European Review for Medical and Pharmacological Sciences 2020; 24: 8493-8501

ANO1 relieves pressure overload-induced myocardial fibrosis in mice by inhibiting TGF-β/Smad3 signaling pathway

J.-C. KONG[1], W.-Q. MIAO[2], Y. WANG[2], S.-F. ZHOU[1]

[1]Department of Cardiac Surgery, Fuwai Yunnan Cardiovascular Hospital, Kunming, China
[2]Department of Cardiology, The 1st People's Hospital of Kunming City, Kunming, China

Jichang Kong and Wenqing Miao contributed equally to this work

Abstract. – OBJECTIVE: The aim of this study was to measure the expression of anoctamin 1 (ANO1) in myocardial tissues of mice with pressure overload-induced myocardial fibrosis, and to further investigate the effect of ANO1 on myocardial fibrosis in mice and its mechanism.

MATERIALS AND METHODS: A total of 40 male C57/B6 mice aged 6-8 weeks old were divided into 2 groups using a random number table, namely sham operation group (Sham group, n=20) and thoracic aortic constriction group (TAC group, n=20). Meanwhile, 20 ANO1 transgenic (TG) mice aged 6-8 weeks old were enrolled for TAC as TAC + ANO1 TG group. At 8 weeks after TAC, ejection fraction (EF%) and fraction shortening (FS%) in each group of mice were detected via echocardiography. Western blotting and immunofluorescence staining assays were conducted to measure the protein expression of ANO1 in myocardial tissues of mice in each group. The pathological changes in myocardial tissues of mice were evaluated through hematoxylin-eosin (H&E) staining. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) assay was performed to measure the messenger ribonucleic acid (mRNA) expression levels of hypertrophy markers atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) in myocardial tissues of mice in each group. The deposition of collagen fibers in heart tissues was determined by Masson staining assay. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) staining assay was carried out to detect the apoptosis of myocardial cells and fibroblasts in heart tissues. Additionally, the protein expressions of oxidative stress markers superoxide dismutase 1 (SOD1) and 4-hydroxynonenal (4-HNE) in myocardial tissues were detected as well. Finally, Western blotting was employed to detect the effect of ANO1 overexpression on the expression of transforming growth factor-β (TGF-β)/Smad3 signaling pathway-related proteins in myocardial tissues of mice.

RESULTS: At 8 weeks after TAC, ANO1 expression was overtly reduced in myocardial tissues of mice ($p<0.05$). Echocardiographic results showed that ANO1 overexpression significantly alleviated TAC-induced cardiac function deterioration in mice ($p<0.05$). The mRNA expression levels of ANP and BNP in myocardial tissues of TAC + ANO1 TG group were evidently lower than those in TAC group ($p<0.05$). Meanwhile, myocardial interstitial collagen deposition was significantly ameliorated in TAC + ANO1 TG group compared with TAC group ($p<0.05$). ANO1 overexpression notably mitigated the apoptosis of myocardial cells and oxidative stress in mice with cardiac pressure overload ($p<0.05$). Western blotting results further indicated that after overexpression of ANO1, the protein levels of TGF-β and phosphorylated Smad3 (p-Smad3) were significantly inhibited in mice undergoing TAC ($p<0.05$).

CONCLUSIONS: In the case of cardiac pressure overload in mice, ANO1 is lowly expressed in myocardial tissues. Meanwhile, its overexpression is able to attenuate pressure overload-induced myocardial fibrosis in mice by repressing the TGF-β/smad3 signaling pathway. All our findings indicate that ANO1 can serve as a potential gene target for the treatment of myocardial fibrosis in the future.

Key Words:
ANO1, Pressure overload, Myocardial fibrosis, TGF-β/Smad3

Introduction

Myocardial fibrosis and hypertrophy are mainly caused by mechanical load, hypertension, myocardial infarction and arrhythmia[1]. Persistent myocardial fibrosis is able to decrease ventricular compliance, thereby inducing heart failure and even sudden cardiac death[2]. It has been

Corresponding Author: Shaofang Zhou, MM; e-mail: zos1981@163.com

confirmed that myocardial fibrosis and hypertrophy are important pathophysiological changes in heart failure[3]. For this reason, further exploration of the occurrence and development of myocardial fibrosis is of crucial significance for the prevention and treatment of heart failure.

Anoctamin-1 (ANO1) is a member of the protein family with 800-1000 amino acid residues, and the family consists of 10 members (ANO1-10)[4]. ANO1 is expressed in various tissues, such as liver, kidneys, lungs, pancreas and heart, with all the functional characteristics of calcium-activated chloride channels[5,6]. Ye et al[7] have shown that overexpression of ANO1 induced by myocardial ischemia is capable of affecting the first-stage repolarization of myocardial cells by regulating calcium-activated chloride channels. This may ultimately result in arrhythmias. It has been also reported[8] that ANO1 can suppress the proliferation and migration of tumor cells. Meanwhile, the proliferation and migration of cardiac fibroblasts are the key mechanisms of myocardial fibrosis. However, the expression and mechanism of ANO1 in pressure overload-induced myocardial fibrosis have not been fully elucidated.

In this study, a model of thoracic aortic constriction (TAC) was first constructed to induce myocardial fibrosis in mice. The expression of ANO1 therein was detected. In addition, ANO1 transgenic (TG) mice were further used to observe the effects of ANO1 overexpression on pressure overload-induced myocardial fibrosis and deterioration of cardiac function in mice, as well as relevant mechanisms.

Materials and Methods

Animal Grouping and Modeling

A total of 40 male C57/B6 mice [6-8 weeks old and (22.03±2.03) g] were divided into 2 groups using a random number table, namely sham operation group (Sham group, n=20) and TAC group (n=20). Additionally, 20 ANO1 TG mice [6-8 weeks old and (22.45±1.29) g] were selected for TAC (TAC + ANO1 TG group). There were no statistically significant differences in basic information, including age and weight among the three groups of mice. TAC was performed as per the following procedures. (1) 3% amobarbital was intraperitoneally injected into mice for anesthesia, and an oral trachea cannula was given for assisted respiration. (2) A horizontal incision was made on the skin between the 2-3 ribs on the mouse chest to separate the descending aorta, and a 26-gauge needle was placed next to the thoracic aorta, followed by ligation. (3) The needle was quickly pulled out, and the muscle and skin were sutured layer by layer. Samples were collected after 8 weeks. This study was approved by the Animal Ethics Committee of Fuwai Yunnan Cardiovascular Hospital Animal Center.

Echocardiographic Examination

To examine the cardiac function of mice in each group, a Mylab 30CV ultrasound system (Esaote S.P.A, Genoa, Italy) and a 10-MHz linear ultrasound transducer were employed to obtain the echocardiogram of each group of mice. Briefly, the anterior chest was first shaved clean. Subsequently, the mice were anesthetized and placed on a 37°C heating plate with the left side facing up. Next, examination of such parameters as ejection fraction (EF%), fraction shortening (FS%) and heart rate (bpm) was performed.

Masson Staining Assay

Paraffin sections were first deparaffinized, dehydrated, and subjected to chromatizing treatment or mercury salt precipitation removal. Next, the sections were successively washed with tap water and distilled water. Regaud hematoxylin or Weigert hematoxylin was added to stain the nuclei for 5-10 min. Thereafter, the sections were sufficiently washed with water and stained with Masson Ponceau Acid Fuchsin liquid for 5-10 min. After that, the sections were soaked in 2% glacial acetic acid aqueous solution for a while, and differentiated with 1% phosphomolybdic acid aqueous solution for 3-5 min. Finally, the sections were treated with 95% alcohol and absolute alcohol, permeabilized with xylene and mounted with neutral balsam.

Hematoxylin-Eosin (H&E) Staining Assay

Heart tissues of mice in each group were first placed in 10% formalin overnight. Subsequently, heart tissues were dehydrated and embedded in paraffin blocks. Myocardial tissues were then cut into 5 μm-thick slices, fixed on a glass slide and baked dry, followed by staining as per the instructions. Next, the slices were soaked in xylene, ethanol with graded concentrations and hematoxylin, respectively, and mounted with resin. Following air drying, morphology of myocardial tissues and interstitium was observed and photographed under an optical microscope.

Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate-Biotin Nick End Labeling (TUNEL) Staining Assay

Myocardial tissue sections were baked in an oven at 60°C for 30 min and deparaffinized with xylene (5 min × 3 times). Subsequently, they were dehydrated with 100% ethanol, 95% ethanol, and 70% ethanol for 3 times each. Thereafter, the sections were incubated with protein kinase K for 0.5 h, rinsed with phosphate-buffered saline (PBS) and added with terminal deoxyribonucleotide transferase and luciferase-labeled deoxyuridine triphosphate, followed by reaction at 37°C for 1 h. After that, the sections were added with horseradish peroxidase-labeled specific antibody for 1 h of incubation at 37°C again. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO, USA), photographed under a fluorescence microscope and counted.

Measurement of Protein Expressions of ANO1 and 4-Hydroxynonenal (4-HNE) in Myocardial Tissues Through Immunofluorescence

Myocardial tissue sections were firstly deparaffinized in an incubator at 60°C for 30 min and subjected to antigen retrieval *via* high-pressure citrate buffer method. Then, myocardial tissues were covered by 3% hydrogen peroxide, incubated for 20 min and blocked with 8% goat serum for 30 min. Subsequently, ANO1 and 4-HNE primary antibodies (diluted in PBS at 1:200) were added dropwise to completely cover the tissues, followed by incubation at 4°C overnight. On the next day, the sections were rewarmed, and added dropwise with secondary antibody B solution for 30 min of incubation at room temperature. After that, the sections were washed and added with diaminobenzidine (DAB) working solution (Solarbio, Beijing, China) in drops. The time of color development was strictly controlled under the optical microscope. Finally, the sections in each group were counterstained with hematoxylin, dehydrated with graded ethanol and mounted, followed by photography under an optical microscope (200×). 10 non-repeated fields of view were randomly selected for each sample.

Detection of Messenger Ribonucleic Acid (mRNA) Expression Level of Myocardial Hypertrophy Genes Via Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNAs were extracted from myocardial tissues of mice in each group by TRIzol method (Invitrogen, Carlsbad, CA, USA). The concentration and purity of extracted RNAs were qualified by an ultraviolet spectrophotometer. A_{260}/A_{280}=1.8-2.0 indicated that the RNAs could be used for the following procedures. Next, RNAs were reversely transcribed into complementary deoxyribonucleic acids (cDNAs) and stored in a refrigerator at -80°C for use. RT-PCR was carried out using the system consisting of 2.5 μL of 10× Buffer, 1 μL of cDNAs, 0.5 μL of forward primers (20 μmol/L), 0.5 μL of reverse primers (20 μmol/L), 10 μL of LightCycler® 480 SYBR Green I Master (2×), and 5.5 μL of ddH_2O. Note: the amplification system of RT-PCR was the same. Primer sequences used in this study were shown in Table I.

Western Blotting Assay

Myocardial tissues of mice in each group were fully ground in lysis buffer. Then, the tissues were subjected to ultrasonic lysis, and the resulting lysate was centrifuged. The supernatant was collected and sub-packaged into Eppendorf (EP; Hamburg, Germany) tubes for use. Protein concentration was determined through the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China) and ultraviolet spectrophotometric assay. All sample proteins were maintained at a constant volume and equal concentration. Next, the proteins were sub-packaged and preserved in a refrigerator at -80°C. Protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and

Table I. Primer sequences of indicators in qRT-PCR.

Target gene		Primer sequence
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Forward	5'-GACATGCCGCCTGGAGAAACCC-3'
	Reverse	5'-AGCCAGGATGCCCTTTAGTCCA-3'
Atrial natriuretic peptide (ANP)	Forward	5'-ACGTTTAGTCGATGTCGTAGTGC-3'
	Reverse	5'-AACGTAGTCGTAGATGTCGTAGT-3'
Brain natriuretic peptide (BNP)	Forward	5'-CCCACACCACACGCTGTAGTCA-3'
	Reverse	5'-ACGATACACACTCTGATGCTAGG-3'

transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Next, the membranes were incubated with primary antibodies at 4°C overnight. On the next day, the membranes were incubated with goat anti-rabbit secondary antibodies in dark for 1 h. Immuno-reactive bands were scanned and quantified using an Odyssey membrane scanner (Seattle, WA, USA). With glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the level of proteins to be tested was corrected.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM Corp., Armonk, NY, USA) was utilized for statistical analysis. Measurement data were expressed as mean ± standard deviation. *t*-test was employed to compare the differences between two groups. $p<0.05$ was considered statistically significant.

Results

Protein Expression Level of ANO1 in Myocardial Tissues of Mice in Each Group

The results of Western blotting and immunofluorescence staining assays (Figure 1) revealed that the protein expression level of ANO1 in myocardial tissues of mice declined significantly at 8 weeks after TAC ($p<0.05$). This implies that ANO1 may participate in TAC-induced myocardial fibrosis in mice.

Effect of ANO1 Overexpression on Cardiac Function of Mice in Each Group

Echocardiographic results (Figure 2) showed that heart rate displayed no statistically significant differences among the three groups, suggesting that differences in EF% and FS% among groups were not caused by different heart rates. Compared with Sham group, the heart had enlarged chambers and thinned walls in TAC group. Meanwhile, ANO1 overexpression visibly alleviated the abnormal changes in heart structure caused by TAC in mice. Subsequently, FS% and EF% were examined in each group of mice. It was uncovered that ANO1 overexpression clearly impeded the decreases in FS% and EF% in mice receiving TAC ($p<0.05$).

Effect of ANO1 Overexpression on Myocardial Hypertrophy of mice in Each Group

H&E staining results indicated that there were disordered myofilaments and remarkably

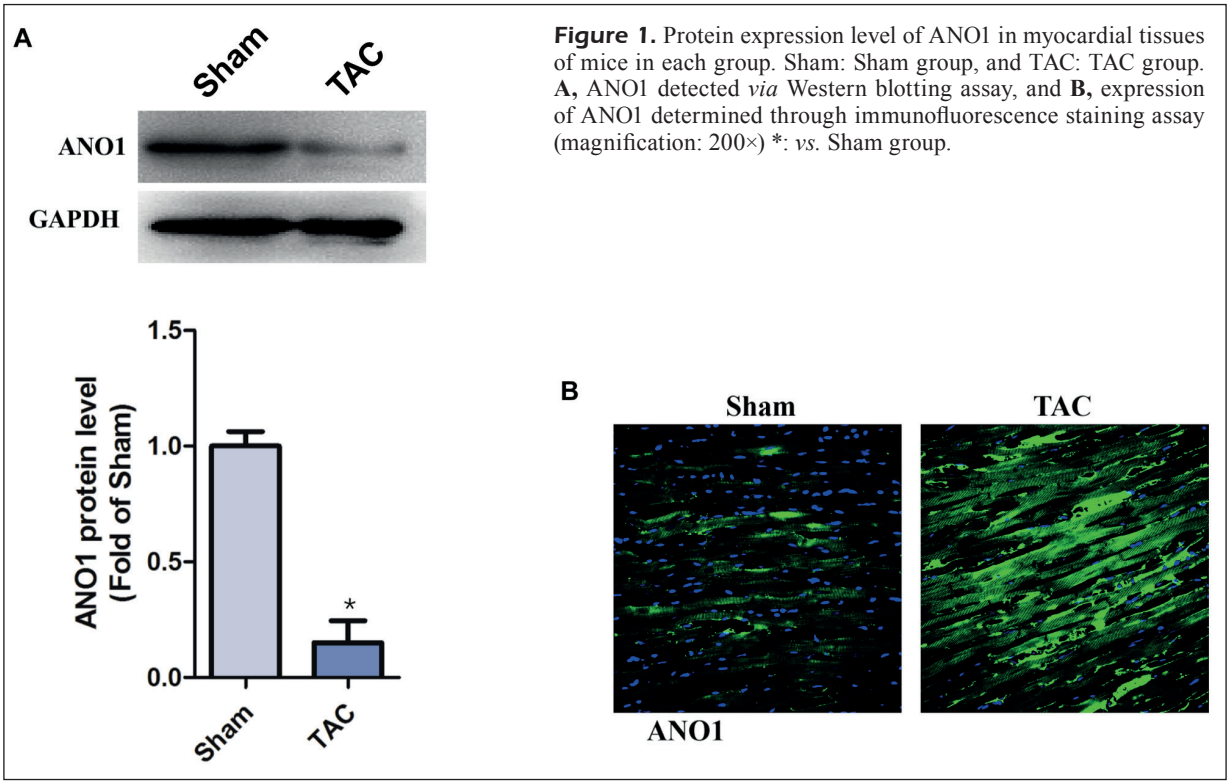

Figure 1. Protein expression level of ANO1 in myocardial tissues of mice in each group. Sham: Sham group, and TAC: TAC group. **A,** ANO1 detected *via* Western blotting assay, and **B,** expression of ANO1 determined through immunofluorescence staining assay (magnification: 200×) *: *vs.* Sham group.

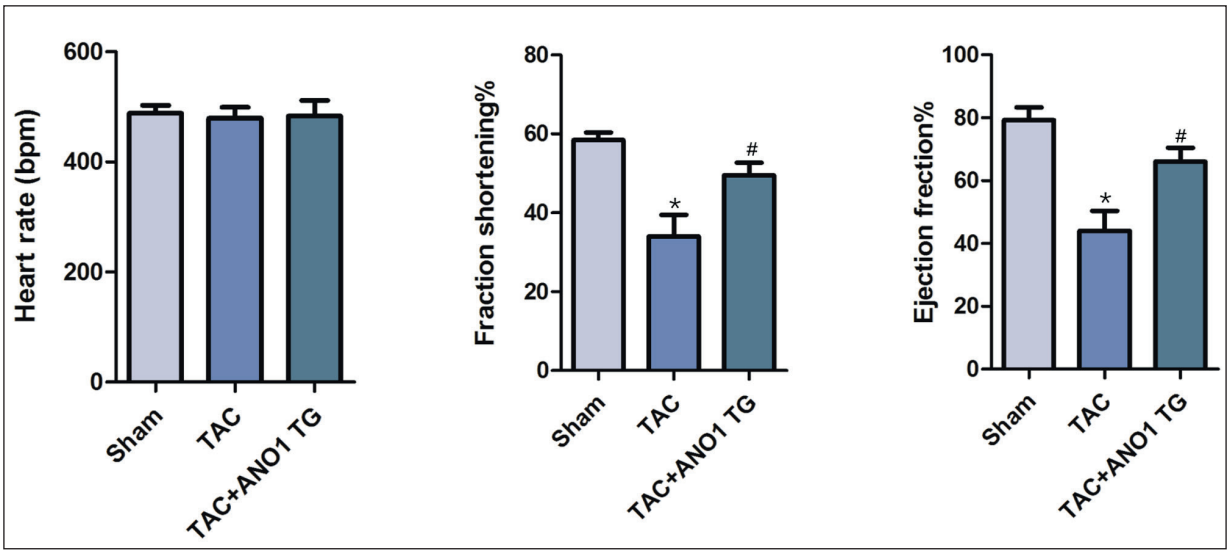

Figure 2. Effect of ANO1 overexpression on cardiac function of mice in each group (*p<0.05 *vs.* Sham group, #p<0.05 *vs.* TAC group).

enlarged myocardial cells in TAC group. However, ANO1 overexpression markedly relieved TAC-induced hypertrophy and injury of myocardial cells in mice (Figure 3). Further experiments demonstrated that after overexpressing ANO1, the mRNA expressions of hypertrophy markers ANP and BNP in myocardial tissues decreased significantly in mice undergoing TAC (p<0.05).

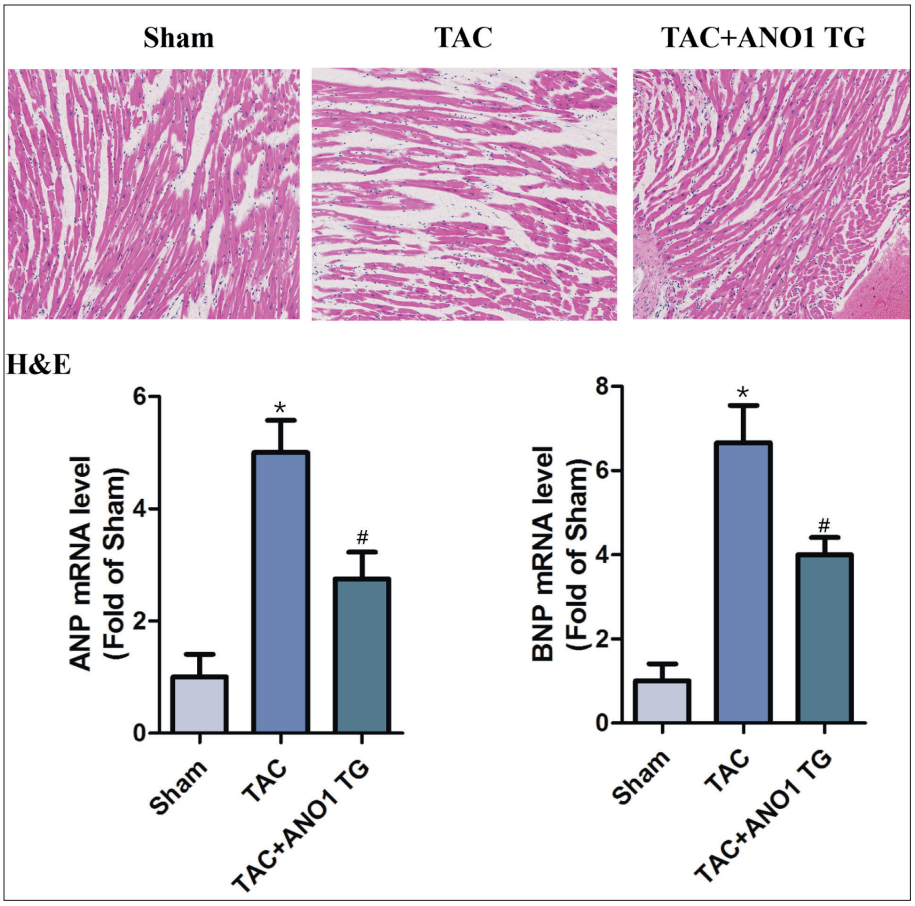

Figure 3. Effect of ANO1 overexpression on myocardial hypertrophy of mice in each group (magnification: 200×) (*p<0.05 *vs.* Sham group, #p<0.05 *vs.* TAC group).

Effect of ANO1 Overexpression on Myocardial Fibrosis of Mice in Each Group

As shown in Figure 4, collagen deposition in myocardial tissues was significantly severer in TAC group than that in Sham group ($p<0.05$). However, collagen content in myocardial tissues of mice undergoing TAC decreased notably after overexpression of ANO1 ($p<0.05$).

Effect of ANO1 Overexpression on Apoptosis of Mouse Myocardial Cells in Each Group

Based on TUNEL staining results (Figure 5), at 8 weeks after TAC, the apoptosis level of myocardial cells in mice increased prominently ($p<0.05$). However, ANO1 overexpression considerably decreased the apoptosis level of myocardial cells in mice undergoing TAC ($p<0.05$).

Effect of ANO1 Overexpression on Oxidative Stress in Mouse Myocardial Tissues in Each Group

In the case of cardiac fibrosis, the level of oxidative stress in myocardial cells and cardiac fibroblasts rises distinctly. In this study, the expression levels of antioxidant enzyme superoxide dismutase 1 (SOD1) and lipid peroxidation product 4-HNE in mouse myocardial tissues in each group were measured. The results (Figure 6) uncovered that ANO1 overexpression significantly suppressed decreased SOD1 expression and increased 4-HNE content induced by TAC ($p<0.05$), suggesting that ANO1 has a potential anti-oxidative stress effect.

Regulatory Effect of ANO1 on Transforming Growth Factor-β (TGF-β)/Smad3 Signal in Mice with Myocardial Fibrosis

The over-activation of the TGF-β/Smad3 signaling pathway is the main mechanism leading to collagen synthesis and phenotypic transformation of cardiac fibroblasts. In this study, it was found that after overexpressing ANO1, the protein level of TGF-β in mouse myocardial tissues was significantly reduced ($p<0.05$). Meanwhile, the content of phosphorylated Smad3 (p-Smad3) was also remarkably inhibited ($p<0.05$) (Figure 7).

Discussion

Cardiovascular disease, one of the leading causes of human death, accounts for 43% of emerging diseases every year[9]. Myocardial fibrosis is the common pathological change of end-stage myocardial infarction, diabetic cardiomyopathy, doxorubicin cardiomyopathy and hypertensive heart disease. In the case of myocardial fibrosis, cardiac fibroblasts proliferate and excessively secrete extracellular matrix in quantity. Meanwhile, various interstitial collagen fibers in the myocardium have increased concen-

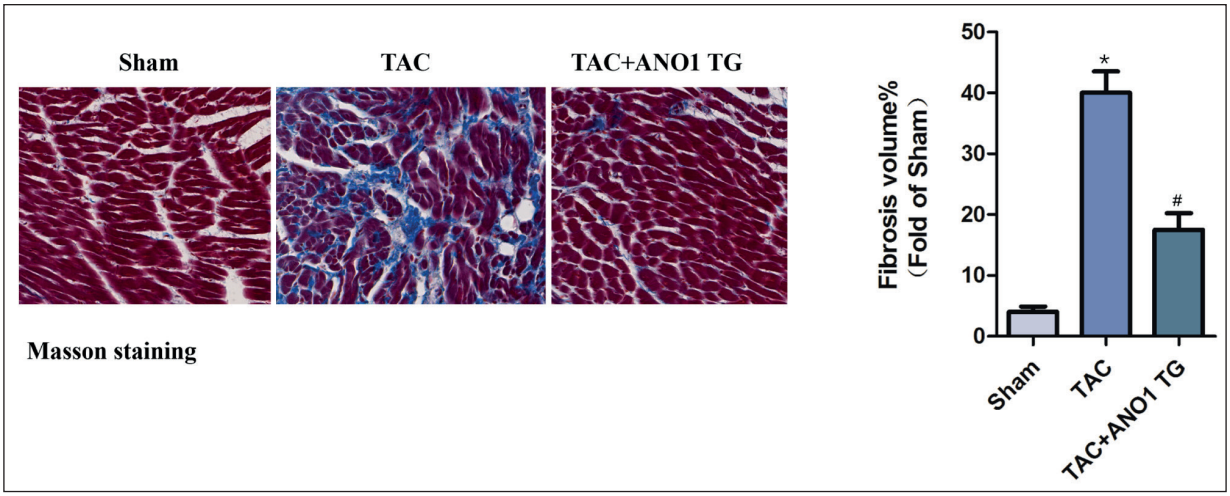

Figure 4. Effect of ANO1 overexpression on myocardial hypertrophy of mice in each group (magnification: 200×) (*$p<0.05$ *vs.* Sham group, #$p<0.05$ *vs.* TAC group).

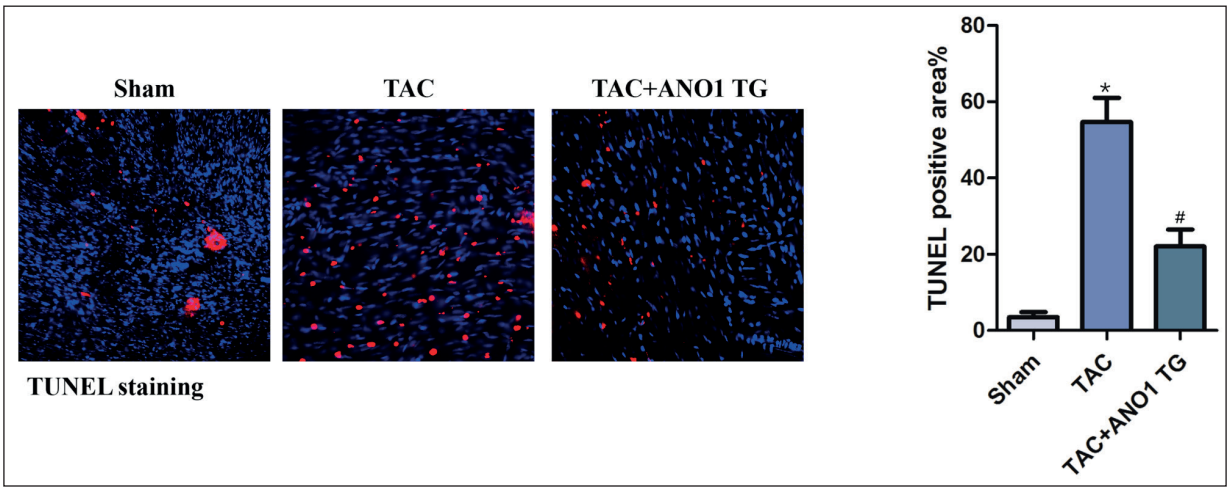

Figure 5. Effect of ANO1 overexpression on apoptosis of mouse myocardial cells in each group (magnification: 200×) (*p<0.05 *vs.* Sham group, #p<0.05 *vs.* TAC group).

tration and unbalanced ratio. Ultimately, this may enhance myocardium stiffness, decrease compliance, weaken the ability of cardiac ejection, and even result in ventricular remodeling and heart failure[10,11]. Myocardial fibrosis is caused by a variety of mechanisms, including: (1) abnormal activation of the renin-angiotensin-aldosterone system (RAAS), (2) production of myocardial collagen due to over-activation of the myocardial oxidation system and upregulation of inflammation levels, (3) differentiation and maturation of cardiac fibroblasts, abnormal aggregation of extracellular matrix, and inhibition of the activity of matrix metalloproteinases because of connective tissue growth factors and TGF-β1[12,13].

Cardiac fibroblasts, one of the main cell types in the heart, can synthesize and secrete collagens once activated by various stimulating

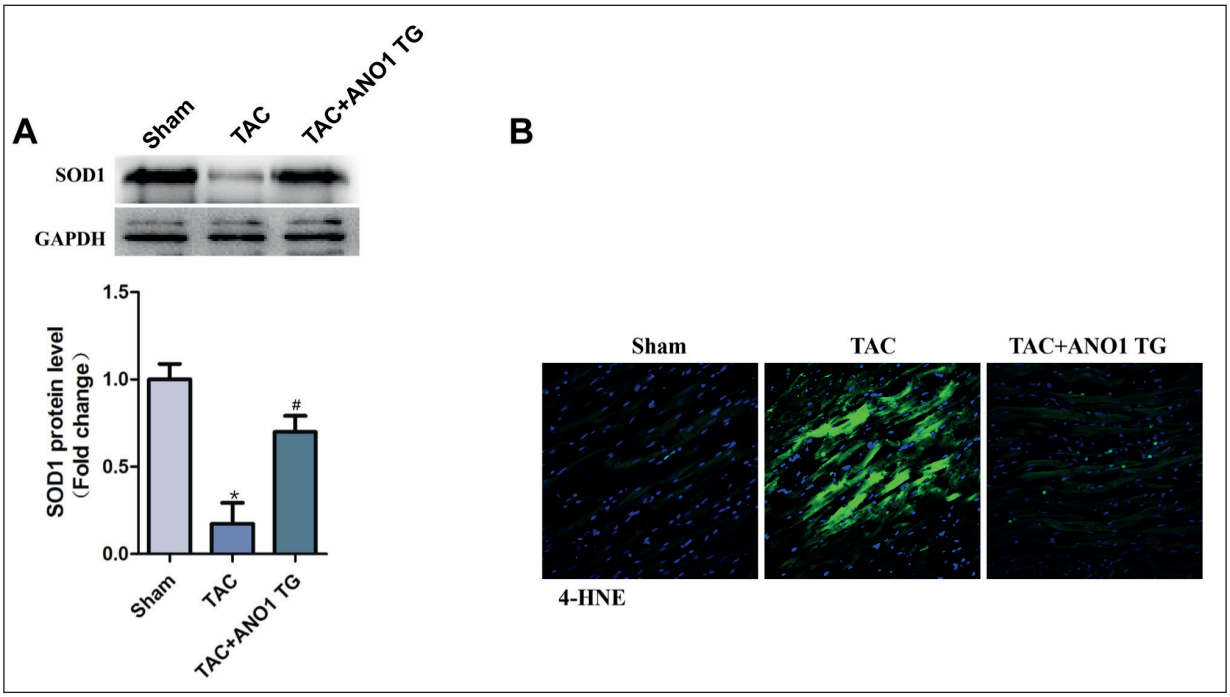

Figure 6. Effect of ANO1 overexpression on oxidative stress in mouse myocardial tissues in each group (magnification: 200×) (*p<0.05 *vs.* Sham group, #p<0.05 *vs.* TAC group).

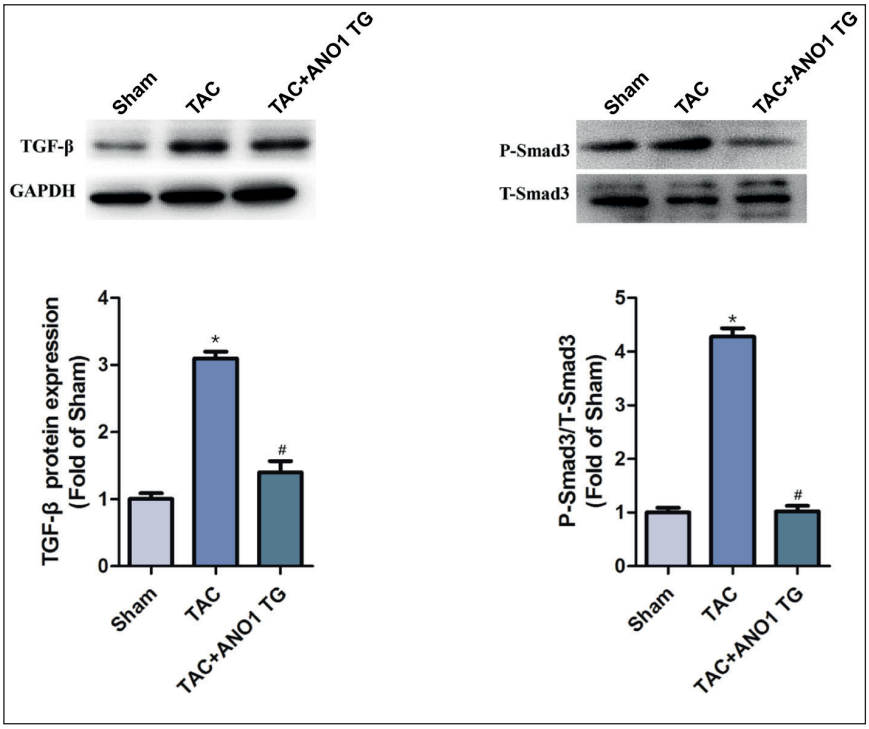

Figure 7. Regulatory effect of ANO1 on TGF-β/Smad3 signal in mice with myocardial fibrosis (*p<0.05 *vs.* Sham group, #p<0.05 *vs.* TAC group).

factors (such as high glucose, isoproterenol, TGF-β and AngII). This may eventually lead to the deposition of extracellular matrix in the myocardium[14]. Besides, extracellular matrix is also synthesized through the conversion of cardiac fibroblasts into myofibroblasts expressing α-SMA[15]. The TGF-β/p-Smad3 signaling pathway is a typical signaling pathway promoting myocardial fibrosis. Current studies have found that it plays a crucial role in the occurrence and development of myocardial fibrosis, which is activated basically as follows. TGF-β binds to TGF-β receptor II to activate TGF-β receptor I, and activated TGF-β receptor I phosphorylates Smad3. P-Smad3 binds to Smad4 and enters the nucleus, directly or indirectly activating fibrosis-related genes like Collagen 1A2, Collagen 3A1, Collagen 5A1, Collagen 6A1, Collagen 6A3 and Collagen 2A1 genes with binding sites to Smad3 in their promoter sequences[16]. The TGF-β/Smad3 signaling pathway has been proven to be closely related to fibrosis in various organs, such as myocardial fibrosis, liver fibrosis, and renal fibrosis[17]. Therefore, targeted inhibition of the TGF-β/Smads signaling pathway is a key strategy for the treatment of myocardial fibrosis.

Previous studies have denoted that ANO1 has a certain expression in cardiac fibroblasts. Meanwhile, its overexpression can significantly inhibit cardiac fibrosis after myocardial infarction probably by the inhibitory effect of ANO1 on the TGF-β/Smad3 signaling pathway[18]. It has also been reported that ANO1 participates in the occurrence and development of spontaneous hypertension and is highly expressed in vascular smooth muscle of rats with spontaneous hypertension. Moreover, the application of ANO1 inhibitors evidently reduces blood pressure and visibly weakens the proliferative ability of vascular smooth muscle cells in rats[19]. Further researches have demonstrated that ANO1 may regulate AngII-dependent vascular remodeling. In this study, it was discovered that the protein expression level of ANO1 in myocardial tissues was significantly inhibited in the mouse model of TAC-induced cardiac pressure overload. Moreover, genetically engineered mice with over-expression of ANO1 were studied. The results showed that overexpression of ANO1 remarkably improved cardiac function and repressed the occurrence of myocardial hypertrophy and fibrosis in mice undergoing TAC. It was also uncovered that ANO1 reduced the levels of apoptosis and oxidative stress in myocardial tissues during pressure overload. Finally, the results indicated that after overexpressing ANO1, the TGF-β/Smad3 signaling pathway

was notably impeded, which might be the underlying mechanism of ANO1 in protecting the myocardium.

Conclusions

For the first time in this study it has been found that ANO1 is lowly expressed in the case of pressure overload-induced myocardial remodeling in mice. Furthermore, ANO1 overexpression inhibits the TGF-β/Smad3 signaling pathway to mitigate pressure overload-induced myocardial fibrosis in mice.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding Acknowledgement

This study was supported by the Project Code of Kunming Health Commission (2019-03-01-011).

References

1) Lim GB. Heart failure: macrophages promote cardiac fibrosis and diastolic dysfunction. Nat Rev Cardiol 2018; 15: 196-197.
2) Morine KJ, Qiao X, York S, Natov PS, Paruchuri V, Zhang Y, Aronovitz MJ, Karas RH, Kapur NK. Bone morphogenetic protein 9 reduces cardiac fibrosis and improves cardiac function in heart failure. Circulation 2018; 138: 513-526.
3) Teekakirikul P, Eminaga S, Toka O, Alcalai R, Wang L, Wakimoto H, Nayor M, Konno T, Gorham JM, Wolf CM, Kim JB, Schmitt JP, Molkentin JD, Norris RA, Tager AM, Hoffman SR, Markwald RR, Seidman CE, Seidman JG. Cardiac fibrosis in mice with hypertrophic cardiomyopathy is mediated by non-myocyte proliferation and requires Tgf-beta. J Clin Invest 2010; 120: 3520-3529.
4) Yu K, Duran C, Qu Z, Cui YY, Hartzell HC. Explaining calcium-dependent gating of anoctamin-1 chloride channels requires a revised topology. Circ Res 2012; 110: 990-999.
5) Buchholz B, Faria D, Schley G, Schreiber R, Eckardt KU, Kunzelmann K. Anoctamin 1 induces calcium-activated chloride secretion and proliferation of renal cyst-forming epithelial cells. Kidney Int 2014; 85: 1058-1067.
6) Bijos DA, Drake MJ, Vahabi B. Anoctamin-1 in the juvenile rat urinary bladder. PLoS One 2014; 9: e106190.
7) Ye Z, Wu MM, Wang CY, Li YC, Yu CJ, Gong YF, Zhang J, Wang QS, Song BL, Yu K, Hartzell HC, Duan DD, Zhao D, Zhang ZR. Characterization of cardiac anoctamin1 Ca(2)(+)-activated chloride channels and functional role in ischemia-induced arrhythmias. J Cell Physiol 2015; 230: 337-346.
8) Jia L, Liu W, Guan L, Lu M, Wang K. Inhibition of calcium-activated chloride channel ano1/tmem16a suppresses tumor growth and invasion in human lung cancer. PLoS One 2015; 10: e136584.
9) Fox CS, Coady S, Sorlie PD, D'Agostino RS, Pencina MJ, Vasan RS, Meigs JB, Levy D, Savage PJ. Increasing cardiovascular disease burden due to diabetes mellitus: the Framingham Heart Study. Circulation 2007; 115: 1544-1550.
10) Kong P, Christia P, Frangogiannis NG. The pathogenesis of cardiac fibrosis. Cell Mol Life Sci 2014; 71: 549-574.
11) Daniels A, van Bilsen M, Goldschmeding R, van der Vusse GJ, van Nieuwenhoven FA. Connective tissue growth factor and cardiac fibrosis. Acta Physiol (Oxf) 2009; 195: 321-338.
12) Lacolley P, Safar ME, Lucet B, Ledudal K, Labat C, Benetos A. Prevention of aortic and cardiac fibrosis by spironolactone in old normotensive rats. J Am Coll Cardiol 2001; 37: 662-667.
13) Zhao W, Zhao T, Chen Y, Ahokas RA, Sun Y. Oxidative stress mediates cardiac fibrosis by enhancing transforming growth factor-beta1 in hypertensive rats. Mol Cell Biochem 2008; 317: 43-50.
14) Porter KE, Turner NA. Cardiac fibroblasts: at the heart of myocardial remodeling. Pharmacol Ther 2009; 123: 255-278.
15) Camelliti P, Borg TK, Kohl P. Structural and functional characterisation of cardiac fibroblasts. Cardiovasc Res 2005; 65: 40-51.
16) Flanders KC. Smad3 as a mediator of the fibrotic response. Int J Exp Pathol 2004; 85: 47-64.
17) Pohlers D, Brenmoehl J, Loffler I, Muller CK, Leipner C, Schultze-Mosgau S, Stallmach A, Kinne RW, Wolf G. TGF-beta and fibrosis in different organs-molecular pathway imprints. Biochim Biophys Acta 2009; 1792: 746-756.
18) Wu MM, Lou J, Song BL, Gong YF, Li YC, Yu CJ, Wang QS, Ma TX, Ma K, Hartzell HC, Duan DD, Zhao D, Zhang ZR. Hypoxia augments the calcium-activated chloride current carried by anoctamin-1 in cardiac vascular endothelial cells of neonatal mice. Br J Pharmacol 2014; 171: 3680-3692.
19) Forrest AS, Joyce TC, Huebner ML, Ayon RJ, Wiwchar M, Joyce J, Freitas N, Davis AJ, Ye L, Duan DD, Singer CA, Valencik ML, Greenwood IA, Leblanc N. Increased TMEM16A-encoded calcium-activated chloride channel activity is associated with pulmonary hypertension. Am J Physiol Cell Physiol 2012; 303: C1229-C1243.