichloro-fluorescein-diacetate fluorescent assay (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions and as described previously (35). The level of H_2O_2 was evaluated using the Hydrogen Peroxide assay kit (Abcam, Cambridge, MA, USA) according to the manufacturer's instructions and using the methods described previously (36). In the presence of horseradish peroxidase, the OxiRed Probe reacted with H_2O_2 to produce product with color (λmax=570 nm) and red fluorescence (excitation/emission=535/587 nm).

Tissue sampling. At the end of 4-week treatment, rats were deeply anesthetized by chloral hydrate (0.4 g/kg; Shanghai Meilian Biological Technology R&D Co., Ltd., Shanghai, China) and then decapitated. The aortae were carefully harvested and washed by distilled water three times. Subsequently, the tissues were frozen and stored at -80°C.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR was performed as described previously (37,38). Total RNA was isolated from frozen tissues and the VSMCs cultured in 6-well dishes using RNAiso Plus reagent (Takara Bio, Inc., Otsu, Japan) and reverse transcribed with M-MLV (Promega Corporation, Madison, WI, USA) into cDNA using the methods described previously (39). The following primers were used for qPCR analysis: TRPM8, forward, 5'-GCTACGGACCAGCATTTCAT-3', and reverse, 5'-GCTTGTCAATGGGCTTCTT-3'; β-actin, forward, 5'-CCCATCTATGAGGGTTACGC-3', and reverse, 5'-TTT AATGTCACGCACGATTTC-3'. β-actin expression was used as an internal control. Quantification of mRNA was performed using the ABI Prism 7500 (Applied Biosystems; Thermo Fisher Scientific, Inc.) using a PrimeScript™ RT-PCR kit (Takara Bio, Inc.). The specificity of the RT-qPCR assay was assessed by melting point analysis and gel electrophoresis. The relative quantities of TRPM8 were calculated using the $2^{-\Delta\Delta Cq}$ method with β-actin as an internal control (40,41).

Western blotting. Western blotting analysis was performed as described previously (42,43). Tissues and the VSMCs cultured in the 6-well dishes were homogenized in radioimmuno-precipitation lysis buffer containing 5% protease inhibitor cocktail (Pierce; Thermo Fisher Scientific, Inc.). Protein quantity was determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology) (44). Protein samples (30-50 μg) were boiled for 15 min and run on 12% SDS-PAGE
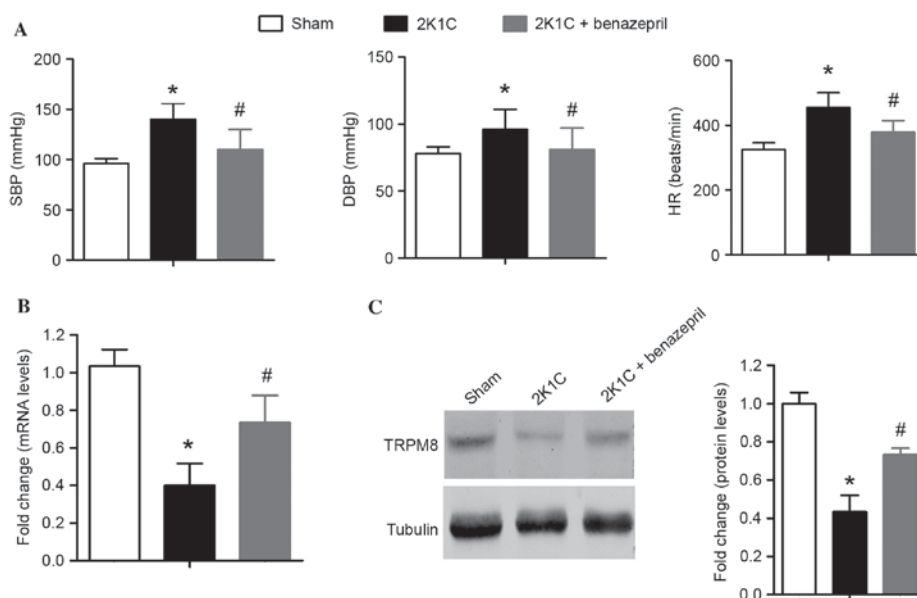
Figure 1. Altered expression of TRPM8 in the aorta media from hypertensive rats. (A) SBP, DBP and HR in Sham-operated, 2K1C and 2K1C+benazepril rats. These parameters were measured at 4 weeks following 2K1C operation. (B) The mRNA expression of TRPM8 in aorta from three groups of rats. (C) The protein expression of TRPM8 in aorta from three groups of rats using western blot analysis. Data are expressed as the mean ± standard error (n=8). *P<0.05 vs. Sham; $^#$P<0.05 vs. 2K1C. TRPM8, transient receptor potential melastatin 8; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; 2K1C, two-kidney one-clip operation.

gels, electroblotted onto nitrocellulose membranes, and immunoblotted at 4°C overnight with anti-TRPM8 (#ab3243; dilution, 1:2,000; Abcam), anti-NADPH oxidase (NOX) 1 (#ab55831; dilution, 1:2,000; Abcam), anti-NOX 4 (#ab60940; dilution, 1:2,000; Abcam), anti-phosphorylated (p)-JAK2 (#ab32101; dilution, 1:1,000; Abcam), anti-total (t)-JAK2 (#ab39636; dilution, 1:1,000; Abcam), anti-RhoA (#sc-32; dilution, 1:600; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-Rock2 (#sc-1851; dilution, 1:800; Santa Cruz Biotechnology, Inc.) and anti-tubulin (#sc-365791; dilution, 1:5,000; Santa Cruz Biotechnology, Inc.) followed by corresponding horseradish peroxidase-conjugated goat anti-mouse IgG (#BA1050; Wuhan Boster Biological Technology, Ltd., Wuhan, China) and and goat anti-rabbit IgG (#BA1054; Wuhan Boster Biological Technology, Ltd.) secondary antibodies (45). The signals were measured using the Amersham ECL Western Blotting assay kit (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) (46). Quantification of blots was conducted using the Quantity One software system, version 4.6.2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each experiment was repeated at least three times.

Statistical analysis. Data are represented as mean ± standard error of the mean. Differences were evaluated by one-way analysis of variance followed by Tukey post hoc analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Downregulation of TRPM8 in the aorta media from hypertensive rats. The systolic blood pressure (SBP), diastolic blood pressure (DBP) and HR of 2K1C rats were significantly higher when compared with that of Sham-operated rats (P=0.0017, P=0.0037 and P=0.0055, respectively; Fig. 1A). Benazepril treatment was associated with a significant reduction in the 2K1C-induced increase in SBP, DBP and HR in 2K1C rats (P=0.0078, P=0.0024 and P=0.0015, respectively; Fig. 1A). In the aortae of 2K1C hypertensive rats, the mRNA level of TRPM8 in de-nuded aortae (without endothelium and perivascular fat pad) was decreased by ~60% compared with that in Sham-operated rats (P=0.0076; Fig. 1B). However, benazepril treatment partially inhibited the downregulation of TRPM8 (Fig. 1B). Similar alterations were observed in TRPM8 protein expression levels (2K1C vs. Sham-operated, P=0.015; Fig. 1C). These results suggest that TRPM8 is downregulated in the aortae of 2K1C hypertensive rats, which may be partially reversed by ACEI treatment. This suggests that TRPM8 downregulation in hypertension may be associated with the Ang II.

Ang II reduces TRPM8 expression in a dose- and time-dependent manner in VSMCs. To determine whether TRPM8 downregulation in hypertension is associated with Ang II, VSMCs were treated with recombinant Ang II. As expected, incubation of cells with 10^{-7} M and 10^{-6} M Ang II for 1 week induced a significant downregulation in TRPM8 mRNA (P=0.014 and P=0.008, respectively; Fig. 2A) and protein expression (P=0.021 and P=0.015, respectively; Fig. 2B). However, treatment of cells with a low concentration of Ang II (10^{-8} M) for 1 week was not associated with a significant reduction in TRPM8 mRNA and protein expression (Figs. 2A and B). The influence of incubation time on the Ang II-induced reduction in TRPM8 expression was then evaluated. Ang II (10^{-7} M) treatment did not modulate TRPM8 mRNA and protein expression at 3 days following treatment (Fig. 2C and D). However, the mRNA and protein levels of
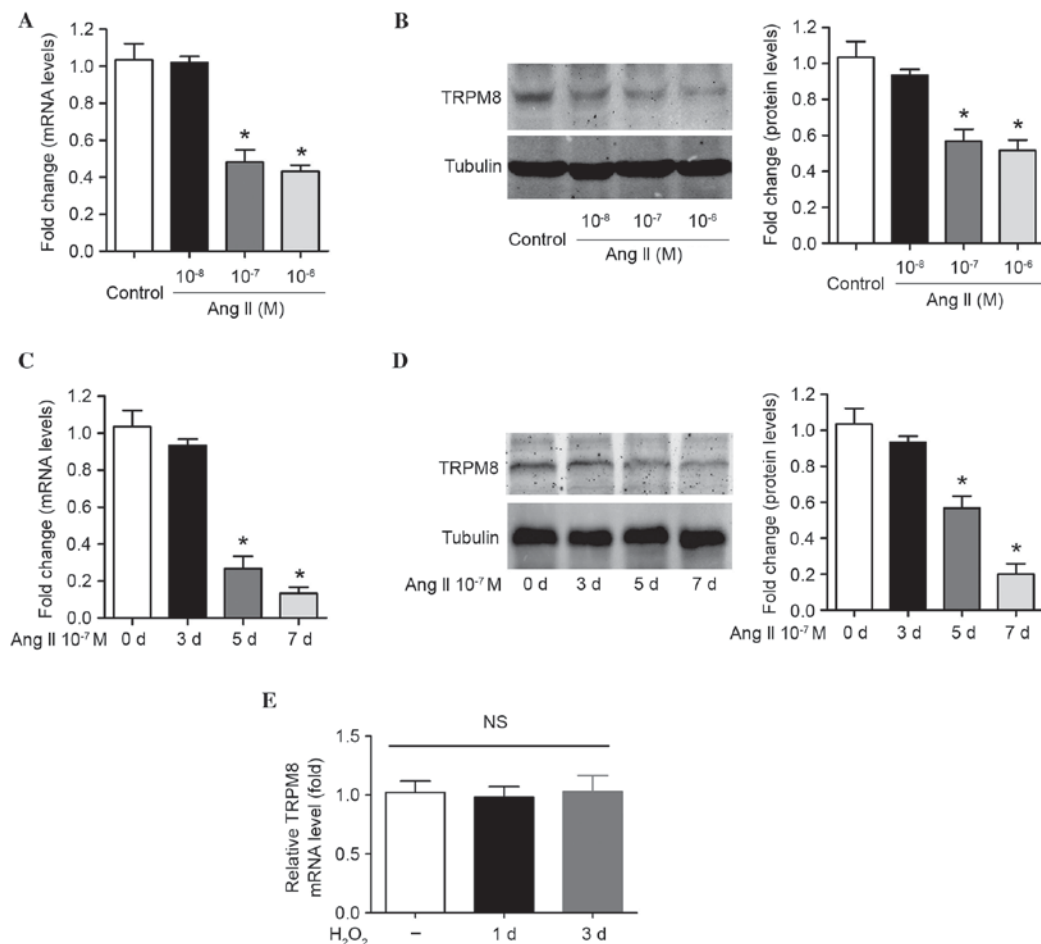
Figure 2. Ang II treatment reduces TRPM8 expression in cultured VSMCs. The effect of different concentrations of Ang II (10^{-8}-10^{-6} M) treatment on TRPM8 (A) mRNA and (B) protein expression levels in cultured rat VSMCs following 7 days (*P<0.05 vs. Control). Effect of Ang II (10^{-7} M) treatment for 3, 5 and 7 days on TRPM8 (C) mRNA and (D) protein expression levels in cultured rat VSMCs (*P<0.05 vs. Control). (E) Effect of H_2O_2 incubation (30 mM) for 1 and 3 days on TRPM8 mRNA expression. Data are expressed as the mean ± standard error (n=8). TRPM8, transient receptor potential melastatin 8; VSMCs, vascular smooth muscle cells; Ang II, angiotensin II; NS, not significant.

TRPM8 at 7 days post-treatment were lower than those at 5 days post-treatment, which reached statistical significance when compared with day 0 (TRPM8 mRNA at day 5 vs. day 0, P=0.019; TRPM8 mRNA at day 7 vs. day 0, P=0.003; TRPM8 protein at day 5 vs. day 0, P=0.014; TRPM8 protein at day 7 vs. day 0, P=0.006; Fig. 2C and D). The data presented in the current study suggest that Ang II reduces TRPM8 expression in a dose- and time-dependent manner in VSMCs.

H_2O_2 was applied to VSMCs directly to increase ROS production, in order to determine whether ROS alters TRPM8 expression. TRPM8 mRNA expression was not altered by H_2O_2 treatment (30 mM) for 1 day or 3 days in VSMCs (Fig. 2E). This suggests that the altered TRPM8 expression was not due to Ang II-induced ROS production.

TRPM8 activation attenuates Ang II-induced oxidative stress and upregulation of NOX enzymes in VSMCs. The effect of TRPM8 downregulation in Ang II-treated VSMCs was investigated. The induction of oxidative stress in VSMCs by Ang II is an important factor that is detrimental to vascular structure (1). Consistent with previous studies (47), significant enhancements of ROS (P=0.00044; Fig. 3A) and H_2O_2 (P=0.000016; Fig. 3B) levels were detected in VSMCs treated

with Ang II (10^{-7} M) for 5 days when compared with untreated controls. Interestingly, activation of TRPM8 by menthol, a selective TRPM8 agonist, significantly reduced the production of ROS (P=0.0018; Fig. 3A) and H_2O_2 (P=0.0076; Fig. 3B) when compared with Ang II-only treated cells, suggesting that activation of TRPM8 attenuates Ang II-induced oxidative stress in VSMCs. As NOX enzymes are major sources of ROS in the cardiovascular system (48), the expression of NOX1 and NOX4 was determined. Treatment of VSMCs with Ang II (10^{-7} M) for 5 days increased NOX1 and NOX4 protein expression by 2-3-fold in VSMCs (Fig. 3C). However, TRPM8 activation by menthol significantly attenuated the Ang II-induced upregulation of NOX1 and NOX4 (P=0.016 and P=0.012, respectively; Fig. 3C).

TRPM8 activation attenuates Ang II-induced activation of RhoA-Rock2 and JAK2 signaling pathways in VSMCs. Previous studies have demonstrated that the RhoA-Rock2 and JAK2 signaling pathway in VSMCs is involved in the pathogenesis of Ang II-dependent hypertension due to the increased presence of ROS (49,50). Therefore, the present study investigated whether TRPM8 activation may affect RhoA-Rock2 and JAK2 activation by Ang II in VSMCs. Consistent with the
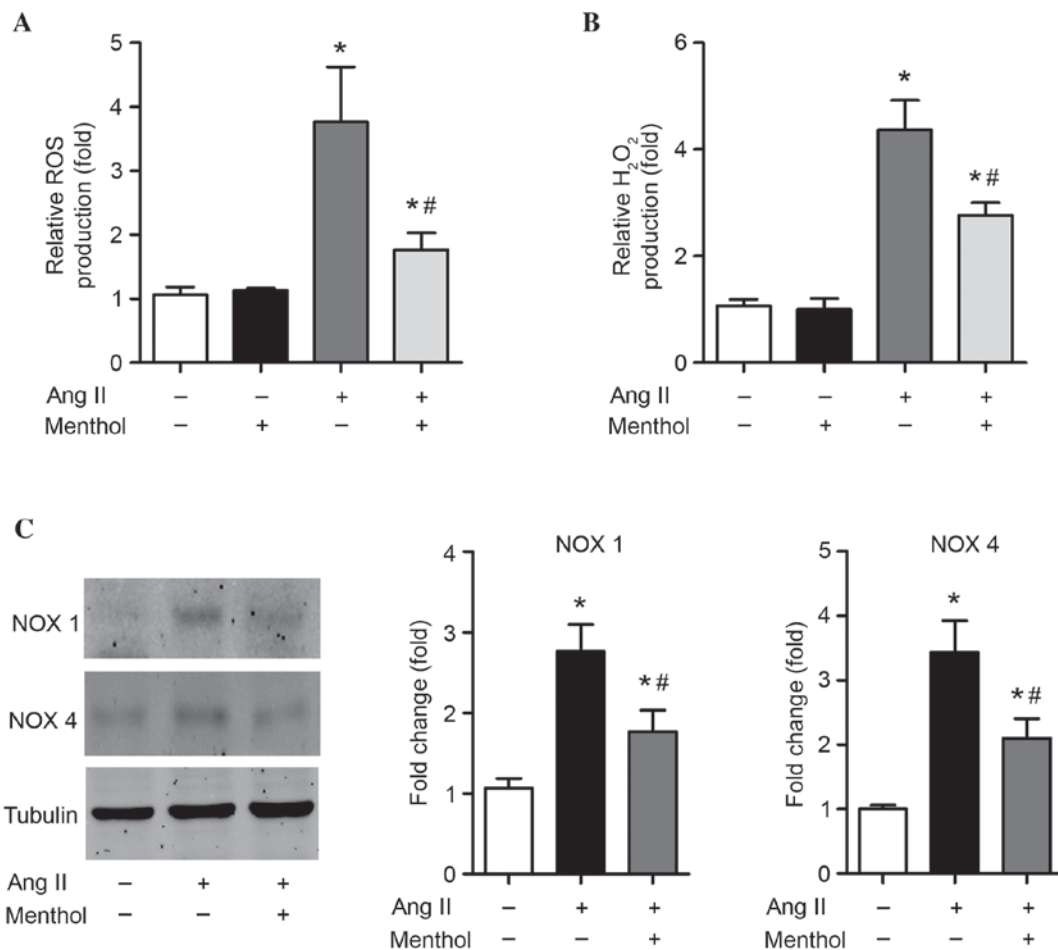
Figure 3. Activation of TRPM8 attenuated Ang II-induced oxidative stress and upregulated NOX1 and NOX4 expression in VSMCs. Effect of TRPM8 activation with the selective TRPM8 agonist, menthol, on Ang II-induced (A) ROS and (B) H_2O_2 production in cultured rat VSMCs. (C) Representative western blotting images and quantitative analysis of NOX1 and NOX4 protein levels in cultured rat VSMCs. Data are presented as the mean ± standard error (n=8). *P<0.05 vs. untreated controls; #P<0.05 vs. Ang II-only treated VSMCs. TRPM8, transient receptor potential melastatin 8; Ang II, angiotensin II; NOX, NAPDH oxidase; VSMCs, vascular smooth muscle cells; ROS, reactive oxygen species.

results of previous studies (49,50), Ang II treatment (10^{-7} M) for 5 days activated RhoA-Rock2 (Fig. 4A) and induced JAK2 phosphorylation (Fig. 4B) in VSMCs. By contrast, TRPM8 activation by menthol partially inhibited Ang II-induced activation of RhoA-Rock2 (Fig. 4A) and JAK2 (Fig. 4B) signaling pathways in VSMCs.

Discussion

The superfamily of TRP channels is composed of proteins that were initially identified in the *Drosophila* eye (29). The first mammalian family of TRP channels identified was the transient receptor potential cation channels (51). In the following years, additional TRP families, such as TRPM (melastatin), TRPML (mucolipidins), TRPV (vanilloid receptor), TRPP (for polycystic kidney disease proteins) and TRPA (for ankyrin-rich proteins) were described (51). The TRPM family consists of 8 members that are subdivided into the following 4 groups based on their sequence homology: TRPM1 and TRPM3; TRPM4 and TRPM5; TRPM6 and TRPM7; and TRPM2 and TRPM8 (29). They regulate a number of cellular functions in normal and pathophysiological conditions (29). Among these members, TRPM7 has

been most intensively studied for its role in the cardiovascular system. Touyz *et al* (52) reported that TRPM7 expression was lower in the VSMCs of spontaneously hypertensive rats in a genetic hypertensive model. Bradykinin, a potent vasodilator that lowers blood pressure, upregulated TRPM7 and its downstream target annexin-1 in VSMCs through phospholipase C-dependent, protein kinase C-dependent, c-Src-dependent and cAMP-independent pathways (53,54). Ang II increased TRPM7 expression in VSMCs and TRPM7 was a functionally important regulator of Mg^{2+} homeostasis in VSMCs (55). In addition, magnesium negatively regulates vascular calcification and osteogenic differentiation through increased TRPM7 activity (56). Furthermore, upregulation of TRPM7 channels by Ang II contributes to the development of the proliferative phenotype of ascending aortic VSMCs (57). However, the possible roles of additional TRPM proteins in the biological functions of VSMCs have been rarely studied.

Recently, it has been reported that TRPM8 has regulatory function in the cardiovascular system, particularly in blood vessels (57). TRPM8 is expressed in pulmonary arterial and aortic smooth muscle (58). Notably, TRPM8 in smooth muscle was demonstrated to not be involved in cold-induced contraction, as it does in the nervous system. TRPM8
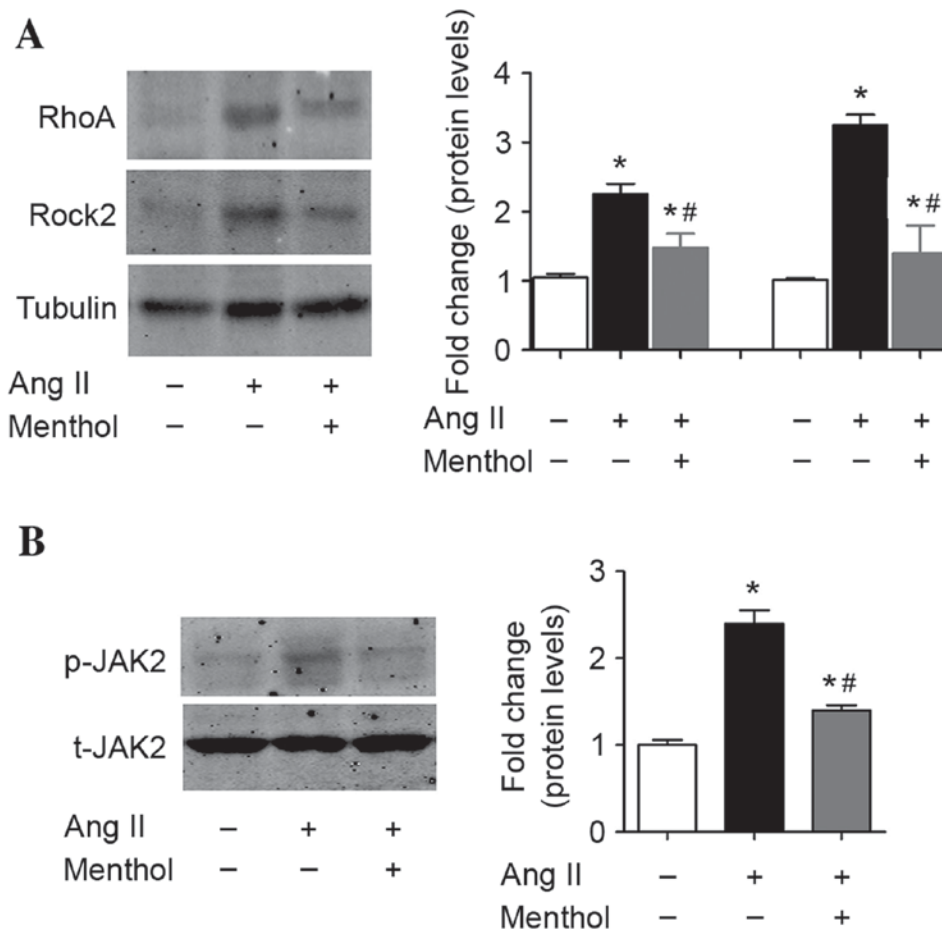
Figure 4. Activation of TRPM8 attenuated Ang II-induced activation of RhoA-Rock2 and JAK2 signaling pathways in VSMCs. (A) Representative western blotting images and quantitative analysis of the effect of TRPM8 activation on Ang II-induced activation of the RhoA-Rock2 pathway in cultured rat VSMCs. (B) Representative western blotting images and quantitative analysis of the effect of TRPM8 activation on Ang II-induced phosphorylation of JAK2 in cultured rat VSMCs. Data are presented as the mean ± standard error (n=8). *P<0.05 vs. untreated controls; #P<0.05 vs. Ang II-only treated VSMCs. TRPM8, transient receptor potential melastatin 8; Ang II, angiotensin II; RhoA, ras homolog gene family, member A; Rock2, rho-associated protein kinase 2; p-JAK2, phosphorylated janus kinase 2; t-JAK2, total janus kinase 2; VSMCs, vascular smooth muscle cells.

activation led to relaxation of vessels (29). In addition, TRPM8 activation by chronic dietary menthol attenuated mesenteric arterial constriction and lowered blood pressure in a genetic model of rat hypertension via inhibition of RhoA-Rho kinase expression and activity in an *in vivo* study (30). The RhoA-Rho kinase cascade serves key roles in pressure overload-induced right ventricular hypertrophy and dysfunction (59) and neointima formation (60). In addition, there is crucial Rho-kinase inhibition during cardiac development in the pathogenesis of hypertension (61,62). These results suggest that TRPM8 is involved in the regulation of vascular tone. However, the precise role of TRPM8 in hypertension and hypertension-associated pathophysiology is not completely known. To the best of our knowledge, the results of the present study are the first to demonstrate that TRPM8 is downregulated in 2K1C rat aortae. The results are consistent with a recent report by Liu *et al* (63), which demonstrated a significant decrease in TRPM8 mRNA and protein expression in the pulmonary arteries of pulmonary hypertensive rats. In the present study, when the hypertension in 2K1C rats was partially reversed by ACEI treatment, the upregulation of TRPM8 was also partially reversed, suggesting the TRPM8 downregulation may be a direct consequence

of hypertension or hypertension-associated factors. In the following experiments, TRPM8 was observed to be downregulated in Ang II-treated VSMCs, which supports the hypothesis that the high blood Ang II concentration may be the cause of TRPM8 downregulation during hypertension.

2K1C is a well-established hypertension animal model. This model has been used for several decades (64-68), and the authors of the present study have used this model in a previous study (69). In this model, not only is the blood level of Ang II increased, the local tissue levels of Ang II are also enhanced. The enhanced Ang II concentrations in the aorta, kidney and brain significantly contribute to the pathogenesis of hypertension (65,68). Benazepril, also known as Lotensin, is an ACEI that was approved by the US Food and Drug Administration in 1991. There are numerous pharmacodynamic data regarding benazepril (70-72). According to an extensive review regarding the pharmacodynamic and pharmacokinetic properties of benazepril (73), benazepril remarkably suppresses human plasma and tissue Ang II levels via inhibiting ACE activity by ~80-90% both *in vitro* and *in vivo*. Mochel *et al* (74) recently provided a comprehensive description of the effect of benazepril on the dynamics of the RAAS in dogs. It was demonstrated that the plasma

Ang II concentration decreased markedly following a bolus administration of benazepril. Consequently, existing data regarding the inhibitory effects of benazepril on plasma and tissue Ang II levels have been considered in the present study. The *in vitro* experiments demonstrated that Ang II treatment reduced TRPM8 expression in cultured VSMCs, while activation of TRPM8 reversed Ang II-induced oxidative stress and upregulated NOX enzymes in VSMCs. These results suggest that Ang II may directly regulate TRPM8.

Of particular note, the results of the present study indicated that activation of TRPM8, using a pharmacological agonist, partially reversed the Ang II-induced oxidative stress and JAK2 signaling activation. Oxidative stress serves an important role in the pathogenesis of hypertension (75,76). Ang II-mediated oxidative stress and DNA damage accelerates cellular senescence in VSMCs (47). During this process, NOX1 and NOX4 are two major sources of ROS in Ang II-induced vascular hypertrophy (48). In addition, RhoA-Rock2 and JAK2 signaling pathways are involved in the pathogenesis of hypertension or vascular remodeling due to the increased presence of ROS (41,49,77). In the present study, activation of TRPM8 significantly inhibited the increase in NOX1 and NOX4 in Ang II-treated VSMCs, and suppressed activation of the RhoA-Rock2 and JAK2 signaling pathways. Recently, Sun *et al* (30) demonstrated that chronic dietary menthol administration for 8 weeks prevented mesenteric arterial constriction and lowered blood pressure in genetic hypertensive rats with high Ang II levels. In addition, this study demonstrated that TRPM8 effects were associated with inhibition of intracellular calcium release from the sarcoplasmic reticulum and RhoA kinase activity in arteries (30). The results of the present study are consistent with this previous study. In addition, the role of TRPM8 in additional vascular cells, such as endothelial cells, may be of important interest. This question requires further investigation in the future.

The RAAS modulates cardiovascular functions, as well as a number of additional biological functions. As some thermoregulatory proteins and RNAs have been demonstrated to be involved in the development of brown and beige fat, which is closely associated with metabolism (78-82), the authors of the present study hypothesize that TRPM8 may be a crucial regulator of adiposity and metabolism. Notably, the RAAS serves an important role in metabolism via multiple molecular mechanisms (83,84). Consequently, the association between the RAAS and TRPM8 may be more complex than assumed in the current study.

In conclusion, the results of the present study demonstrated that TRPM8 may be involved in the pathophysiology of hypertension, and support the notion that pharmacological activation of TRPM8 may be a novel approach for treatment of hypertension and other Ang II-induced vascular injuries, such as aneurysm.

Acknowledgements

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