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Original Article

Effects of ACE2-Ang (1-7)-Mas axis on primary myocardial cell apoptosis: an *in vitro* study

Haomiao Rui, Chenghao Cao, Huichao Zhang, Zhentao Wang

Department of Cardiology, Henan Province Hospital of TCM, Zhengzhou, China

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Abstract: Angiotensin converting enzyme (ACE) and its catalysate angiotensin II (Ang II) are important effectors of rennin-angiotensin system (RAS) that exert deleterious effects in myocardial injury. However, it is not clear whether up-regulation of the ACE2/Ang (1-7)/Mas axis prevents Ang II-induced injury in myocardial cells. This study explored the biological effects of ACE2-Ang (1-7)-Mas axis on myocardial cells apoptosis and its possible underlying mechanism. Primary cultured rat myocardial cells were transduced with adenovirus-borne Ace2, and then were treated with Mas receptor blocker (A779) before exposure to Ang II. The cells were differentiated into seven groups as control, Ang II, Ang II+Ang (1-7), Ang II+pAAV9-ACE2, Ang II+pAAV9-EGFP and Ang II+pAAV9-ACE2+A779. MTT assay, apoptosis assay, qRT-PCR and Western blotting were also performed to analyze the expression of ACE2. Result showed that overexpression of Ang (1-7) and ACE2 promoted cell survival and suppressed Ang II induced cardiac apoptosis ($P<0.05$). A779, the competitive antagonist of the Mas receptor, significantly inhibited the protective effects of ACE2 on Ang II-induced myocardial injury. ACE2-Ang (1-7)-MAS axis down-regulated the apoptotic factors such as Bax, Cytochrome *c,* and active caspase-3 and up-regulated Bcl-2, thus inhibiting cell apoptosis. Also, ACE2-Ang (1-7)-MAS axis activated PI3K/AKT and eNOS pathways (PI3K/AKT pathway is an upstream of eNOS pathway) and resulted in reduction of myocardial injury. These findings clarified that ACE2-Ang (1-7)-MAS axis has cardioprotective effects against Ang II-induced myocardial injury by activating the PI3K/AKT/eNOS pathways. This can also act as a novel drug target which needs to be further explored in future studies.

Keywords: Primary myocardial cells, ACE2-Ang (1-7)-Mas axis, apoptosis, PI3K/AKT/eNOS pathways

Introduction

Cardiovascular diseases (CVDs) remain to be one of the leading causes of death worldwide and of the various forms of CVDs, congestive heart failure (CHF) is one of the most common conditions. CHF is generally caused by impairing the ability of ventricle's to fill with or eject blood that supplies to the heart muscle itself (i.e. myocardium). There were 23 million people suffering from CHF worldwide with a trending rise in the mortality rates [1]. Therefore, it is necessary to identify, validate, and develop effective drugs for the treatment of CHF.

It is well established that excessive activation of renin-angiotensin-system (RAS) plays a vital role in myocardial remodeling and is an important regulator of cardiovascular homeostasis [2, 3]. Angiotensin converting enzyme (ACE) is the core enzyme of RAS, and its catalysate, *i.e.*, angiotensin II (Ang II), is a key effector of RAS [4, 5]. In CHF, the activated Ang II directly induces myocardial remodeling, and contributes to the pathogenesis and progression of the disease [5]. Angiotensin converting enzyme 2 (ACE2) hydrolyzes Ang II, and produces angiotensin1-7 (Ang 1-7). Ang (1-7) combines with theirs specific receptor MAS, and then opposes the actions of Ang II [6-8]. ACE2 protects cardiac function in CHF because of its vasodilatory properties and improvement of endothelial functions due to suppression of hyperplasia, inflammation and myocardial fibrosis and regulation of water salt balance in the body [9-11]. Literature search reveals that, the novel axis, ACE2-Ang (1-7)-Mas axis, an intrinsic part of the traditional ACE-Ang II-AT1 axis in RAS plays a negatively regulative role in the metabolic pathways of RAS [12]. These findings provided a

novel drug target for treatment of CHF. Studies have demonstrated that, ACE2 is up-regulated in myocardial tissues, during myocardial infarction and cardiac failure [13, 14]. Suppressors of RAS, such as ACEI, ARB, and aldosterone, enhance the potency of the drugs for cardiac failure at least in part via up-regulation of ACE2 and Ang (1-7) [12]. Recently, many ACE2 knockout studies further confirmed that ACE2 has an inhibitory effect in myocardial remodeling in CHF [14, 15]. Ang (1-7) exerts cardio-protective effects through activation of MAS receptors, although the underlying molecular mechanisms are still unclear. Many studies indicate-that a variety of pathways might be implicated in the interaction between Ang (1-7) and its receptors. ACE2 exerts its cardio-protective actions via phosphatidylinositol 3-kinase-protein kinase B-endothelial NO synthase (PI3K-Akt-eNOS) signaling pathway and regulating the expressions of a number of factors regulating apoptosis such as Bax, Bcl-2, cleaved/pro-caspase-3, cytochrome c in myocardial cells which in turn regulates cardiovascular homeostasis and vessel integrity [16, 17]. Sampaio et al demonstrated the protective role of Ang-(1-7) on endothelial cells via phosphorylation of endothelial NO synthase (eNOS) and release of NO via the PI3K-protein kinase B Akt-dependent pathway. Ang-(1-7) via Mas receptors also reduces the overall growth of myocytes [18, 19].

In this study, primary myocardial cells separated from rats were employed to identify the effects of ACE2/Ang (1-7)/Mas axis on myocardial cell apoptosis along with exploration of the probable underlying molecular mechanism.

Materials and methods

Animals

Wistar rats weighing 200-250 g were obtained from the Department of Cardiology, Henan Provincial People's Hospital, Henan, China. All animals were handled in accordance with the protocol approved by the Ethics Committee of Henan Provincial People's Hospital, Henan, China.

Primary myocardial cell culture

Neonatal Wistar rats underwent thoracotomy under sterile conditions to remove the heart using the iodophor disinfectant. Myocardial cells were separated by digestion with trypsin and type I collagen enzyme combined digestive method as well as by differential adhesion method. Cells were then planted onto the gelatin-covered 24-well plates at a density of 1×10^5/mL. Brdu (0.1 mmol/L) was added into each well to inhibit the growth of non-myocardial cells. Cells were cultured in an incubator with 5% CO_2 at 37°C. After 2 days of incubation, the medium was replaced by DMEM medium supplemented with 20% fetal calf serum. The concentrations of Ang II, Ang (1-7), and MAS receptor antagonist were measured at 100 nM.

Plasmids transfection

According to the sequence of rat ACE2 mRNA (NM_001012006.1), we designed a siRNA sequence (5'-GGTCACAATGGACAACTTC-3') targeting the ACE2 coding region. The corresponding oligonucleotide templates of the shRNA were chemically synthesized and cloned into the pSIH1-H1-copGFP shRNA Vector (System Biosciences, California, USA), which was digested by BamHI and EcoRI and purified by agarose gel electrophoresis. A scrambled RNAi sequence (5'-GAAGCCAGATCCAGCTTCC-3') was used as the negative control. The resultant plasmids were selected and confirmed by direct DNA sequencing.

Total RNA was extracted from rat myocardial cells, and reversely transcribed into cDNA using M-MLV reverse Transcriptase (Takara BIO, Japan), which was used to amplify the ACE2 coding sequence using the following primers: Forward primer: 5'-GCTCTAGAGCCACCATGTCAAGCTCCTGCTGGC-3' and Reverse primer: 5'-CGGGATCCTTAGAATGAAGTTTGAGC-3'. The product was purified and then ligated to the linear lentivector pcDNA-CMV-copGFP cDNA Vector (System Biosciences, USA). The ligation mixture was transformed into a competent DH5α strain and the positive clones were selected. The plasmids were extracted and then analyzed by PCR and sequencing.

An ACE2 expression vector (pAAV9-ACE2) was constructed by sub-cloning the full-length wild-type ACE2 coding sequence into pAAV9-EGFP which was confirmed by sequencing. The empty construct pAAV9-EGFP was transfected as a control. Cell transfections were conducted using Lipofectamine 3000 reagent (Invitrogen, CA, USA) following the manufacturer's protocol.

The rat myocardial cells, whether normal or infected were grouped as (1) normal cells in PBS and untreated (control); (2) normal cells when induced with Ang II; (3) normal cells, treated with Ang II via Ang (1-7); (4) cells treated with Ang II+Ang (1-7)+A779; (5) cells treated with Ang II and infected with pAAV9-ACE2; (6) cells induced by Ang II and infected with pAAV9-ACE2 and combined with A779; (7) cells infected with pAAV9-EGFP induced by Ang II.

MTT assay

The cell proliferative and invasive capacities were determined using a 3-(4, 5-dimethylthiazol-2-yl)-2 5-diphenyl-2H tetrazolium bromide (MTT) colorimetric assay and a Matrigel invasion chamber assay, respectively, according to standard methods described. Each experiment was performed three times.

Ang II-induced injury

Myocardial injury was induced by single intravenous injection of Ang II as previously described 28. Control rats received 0.9% NaCl solution (500 μl) through tail vein. In treatment groups, A779 (10 μg/Kg) was injected via rat tail vein 30 min before the induction of myocardial injury. All animals were breathing spontaneously during the experimental protocol. Eight hours after Ang II administration, animals were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/Kg) and euthanized by exsanguination. Serum sample were collected and stored at -80°C.

To further investigate the role of PI3K/AKT pathway in the development of CHF and the effects of ACE2 overexpression, PI3K/AKT specific inhibitors (LY294002 and L-NAME) were administered intraperitoneally 10 min before Ang II administration.

Apoptosis assay

Flow cytometry was performed to identify and quantify the apoptotic cells by Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China) in accordance with the manufacturer's protocol. The cells (100,000 cells/well) were seeded in 6 well-plates and the treated cells were washed twice with cold PBS and resuspended in buffer. The adherent and floating cells were combined and treated according to the manufacturer's instructions and then were measured on the flow cytometry (Beckman Coulter, USA) to differentiate apoptotic cells (Annexin-V positive and PI-negative) from necrotic cells (Annexin-V and PI-positive).

qRT-PCR

Total RNA was isolated from transfected cells by using TRIzol reagent (Invitrogen, OR, USA) and treated with DNaseI (Promega). Reverse transcription was performed by using the Multiscribe RT kit (Applied Biosystems) and random hexamers or oligo (dT). The reverse transcription conditions were 10 min at 25°C, 30 min at 48°C, and a final step of 5 min at 95°C.

Western blot

The protein was extracted using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Guangzhou, China). The proteins were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The western blot system was established using a Bio-Rad Bis-Tris Gel system according to the manufacturer's instructions. Primary antibodies were prepared in 5% blocking buffer at a dilution of 1:1000. Primary antibody was incubated with polyvinylidene difluoride (PVDF) membrane at 4°C overnight, followed by washing with TBST and the blots were incubated in secondary antibody marked by horseradish peroxidase at room temperature for 1 hour. The blots were probed with a primary antibody against rat ACE2 (1:500), ERK1/2 (1:400), SAPK/JNK (1:500), p38 MAPK (1:600), PI3K (1:500), AKT (1:300), and eNOS (1:500). After rinsing, the PVDF membrane carried blots and antibodies were transferred onto the Bio-Rad ChemiDoc™ XRS system, and then 200 μl Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, China).

Statistical analysis

All data are presented as mean ± standard deviation (SD) and all experiments were repeated at least thrice. Statistical analyses were performed using Prism 5.0 software package. Data were analyzed using one-way analysis of vari-

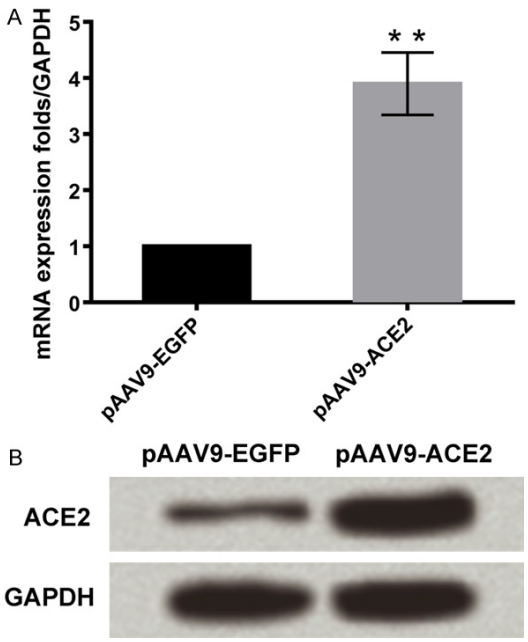

Figure 1. Effects of transfection on ACE2 expression. The expression of ACE2 in cells was overexpressed, and the mRNA (A) and protein (B) levels of ACE2 were determined to verify the transfection efficiency. ***P*<0.01.

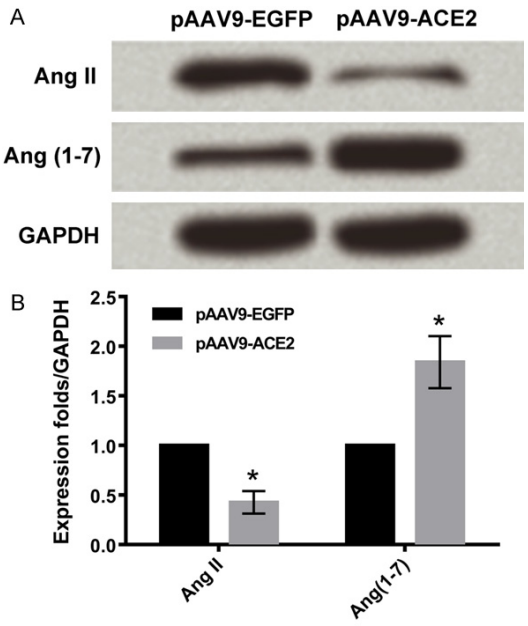

Figure 2. Effects of ACE2 overexpression on the Ang (1-7) and Ang II expression of in myocardial cells. After ACE2 was overexpressed in myocardial cells, AngII was down-regulated, while Ang (1-7) were up-regulated. **P*<0.05.

ance (ANOVA). *P*<0.05 was considered statistically significant.

Results

Effects of transfection on ACE2 expression

Compared to the pAAV9-EGFP control, the mRNA expression (**Figure 1A**) and protein levels (**Figure 1B**) of pAAV9-ACE2 was significantly increased (*P*<0.01). These results showed high efficiency of *ACE2* gene transfer into the rat myocardial cells.

Effects of overexpression of ACE2 on the expression of Ang (1-7) and Ang II in myocardial cells

Ang II levels in pAAV9-ACE2 cells when compared to that in pAAV9-EGFP control cells, showed a significant (*P*<0.05) decrease (down-regulated), whereas Ang (1-7) level was significantly (*P*<0.05) increased (up-regulated) in pAAV9-ACE2 cells in comparison to that in pAAV9-EGFP control cells. The results were shown in **Figure 2**.

ACE2 protected myocardial cells from AngII induced injury via up-regulation of Ang (1-7) and combination with MAS receptor

Activated Ang II directly induces myocardial remodeling, and accelerates the development and deterioration of CHF. Cell viability was examined by MTT assay where overexpression of Ang (1-7) and ACE2 antagonized Ang II, and protect myocardial cells from injury. ACE2 overexpression in rat heart markedly attenuated Ang II-induced cardiac injury and silencing of ACE2 expression noticeably increased Ang II-induced cardiac injury. Furthermore, pretreatment with either A779, a potent and competitive antagonist of Mas receptor (Ang (1-7) being the agonist of Mas receptor), significantly reversed the protective effects of ACE2 on Ang II-induced cardiac injury. It indicates that the effect of Ang (1-7) is mediated via MAS receptors. The groups of cells expressing Ang II+Ang (1-7)+A779 and Ang II+pAAV9-ACE2+A779 showed significant (*P*<0.05) reduction in cell viability in comparison to those of other cells (**Figure 3**).

ACE2 suppressed myocardial cells apoptosis via up-regulation of Ang (1-7) and combination with MAS receptor

The rate of apoptosis was determined using flow cytometry. The rates of apoptosis of myocardial cells in the groups Ang II+Ang (1-7)+A779

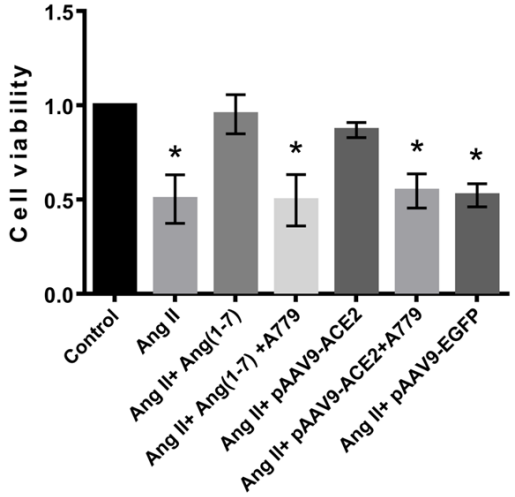

Figure 3. ACE2 protected myocardial cells from Ang II induced injury via up-regulation of Ang (1-7) following binding with MAS receptor. MTT assay displayed that, overexpression of Ang (1-7) and ACE2 could antagonize Ang II, and protect myocardial cells from injury. However, after addition of A779, antagonist of MAS receptor, the protective effects were inhibited, indicating that the effects of Ang (1-7) are mediated via MAS receptors. **P*<0.05.

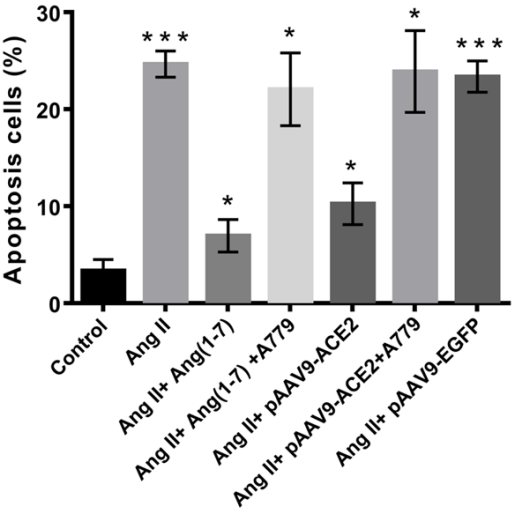

Figure 4. ACE2 suppressed myocardial cells apoptosis via up-regulation of Ang (1-7) and following binding with MAS receptor. Results from flow cytometry showed that, overexpression of Ang (1-7) and ACE2 could antagonize Ang II effects and suppress myocardial cells apoptosis. However, after addition of A779, the anti-apoptosis effects were inhibited. **P*<0.05; ****P*<0.001.

(Mean = 22.0667) and Ang II+pAAV9-ACE2+A779 (Mean = 23.9) were significantly (*P*<0.05) enhanced in comparison to those of myocardial cells of other groups (**Figure 4**). These indicated that the overexpression of Ang (1-7) and ACE2 antagonize Ang II effects resulting in suppression of cell apoptosis. However, the anti-apoptosis effects of Ang (1-7) were inhibited after addition of A779.

ACE2 blocked apoptosis pathways via up-regulation of Ang (1-7) following binding with MAS receptor

Bax, Bcl-2, cleaved/pro-caspase-3, and cytochrome c are the apoptosis related factors and the expression of these factors were observed under ACE2 overexpression. The results showed that overexpression of Ang (1-7) and ACE2 blocked the apoptotic pathways in the myocardial cells (**Figure 5**) through down-regulation of Bax, cytochrome *c* and pro-caspase-3 and up-regulation of Bcl-2. Increase in the pro-apoptotic factor such as Bax and other apoptotic proteins like cytochrome c and pro-caspase and decrease in the anti-apoptotic factor Bcl-2 in the cells treated with A779 showed enhanced apoptosis. The apoptotic rates of Bax, cytochrome c, pro-caspase, and Bcl-2 were not significant. Therefore, the protective effects of ACE2 overexpression on Ang II-induced apoptosis and the cardiac injury are mediated through regulation of the ACE2/Ang (1-7)/Mas receptor.

Effects of ACE2-Ang (1-7)-MAS axis on downstream pathways

The western blot results showed that Ang (1-7) and ACE2 were overexpressed in myocardial cells, and then the downstream pathways in the cells were detected. Results indicated that, ACE2-Ang (1-7)-MAS axis activated PI3K/AKT/eNOS pathway, while this axis could not affect ERK/JNK/p38MAPK pathway (**Figure 6**). Ang-(1-7) activates endothelial nitric oxide synthase (eNOS) through an Akt-dependent mechanism via the Mas receptor.

ACE2-Ang (1-7)-MAS axis protected myocardial cell from injury via regulating eNOS/ERK/JNK pathway

LY294002, a PI3K/AKT pathway inhibitor, was added into the cells, and observed that eNOS pathway was completely blocked (**Figure 7A**). However, after L-NAME (eNOS pathway inhibitor) was added, no influence was found in PI3K/AKT pathway (**Figure 7B**). These results indicated that PI3K/AKT pathway is upstream of eNOS

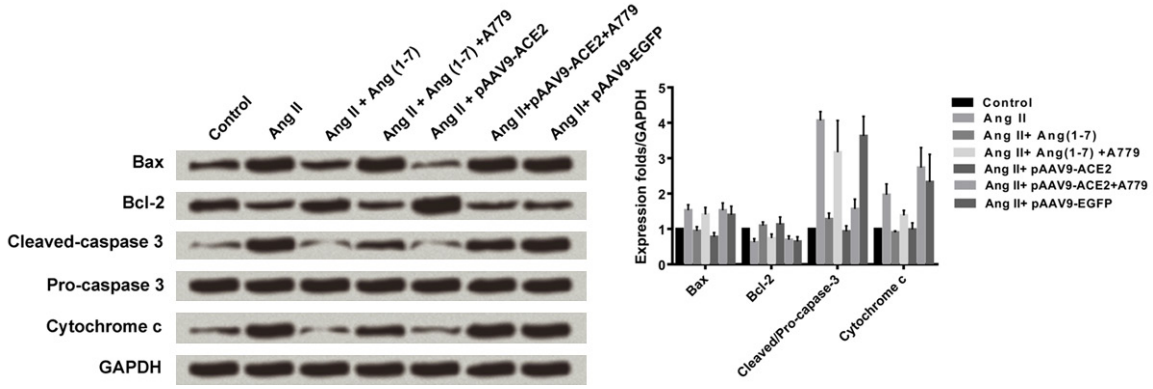

Figure 5. ACE2 blocked apoptosis pathways via up-regulation of Ang (1-7) following binding with MAS receptor. Overexpression of Ang (1-7) and ACE2 could block apoptosis pathways in myocardial cells.

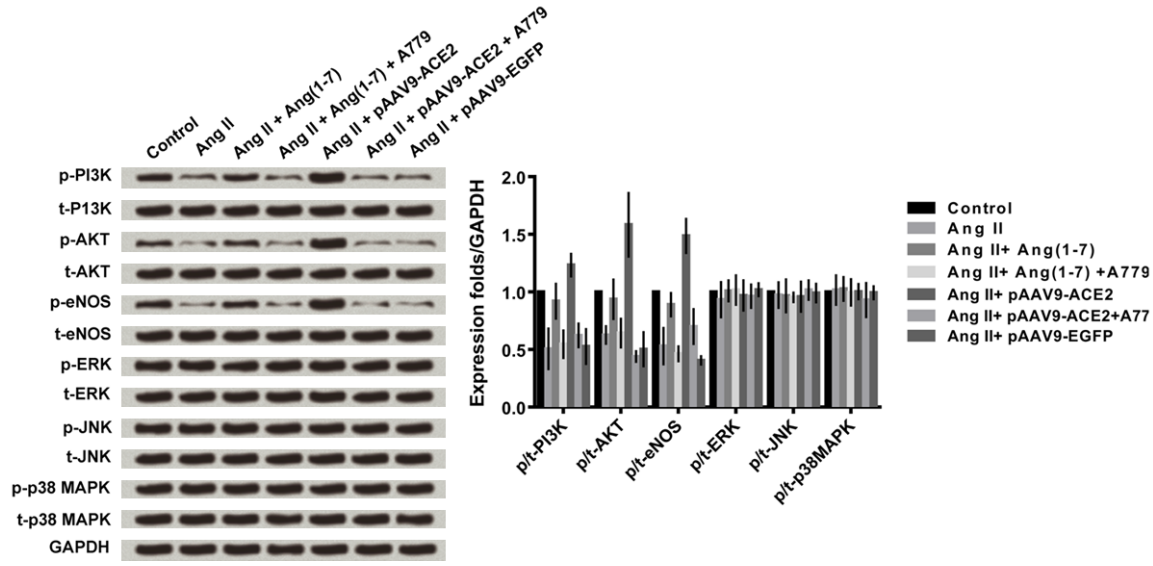

Figure 6. Effects of ACE2-Ang (1-7)-MAS axis on downstream pathways. Ang (1-7) and ACE2 were overexpressed in myocardial cells, and then the downstream pathways in cells were detected. Results indicated that, ACE2-Ang (1-7)-MAS axis activated PI3K/AKT/eNOS pathway, while this axis could not affect ERK/JNK/p38MAPK pathway.

pathway. Ang (1-7) increased the expression of p-Akt and p-eNOS, co-administration of Ang (1-7) with Ang II increased p-Akt and p-eNOS expression in the myocardial cells. In addition, ACE2/Ang (1-7)/Mas and LY294002 were able to block those effects of Ang (1-7)-induced injury, thus protecting the myocardial cells from injury.

Discussion

In this study, we investigated the effects of overexpressed ACE2 on Ang II induced myocardial injury contributes to the etiology and pathogenesis of CHF. We also explored the biological effects and possible underlying mechanism of ACE2/Ang (1-7)/MAS axis on myocardial cells apoptosis *in vitro*. ACE2 hydrolyzes Ang II, and produces Ang (1-7); the biological effect of Ang (1-7) is mediated via MAS receptor. Overexpression of Ang (1-7) and ACE2 promoted myocardial cell survival and suppressed Ang II induced myocardial apoptosis ($P<0.05$). ACE2-Ang (1-7)-MAS axis down-regulates Bax, cytochrome *c* and active caspase-3 and up-regulates Bcl-2, thus inhibiting myocardial cell apoptosis. We found that ACE2-Ang (1-7)-MAS axis activated PI3K/AKT and eNOS pathways, where PI3K/AKT pathway acts upstream of eNOS pathway. Thus, ACE2-Ang (1-7)-MAS axis promoted cell

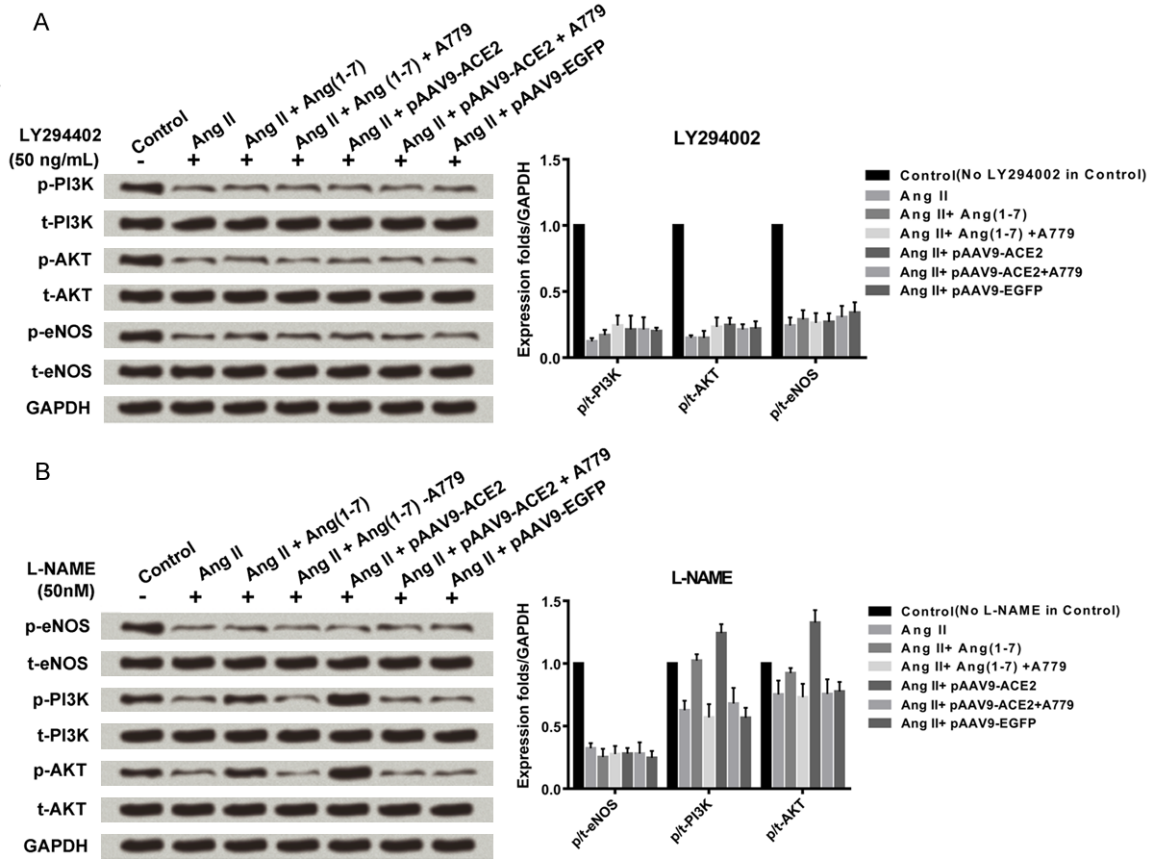

Figure 7. ACE2-Ang (1-7)-MAS axis protected myocardial cell from injury via regulating eNOS/ERK/JNK pathway. A: LY294002, a PI3K/AKT pathway inhibitor, was added into cells, and we found that eNOS pathway was completely blocked. B: Addition of L-NAME (eNOS pathway inhibitor) did not affect the PI3K/AKT pathway. These results indicated that PI3K/AKT pathway is upstream of eNOS pathway.

survival and suppressed cell apoptosis via activation of PI3K/AKT/eNOS pathway. Together, these findings clarified the functional role and the possible underlying mechanism of ACE2-Ang (1-7)-MAS axis on myocardial cells apoptosis and provide a novel drug target for treating CHF.

RAS plays an important role in blood pressure regulation as well as water balance. Ang II is one of the main components of RAS that has its function in cardiovascular system. Ang (1-7) can be directly formed from Ang I or II by ACE2. ACE2 with Ang (1-7) and Mas [a G-protein coupled receptor for Ang (1-7)] forms a complex and is considered as a biologically active component of RAS. This complex further shows its function in cardiomycytes during physiological and pathological conditions. Since ACE2 converts Ang II to Ang-(1-7), it is shown that down-regulation of ACE2 by Ang II favors Ang II-mediated responses, by preventing its degradation to Ang-(1-7) [20]. Therefore, these conditions exert the generation of excess Ang II and reduce Ang II breakdown which might lead to more deleterious effects on the heart. Grobe *et al* in their study showed that Ang-(1-7) administration prevented cardiac fibrosis induced by Ang II in Sprague-Dawley rat cardiac fibrosis models [21]. In another study by Shah, *et al*, which included two-kidney one-clip hypertensive rats, it was demonstrated that Ang-(1-7) decreased fibrosis and up-regulated Mas, AT2, and endothelial NOS phosphorylation in the heart [22]. In other studies which involved ACE2-deficient mice, demonstrated lower levels of Ang-(1-7) with evidences of early cardiac hypertrophy [23] and adverse ventricular remodeling after myocardial infarction [24]. Few studies supported that ACE2 deficiency showed progressive increase in cardiac fibrosis and cardiac pressure overload [25-27]. Similarly some studies showed that ACE2 overexpression reversed

cardiac hypertrophy and fibrosis in mice [28, 29]. The addition of Mas receptor showed anti-proliferative and anti-fibrotic effects in the myocytes [30]. Our study results were consistent with these results.

Our study demonstrated that Ang-(1-7) plays an important role in the regulation of the cardiovascular system. Dysregulation of the Ang-(1-7) and the signaling pathways may contribute to the myocardial cell injury. Ang-(1-7)/Mas activates the PI3K/Akt/eNOS pathway, leading to the production of NO in myocardial cells. These further confirmed that eNOS and Akt act as important downstream effectors of Ang-(1-7)/Mas-mediated pathway in the myocardial cells and resulted in the beneficial effects in the heart with the help of Ang-(1-7). Our data demonstrated that PI3K/Akt/eNOS signaling pathway was associated with Ang-(1-7)/Mas axis in myocardial cells that regulates myocardial functions.

In conclusion, the data from the previous studies and the current study suggests that AEC2 has cardio protective properties mediated by generation of Ang-(1-7), which in turn stimulates ACE2/Ang (1-7)/MAS axis-mediated signaling pathway. This study provides a theoretical basis for development of a novel drug target in the treatment of CHF.

Disclosure of conflict of interest

None.

Address correspondence to: Zhentao Wang, Department of Cardiology, Henan Province Hospital of TCM, 6 Dongfeng Road, Zhengzhou 450002, China. E-mail: wangzhentao0028@126.com

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