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Research paper

TRPA1 inhibition ameliorates pressure overload-induced cardiac hypertrophy and fibrosis in mice

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ABSTRACT

Background: Recent evidence has indicated that the transient receptor potential ankyrin 1 (TRPA1) is expressed in the cardiovascular system and implicated in the development and progression of several cardiovascular diseases. However, the effects of TRPA1 on cardiac hypertrophy development remain unclear. The aim of this study was to determine the role of TRPA1 in cardiac hypertrophy and fibrosis development.
Methods: C57BL/6J mice were subjected to transverse aortic constriction (TAC) and were orally treated with the TRPA1 selective inhibitors HC-030031 (HC) and TCS-5861528 (TCS). Morphological assessments, echocardiographic parameters, histological analyses and flow cytometry were used to evaluate cardiac hypertrophy and fibrosis.
Results: Human and mouse hypertrophic hearts presented with noticeably increased TRPA1 protein levels. Inhibition of TRPA1 by HC and TCS attenuated cardiac hypertrophy and preserved cardiac function after chronic pressure overload, as evidenced by increased heart weight/body weight ratio, cardiomyocyte cross-sectional area and mRNA expression of hypertrophic markers, including ANP, BNP and β-MHC. Dramatic interstitial fibrosis was observed in the mice subjected to TAC surgery, and this was markedly attenuated in the HC and TCS treated mice. Mechanistically, the results revealed that TRPA1 inhibition ameliorated pressure overload-induced cardiac hypertrophy by negatively regulating Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and calcineurin signaling pathways. We also demonstrated that blocking TRPA1 decreased the proportion of M2 macrophages and reduced profibrotic cytokine levels, thereby improving cardiac fibrosis.
Conclusions: TRPA1 inhibition protected against cardiac hypertrophy and suppressed cardiac dysfunction via Ca^{2+}-dependent signal pathways and inhibition of the M2 macrophages transition. These results suggest that TRPA1 may represent a potential therapeutic drug target for cardiac hypertrophy and fibrosis.

1. Introduction

Pathological cardiac hypertrophy, which is defined as cardiomyocyte enlargement and cardiac contractile dysfunction, is an independent risk factor for cardiovascular events and eventually leads to heart failure (HF) and malignant arrhythmia [1, 2]. At the cellular and molecular levels, myocyte enlargement involves three basic processes, namely, extracellular stimulating signals, intracellular signal transduction and nuclear gene activation, which eventually induce the phenotypic changes associated with cell hypertrophy. However, the molecular mechanisms underlying cardiac hypertrophy are uncertain.

The transient receptor potential (TRP) superfamily has at least 28 members and is involved in diverse aspects of cellular function, including sensory perception and signal transduction [3, 4]. Evidence suggests that TRPC channels play a pivotal role in the development of cardiac hypertrophy [5, 6]. Transgenic mice with cardiac-specific TRPC6 overexpression exhibit aggravated pressure overload-induced cardiac hypertrophy and HF [7]. Similarly, TRPC1 knockout, in coordination with calcineurin/NFAT signaling, preserves heart structure and function in pressure overload mouse models [8]. Transient receptor potential ankyrin 1 (TRPA1), which belongs to the TRP channel superfamily, is an important target in oxidative stress and inflammatory diseases [9, 10]. Inhibiting the TRPA1 channel has been shown to attenuate fibrosis and the inflammatory response in ocular fibroblasts [11]. Increasing

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Research in context

Cardiac hypertrophy is an independent risk factor for cardiovascular events and is characterized as an increase in cardiomyocyte size and cardiac dysfunction. Hence, there is an ongoing need to further investigate and develop efficient therapeutic agents to combat cardiac hypertrophy and fibrosis. TRPA1, which is characterized as a nonselective cation channel, was originally thought to be predominately expressed in primary nociceptive sensory neurons. Recent studies have shown that the TRPA1 was also widely expressed in the cardiovascular system and was involved in the regulation of several cardiovascular diseases. This study aimed to investigate the potential role of TRPA1 in cardiac hypertrophy and fibrosis development.

evidence has shown that TRPA1 is a modulator of physiological and pathophysiological cardiovascular events [12, 13]. However, whether the TRPA1 channel plays a role in cardiac hypertrophy and fibrosis remains unclear.

In the present study, we observe increased TRPA1 protein levels in the left ventricle of patients with dilated cardiomyopathy (DCM) and in a mouse model of pressure overload-induced cardiac hypertrophy. We demonstrate that TRPA1 channel inhibition protects the heart against hypertrophy and dysfunction and that macrophage differentiation and Ca^{2+}-dependent signaling pathways are involved.

2. Materials and methods

2.1. Human heart tissues

Explanted, failing hearts were obtained from the left ventricles of DCM patients. Normal heart tissues were obtained from prospective multiorgan donors that were unsuitable for transplantation for noncardiac reasons. Written informed consent was obtained from the patients and donor families. All experimental procedures that involved human samples complied with the Declaration of Helsinki and were approved by the Human Research Ethics Committees of Renmin Hospital of Wuhan University (Wuhan, China).

2.2. Reagents

An anti-TRPA1 antibody (1:500 dilution) was obtained from Novus Biologicals (Littleton, CO, USA). The selective TRPA1 antagonists HC-030031 (HC) and TCS-5861528 (TCS) were purchased from R&D Systems (Minneapolis, MN, USA). Wheat germ agglutinin (WGA, 1:200 dilution) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies for T-CaMKII (1:1000 dilution), calcineurin (1:1000 dilution), GAPDH (1:2500 dilution), α-smooth muscle actin (α-SMA, 1:500 dilution), CD3 (1:200 dilution), and CD68 (1:200 dilution) were purchased from Abcam (Cambridge, MA, USA). An antibody for p-CaMKII (1:1000 dilution) was purchased from GeneTex (Irvine, CA, USA). Antibodies for CD206 (1:200 dilution) were purchased from R&D Systems (Minneapolis, MN, USA). Fluorescein isothiocyanate (FITC)-conjugated anti-CD206, allophycocyanin (APC)-conjugated anti-F4/80, phycoerythrin (PE)-conjugated anti-CD45 and FITC-conjugated anti-CD3 were purchased from BioLegend (San Diego, CA, USA). Secondary antibodies and goat anti-rabbit IgG were obtained from LI-COR Biosciences (Lincoln, NE, USA). All other chemicals were of analytical grade.

2.3. Animals and treatments

All experiments involving animals were approved by the Animal Care and Use Committee of Renmin Hospital of Wuhan University (Wuhan, China) and were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Male C57BL/6J mice (8–10 weeks old; male; body weights of 23.5–27.5 g) were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and were housed with controlled temperature and humidity conditions. The mice were allowed free access to food and water under a 12-h light-dark cycle in the Cardiovascular Research Institute of Wuhan University (Wuhan, China). The animals were randomly assigned into either a sham surgery group or a transverse aortic constriction (TAC) group. Subsequently, the mice in the TAC group were also randomly assigned into three groups. The four groups were named as follows: sham + vehicle (DMSO), TAC, TAC + HC and TAC + TCS. HC (10 mg/kg) and TCS (3 mg/kg) were administered for 4 weeks by gastric gavage daily. At the end of the study, the hearts and lungs were harvested and weighed to compare the heart weight/body weight (HW/BW, mg/g), lung weight to body weight (LW/BW, mg/g), heart weight to tibia length (HW/TL, mg/mm) and heart weight/tibia length (HW/TL, mg/mm) ratios in the mice. Then, left ventricle (LV) tissues were collected for further experiments.

2.4. Echocardiography and hemodynamic analysis

Echocardiography was performed in anesthetized (1.5–2% isoflurane) mice using a Mylab 30CV ultrasound (Biosound Esaote) equipped with a 10-MHz linear array ultrasound transducer. The LV dimensions were assessed in the parasternal short-axis at the level of the papillary muscles. The heart rate (HR), LV end-systolic diameter (LVESd), LV end-diastolic diameter (LVEDd), interventricular septal thickness at end-diastole (IVSd), interventricular septal thickness at end-systole (IVSs), LV posterior wall thickness at end-diastole (LVPWd), LV posterior wall thickness at end-systole (LVPWs) and fractional shortening (FS) were measured.

Hemodynamics was measured in the mice using cardiac catheterization, and then a microtip catheter transducer (Millar Instruments, Houston, TX, USA) was inserted into the right carotid artery and advanced into the LV. Fifteen minutes after stabilization, the data were continuously recorded with a Millar Pressure-Volume System (Millar Instruments, Houston, USA) and measured using PVAN data analysis software.

2.5. Histological analysis

The hearts were fixed by perfusion with 10% formalin and embedded in paraffin. Subsequently, these tissues were cut transversely into 4- to 5-μm slides. To evaluate the cardiomyocyte cross-sectional area (CSA), the slides were stained with hematoxylin and eosin (HE) and WGA. Picrosirius red (PSR) staining was performed to assess the extent of fibrosis. The CSA and average collagen volume were determined using a digital image analysis system (Image-Pro Plus 6.0, Media Cybernetics, Bethesda, MD, USA).

2.6. Immunohistochemistry and immunofluorescence

For immunohistochemistry, paraffin-embedded hearts were cut transversely into 4- to 5-μm sections. Next, the sections were deparaffinized and blocked with 10% bovine serum albumin. Then, the sections incubated overnight at 4 °C with primary antibodies and anti-rabbit HRP reagent (Gene Tech, Shanghai, China) at 37 °C for 60 min. Finally, the sections were visualized with diaminobenzidine (DAB) (Gene Tech, Shanghai, China) for 2 min at 37 °C and mounted with neutral gums. The sections were examined under a light microscope (Nikon H550L, Tokyo, Japan). For immunofluorescence, the sections were autoclaved for antigen retrieval and then blocked with 10% goat serum for 10 min. Next, the sections were incubated with primary antibodies against CD206 overnight at 4 °C. The sections were rinsed with PBS for 20 min before incubating with two different IRDye® 800CW-

Table 1
Primers for quantitative polymerase chain reaction.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
ANP	ACCTGCTAGACCACCTGGAG	CCTTGGCTGTTATCTTCGGTACCGG
BNP	GAGGTCACTCCTATCCTCTGG	GCCATTTCCTCCGACTTTTCTC
β-MHC	CCGAGTCCCAGGTCAACAA	CTTCACGGGCACCCTTGGA
Collagen I	TGGCCTTGGAGGAAACTTTG	CTTGGAAACCTTGTGGACCAG
Collagen III	GTCAGCTGGATAGCGACA	GAAGCACAGGAGCAGGTGTAGA
CTGF	TGTGTGATGAGCCCAAGGAC	AGTTGGCTCGCATCATAGTTG
IL-4	GGTCTCAACCCCCAGCTAGT	GCCGATGATCTCTCTCAAGTGAT
IL-10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
TGF-β	GACATGCCGCCTGGAGAAAC	AGCCCAGGATGCCCTTTAGT
GAPDH	AACTTTGGCATTGTGGAAGG	CACATTGGGGGTAGGAACAC

conjugated secondary antibodies for 60 min and subsequently counter-stained with the SlowFade Gold antifade reagent containing DAPI.

2.7. Calcineurin activity assay

The cardiac tissues were homogenized and centrifuged at 3000*g* for 5 min at 4 °C, and the total protein concentration of the supernatant was determined. The calcineurin activity was measured using a calcineurin activity assay kit (Abcam, MA, USA) following the manufacturer's instructions.

2.8. Quantitative real-time RT-PCR

Total RNA was extracted from frozen mouse LV tissues using the TRIzol reagent. The RNA was reverse-transcribed into cDNA using oligo(dT) primers and the Transcriptor First Strand cDNA Synthesis kit (04896866001; Roche). In all groups, PCR amplification was quantified using the LightCycler 480 SYBR Green 1 master mix (04707516001; Roche), and the results were analyzed with the $2^{-\Delta\Delta Ct}$ method and normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression. The primer details are presented in Table 1.

2.9. Western blotting

Proteins were extracted from the cardiac tissue using radioimmunoprecipitation assay (RIPA) buffer, and the protein concentration was measured using a BCA protein assay kit. The proteins (50 μg) were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), subsequently transferred onto an Immobilon-P membrane (Millipore, Beijing, China) by a gel transfer device (Invitrogen) and incubated with different primary antibodies. The membrane was incubated with secondary antibodies at room temperature for 1 h. The blots were scanned by a two-color infrared imaging system (Odyssey; LI-COR) to quantify protein expression. The protein expression levels were normalized to the corresponding GAPDH levels.

2.10. Flow cytometry

Mouse hearts were harvested, minced into small pieces and then digested with collagenase II and dispase II (Sigma-Aldrich, MO, USA) in PBS for 30 min at 37 °C. The cell suspension was filtered, centrifuged, resuspended and blocked with a CD16/32 antibody. Then, the cells were stained with primary antibodies in FACS buffer for 30 min at 4 °C in the dark. Flow cytometry analysis was performed on a BD FACS Calibur using Diva 6 Software (BD Biosciences, San Jose, CA, USA). The results were analyzed using FlowJo Software V10.2 (TreeStar, OR, USA).

2.11. Cell culture

Bone marrow-derived macrophages (BMDMs) were isolated and plated in complete DMEM supplemented with murine macrophage colony-stimulating factor (50 ng/mL) and cultured for 5 days. The BMDMs were treated with HC (100 μM, dissolved in DMSO) or TCS (50 μM, dissolved in DMSO) with or without Ang II (100 nM) for 24 h to extract cellular RNA for mRNA analysis.

2.12. Statistical analysis

The results are expressed as the means ± standard deviation (SD). Statistical differences between 2 groups were determined by Student's *t*-test. Statistical comparisons among multiple groups were performed with one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. P values <0.05 were considered significant.

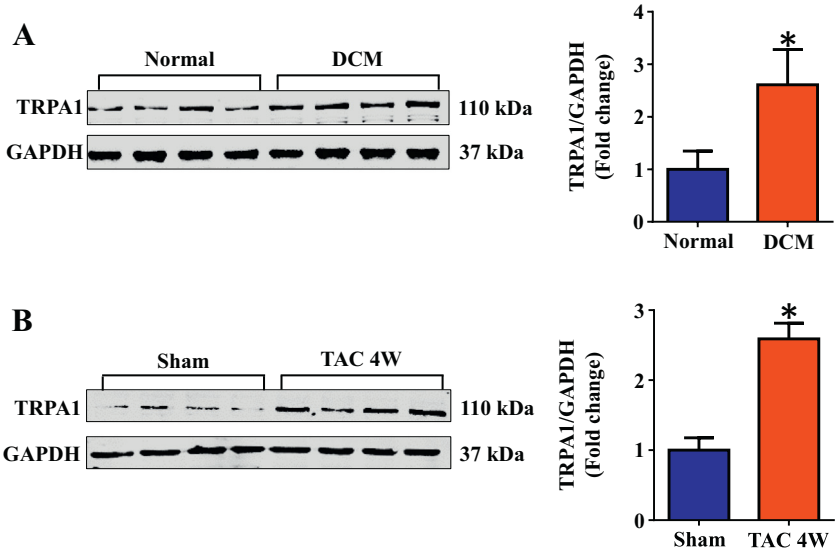

Fig. 1. TRPA1 expression is upregulated in the hypertrophic myocardium. (A) Representative Western blot and quantitative results of the TRPA1 protein levels in human heart samples from normal donors and patients with dilated cardiomyopathy (DCM) (n = 8; *P < 0.05 vs normal hearts). (B) Western blot analysis and quantitative results of TRPA1 expression in a murine model of cardiac hypertrophy induced by transverse aortic constriction (TAC). (n = 8, *P < 0.05 vs sham group).

Table 2
Echocardiographic and and hemodynamic parameters in mice of each group.

Groups	Sham (n = 12)	TAC (n = 12)	TAC + HC (n = 12)	TAC + TCS (n = 8)
HR (beats/min)	520.3 ± 36.2	519.3 ± 41.4	527.1 ± 33.2	508.6 ± 39.1
LVESd (mm)	2.02 ± 0.16	3.12 ± 0.27*	2.57 ± 0.21#	2.48 ± 0.17#
LVEDd (mm)	3.49 ± 0.22	4.28 ± 0.34*	3.87 ± 0.31#	3.92 ± 0.29#
IVSd (mm)	0.68 ± 0.03	0.91 ± 0.05*	0.74 ± 0.04#	0.77 ± 0.04#
IVSs (mm)	1.08 ± 0.04	1.41 ± 0.07*	1.22 ± 0.06#	1.24 ± 0.06#
LVPWd (mm)	0.61 ± 0.03	0.86 ± 0.05*	0.72 ± 0.04#	0.75 ± 0.04#
LVPWs (mm)	1.04 ± 0.04	1.46 ± 0.08*	1.20 ± 0.09#	1.22 ± 0.10#
FS (%)	42.01 ± 2.35	26.42 ± 2.58*	33.75 ± 2.32#	35.25 ± 2.27#
ESP (mmHg)	102.4 ± 4.26	141.1 ± 6.31*	143.9 ± 6.53*	140.3 ± 6.02*
EDP (mmHg)	11.34 ± 2.12	21.25 ± 2.34*	16.01 ± 2.23#	16.78 ± 2.25#
dP/dtmax (mmHg/s)	8951 ± 473	5608 ± 412*	6487 ± 398#	6323 ± 376#
dP/dt min (mmHg/s)	−8564 ± 322	−5981 ± 402*	−6893 ± 369#	−6641 ± 345#

HR, heart rate; LVESd, LV end-systolic diameter; LVEDd, LV end-diastolic diameter; IVSd, interventricular septal thickness at end-diastole; IVSs, interventricular septal thickness at end-systole; LVPWd, LV posterior wall thickness at end-diastole; LVPWs, LV posterior wall thickness at end-systole; FS, fractional shortening; ESP, end-systolic pressure; EDP, end-diastolic pressure; dp/dtmax, maximal rate of pressure development; dp/dtmin, maximal rate of pressure decay.

* P < 0.05 vs sham group.

\# P < 0.05 vs TAC group.

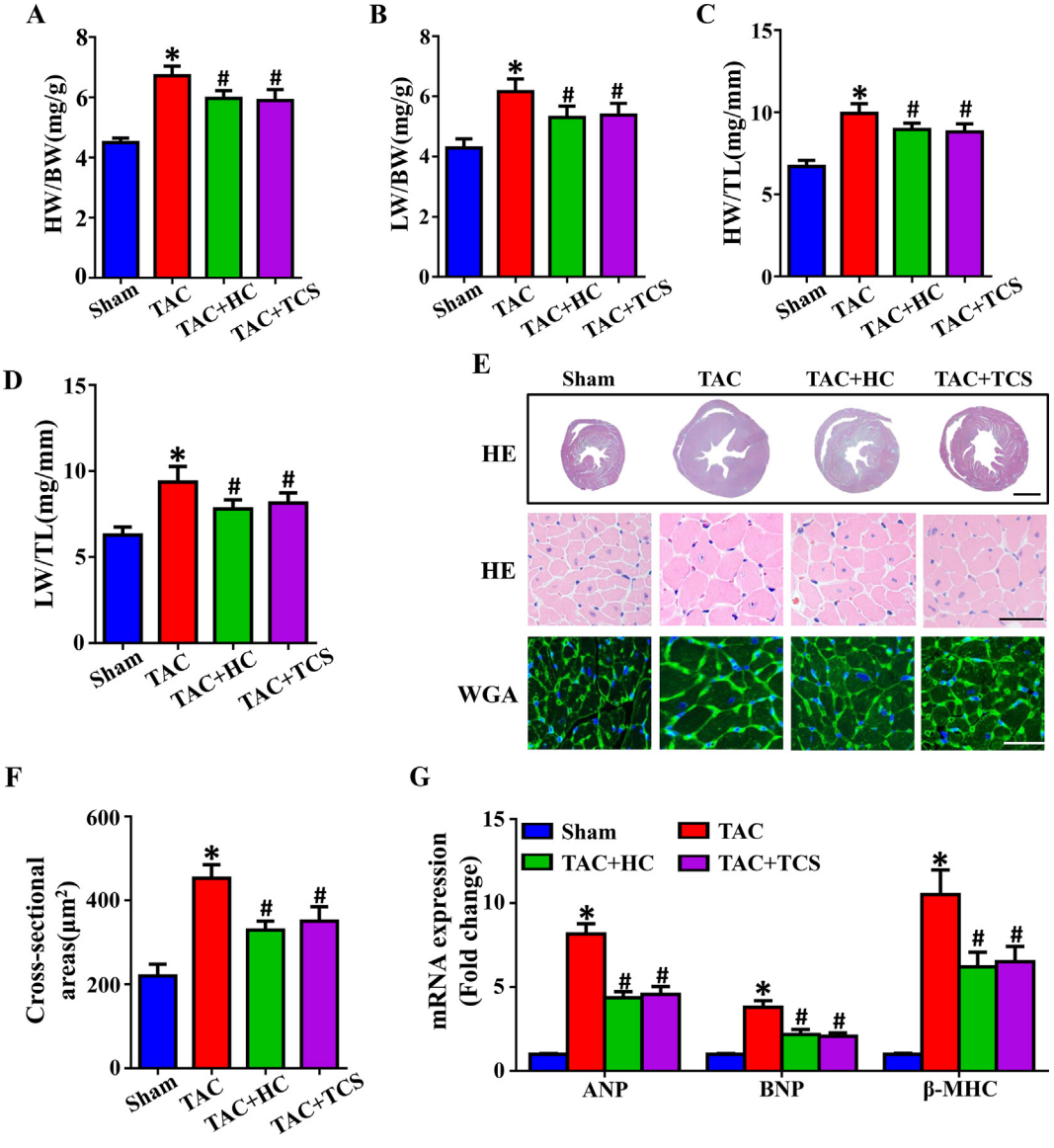

Fig. 2. TRPA1 inhibition ameliorates pressure overload-induced cardiac hypertrophy. (A-D) The heart weight to body weight (HW/BW) (A), lung weight to body weight (LW/BW) (B), heart weight to tibia length (HW/TL) (C), and lung weight to tibia length (LW/TL) (D) ratios were determined in the indicated groups 4 weeks after TAC surgery (n = 10). (E) Histological analysis of heart slices was performed by HE and fluorescein isothiocyanate-labeled wheat germ agglutinin (WGA) staining to assess the cardiomyocyte cross-sectional areas 4 weeks after TAC surgery (n = 7; scale bar, 1000 μm for the upper set of panels and 50 μm for the lower panels). (F) Statistical results of the cardiomyocyte cross-sectional areas in different groups. (G) The relative mRNA levels of atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and β-myosin heavy chain (β-MHC) in the left ventricles of mice from the indicated groups (n = 8). *P < 0.05 vs sham group; #P < 0.05 vs TAC group.

3. Results

3.1. TRPA1 expression is increased in hypertrophic hearts

To investigate the potential role of TRPA1 in pathological cardiac hypertrophy development, we first explored TRPA1 expression in failing human hearts and hypertrophic mouse hearts. The results indicated that TRPA1 expression was upregulated in human hearts with DCM (Fig. 1A). At 4 weeks after TAC surgery, the TRPA1 protein levels were significantly increased in mouse hearts in the experimental hypertrophic model compared with those in the sham-operated control model (Fig. 1B). Collectively, these data suggest that TRPA1 may be implicated in cardiac hypertrophy.

3.2. TRPA1 inhibition improves impaired cardiac function after TAC

To further investigate the protective effect of HC and TCS against TAC-induced cardiac dysfunction, echocardiography and hemodynamic analysis were used to assess changes in cardiac function. Four weeks after TAC, the animals exhibited significantly increased LVEDd, LVESd, IVSd, IVSs, LVPWs and LVPWd and decreased FS. However, HC and TCS treatment markedly attenuated the cardiac dilation and LV dysfunction (Table 2). The hemodynamic results showed that HC and TCS treatment improved cardiac contractility and diastolic function after TAC surgery (Table 2). No significant difference in cardiac function was observed between the HC and TCS groups. These data indicate that TRPA1 inhibition blocks the pathological cardiac dysfunction induced by chronic pressure overload.

3.3. TRPA1 inhibition attenuates cardiac hypertrophy in vivo

Next, we determined whether HC and TCS administration attenuates cardiac hypertrophy and HF. As shown in Fig. 2, substantial cardiac hypertrophy was observed in the mice subjected to TAC surgery compared with the sham-treated mice, as evidenced by the increased HW/BW and HW/TL ratios and CSA at the end of 4 weeks. Interestingly, compared with the TAC-treated mouse hearts, the HC- and TCS-treated mouse hearts exhibited markedly attenuated cardiac hypertrophy (Fig. 2A to F). Similarly, after TAC surgery, the mRNA expression levels of several hypertrophic markers, including atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and β-myosin heavy chain (β-MHC), were also significantly reduced in the HC and TCS-treated mice compared with the TAC-treated mice (Fig. 2G). No significant difference was observed between the HC and TCS groups. Together, these results indicate that TRPA1 inhibition prevents pathological cardiac hypertrophy.

3.4. TRPA1 inhibition ameliorates pressure overload-induced cardiac fibrosis

We performed PSR staining on cardiac tissue sections to evaluate fibrosis in mice from each group. Dramatic interstitial fibrosis was observed in the mice subjected to TAC surgery, but HC and TCS administration markedly attenuated this interstitial fibrosis (Fig. 3A and B). As shown in Fig. 3C, the mRNA levels of the fibrotic markers connective tissue growth factor (CTGF), collagen I and collagen III were markedly decreased in the hearts of the HC and TCS-treated mice compared with the hearts of the TAC-treated mice. No significant difference was observed between the HC and TCS groups. These data indicate that TRPA1

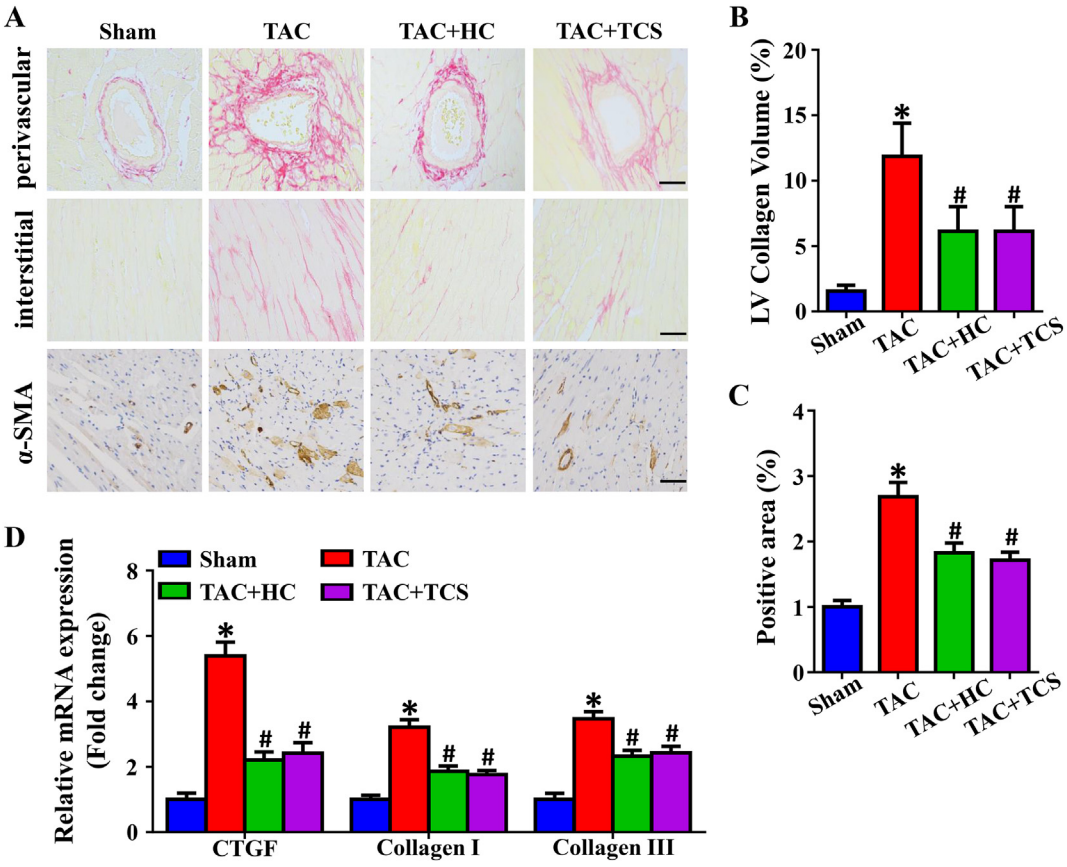

Fig. 3. TRPA1 inhibition attenuates fibrosis in pressure-overloaded hearts. (A) Histological analysis of heart slices by PSR staining and α-smooth muscle actin (α-SMA) immunostaining to assess cardiac fibrosis (n = 7; scale bar, 25 μm for the upper set of panels and 50 μm for the lower panels). (B) Statistical results for the fibrotic areas in the indicated groups (n = 7). (C) Statistical results for α-SMA immunostaining in the indicated groups (n = 7). (D) The relative connective tissue growth factor (CTGF), collagen I and collagen III mRNA levels in the left ventricles of mice from the indicated groups (n = 8). *P < 0.05 vs sham group; #P < 0.05 vs TAC group.

inhibition blocks the pathological cardiac fibrosis induced by chronic pressure overload.

3.5. TRPA1 regulates cardiac hypertrophy via Ca^{2+}/CaMKII/calcineurin signaling

Recent evidence has strongly suggested that Ca^{2+}-dependent signaling pathways, such as CaMKII and calcineurin, are critical regulators of pathological hypertrophy in the heart [14, 15]. Thus, we investigated whether TRPA1 regulation in pressure overload-induced cardiac hypertrophy is associated with Ca^{2+}-dependent signaling pathways. The results revealed that the pressure overload significantly increased calcineurin activity and protein expression in the myocardial tissue, while HC and TCS treatment significantly attenuated calcineurin activity and protein expression (Fig. 4A–B). Similarly, HC and TCS treatment significantly reduced pressure overload-induced CaMKII autophosphorylation (Fig. 4C). These data suggest that Ca^{2+}/CaMKII and calcineurin signaling activation may be essential for TRPA1-mediated cardiac hypertrophy.

3.6. TRPA1 inhibition attenuated macrophage infiltration in cardiac tissues

Many studies have provided strong evidence that inflammatory cells are involved in the progression of pathological hypertrophy. Specifically, recent data have revealed that T cells and macrophages play a fundamental role in cardiac hypertrophy and fibrosis [16–18]. Consistent with previous reports, T cell accumulation was markedly induced by pressure overload in the heart after TAC surgery (Fig. 5A). Interestingly, flow cytometry revealed that HC and TCS treatment did not influence T cell infiltration (Fig. 5C). Therefore, we next examined whether TRPA1 inhibition regulated macrophage recruitment. As shown in Fig. 5A, pressure overload significantly increased macrophage infiltration into the hearts of the TAC-treated mice; however, relatively reduced macrophage infiltration was found in the hearts of the HC and TCS-treated mice (Fig. 5B). These results indicated that TRPA1 might regulate cardiac fibrosis through impacting macrophages.

3.7. TRPA1 regulates cardiac fibrosis via M2 macrophages polarization

Considering that cardiac macrophages polarization plays a critical role in cardiac fibrosis [19, 20], we characterized the effect of TRPA1 inhibition on macrophages polarization. The immunofluorescence results revealed that pressure overload significantly increased the expression of CD206 (a marker of M2 macrophages) in hearts, whereas this effect was markedly attenuated by HC and TCS administration (Fig. 6A). Flow cytometry revealed that HC and TCS administration reduced pressure overload-induced M2 macrophages ($CD45^+F4/80^+CD206^+$cells) accumulation (Fig. 6B). To determine the role of TRPA1 inhibition in regulating macrophage differentiation, we cultured BMDMs with Ang II stimulation and detected the expression of M2 cytokines by RT-PCR. The results showed that Ang II treatment significantly increased the levels of M2 cytokines (IL-4, IL-10 and TGF-β) and that TRPA1 inhibition markedly attenuated these levels (Fig. 6C). Therefore, TRPA1 is responsible for driving macrophages toward the M2 phenotype after pressure overload in hearts.

4. Discussion

In this study, we clearly identified a new function and related potential mechanism for TRPA1 in pressure overload-induced cardiac

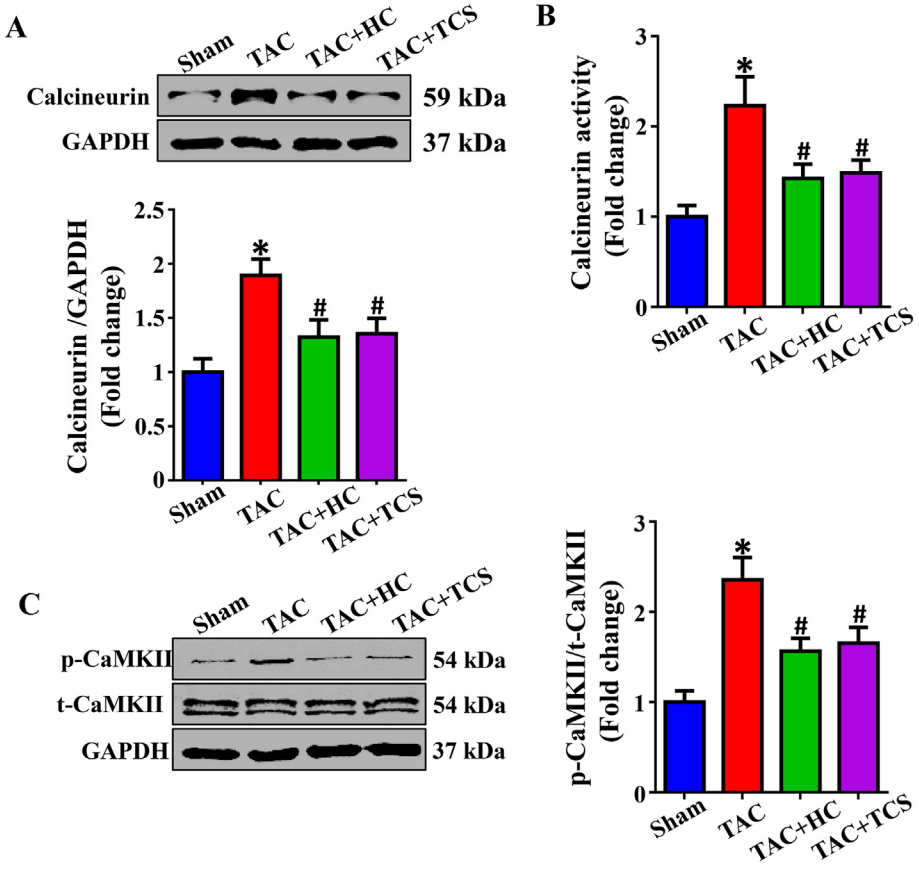

Fig. 4. Ca^{2+}/CaMKII/calcineurin signaling is involved in TRPA1-regulated cardiac hypertrophy. (A) Western blots showing calcineurin expression in mice after TAC surgery (n = 8). (B) Measurement of calcineurin activity in the 4 groups. (C) Western blots showing the phosphorylation and total protein levels of CaMKII in mice after TAC surgery (n = 8). *P < 0.05 vs sham group; #P < 0.05 vs TAC group.

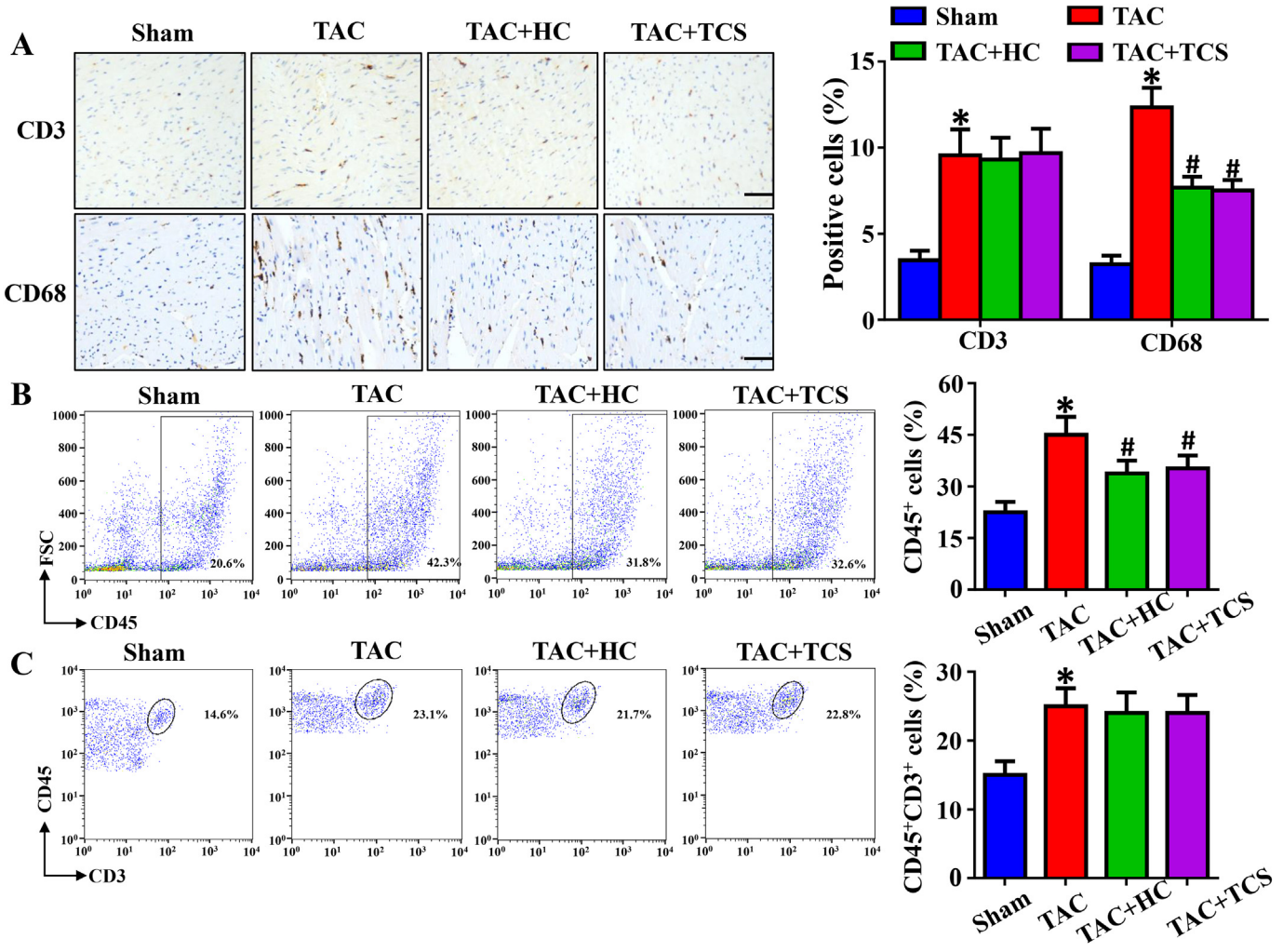

Fig. 5. TRPA1 inhibition prevents pressure overload-induced macrophage infiltration in cardiac tissues. (A) Immunohistochemical analysis of CD3 and CD68 in heart sections (n = 7, scale bar, 50 μm). (B–C) Flow cytometry analysis of $CD45^+$ cells and T cells ($CD45^+CD3^+$) in mice at 4 weeks after TAC surgery (n = 4). *P < 0.05 vs sham group; #P < 0.05 vs TAC group.

hypertrophy and fibrosis. First, our study demonstrated that TRPA1 expression was noticeably upregulated in human pathological hearts and mouse hypertrophic hearts, suggesting that TRPA1 might be implicated in the processes of cardiac hypertrophy and failure. Moreover, we provided evidence that TRPA1 inhibition ameliorated cardiac hypertrophy development and improved cardiac performance, as well as cardiac fibrosis, in mice subjected to pressure overload. Importantly, the cardioprotective effects of TRPA1 inhibition were associated with suppression of M2 macrophage polarization, which resulted in decreased hypertrophy and cardiac remodeling and ultimately improved cardiac function. Furthermore, we noticed that TRPA1 inhibition induced the attenuation of Ca^{2+}-dependent signaling pathways, including the CaMKII and calcineurin signaling pathways, leading to improved cardiac function.

TRPA1, which is characterized as a calcium-permeable cation channel, was originally thought to be predominately expressed in primary nociceptive sensory neurons and to mediate pain and inflammation [10, 21]. Evidence has strongly suggested that TRPA1 is expressed in a variety of tissues and cell types, including those of the cardiovascular system [22–25]. An elegant study by Andrei et al. showed that TRPA1 was expressed in cardiomyocytes and that TRPA1 activation increased cardiomyocyte contractile function via a CaMKII-dependent pathway [26, 27]. Moreover, Oguri et al. demonstrated that TRPA1 expression in human cardiac fibroblasts and HC administration suppressed methylglyoxal (MG)-induced cardiac fibroblast proliferation and α-SMA expression [28]. In the present study, pathological hypertrophic responses and dramatic fibrosis were observed in mice subjected to TAC surgery. Furthermore, our study demonstrated that TRPA1 inhibition markedly blunted pressure overload-induced cardiac hypertrophy and fibrosis. This study indicates that TRPA1 represents an essential mediator of the pathological cardiac hypertrophy and fibrosis induced by pressure overload.

Ca^{2+}-regulated signaling pathways have been shown to contribute to pathological cardiac hypertrophy and fetal gene activation. The Ca^{2+}/calmodulin-dependent signaling pathways, including CaMKII and calcineurin, are considered central pro-hypertrophic signaling effectors in the myocardium [29, 30]. In the cytoplasm, activated calcineurin directly binds and dephosphorylates nuclear factor of activated T cells (NFAT), permitting its translocation into the nucleus, where it participates in hypertrophic gene expression [31, 32]. Ca^{2+}/CaMKII can cross-talk with the calcineurin signaling pathway to regulate hypertrophic processes [33]. CaMKII is known to activate myocyte enhancer factor 2 (MEF2) on class II histone deacetylases (HDACs) via phosphorylation, thereby allowing MEF2 to induce hypertrophic gene expression [29, 31]. A previous report indicated that TRPC channels act as critical regulators to control pathological cardiac hypertrophy, which depends on CaMKII and calcineurin pathway integrity [34–36]. In this study, we hypothesized that TRPA1 experiences mechanical stress and activates pro-hypertrophic signaling responses. We found that CaMKII and calcineurin activity was upregulated in TAC-induced hypertrophic hearts. TRPA1 inhibition suppressed Ca^{2+}-activated CaMKII and calcineurin. This evidence supports a key role of TRPA1 in

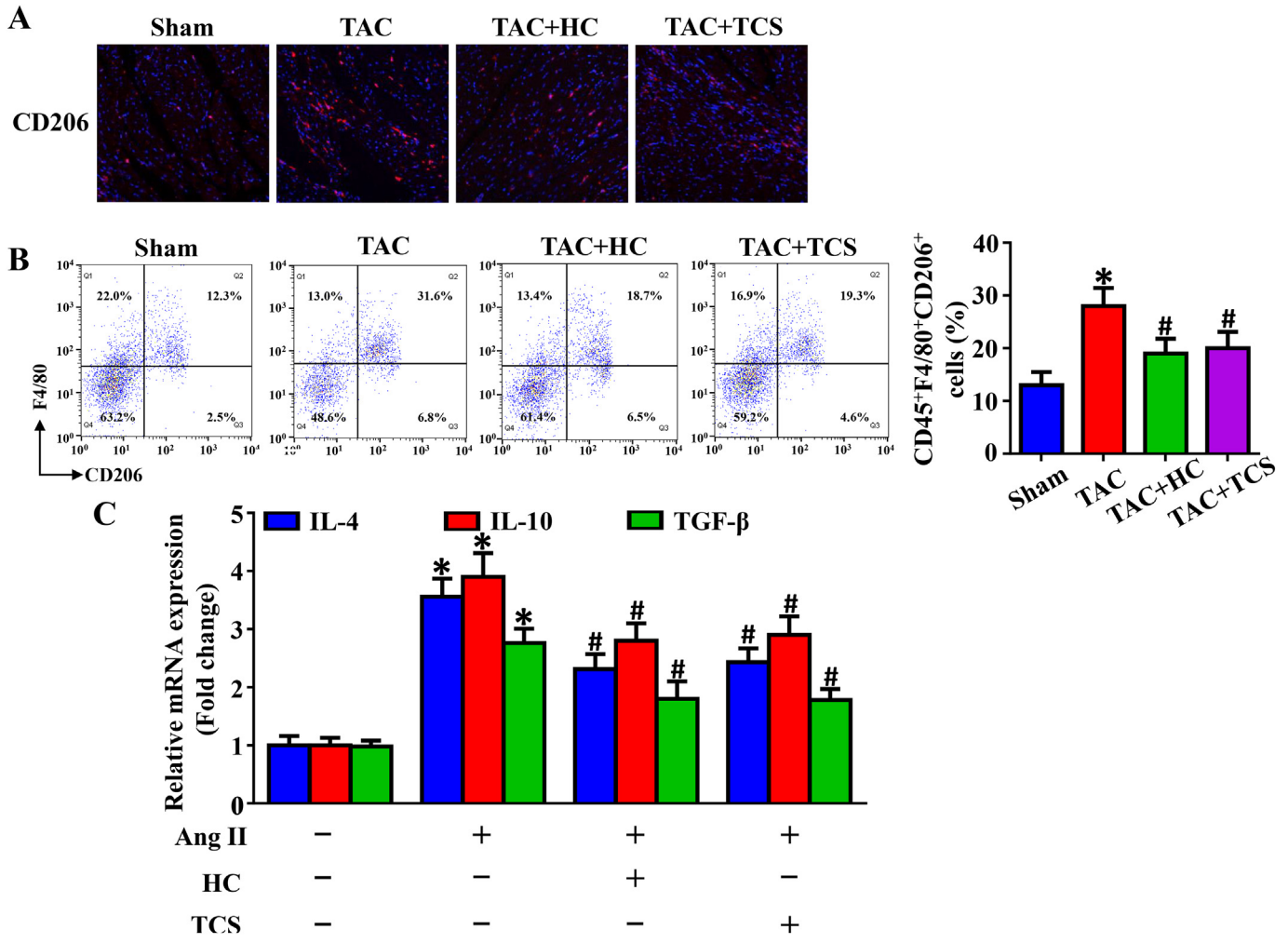

Fig. 6. TRPA1 inhibition reduces M2 macrophage polarization. (A) Immunofluorescence analysis of M2 macrophages (anti-CD206) in mice at 4 weeks after TAC surgery (n = 7). (B) Flow cytometry analysis of M2 macrophage ($CD45^+F4/80^+CD206^+$) expression in mice at 4 weeks after TAC surgery (n = 4). (C) RT-PCR analysis of interleukin-4 (IL-4), IL-10 and transforming growth factor-β (TGF-β) expression in bone marrow–derived macrophages (BMDMs) cultured with and without Ang II (n = 8). *P < 0.05 vs sham group; #P < 0.05 vs TAC group.

mediating Ca^{2+} signaling pathways during cardiac hypertrophy development.

Inflammatory responses are important for the initiation and progression of pathological cardiac hypertrophy. Cardiac tissue consists of several types of inflammatory cells, including primarily T lymphocytes and macrophages. T cells and macrophages are recruited to the heart during cardiac disease pathophysiology and have emerged as important cellular components for targeting cardiac hypertrophy and HF [37]. Nevers et al. demonstrated that T cells are activated and infiltrate the heart in experimental mouse models of pressure overload [38]. Similarly, cardiac macrophages are specifically increased in response to pressure overload after TAC [39, 40]. Consistent with these studies, substantial T cells and macrophages infiltration were observed in mice subjected to TAC surgery. TRPA1 inhibition, however, significantly decreased macrophages infiltration but did not influence T cells levels.

Recent evidence has demonstrated that macrophages are heterogeneous immune cell populations and may be master regulators of inflammation and fibrosis. Modulating the diversity of macrophage phenotypes and functionality is an enticing strategy and could be an important therapeutic approach [40]. M1 macrophages are associated with inflammation and tissue destruction, whereas M2 macrophages are associated with tissue repair and fibrotic properties [41]. Several studies have demonstrated that activation of M2 macrophages is closely associated with enhanced fibrosis, whereas blocking M2 macrophage activation is associated with decreased cardiac fibrosis [19, 20]. In our study, we analyzed the macrophage phenotype in mice. The results revealed that M2 macrophage expression was increased in mice subjected to TAC surgery, whereas this effect was markedly attenuated by HC and TCS treatment. Emerging evidence has demonstrated that M2 macrophages secrete cytokines that promote fibrotic remodeling, including IL-4, IL-10 and TGF-β [19, 42]. Consistent with these studies, Ang II treatment significantly increased the levels of M2 cytokines, and TRPA1 inhibition markedly attenuated these levels. These results suggested a novel function of TRPA1 in regulating M2 macrophage polarization during pressure overload-induced cardiac fibrosis.

This study is subject to the following limitations. First, although HC and TCS are two of the most widely used TRPA1 blockers and nonspecific effects of these compounds have not been reported, TRPA1 KO mice may more precisely demonstrate the important function of TRPA1 in cardiac hypertrophy and fibrosis. Second, an increasing amount of evidence has demonstrated that the activation of inflammatory cells, such as macrophages, T cells, neutrophils and mast cells, plays a fundamental role in cardiac hypertrophy and fibrosis [40, 43, 44]. However, since T cells and macrophages are the most intensely investigated cells in cardiac tissue, we evaluated the roles of only T cells and macrophages in cardiac hypertrophy and fibrosis. Third, previous studies have thoroughly demonstrated the role of Ca^{2+}-dependent signaling and its downstream signaling pathways in hypertrophic responses both in vivo and in vitro. Thus, we did not repeat this research.

In conclusion, we have provided novel insights into the roles of TRPA1 in regulating compensatory myocardial hypertrophy and fibrosis. Specifically, TRPA1 regulates pro-hypertrophic CaMKII and calcineurin signaling pathway activation in cardiomyocytes. Our study identified that TRPA1 inhibition protects against cardiac fibrosis by modulating M2 macrophage differentiation. Our data indicate that TRPA1 may be an attractive target for treating pathological cardiac hypertrophy and fibrosis.

Conflicts of interest

No conflicts of interests are declared by the authors.

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Author contributions

Zhen Wang, Yao Xu, Menglong Wang, Jianfang Liu and Jing Ye performed the study, analyzed the data, and wrote the manuscript. Zhen Wang, Yao Xu, Jing Ye, Menglong Wang, Huimin Jiang and Di Ye contributed to the acquisition of data and to manuscript preparation and revision. Zhen Wang, Yao Xu, Menglong Wang and Jun Wan conceived of the hypothesis and participated in the experimental design, data interpretation, and manuscript preparation and revision. All authors approved the final version of the manuscript.

References

[1] Lyon RC, Zanella F, Omens JH, Sheikh F. Mechanotransduction in cardiac hypertrophy and failure. Circ Res 2015;116(8):1462–76.
[2] Hou J, Kang YJ. Regression of pathological cardiac hypertrophy: signaling pathways and therapeutic targets. Pharmacol Ther 2012;135(3):337–54.
[3] Earley S, Brayden JE. Transient receptor potential channels in the vasculature. Physiol Rev 2015;95(2):645–90.
[4] Watanabe H, Murakami M, Ohba T, Takahashi Y, Ito H. TRP channel and cardiovascular disease. Pharmacol Ther 2008;118(3):337–51.
[5] Watanabe H, Iino K, Ohba T, Ito H. Possible involvement of TRP channels in cardiac hypertrophy and arrhythmia. Curr Top Med Chem 2013;13(3):283–94.
[6] Eder P, Molkentin JD. TRPC channels as effectors of cardiac hypertrophy. Circ Res 2011;108(2):265–72.
[7] Kuwahara K, Wang Y, McAnally J, Richardson JA, Bassel-Duby R, Hill JA, et al. TRPC6 fulfills a calcineurin signaling circuit during pathologic cardiac remodeling. J Clin Invest 2006;116(12):3114–26.
[8] Seth M, Zhang ZS, Mao L, Graham V, Burch J, Stiber J, et al. TRPC1 channels are critical for hypertrophic signaling in the heart. Circ Res 2009;105(10):1023–30.
[9] Numata T, Takahashi K, Inoue R. "TRP inflammation" relationship in cardiovascular system. Semin Immunopathol 2016;38(3):339–56.
[10] Nilius B, Szallasi A. Transient receptor potential channels as drug targets: from the science of basic research to the art of medicine. Pharmacol Rev 2014;66(3):676–814.
[11] Okada Y, Shirai K, Reinach PS, Kitano-Izutani A, Miyajima M, Flanders KC, et al. TRPA1 is required for TGF-beta signaling and its loss blocks inflammatory fibrosis in mouse corneal stroma. Lab Invest 2014;94(9):1030–41.
[12] Bodkin JV, Brain SD. Transient receptor potential ankyrin 1: emerging pharmacology and indications for cardiovascular biology. Acta Physiol (Oxf) 2011;203(1):87–98.
[13] Pozsgai G, Bodkin JV, Graepel R, Bevan S, Andersson DA, Brain SD. Evidence for the pathophysiological relevance of TRPA1 receptors in the cardiovascular system in vivo. Cardiovasc Res 2010;87(4):760–8.
[14] Anderson ME, Brown JH, Bers DM. CaMKII in myocardial hypertrophy and heart failure. J Mol Cell Cardiol 2011;51(4):468–73.
[15] Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, et al. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell 1998;93(2):215–28.
[16] Li C, Sun XN, Zeng MR, Zheng XJ, Zhang YY, Wan Q, et al. Mineralocorticoid receptor deficiency in T cells attenuates pressure overload-induced cardiac hypertrophy and dysfunction through modulating T-cell activation. Hypertension 2017;70(1):137–47.
[17] Wang H, Kwak D, Fassett J, Hou L, Xu X, Burbach BJ, et al. CD28/B7 deficiency attenuates systolic overload-induced congestive heart failure, myocardial and pulmonary inflammation, and activated T cell accumulation in the heart and lungs. Hypertension 2016;68(3):688–96.
[18] Schulze PC, Lee RT. Macrophage-mediated cardiac fibrosis. Circ Res 2004;95(6):552–3.
[19] Yang M, Zheng J, Miao Y, Wang Y, Cui W, Guo J, et al. Serum-glucocorticoid regulated kinase 1 regulates alternatively activated macrophage polarization contributing to angiotensin II-induced inflammation and cardiac fibrosis. Arterioscler Thromb Vasc Biol 2012;32(7):1675–86.
[20] Li Y, Zhang C, Wu Y, Han Y, Cui W, Jia L, et al. Interleukin-12p35 deletion promotes CD4 T-cell-dependent macrophage differentiation and enhances angiotensin II-induced cardiac fibrosis. Arterioscler Thromb Vasc Biol 2012;32(7):1662–74.
[21] Bautista DM, Pellegrino M, Tsunozaki M. TRPA1: a gatekeeper for inflammation. Annu Rev Physiol 2013;75:181–200.
[22] Yue Z, Xie J, Yu AS, Stock J, Du J, Yue L. Role of TRP channels in the cardiovascular system. Am J Physiol Heart Circ Physiol 2015;308(3):H157–82.
[23] Earley S. TRPA1 channels in the vasculature. Br J Pharmacol 2012;167(1):13–22.
[24] Wang Z, Wang M, Liu J, Ye J, Jiang H, Xu Y, et al. Inhibition of TRPA1 attenuates doxorubicin-induced acute cardiotoxicity by suppressing oxidative stress, the inflammatory response, and endoplasmic reticulum stress. Oxid Med Cell Longev 2018;2018:5179468.
[25] Viana F. TRPA1 channels: molecular sentinels of cellular stress and tissue damage. J Physiol 2016;594(15):4151–69.
[26] Andrei SR, Sinharoy P, Bratz IN, Damron DS. TRPA1 is functionally co-expressed with TRPV1 in cardiac muscle: co-localization at z-discs, costameres and intercalated discs. Channels (Austin) 2016;10(5):395–409.
[27] Andrei SR, Ghosh M, Sinharoy P, Dey S, Bratz IN, Damron DS. TRPA1 ion channel stimulation enhances cardiomyocyte contractile function via a CaMKII-dependent pathway. Channels (Austin) 2017:1–17.
[28] Oguri G, Nakajima T, Yamamoto Y, Takano N, Tanaka T, Kikuchi H, et al. Effects of methylglyoxal on human cardiac fibroblast: roles of transient receptor potential ankyrin 1 (TRPA1) channels. Am J Physiol Heart Circ Physiol 2014;307(9):H1339–52.
[29] Kreusser MM, Lehmann LH, Keranov S, Hoting MO, Oehl U, Kohlhaas M, et al. Cardiac CaM kinase II genes delta and gamma contribute to adverse remodeling but redundantly inhibit calcineurin-induced myocardial hypertrophy. Circulation 2014;130(15):1262–73.
[30] Chung E, Yeung F, Leinwand LA. Calcineurin activity is required for cardiac remodelling in pregnancy. Cardiovasc Res 2013;100(3):402–10.
[31] Wilkins BJ, Molkentin JD. Calcium-calcineurin signaling in the regulation of cardiac hypertrophy. Biochem Biophys Res Commun 2004;322(4):1178–91.
[32] Wilkins BJ, Dai YS, Bueno OF, Parsons SA, Xu J, Plank DM, et al. Calcineurin/NFAT coupling participates in pathological, but not physiological, cardiac hypertrophy. Circ Res 2004;94(1):110–8.
[33] MacDonnell SM, Weisser-Thomas J, Kubo H, Hanscome M, Liu Q, Jaleel N, et al. CaMKII negatively regulates calcineurin-NFAT signaling in cardiac myocytes. Circ Res 2009;105(4):316–25.
[34] Morales S, Diez A, Puyet A, Camello PJ, Camello-Almaraz C, Bautista JM, et al. Calcium controls smooth muscle TRPC gene transcription via the CaMK/calcineurin-dependent pathways. Am J Physiol Cell Physiol 2007;292(1):C553–63.
[35] Harada M, Luo X, Murohara T, Yang B, Dobrev D, Nattel S. MicroRNA regulation and cardiac calcium signaling: role in cardiac disease and therapeutic potential. Circ Res 2014;114(4):689–705.
[36] Wu X, Eder P, Chang B, Molkentin JD. TRPC channels are necessary mediators of pathologic cardiac hypertrophy. Proc Natl Acad Sci U S A 2010;107(15):7000–5.
[37] Hofmann U, Frantz S. Role of lymphocytes in myocardial injury, healing, and remodeling after myocardial infarction. Circ Res 2015;116(2):354–67.
[38] Nevers T, Salvador AM, Grodecki-Pena A, Knapp A, Velazquez F, Aronovitz M, et al. Left ventricular T-cell recruitment contributes to the pathogenesis of heart failure. Circ Heart Fail 2015;8(4):776–87.
[39] Kamo T, Akazawa H, Komuro I. Cardiac nonmyocytes in the hub of cardiac hypertrophy. Circ Res 2015;117(1):89–98.
[40] Frieler RA, Mortensen RM. Immune cell and other noncardiomyocyte regulation of cardiac hypertrophy and remodeling. Circulation 2015;131(11):1019–30.
[41] Liao X, Sharma N, Kapadia F, Zhou G, Lu Y, Hong H, et al. Kruppel-like factor 4 regulates macrophage polarization. J Clin Invest 2011;121(7):2736–49.
[42] Wang Y, Harris DC. Macrophages in renal disease. J Am Soc Nephrol 2011;22(1):21–7.
[43] Wu Y, Li Y, Zhang C, A X, Wang Y, Cui W, et al. S100a8/a9 released by CD11b+Gr1+ neutrophils activates cardiac fibroblasts to initiate angiotensin II-induced cardiac inflammation and injury. Hypertension 2014;63(6):1241–50.
[44] Zhang W, Chancey AL, Tzeng HP, Zhou Z, Lavine KJ, Gao F, et al. The development of myocardial fibrosis in transgenic mice with targeted overexpression of tumor necrosis factor requires mast cell-fibroblast interactions. Circulation 2011;124(19):2106–16.