



Research article

Tumstatin (69–88) alleviates heart failure via attenuating oxidative stress in rats with myocardial infarction

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ABSTRACT

Background: This study aimed to elucidate the effects of tumstatin (69–88) on heart failure and the underlying mechanism.**Materials and methods:** Myocardial infarction (MI) was induced by ligating the left coronary artery in rats to trigger heart failure.**Results:** Tumstatin (69–88) can reduce cardiac insufficiency in rats with heart failure. The increased cardiac fibrosis in MI rat was attenuated by tumstatin (69–88). Increase of cardiac atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) in rats with myocardial infarction, and Ang II-treated NRCMs or H9C2 cells was inhibited by tumstatin (69–88). In the heart of MI rats, and Ang II-treated NRCMs or H9C2 cells, the superoxide anions and NADPH oxidase (Nox) activity rose and the superoxide dismutase (SOD) activity was reduced, which was inhibited by tumstatin (69–88). Diethyldithiocarbamate, an SOD inhibitor, increased the ANP and BNP in NRCMs or H9C2 cells. Tumstatin (69–88) inhibited the Ang II-induced raises of ANP and BNP in NRCMs or H9C2 cells, which was reversed by DETC.**Conclusions:** These results indicate that tumstatin (69–88) alleviates cardiac dysfunction of heart failure. Tumstatin (69–88) improves the hypertrophy of cardiomyocytes via attenuation of oxidative stress. Tumstatin (69–88) may be a potential drug for heart failure in the future.

1. Introduction

Cardiovascular diseases are still a main cause of death globally [1, 2, 3]. In cardiovascular diseases, the disordered structure or function of the heart can lead to heart failure [4]. Heart failure is still the most common cause of morbidity and mortality in the world, though achievements have been made in its diagnosis [5]. Heart failure is induced by left ventricular (LV) remodeling, which is characterized by myocardial hypertrophy and interstitial fibrosis, manifested by the increased expression of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and collagen I and III [6, 7, 8].

Tumstatin, the NC1 domain of alpha3 chain of type IV collagen, can inhibit growth of tumor [9]. Three active subfragments of tumstatin, tum-5 (54–132 amino acids), T3 peptide (69–88 amino acids) and T7 peptide (74–98 amino acids), can regulate anti-angiogenetic activity [10]. T3 peptide has been shown to inhibit hydrogen peroxide

(H₂O₂)-induced apoptosis of H9C2 cardiomyoblasts [11]. However, it is still unclear whether tumstatin (69–88) can alleviate heart failure.

Heart-related pathology causes an imbalance between antioxidants and oxidants [12]. This imbalance enhances oxidative stress, which participates in pathological cardiac remodeling and then induces heart failure [2, 13, 14]. A previous study found that tumstatin (69–88) inhibited oxygen and glucose deprivation/reoxygenation-induced H9C2 cardiomyoblast apoptosis by suppressing mitochondrial reactive oxygen species (ROS) production and dysfunction [15]. Nevertheless, it is unknown whether oxidative stress is involved in the effects of tumstatin (69–88) on the heart failure.

In summary, the purpose of this study is to clarify the protective effects of tumstatin (69–88) on myocardial infarction (MI)-induced heart failure in rats, and the involvement of oxidative stress in the alleviating influences of tumstatin (69–88) on hypertrophy of cardiomyocytes.

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2. Materials and methods

2.1. Animals

The experiment was carried out in Animal Core Facility of Nanjing Medical University. The Sprague-Dawley (SD) rats (male, 180–200 g; Vital River, Beijing, China) were housed in room temperature ($22 \pm 1^\circ\text{C}$) under a 12/12 h light/dark cycle. It was unrestricted to access to food and water was unrestricted. The procedures and ethics were approved by the Experimental Animal Care and Use Committee of Xuzhou Medical University (Xuzhou, China) in May 2018, and carried out following the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85–23, 1996).

2.2. Myocardial infarction (MI) model and treatment

MI model of the rats was induced via ligating the coronary artery as previously described [16]. After sodium pentobarbital anesthesia (50 mg/kg, i. p.), the rats (180–200g) were randomized to receive sham operation or the left coronary artery (LCA) ligation. Used left intercostal thoracotomy to expose the heart and the LCA was looped by a single nylon suture. Treated the sham rats in the same manner except that no ligation was performed. On the same day, tumstatin (69–88) (1.0 $\mu\text{g}/\text{kg}/\text{d}$, Phoenix Pharmaceuticals Inc., CA, USA) or the same volume of saline (Solvent; 200 μl) was administrated for 28 days (i. p.).

2.3. Echocardiography

After 28 days, transthoracic echocardiography (M-mode, long axis) was conducted on rats under 3.0% isoflurane anesthesia with an ultrasound system (VisualSonics, Canada) as previously reported [17]. The mean value of three consecutive cardiac cycles was measured. The ejection fraction (EF) and fractional shortening (FS) in left ventricular, LV volume in systole (LVVs) and in diastole (LVVd), Obtained LV internal diameter at end-systole (LVIDs) and at end-diastole (LVIDd) in rats.

2.4. Hemodynamic monitoring

After 3.0% isoflurane anesthesia, inserted a conductance 1.4F micromanometer catheter (Millar Instruments, TX, USA) into the rat LV through the carotid artery for hemodynamic monitoring. LV systolic pressure (LVSP), maximum value of the first differential of LV pressure ($\text{LV} + \text{dp}/\text{dt}_{\text{max}}$), and LV end-diastolic pressure (LVEDP) were measured with the PowerLab system (AD Instruments, Australia).

2.5. Sirius red staining

After hemodynamic monitoring, the rats were sacrificed with cervical dislocation after 3.0% isoflurane anesthesia. The heart was removed after PBS perfusion. The heart tissues were fixed with paraformaldehyde (4%) for 24h, and dehydrated by gradient alcohols, embedded in paraffin and made into 5- μm -thick. Employed sirius red staining to evaluate the fibrosis of LV. And chosen 3–5 random fields from each of 3 sections from each animal and observed using a light microscope (Carl Zeiss GmbH,

Germany). Used Image-Pro Plus software (Media Cybernetics, Inc., MD, USA) to analyze the images.

2.6. Detection of reactive oxygen species (ROS)

ROS level was detected by dihydroethidium staining (DHE; Beyotime Biotechnology, Shanghai, China). Subsequently, the intensity of fluorescence was detected using the Zeiss fluorescence inverted microscope (Carl Zeiss, Jena, Germany). The levels of ROS were measured by the Image J software (National Institutes of Health, Bethesda, Maryland, USA).

2.7. Quantitative real time-PCR (qRT-PCR)

Extracted the total RNA from LV or cells with TRIzol (Ambion, TX, USA). Extracted the cDNA from the RNA with reverse transcription using 10 μl random primers (Genscript, Nanjing China, Table 1) with the PrimeScript™ RT Master Mix (37 $^\circ\text{C}$, 15 min; 85 $^\circ\text{C}$, 5 s; Takara Biotechnology Co., Ltd., China). Stored the cDNA at -80°C before use. ANP, BNP, collagen I and III were examined using SYBR Green I fluorescence. Amplified all samples in triplicates for 40 cycles in 384-well plates (1 cycle of 95 $^\circ\text{C}$ for 10min; 40 cycles of 95 $^\circ\text{C}$ for 10 s, and 60 $^\circ\text{C}$ for 1min). The relative expressions of genes were calculated and Δ Cycle threshold (Δ CT) value was used as the relative amount of endogenous control. GAPDH was as a control to ANP, BNP, collagen I and collagen III. The primers were shown in Table 1.

2.8. Western blotting

Sonicated LV tissues or cell samples in RIPA lysis buffer and homogenized. The supernatant was obtained after centrifugation at 4 $^\circ\text{C}$ (12,000 g, 10 min) and debris removal. After electrophoresis, transferred the proteins to a nitrocellulose membrane, probed with primary antibodies of collagen I, collagen III and GAPDH (Abcam, MA, USA), and incubated with secondary antibodies (Abcam). The bands were visualized by chemiluminescence substrate (Beyotime). Normalized the target protein level to the GAPDH level.

2.9. Culture of neonatal rat cardiomyocytes

Primary neonatal rat cardiomyocytes (NRCMs) were obtained from 1- to 2-day-old Sprague-Dawley rats [18]. Removed and digested the heart in PBS containing collagenase type II (Worthington, NJ, USA) and pancreatin (Sigma, MO, USA). Deserted the atria and great vessels. Minced and digested the ventricles with collagenase type II and pancreatin. Collected and cultured Cells from digestion in DMEM (GIBCO, Invitrogen Inc.) for 2–4 h to decrease fibroblasts and increase cardiomyocytes. Cultured the cardiomyocytes at 37 $^\circ\text{C}$ with 5% CO_2 . The cells were received treatment when the cells were grown to 80–90% confluence. Firstly, divided and treated the primary cardiomyocytes with PBS, Ang II (10^{-6} M; Sigma), tumstatin (69–88) (10^{-6} M) and Ang II + tumstatin (69–88), respectively. Secondly, the primary cardiomyocytes were divided and treated with PBS, Ang II, Ang II + tumstatin (69–88), diethyldithiocarbamate (DETC; 10^{-6} M; Sigma) and DETC + Ang II +

Table 1. List of utilized primers for qRT-PCR.

Gene	Species	Forward primer	Reverse primer
Collagen I	Rat	TCAAGATGGTGGCCGTAC	CTGCGGATGTTCTCAATCTG
Collagen III	Rat	CGAGATTAAGCAAGAGGAA	GAGGCTTCTTACATACCAC
ANP	Rat	GAGCAAATCCCGTATACAGTGC	ATCTTCTACCGCATCTCCTCC
BNP	Rat	GCTGCTGGAGCTGATAAGAGAA	GTTCTTTGTAGGCGCTTGGTC
GAPDH	Rat	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA

ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

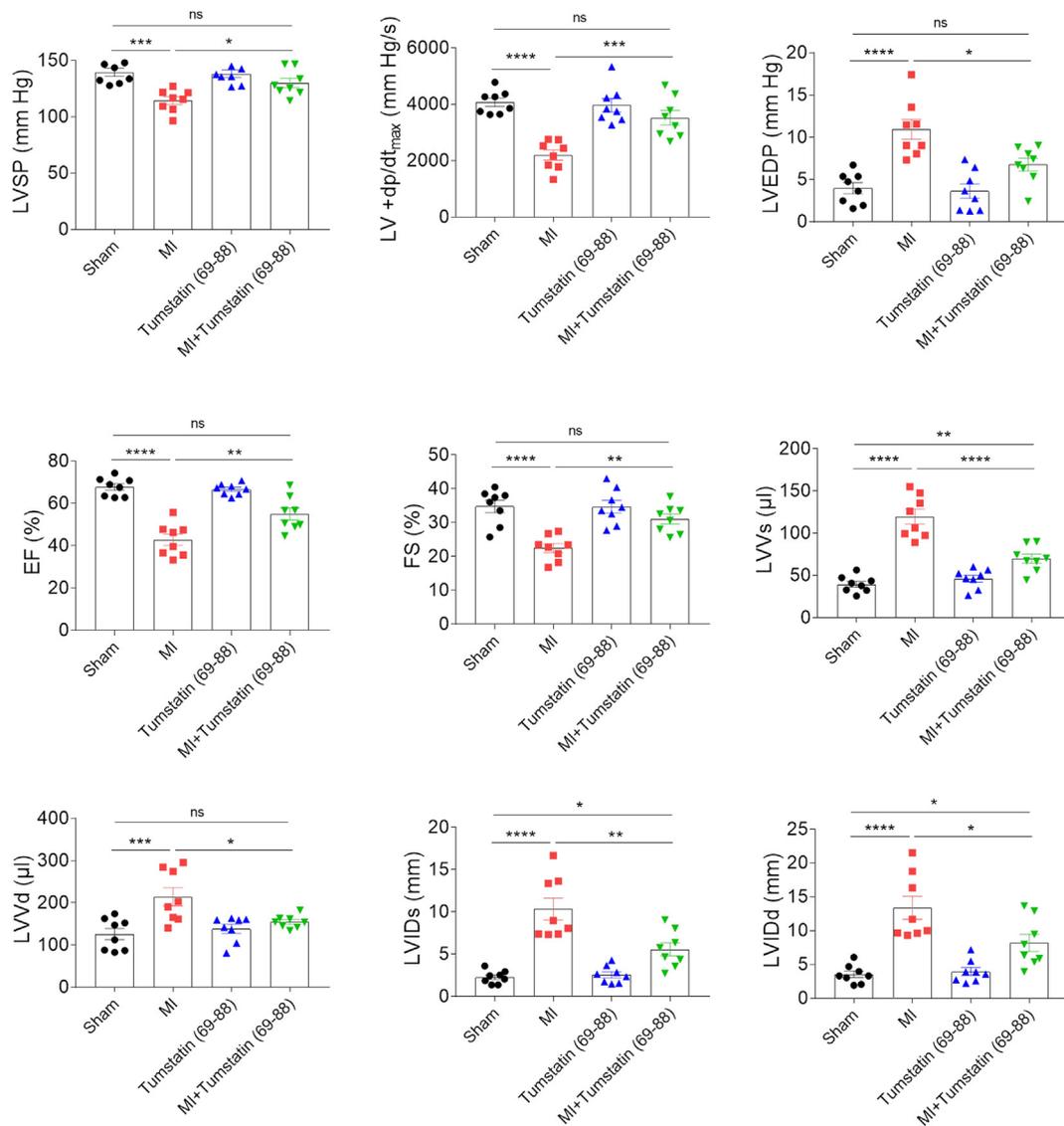


Figure 1. Tumstatin (69–88) attenuated cardiac dysfunction of myocardial infarction rats. Tumstatin (69–88) reversed the decreases of LVSP, LV + dp/dt_{max}, EF and FS, and the increases of LVEDP, LVVs, LVVd, LVIDs and LVIDd of myocardial infarction rats. n = 8 for each group. The results are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

tumstatin (69–88), respectively. Thirdly, divided and treated the primary cardiomyocytes with PBS, Ang II, Tiron (antioxidant; 10^{-5} M; Sigma) and Ang II + Tiron. All the reagents added into the medium at the same time for 24 h.

2.10. Culture of H9C2 cells

Cultured the rat myoblast cell line H9C2 in DMEM with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Seeded cells in 6-well plates (2×10^4 cells/well). The H9C2 cells were exposed to reagent treatment as NRCMs.

2.11. Measurement of superoxide anions

Measured superoxide anion levels in the heart and cultured cells with lucigenin-derived chemiluminescence. Detected the protein consistency with a BCA kit (Beyotime Biotechnology). Added dark adapted fluorescein (5 μM) to each sample to generate photon emission, and measured this with a microplate reader (BioTek) every minute for 10 min. The superoxide anions level was expressed as mean light units (MLU) per milligram of protein per minute.

2.12. Measurement of NADPH oxidase activity

Measured the NADPH oxidase (Nox) activity in the heart and cultured cells with enhanced fluorescein chemiluminescence. As a substrate, NADPH (100 μM; Sigma) was added into the media, and reacted with Nox to generate superoxide anions. Determined the light emission produced by the reaction of lucigenin (5 μM) and superoxide anions with a microplate reader (BioTek) once a minute for 10 min. The Nox activity level was presented as the MLU per milligram protein per minute.

2.13. Determination of superoxide dismutase activity

The heart and cultured cells samples were homogenized in lysis buffer (Thermo Fisher Scientific). The activity of superoxide dismutase (SOD) was determined according to the manufacturer's instructions (Beyotime Biotechnology) via a microplate reader (BioTek, VT, USA).

2.14. Statistical analyses

Data are presented as mean ± SEM. With GraphPad Prism 6.0 (GraphPad software Inc., CA, USA), the statistical significance between

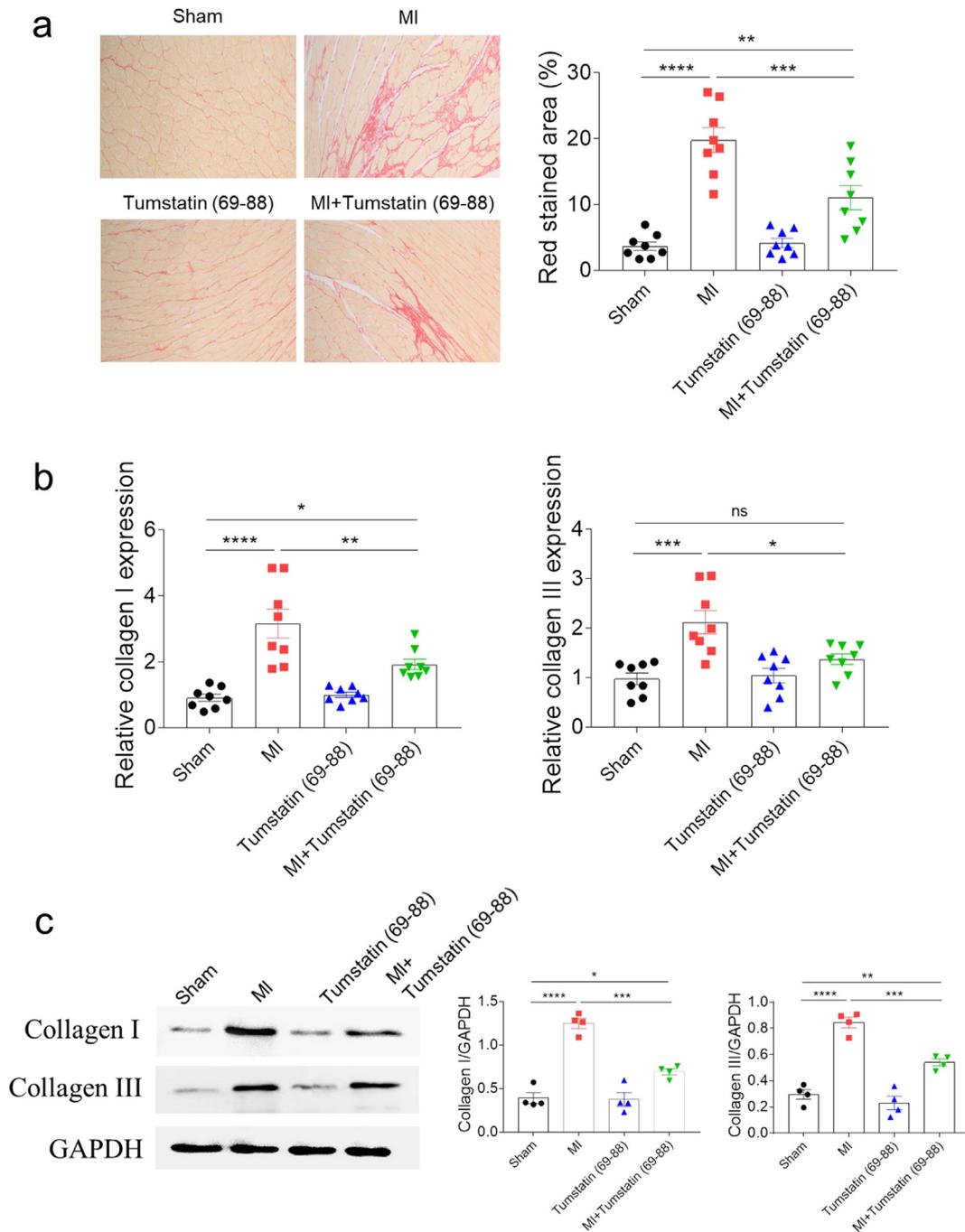


Figure 2. Tumstatin (69–88) alleviated cardiac fibrosis of myocardial infarction rats. a, Tumstatin (69–88) alleviated the increase of myocardial fibrosis in rats with myocardial infarction using sirius red staining. b, Tumstatin (69–88) inhibited the increases of collagen I and III mRNA in the heart of myocardial infarction rats. c, Tumstatin (69–88) inhibited the raises of collagen I and III protein in the heart of myocardial infarction rats. n = 8 for each group. The uncropped versions of western blots used for the analysis are presented in Supplementary Figure 1.

groups was evaluated by one-way analysis of variance (ANOVA) of Bonferroni post test. A two-tailed P-value <0.05 was defined as statistically significant.

3. Results

3.1. Tumstatin (69–88) improved cardiac dysfunction in MI rats

LVSP and LV + dp/dt_{max} were reduced in heart failure rats induced by myocardial infarction, which was reversed after tumstatin (69–88) administration. Tumstatin (69–88) treatment inhibited the elevated

LVEDP in MI rats. EF and FS in MI rats were reduced, which were reversed after tumstatin (69–88) administration. The MI-induced raises of LVVs, LVVd, LVIDs and LVIDd in rats were attenuated by tumstatin (69–88) treatment (Figure 1).

3.2. Tumstatin (69–88) alleviated cardiac fibrosis in MI rats

The cardiac fibrotic level was enhanced in MI rats by sirius red staining, and this enhancement was suppressed by tumstatin (69–88) (Figure 2a). The expressions of collagen I and III mRNA were elevated in the hearts of rats with myocardial infarction, which were inhibited after

tumstatin (69–88) treatment (Figure 2b). The protein levels of collagen I and III were raised in the hearts of rats with myocardial infarction, and these increases were appressed after tumstatin (69–88) administration (Figure 2c).

3.3. Tumstatin (69–88) alleviated cardiac hypertrophy

The mRNA expressions of ANP and BNP were elevated in the heart of MI rat, which were reversed after tumstatin (69–88) administration (Figure 3a). In NRCMs, Ang II treatment enhanced the mRNA levels of

ANP and BNP, which was attenuated after tumstatin (69–88) treatment (Figure 3b). The mRNA expressions of ANP and BNP rose in Ang II-treated H9C2 cells, which was reversed by tumstatin (69–88) treatment (Figure 3c).

3.4. Tumstatin (69–88) attenuated oxidative stress in heart failure

DHE staining showed that ROS level was increased in the heart of MI rats, which was inhibited by tumstatin (69–88) treatment (Figure 4a). The levels of superoxide anions and Nox activity were

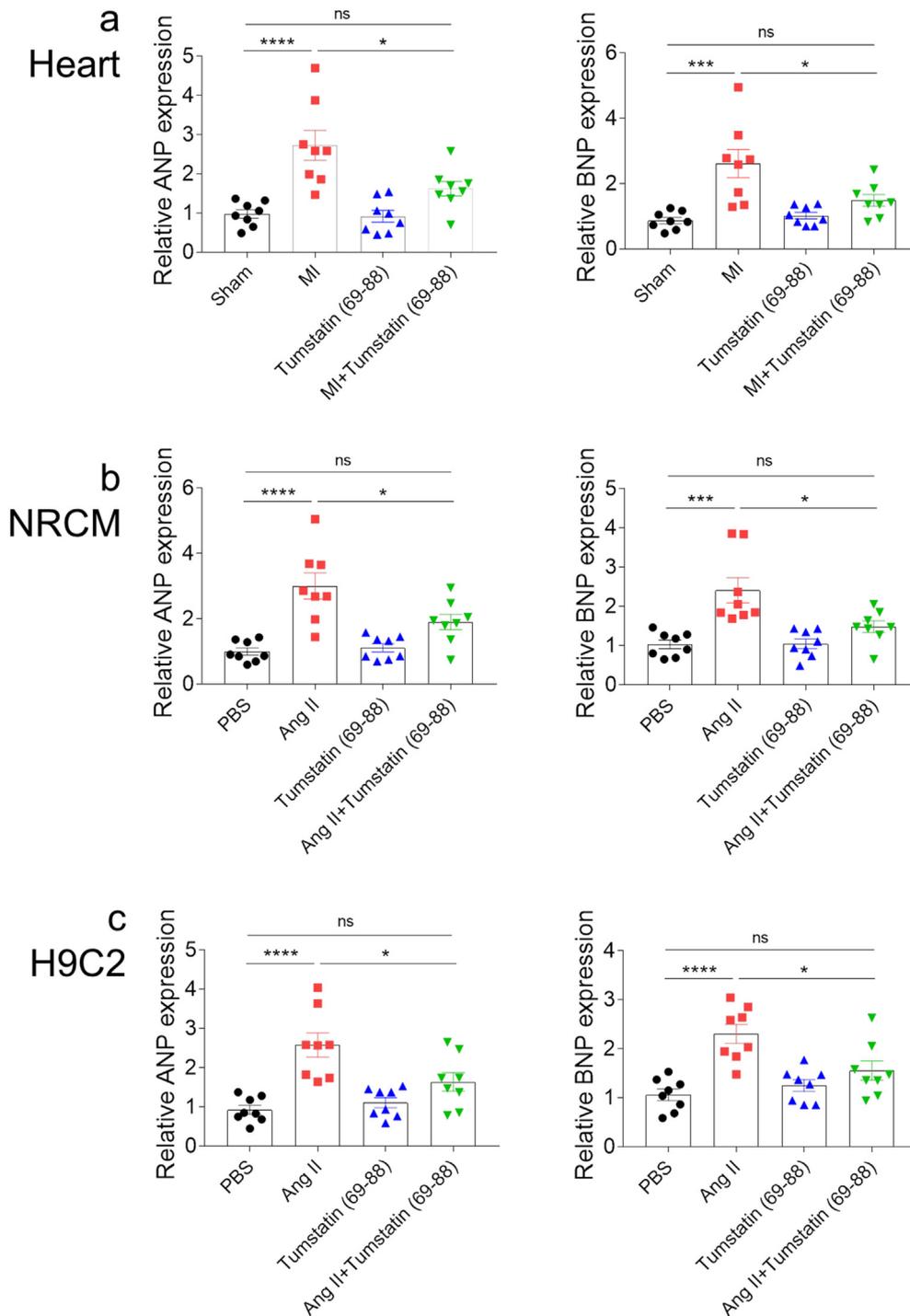


Figure 3. Tumstatin (69–88) alleviated cardiac hypertrophy. a, Tumstatin (69–88) inhibited the raised ANP and BNP in the heart of myocardial infarction rats. b, Tumstatin (69–88) inhibited the Ang II-induced raises of ANP and BNP in neonatal rat cardiomyocytes (NRCMs). c, Tumstatin (69–88) inhibited the Ang II-induced raises of ANP and BNP in H9C2 cells. n = 8 for each group.

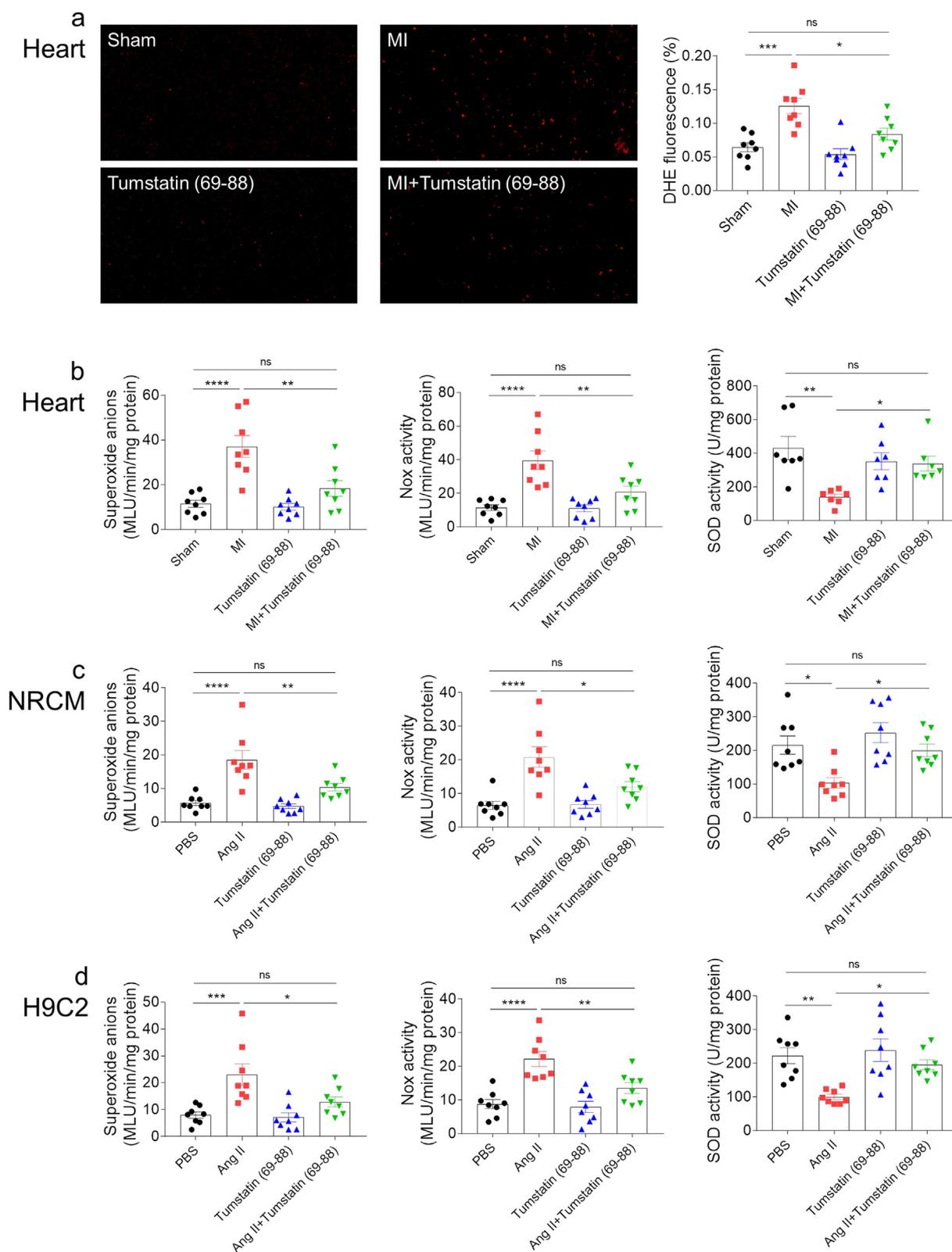


Figure 4. Tumstatin (69–88) attenuated oxidative stress of heart failure. a, Tumstatin (69–88) reversed the increased ROS level in the heart of myocardial infarction rats. b, Tumstatin (69–88) reversed the increased superoxide anions and Nox activity, and the decreased SOD activity in the heart of myocardial infarction rats. c, Tumstatin (69–88) reversed the Ang II-induced increases of superoxide anions and Nox activity, and the decreased SOD activity in NRCMs. d, Tumstatin (69–88) reversed the Ang II-induced raises of superoxide anions and Nox activity, and the decreased SOD activity in H9C2 cells. n = 8 for each group.

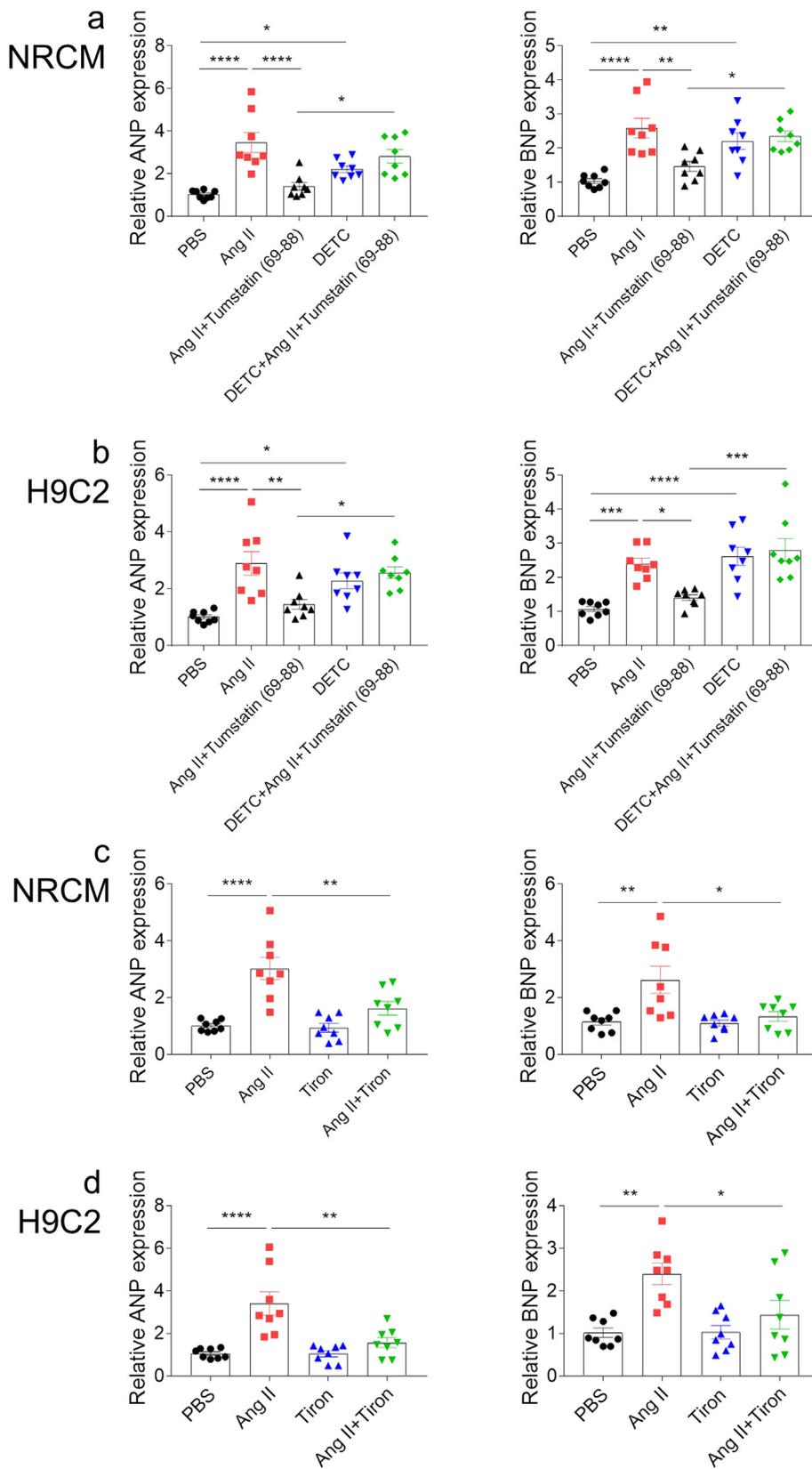


Figure 5. Effects of SOD inhibitor and antioxidant tiron on cardiomyocyte hypertrophy. a, SOD inhibitor DETC reversed the mitigation influences of tumstatin (69–88) on the raised ANP and BNP which were induced by Ang II in NRCMs. b, DETC reversed the alleviating effects of tumstatin (69–88) on the raises of ANP and BNP in H9C2 cells which were induced by Ang II. c, Antioxidant tiron inhibited the raises of ANP and BNP in NRCMs which were induced by Ang II. d, Tiron inhibited the raises of ANP and BNP in H9C2 cells which were induced by Ang II. n = 8 for each group.

elevated, and the activity of cardiac SOD decreased in rats with MI, which were reversed by tumstatin (69–88) administration (Figure 4b). In NRCMs, Ang II increased superoxide anions and Nox activity, and reduced SOD activity, and these changes were reversed

by tumstatin (69–88) treatment (Figure 4c). In H9C2 cells, Ang II treatment increased superoxide anions and Nox activity, and decreased SOD activity, which were reversed by tumstatin (69–88) treatment (Figure 4d).

3.5. SOD inhibitor reversed inhibitory effects of tumstatin (69–88) on cardiomyocyte hypertrophy

DETC, an inhibitor of SOD, increased the mRNA expressions of ANP and BNP in NRCMs; tumstatin (69–88) attenuated the Ang II-induced increases of ANP and BNP, which were reversed by DETC (Figure 5a). In H9C2 cells, DETC treatment increased the mRNA expressions of ANP and BNP; the Ang II-induced raises of ANP and BNP were inhibited by tumstatin (69–88), which was reversed by DETC (Figure 5b).

3.6. Antioxidant tiron attenuated ang II-induced cardiomyocyte hypertrophy

Tiron, an antioxidant, inhibited Ang II-induced raises of ANP and BNP in NRCMs (Figure 5c). The raises of ANP and BNP in H9C2 cells induced by Ang II were suppressed by tiron (Figure 5d).

4. Discussion

Oxidative stress was enhanced in cardiovascular diseases, including heart failure and cardiac hypertrophy [19, 20]. Tumstatin (69–88) is one of the active subfragments of tumstatin [10]. The novel findings of this study were that tumstatin (69–88) could alleviate cardiac dysfunction of heart failure. Tumstatin (69–88) attenuated the increased heart fibrosis in heart failure and it also alleviated cardiomyocyte hypertrophy via inhibiting oxidative stress.

In cardiac disorders, the changed structure and function of the heart weaken the ventricle's ability to fill with or eject blood, thus leading to heart failure [21, 22]. Medical interventions, though effective, cannot significantly reduce the morbidity and mortality of heart failure [23]. We presently found that tumstatin (69–88) alleviated the decreased LVSP, LV + dp/dt_{max}, EF and FS, and the increased of EF, FS, LVEDP, LVVs, LVVd, LVIDs and LVIDd. These results indicate that tumstatin (69–88) could improve the impaired cardiac hemodynamics and cardiac dysfunction. Tumstatin (69–88) is a potential drug for heart failure therapy in the future.

The pathophysiology of heart failure is multifaceted and may partially be attributed to heart fibrosis and cardiomyocyte hypertrophy [24, 25]. Myocardial interstitial fibrosis is found at the advanced stage of most cardiovascular diseases, especially heart failure [26]. Heart failure usually precedes myocardial hypertrophy, as a result of the response of the heart to excessive workload in conditions like a genetic mutation or heart attack [27]. In this study, we found that tumstatin (69–88) could prevent the raised collagen I, collagen III, ANP and BNP in the MI rat heart. In NRCMs, tumstatin (69–88) treatment suppressed the Ang II-induced raises of ANP and BNP. These results demonstrate that tumstatin (69–88) can alleviate cardiac fibrosis and hypertrophy in heart failure.

Under physiological conditions, a small amount of reactive oxygen species (ROS) is generated and soon transformed to H₂O by superoxide dismutase. However, under stress, excessive ROS and superoxide production beyond the antioxidant defense ability of the body are toxic to the organs [28]. Oxidative stress plays a pivotal role in cardiac remodeling and heart failure [29, 30]. The current study found that tumstatin (69–88) reversed the increased superoxide anions and Nox activity, and the decreased SOD activity in MI rat heart. In cardiomyocytes, the Ang II-induced raises of superoxide anions and Nox activity were reversed by tumstatin (69–88) treatment, and the decrease of SOD activity. Also in cardiomyocytes, DETC, an inhibitor of SOD, reversed the mitigation effects of tumstatin (69–88) on the Ang II-induced increases of ANP and BNP. These results indicate that tumstatin (69–88) could alleviate cardiomyocyte hypertrophy of via attenuating oxidative stress.

Antioxidant is being considered as a treatment option of cardiovascular diseases [31, 32]. Previous studies showed that superoxide anion scavenger tempol improved chronic pressure overload-induced heart hypertrophy [33]. Tiron is a potent antioxidant, and can reduce the intracellular reactive oxygen species [34]. We present found that tiron

could inhibit the raises of ANP and BNP in NRCMs and H9C2 cells induced by Ang II. The outcomes showed that inhibition of oxidative stress could improve hypertrophy of cardiomyocyte.

There are still some limitations in our present study. Firstly, we showed that tumstatin (69–88) attenuated oxidative stress in Ang II-induced hypertrophic cardiomyopathy, but the detailed mechanism was not explored. Secondly, only one model of hypertrophic cardiomyopathy was explored in this study. In the future, other models of cardiac hypertrophy will be used to investigate the roles of tumstatin (69–88) on cardiac hypertrophy, and the more accurate mechanisms also will be further probed.

In conclusions, tumstatin (69–88) can attenuate heart failure by alleviating cardiac fibrosis and hypertrophy. Tumstatin (69–88) alleviates cardiomyocyte hypertrophy via suppressing oxidative stress. Attenuation of oxidative stress could alleviate hypertrophy of cardiomyocyte. Tumstatin (69–88) may be a potential drug for the treatment of heart failure in the future.

Declarations

Author contribution statement

Congfei Zhu and Zhi Zuo: Conceived and designed the experiments; Performed the experiments.

Cheng Xu and Mingyue Ji: Analyzed and interpreted the data.

Junjie He: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Jinshuang Li: Conceived and designed the experiments; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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