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TRPV1 inhibits smooth muscle cell phenotype switching in a mouse model of abdominal aortic aneurysm

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ABSTRACT

The natural outcome of abdominal aortic aneurysm (AAA) is that of slow progression and ultimate rupture, then a life-threatening hemorrhage consequently. Ruptured AAA is a dramatic catastrophe and constitutes one of the leading causes of acute death in elderly men. However, the mechanism of AAA is still unclear. Transient receptor potential vanilloid (TRPV) family has protective effects in cardiovascular diseases. In this study, we revealed the expression and the pathogenesis of TRPV1 in a mouse AAA model. The results presented here identify TRPV1 could be a potential therapeutic target for AAA treatment.

Introduction

Abdominal aortic aneurysm (AAA) is a degenerative disease characterized by destruction and progressive expansion of the abdominal aortic wall. Pathological changes in AAA include chronic inflammatory infiltration, destruction and remodeling of extracellular matrix (ECM), and degradation of collagen and elastin. In the United States, AAA rupture is the 13th leading cause of death, with an estimated annual death of approximately 15,000 [1]. According to reports, when AAA ruptures, the mortality rate exceeds 80% [2]. Currently, there is no effective medical treatment to prevent progression or rupture of AAA, and intravascular or open surgical repair is the only medical choice. Therefore, it is urgent to clarify the pathogenesis of AAA and uncover its therapeutic target.

Matrix metalloproteinases (MMPs) are a large family and are named for their need for metal ions, such as Zn^2+ or Ca2+, as cofactors [3]. The main function of MMPs is to degrade various protein components in the extracellular matrix [4,5]. In total, 24 members of the MMP family have been identified according to their structure and function and classified into 6 classes according to their substrates: collagenases [6], gelatinases [7], stromelysins [7,8], matrilysins [9] and other secretory MMPs. Vascular smooth muscle cells (VSMCs) in the media and adventitia of the aortic wall are the main source of MMP-2 [10,11]. It is now believed that MMP-2 can degrade both elastin and collagen, and by inhibiting MMP-2, AAA could be abolished [12–14].

Some studies reported that in the cardiovascular system, transient receptor potential vanilloid (TRPV) family involves in the regulation of pathological conditions, including atherosclerosis [15–17], congestive heart failure [16,18], systemic hypertension [15,19,20], and pulmonary hypertension [21]. TRPV1 channel is a nonselective cation channel with significant permeability to H^+, Na^+, Ca^{2+}, and Mg^{2+} plasmas and it plays a significant role in vascular remodeling. However, whether TRPV1 participates in the AAA process is still unclear. We reasoned that this study would argue for, or against that TRPV1 as a therapeutic target in AAA, since recent studies have shown that by activating TRPV1, intracranial arteriole remodeling can be attenuated [22].

Results

ApoE$^{-/-}$ mice were infused with angiotensin II (AngII) (1.44 mg/kg/day infused by osmotic mini-pump) for 4 weeks to induce the AAA model (Figure 1(a)). The incidence of AAA in AngII group was 50%, while no AAA was observed in
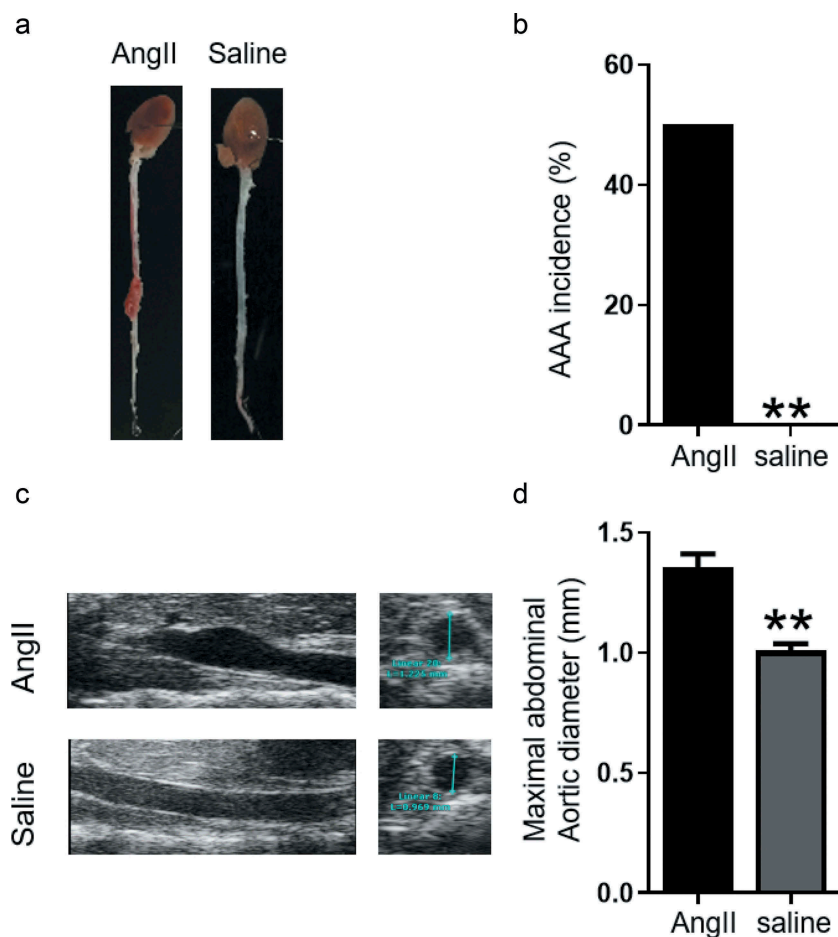

Figure 1. ApoE$^{-/-}$ mice were infused with Saline or AngII for 28 d by embedding osmotic minipump subcutaneously (n = 10 per group). (a) Representative photographs showing the features of AngII-induced aneurysm compared with normal abdominal aorta. (b) The incidence of AngII-induced AAA in ApoE$^{-/-}$ mice. (c) Representative ultrasound images showing the abdominal aortas of each group. (d) Graphical representation of the ultrasound biomicroscopy technique used for the determination of maximal abdominal aortic diameter of each group. Data are presented as mean ± SEM (n = 10). **P < 0.01 vs. saline group.

control group (Figure 1(b)). The diameter of the maximal abdominal aorta was significantly larger than that of the control group (1.355 vs 1.009, P < 0.01, Figure 1(c and d)). More elastin and collagen fragmentations were observed in the AngII group than in the control group (Figure 2 (a–c)). Immunohistochemistry staining showed decreased expression of TRPV1 (Figure 2(d)). RT-PCR and western blotting showed that in AAA tissue, mRNA level and protein expression of TRPV1 were significantly lower (Figure 3(a, c and e)). On the contrary, MMP-2 was higher than in normal aorta tissue (Figure 3(b, d and e)), which is the marker of AAA [14]. In vitro, the mRNA level and protein expression of TRPV1 and MMP-2 showed similar results in VSMCs infused with AngII. However, overexpression of TRPV1 by transfecting adenovirus could decrease MMP-2 level

(Figure 4(a-d, g)). Furthermore, representative vaso-constrictive proteins such as α-SMA and SM22α were decreased in response to AngII, which could be converted by overexpression of TRPV1 (Figure 4 (e and f)). Cell scrape assay was performed to identify whether the overexpression of TRPV1 can affect the migration of VSMCs in response to AngII. The result showed that in VSMCs infused by AngII, migration was increased while it can be attenuated by overexpression of TRPV1 and down-regulation of TRPV1 promoted the migration (Figure 5).

Discussion

Our study found that TRPV1 cation channel was downregulated in a mouse model of AAA in vivo. In vitro, TRPV1 expression was downregulated and MMP-2 was upregulated in VSMCs infused by AngII.
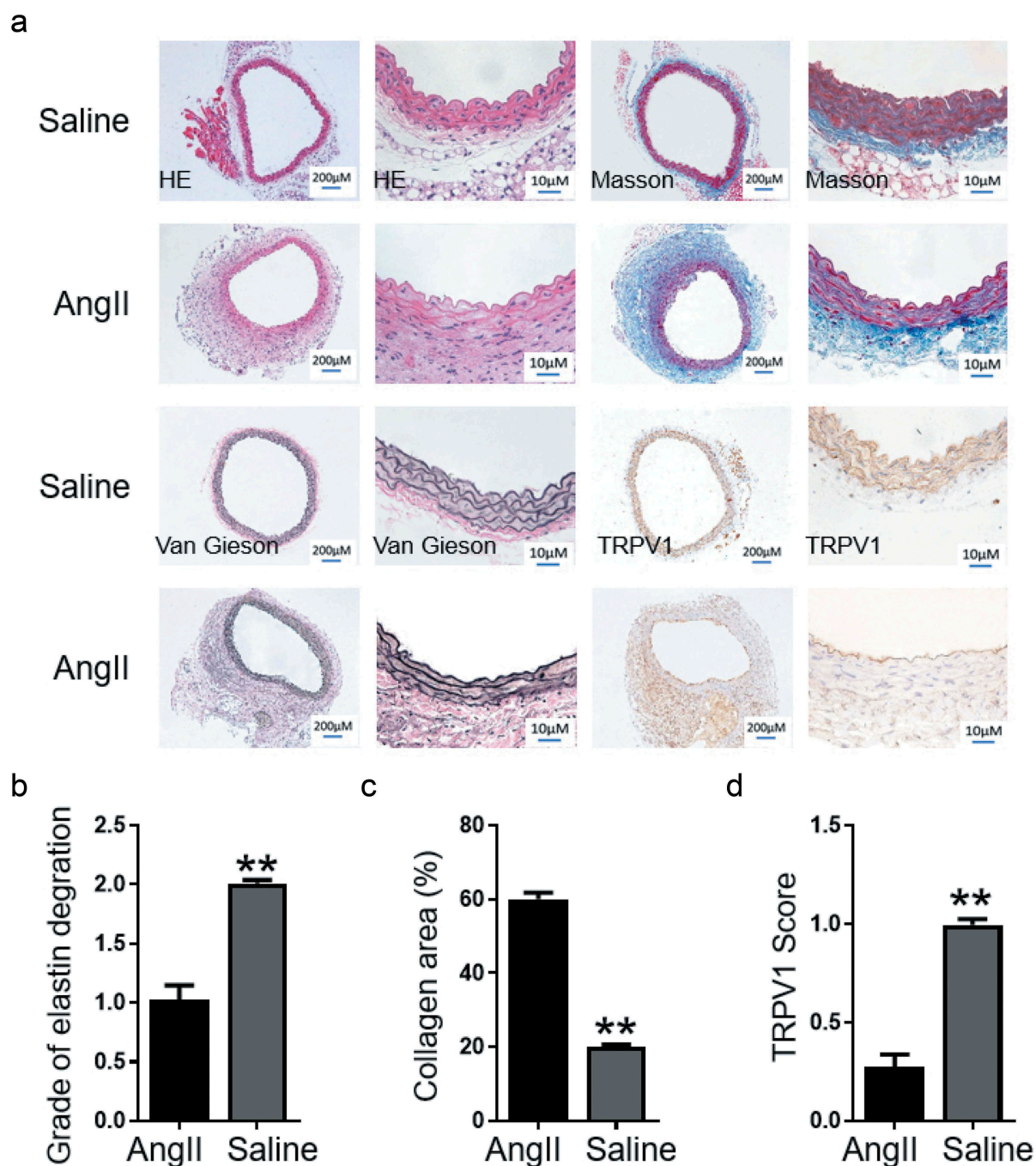
Figure 2. Decreased TRPV1 expression in AngII-induced ApoE$^{-/-}$ mice aneurysms. ApoE$^{-/-}$ mice were infused with Saline or AngII for 28 d by embedding osmotic minipump subcutaneously (n = 10 per group). (a) Representative staining with hematoxylin and eosin (H&E), Van Gieson (elastin), Masson Trichrome (collagen), and representative immunohistochemical staining for TRPV1 in the suprarenal aortas of mice after saline or AngII infusion. (b) Grade of elastin degradation. (c) Collagen deposition in the aortic wall of mice after saline or AngII infusion. (d) Quantification for TRPV1 in the suprarenal aortas of saline- or AngII-infused mice. Data are presented as mean ± SEM (n = 10). **P < 0.01 vs. saline group.

Overexpression of TRPV1 could inhibit the secretion of MMP-2 and attenuate the down-regulated SM22α and α-SMA caused by AngII infusion. Meanwhile, the migration of VSMCs induced by AngII was also converted by overexpression of TRPV1. In contrast, down-regulation of TRPV1 promoted the migration.

Nowadays, TRP family has been classified into six subfamilies including vanilloid (TRPV), canonical (TRPC), melastatin (TRPM), polycystin (TRPP), mucoloipin (TRPML) and ankyrin (TRPA), depending on the sequence of amino acids homology [23]. TRPV1 was believed to have various distributions in
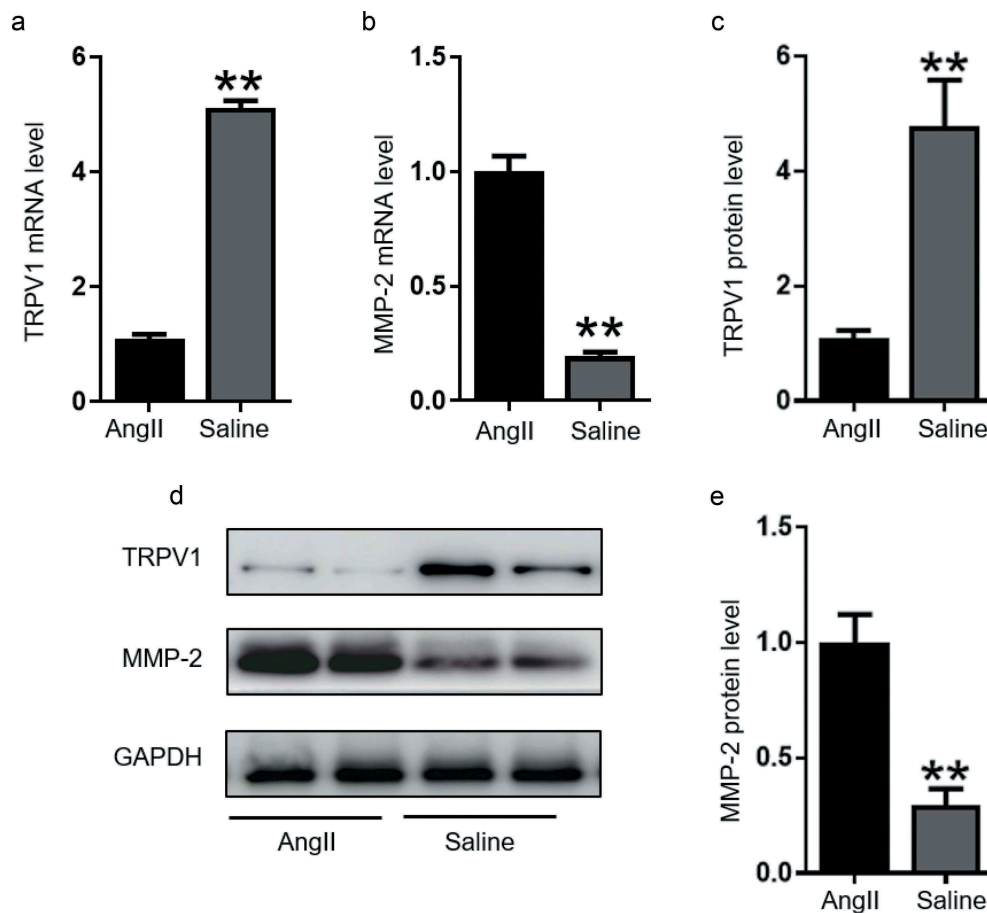
Figure 3. Decreased TRPV1 expression and increased MMP-2 expression in AngII-induced ApoE$^{-/-}$ mice aneurysms. (a) TRPV1 mRNA level. (b) MMP-2 mRNA level. (c–e) TRPV1 and MMP-2 protein expressions level. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control in the suprarenal aortas of saline- or AngII-infused mice. Data are presented as mean ± SEM (n = 10). **P < 0.01 vs. saline group.

organs including heart, liver, lungs, kidney, intestine, brain, uterus, testis, salivary glands, dorsal root ganglia, and sensory neurons [16,24,25]. One study gave experimental evidence that continuous activation of TRPV1 could attenuate atherosclerosis evoked by a high-fat diet. They declared that TRPV1 activation regulates ABCA1/LRP1 expression through calcium-evoked calcineurin- and PKA-dependent mechanisms in VSMC [26]. Similarly, another study stated that TRPV1 activation attenuated foam cell formation by inducing autophagy in oxLDL-treated VSMCs. Activation of TRPV1 by capsaicin-induced autophagy through AMP-activated protein kinase (AMPK) signaling pathway [15]. Besides, TRPV1 activation by dietary capsaicin prevented high-salt diet-induced cardiac hypertrophy by ameliorating cardiac mitochondrial dysfunction. TRPV1 activation upregulated sirtuin 3 in vivo, thus protecting mitochondria from dysfunction [18]. In my study,

TRPV1 showed decreased expression in heart tissue from ApoE$^{-/-}$ mice infused with AngII compared with ApoE$^{-/-}$ mice infused with Saline (Figure S1). These results suggested that TRPV1 has a protective effect in cardiovascular diseases. Although one study demonstrated that TRPV1 expression was increased in patients with ascending aortic aneurysm, which seemed to be contradictory with our results [27], it should be noted that the origin of VSMCs in the ascending aorta was different from the abdominal aorta [28]. Nogi M et al. reported that disruption and degradation of medial elastic lamina in the ascending aorta were more severe in ApoE$^{-/-}$SmgGDS$^{\pm}$ mice compared with ApoE$^{-/-}$ mice after AngII infusion for 4 weeks. However, there was no significant difference in the disruption and degradation of the medial elastic lamina in the abdominal aorta between the two genotypes after AngII infusion [29]. Therefore, it was no surprising to find that
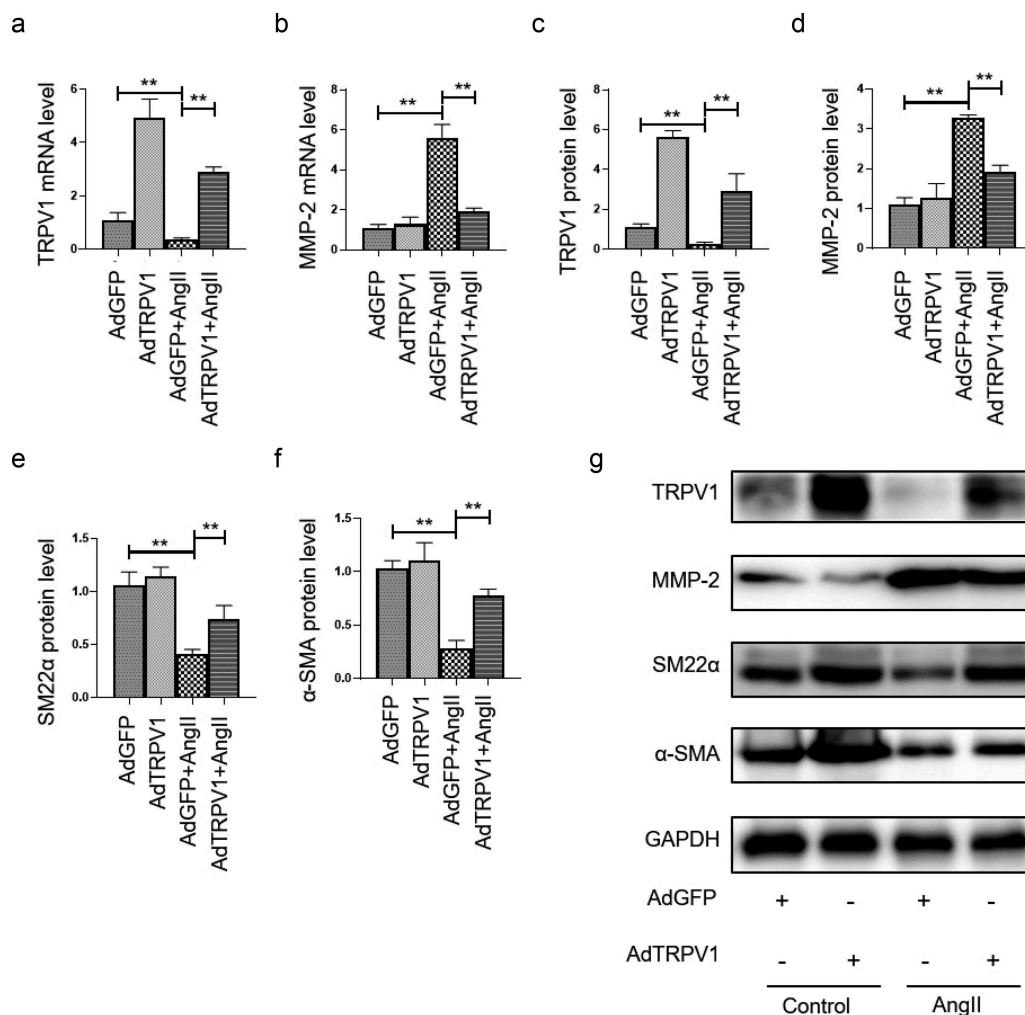
Figure 4. TRPV1 overexpression inhibits MMP-2 in AngII-induced VSMCs. (a–g) Cultured primary VSMCs transfected with AdGFP or AdTRPV1 for 24 h, then infused with or without AngII for 24 h. (a) TRPV1 mRNA level and (b) MMP-2 mRNA level in various groups detected by RT-PCR. (c) A bar graph showing relative levels of TRPV1, (d) MMP-2, (e) SM22α and (f) α-SMA proteins. (g) TRPV1, MMP-2, SM22α, and α-SMA protein expression from groups were detected by western blotting. Data are presented as mean ± SEM (n = 10). **P < 0.01 vs. saline group.

TRPV1 expression was decreased in AngII-induced abdominal aortic aneurysm mouse models.

MMP family played an important role in the initiation of AAA. The main function of MMPs was to degrade various protein components in the extracellular matrix [4,5]. Besides, MMPs maintained the normal structure and played an important role in the regulation of various soluble factors. Most studies reported that under AngII stimulation, VSMCs changed from contractile phenotype to secretory phenotype, accompanied by increased MMP-2 secretion, which was a significant pathological process of AAA [11]. VSMCs in the media and adventitia of the aortic wall were the main source of MMP-2. It was now believed that MMP-2 could degrade both elastin and collagen [14]. Normally, VSMCs had a protective effect on the aortic tissue. VSMCs synthesized collagen, elastin, and proteoglycans. Under the conditions of hypertension, atherosclerosis, or some other vascular diseases that could cause inflammation, VSMCs confined the inflammation to the adventitia to limit proteolysis. Therefore, it was reasonable that the release of proteolytic enzymes, such as MMP-2, might result in disruption of the medial membrane of the aorta and aneurysm formation eventually [30]. The MT1-MMP/TIMP2/MMP-2 enzyme complex was additional evidence that MMP-2 played a key role in ECM remodeling and medial disruption in AAA [31]. TRPV1 was reported to have the potential regulatory effect of MMP. Previous studies
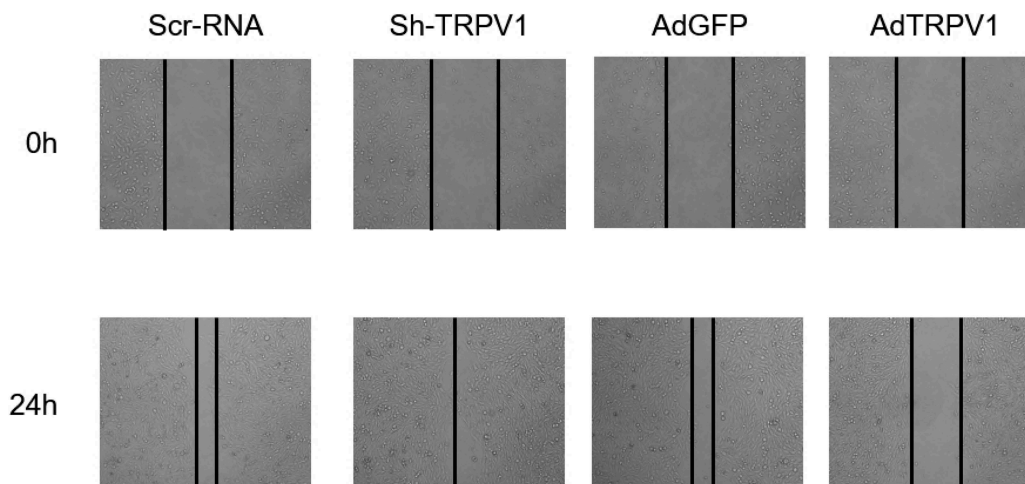
Figure 5. TRPV1 overexpression inhibits migration and down-regulation of TRPV1 promotes the migration of VSMCs infused with AngII. Representative images show cultured primary VSMCs transfected with Scr-RNA, Sh-TRPV1, AdGFP, or AdTRPV1 for 24 h, then infused with AngII for 24 h.

demonstrated that TRPV1 inhibition could inhibit the expression of MMP-1 and MMP-2 in skin fibroblasts and TRPV1 increased activity is associated with the up-regulation of MMP-2 levels in other models [32,33]. However, another study concerning myocardial fibrosis stated that TRPV1 activation attenuated pressure overload-induced overexpression of MMP-2, MMP-9, and MMP-13 in Wild-Type mice. This discrepancy might be due to different cell types which need to be elucidated in the future study. In my study, overexpression of TRPV1 could inhibit MMP-2 expression. Up-regulation or down-regulation of TRPV1 has no influence on the expression of MMP-1 (Figure S2).

The phenotypic regulation of contractile VSMCs was considered to be the key causing of arterial remodeling and played an important role in the formation of AAA [34]. Consequently, inhibiting the conversion of VSMCs to secretory phenotypes, especially inhibiting the increased MMP-2 secretion in AAA formation was an important treatment strategy. In this study, low TRPV1 expression was detected in the AAA tissue, accompanied by high MMP-2 expression. Besides, elastin and collagen fiber were found degraded in AAA compared with the control group (Figure 2(a–c)). These findings were consistent with in vitro results. In AngII-infused VSMCs, representative vasoconstrictive proteins such as α-SMA and SM22α were upregulated. Our study delineated that overexpression of TRPV1 by using adenovirus inhibited the expression of MMP-2, indicating that TRPV1

had a potential effect to attenuate AAA. The complex role of TRPV1 in AAA progression required further investigation. Meanwhile, reduced SM22α and SMA in AngII-infused VSMCs was converted by overexpression of TRPV1. Migration of VSMCs played a crucial role in AAA formation. Maegdefessel L et al. reported that the migration of VSMCs was increased in both human specimens and animal models of AAA and inhibition of migration may attenuate AAA[35]. Our study verified that the hypothesis and the migration of VSMCs induced by AngII infusion could be inhibited by TRPV1 overexpression. In summary, our findings suggested that TRPV1 could be a potential effective target for AAA.

Methods

Animals All experiments were approved by the Ethics Committee of Shengjing Hospital and conducted in accordance with existing guidelines on the care and use of laboratory animals. Our investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). The mice were housed at 18°C to 22°C with bedding and nest material and purified water (Milli-Q system; Millipore, Glostrup, Denmark) ad libitum. Mice were kept under conditions of a 12-h light: dark cycle. Experiments began after 1 week of acclimatization.

Twenty 8-week-old male ApoE$^{-/-}$ mice were divided into two groups randomly, which was infused with either AngII (1.44 mg/kg/day) or physiological saline (0.9% sodium chloride) by osmotic pumps (Alzet model 1004; AlzaCorp., Mountain View, CA) for 4 weeks. Vascular ultrasound (Vevo 2100 apparatus) was performed by using small animal ultrasound system to examine the incidence of AAA and the maximal abdominal aorta in each group. The abdominal aortic tissue was removed at day 28 and kept at −80°C for the following studies.

Ultrasound imaging

A Vevo 2100 apparatus (Visual Sonics, Toronto, Canada) equipped with a 30-MHz probe was utilized in this study. Mice abdomen were shaved before the ultrasound examination of the abdominal aorta. After isoflurane anesthesia, the mice were placed on a platform that could be heated and kept warm, and 2% isoflurane was continuously inhaled throughout the ultrasound examination. Monitor heart rate through electrocardiogram system, maintain heart rate at 400–500 bpm and keep constant. Firstly, apply the probe on short axis to locate aorta, distinguishing it from vena cava by pulsatile property of the blood vessel. Secondly, move the probe slightly to ensure that the aorta is precisely parallel to the device, then measure the aortic diameter on long axis. A blinded investigator measured three representative sites of the aorta including above the upper renal artery, on maximal aortic dilation and below the lower renal artery.

Morphometric analysis

The mean vascular diameter was calculated as the circumference of the inner elastic lamina/π. The dissected abdominal aortas were fixed in 4% buffered paraformaldehyde for 48 h. After fixation, tissues were paraffin embedded, sectioned (5-μm thickness), and stained with Hematoxylin and Eosin (HE), Masson's trichrome (Sigma, HT15) and Verhoeff van Gieson Elastic (H Sigma, T25A) stain. Collagen stained with brown in Masson's trichrome and elastin stained with black in Verhoeff van Gieson Elastic. Eight sequential sections per thoracic aorta were measured for each mouse and used for analysis.

Immunohistochemistry staining

Sections (5-μm thickness) were air-dried, fixed in 10% buffered formalin for 5 min, and washed in PBS for 10 min. Sections were then blocked with 5% goat serum (X0907, Dako, Kyoto, Japan) in phosphate-buffered saline. Sections were reacted with anti-TRPV1 or anti-MMP-2 antibody overnight at 4°C, incubated with a secondary antibody the next day. Sections were then stained for peroxidase activity with 0.01% hydrogen peroxide and 0.05% 3,3-diaminobenzidine tetrahydrochloride (Dojindo, Kumamoto, Japan) as substrates, followed by light counterstaining with hematoxylin to identify the nuclei. A brown reaction product indicated localization of TRPV1 or MMP-2. All sections were counterstained with hematoxylin, dehydrated, mounted, and examined by light microscopy.

Vascular smooth muscle cells isolation and culture

Mouse primary vascular smooth muscle cells (VSMCs) were isolated from the freshly dissected aortas. After dissection, remove the peri-adventitial fat. Then, aortic samples were digested in enzymatic mix (10 mg/ml type II collagenase, Beyotime, Shanghai, China and 1 mg/ml elastase, Beyotime) at 37°C for 10 min. After the adventitia was gently removed under a dissecting microscope, we put the remaining tissue into the enzymatic mix again for 50 min. Finally, the digested aortas were mechanically disrupted and passed through a 70 μm cell strainer (340607, BD Biosciences) for purification. All the collected cells were cultured in DMEM-10%FBS-1% PS.

Up-regulation of TRPV1 by adenovirus transfection and down-regulation of TRPV1 by small interfering RNA transfection

Adenoviral GFP (AdGFP) control and recombinant adenoviral vectors expressing TRPV1 (AdTRPV1) were constructed by Hanbio Biotechnology Ltd (Shanghai, China). VSMCs were cultured in serum-free DMEM containing adenovirus (10 μl/ml, MOI: 100:1) for 24 h. Then, the medium was replaced and the VSMCs were harvested and lysed for Western blot, PCR and cell scrape assay. For down-regulation

of TRPV1, VSMCs were transfected at 50–60% confluence using siRNA transfection reagent (sc-29528; Santa Cruz Biotechnology, Santa Cruz, CA, USA) with TRPV1 siRNA (223138; Thermo Fisher Scientific, Waltham, MA) diluted into siRNA transfection medium (sc-36868; Santa Cruz Biotechnology) at a final concentration of 100 nM according to the manufacturer's instructions. VSMCs that were transfected with scrambled control siRNA (4404020; Thermo Fisher Scientific, Waltham, MA) at a concentration of 100 nM were served as a negative control. Cells were transfected for 24 h for the TRPV1 knockdown study prior to cell scrape assay.

TRPV1 and MMP-2 mRNA expression analysis

Total RNA was extracted from frozen, pulverized mouse abdominal aorta tissue using Trizol (Invitrogen, Carlsbad, CA, USA). Then, RNA (2 μg) was reverse-transcribed into cDNA using a PrimeScript RT reagent kit (RR036A; Takara, Shiga, Japan). We quantitated PCR amplifications using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and normalized results against glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene expression on an ABI 7900HT Fast Real-Time PCR System (Life Technologies) using the following cycling conditions: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C, 1 min at 60°C. The following primers were used: for mouse TRPV1, 5'-CCTACGCTCTGCTGACTA-3' (forward) and 5'-CACAACCTGATTCTGATGGA-3' (reverse); for mouse MMP-2, 5'-CAAGTTCCCC GGCGATGTC-3' (forward) and 5'-TTCTGGTCA AGGTCACCTGTC-3' (reverse); for mouse GAP DH, 5'-ACATCATCCCTGCATCCACT-3' (forward) and 5'-CCTGCTTCACCACCTTCTTG-3' (reverse);

Western-blot analysis

The Abdominal aortas of each mouse were dissected, combined, and homogenized. The homogenized tissue or cells were lysed in RIPA buffer (Thermo Scientific, USA). Proteins were separated by electrophoresis on SDS-polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. Blots were incubated with primary antibodies (Abcam)

including anti-TRPV1 (1:2000 dilution), anti-SM22α (dilution 1:1000), anti-MMP-2 (dilution 1:2000), anti-GAPDH (dilution 1:2000), anti-α-SMA (dilution 1:2000) overnight at 4°C; this was followed by incubation with secondary antibody (Zhongshan Golden Bridge Biotechnology, Beijing, China) for 1 h at 37°C, were applied before revelation with Clarity Western ECL (#170-5060, Bio-Rad). Bands were then quantified using a Gel Doc 2000 UV Gel imaging system (Bio-Rad, Hercules, CA, USA). The band intensity was measured and normalized over GAPDH mean band intensity.

Statistical analysis

All values are expressed as mean ± SEM. Data were evaluated using Prism 7 software (GraphPad software Inc., CA, USA). Differences between the two groups were compared using unpaired Student's t-tests. Differences among three or more than three groups were compared using one-way ANOVA followed by Tukey's post hoc test. A p value < 0.01 was considered statistically significant.

Disclosure statement

No potential conflict of interest was reported by the authors.

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