Silencing of angiotensin-converting enzyme by RNA interference prevents H9c2 cardiomyocytes from apoptosis induced by anoxia/reoxygenation through regulation of the intracellular renin-angiotensin system

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Abstract. Inhibition of the angiotensin-converting enzyme (ACE) attenuated apoptotic cardiomyocytes induced by ischemic reperfusion (I/R). However, it is difficult to evaluate the effects of inhibition of the intracellular ACE *in vivo*. The objective of this study was to determine whether the apoptosis in H9c2 cardiomyocytes following anoxia/reoxygenation (A/R) would be improved by the silencing of intracellular ACE by RNA interference (RNAi). H9c2 cardiomyocytes were subjected to A/R 48 h following transfection with ACE-shRNA plasmid. The results showed that the gene silencing of intracellular ACE significantly inhibited the decrease of cell viability and the increase of apoptotic H9c2 cardiomyocytes undergoing A/R. Additionally, the gene silencing of intracellular ACE significantly promoted the expression of ACE2, decreased caspase-3 activity and Bax levels, and enhanced the expression of Bcl-2 in H9c2 cardiomyocytes subjected to A/R. The results suggest that the gene silencing of intracellular ACE holds great potential in the treatment of cardiomyocyte apoptosis following I/R injury through the regulation of the intracellular renin-angiotensin system, thereby regulating the intrinsic pathway of apoptosis.

Introduction

Early reperfusion has been widely utilized for impending acute myocardial infarction (AMI) by thrombolysis or primary percutaneous coronary angioplasty (1,2). Although ventricular remodeling and dysfunction subsequent to AMI are considerably improved by restoration of the blood flow, reperfusion itself results in additional myocardial ischemia-reperfusion (I/R) injury (3-5), which counteracts the protective effect of reperfusion therapy. Mounting evidence has demonstrated that apoptosis contributes significantly to myocardial I/R injury (4,5).

Angiotensin converting enzyme (ACE) is an important member of renin-angiotensin system (RAS). ACE cleaves angiotensin I (Ang I) to produce the biologically active angiotensin II (Ang II), a vital peptide molecule in RAS, which may stimulate apoptosis following I/R (6). In general, the RAS exists as classical circulating RAS and as a local tissue RAS (7,8). Classically, Ang II can be generated in the circulation by ACE and delivered to target tissues or cells. Furthermore, ACE2 is capable of hydrolyzing Ang II to Ang-(1-7) and Ang I to Ang-(1-9) (9-11), and regulating the balance of RAS activation. Findings of a previous study demonstrated that ACE inhibitors prevented cardiomyocytes from apoptosis induced by I/R via the inhibition of ACE with a decrease of Ang II levels (12). However, the effect of ACE cannot be completely blocked as ACE inhibitors may upregulate ACE levels in myocardium and plasma through a feedback mechanism (13). In addition, the local tissue RAS includes two forms: intracellular and extracellular (7,8). Evidence revealed that the morbidity and mortality were significantly reduced by using ACE inhibitors in patients with MI (14-16). Notably, it was found that the concentrations of Ang II in local tissue may exceed those in plasma. However, it is difficult to separate the effects of inhibiting intracellular ACE from systemic ACE inhibition *in vivo*, whereas it is easy to evaluate the effects of intracellular RAS inhibition *in vitro*.

Gene therapy has been increasingly applied to inherited or acquired diseases at their genetic levels (17,18). As a new knocking gene-technique, RNA interference (RNAi) has been demonstrated to be useful for the treatment of diseases by reducing the production of target RNA and proteins that are a hindrance to identification of disease (19). To avoid the feedback elevation of ACE when using ACE inhibitors and more completely block the effects of ACE, silencing of the ACE gene by RNAi may be an ideal option.

Results of previous studies demonstrated that the injury of cardiomyocytes induced by anoxia/reoxygenation (A/R) is

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a useful *in vitro* model for examining myocardial I/R injury (20,21). The aim of this study was to investigate whether the apoptosis of H9c2 cells subjected to A/R would be improved through the silencing of intracellular ACE by RNAi in order to regulate the intracellular RAS, thereby regulating the intrinsic pathway of apoptosis.

Materials and methods

ACE-shRNA plasmid construction. A series of 21-nucleotide siRNA duplexes (AAC CTA ACA TGT CAG CCT CTG) against the rat ACE consensus coding sequence (GenBank accession no. NM012544) was designed. Sequences were determined to be unique to the rat gene by basic local alignment search tool (BLAST) searches of the GenBank database. The target sequence was designed with a randomly selected nonsense sequence to serve as the negative control. The series was designed into a shRNA oligonucleotide template consisting of sense, hairpin loop, antisense and terminator sequences, all of which were flanked by restriction enzyme sites to facilitate directional subcloning. The DNA sequence coding for the rat ACE-shRNA was synthesized and cloned into a pGenesil-1 plasmid encoding for enhanced green fluorescent protein (EGFP). The shRNA was confirmed by sequencing.

Cell culture and transfection. H9c2 cardiomyocytes (Chinese Academy of Sciences Cell Bank, Shanghai, China), a subclone of the original cell line derived from embryonic BD1X rat heart tissue, were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, UT, USA) containing 10% (v/v) fetal bovine serum (FBS, Invitrogen Corp., Carlsbad, CA, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were maintained at 37˚C in a humidified atmosphere with 5% CO_2. The medium was replaced every 2-3 days, and cells were subcultured or subjected to experimental procedures.

H9c2 cardiomyocytes were seeded ($1.0x10^5$ cells/well) in 6-well plates 1 day prior to transfection. Transfection was performed strictly according to the manufacturer's instructions regarding the Lipofectamine® LTX and Plus™ transfection reagent (Life Technologies, Grand Island, NY, USA). Six hours after transfection, the medium was replaced with fresh DMEM (10% FBS, v/v). The cells were incubated for 48 h and was observed under a fluorescence microscope (Olympus IX51; Olympus, Tokyo, Japan). The percentages of EGFP-expressing cells following the plasmid transfection were quantified using a flow cytometer (FACSort; Becton-Dickinson, San Jose, CA, USA). H9c2 cardiomyocytes were collected for EGFP assay at 48 h post-transfection. EGFP fluorescence was calculated as the percentage of EGFP cells present in a total of 10^4 cells.

A/R injury model. The A/R procedures used in this study were similar to those described in a previous study (22). H9c2 cardiomyocytes were washed with PBS and incubated in serum-free DMEM. The cells in DMEM were placed in a gas transfusive apparatus (Changjing Biotech Co., Beijing, China), and anoxic gas (95% N_2/5% CO_2) was flushed into the gas transfusive apparatus to reduce the pO_2 to 0 mmHg. Subsequent to 3 h of anoxia at 37˚C, reoxygenation was achieved by changing the medium into DMEM with 10% (v/v) FBS followed by exposure of cells to room air (CO_2 incubator).

Experimental groups and protocols. The cultured H9c2 cardiomyocytes were randomly divided into different groups. In the control (Con) group, the H9c2 cardiomyocytes were cultured under normal conditions for 12 h. The A/R group was managed as described in the preceding section. In the negative control (NC) group and the ACE-shRNA plasmid-treated group (shRNA), the H9c2 cardiomyocytes were subjected to A/R 48 h following transfection with the negative control ACE-shRNA plasmid or ACE-shRNA plasmid.

Cell viability assay. Cell viability was determined by the cell counting kit (CCK)-8 assay (Dojindo, Tokyo, Japan). The experimental procedure was conducted according to the manufacturer's instructions. H9c2 cardiomyocytes were subcultured at $1x10^4$ cells/well in 96-well plates and incubated for 1 day prior to being divided into the Con, A/R, NC and shRNA groups. Each group included 9 assays and each assay was averaged from the absorbance of 4 wells. Prior to the anoxia or following the A/R, 10 μl of WST-8 solution (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) was added to each well, and the H9c2 cardiomyocytes were incubated for an additional 2 h at 37˚C. The absorbance of each well at 450 nm was measured with a reference at 630 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The percentage of cell viability was calculated using the formula: cell viability (%) = (At/Ac) x 100, where At is the mean absorbance in test wells and Ac is the mean absorbance in control well.

Apoptosis. Apoptosis was determined by Annexin V and propidium iodide (PI) double staining. H9c2 cardiomyocytes were centrifuged to remove the medium, washed with PBS, and stained with Annexin V and PI in binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$), according to the manufacturer's instructions (BioVision, Inc., Palo Alto, CA, USA). Ten thousand events were collected for each sample. Stained cells in the FL1-H and FL2-H channels were analyzed using a flow cytometer (FACSort, Becton-Dickinson).

Measurement of caspase-3 activity. Caspase-3 activity was evaluated by using caspase-3 colorimetric assay kit (BioVision, Inc.). A total of 10^6 cells were collected by centrifugation, and the pellet was resuspended in lysis buffer. Protein levels were determined with bicinchoninic acid assay (Beyotime Biotechnology, Co., Ltd., Shanghai, China). Caspase-3 activity was detected in equal amounts of cell lysates with synthetic peptide substrate Ac-DEVD-pNA, as described in the manufacturer's instructions. Caspase-3 activity was expressed as the optical density, with absorbance at 405 nm of the released pNA being monitored using a spectrophotometer (UV762; Shanghai Precision and Scientific Instrument Co., Shanghai, China).

Quantitative reverse transcription PCR analysis (qRT-PCR). Total-RNA was prepared from cells with TRIzol reagent (Invitrogen Corp.). For quantitative PCR analysis, reverse transcription was performed to produce cDNA from total RNA with oligo(dT), and the fragments were amplified with SYBR-Green-based assays kit (Invitrogen Life Technologies) according to the manufacturer's instructions. The RT-PCR conditions were 42˚C/15 min, 95˚C/2 min for reverse transcription; 95˚C/30 sec,

58.9˚C (ACE) or 60˚C (GAPDH)/30 sec, and 72˚C/60 sec, over 40 cycles for PCR. ACE mRNA levels were calculated based on the method of $^{2-\Delta\Delta}$CT between the intervention and control groups. GAPDH was used for normalization, and the comparative threshold method was used to assess the relative abundance of ACE mRNA. The specific primer sequence and amplicon size of the selected genes used were: ACE, sense: 5'-GCCTCCCAACGAGTTAGAAGAG-3', antisense: 5'-CGGGACGTGGCCATTATATT-3'; GAPDH, sense: 5'-GACAACTTTGGCTCGTGGA-3', antisense: 5'-ATGC AGGGGTTCTGG-3'. Primers were synthesized by Shanghai Sangon Biological Engineering Technology Company Limited. Correctness of the gene order was proven in GenBank.

Western blot analysis. Membranous protein was prepared using membranous extraction reagents (Pierce Biotechnology, Inc., Rockford, IL, USA) and mitochondrial protein was extracted using a mitochondria/cytosol fractionation kit (BioVision, San Francisco, CA, USA) according to the manufacturer's instructions. Protein concentration was determined by the bicinchoninic acid protein assay (Beyotime Biotechnology, Co., Ltd.,). Equal amounts (50 μg) of denatured proteins were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membrane. The membranes were blocked with 5% non-fat dry milk in TBST (containing 0.05% Tween-20), and incubated overnight at 4˚C with the primary antibody (ACE, 1:500, Cell Signaling Technology, Inc., Beverly, MA, USA; ACE2, 1:200, Cell Signaling Technology, Inc.; Bax, 1:500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; Bcl-2, 1:500, Santa Cruz Biotechnology, Inc.). The blots were then washed and incubated with horseradish peroxidase-conjugated second antibody (goat anti-rabbit IgG, 1:2,000, Beyotime Biotechnology) for 1 h under room temperature. Immunoreactivity was enhanced by chemiluminescence kit (Beyotime Biotechnology, Co., Ltd.) and exposed to film. β-actin was used as an internal control to correct the variations of different samples. The density of bands on western blots was quantified by using a Bio-Rad image system.

Ang II measurement. The levels of Ang II in the culture medium were measured by enzyme-linked immunosorbent assay (ELISA), using commercially available kits (Zhong Shan-Golden Bridge Biological Technology Co., Beijing, China) according to the manufacturer's instructions.

Statistical analysis. Data are presented as the means ± SD. Statistical analyses of data were performed by one-way ANOVA followed by the Student-Newman-Keuls test. P<0.05 was considered statistically significant.

Results

Efficiency of H9c2 cells transfection. Forty-eight hours after ACE-shRNA plasmid transfection, (76.8±5.1)% of H9c2 cardiomyocytes were positive for green fluorescence (Fig. 1).

Cell viability. CCK-8 assay was performed to investigate the cytotoxicity of plasmid-LTX-Plus in H9c2 cardiomyocytes. The results demonstrated that there were no significant differences in viability between experimental groups prior to A/R (Fig. 3). However, transfection of the ACE-shRNA plasmids significantly prevented the loss of H9c2 cardiomyocyte viability induced by A/R (P<0.05) (Fig. 2).

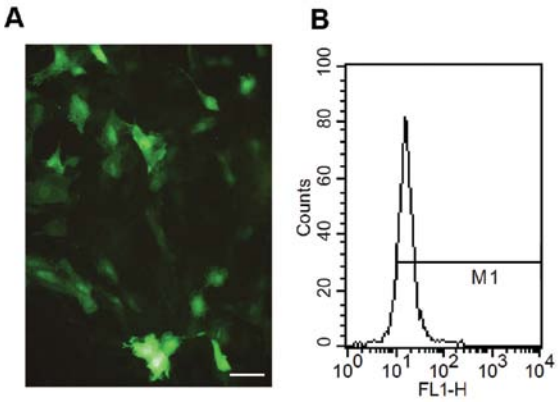

Figure 1. Positively transfected cells under fluorescence microscopy and the transfection rate in H9c2. (A) Representative images of the cells emitting green fluorescence under a fluorescence microscope; scale bar, 5 μm. (B) Representative images of the transfection rate in H9c2 cells at 48 h *in vitro* by flow cytometry.

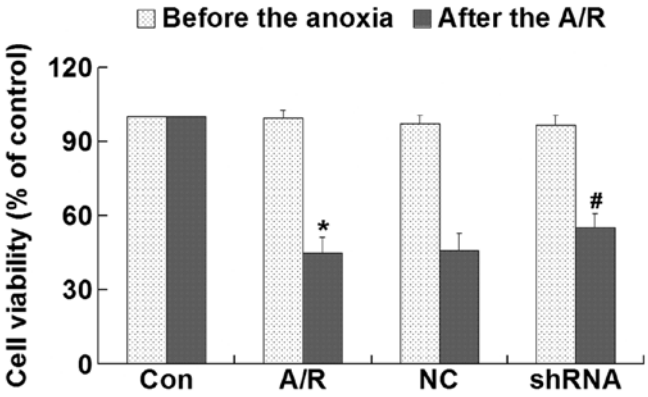

Figure 2. Angiotensin-converting enzyme (ACE)-shRNA plasmid transfection protects against anoxia/reoxygenation (A/R)-induced cell death in H9c2 cardiomyocytes. After the H9c2 cardiomyocytes were transfected with ACE-shRNA plasmids under normal conditions or undergoing A/R, cell viability was measured by CCK-8 assay. Data are expressed as means ± SD (n=9). *P<0.05 vs. the control (Con) group; #P<0.05 vs. the A/R group.

ACE mRNA and protein expression. The expression of ACE mRNA was significantly increased in H9c2 cardiomyocytes undergoing A/R (P<0.05). However, ACE-shRNA plasmid transfection significantly decreased the upregulation of ACE mRNA expression induced by A/R in H9c2 cardiomyocytes (P<0.05) (Fig. 3A). Similar to the qRT-PCR result, western blot analysis revealed that the ACE protein level was significantly lower in the shRNA group compared with that of the A/R group (P<0.05) (Fig. 3B and C). The negative control ACE-shRNA plasmid transfection did not affect A/R-induced ACE mRNA and protein expression.

ACE2 protein expression. The ACE2 protein level was upregulated in H9c2 cardiomyocytes following A/R (P<0.05). However, transfection of ACE-shRNA plasmids significantly promoted elevation of the ACE2 level induced by A/R

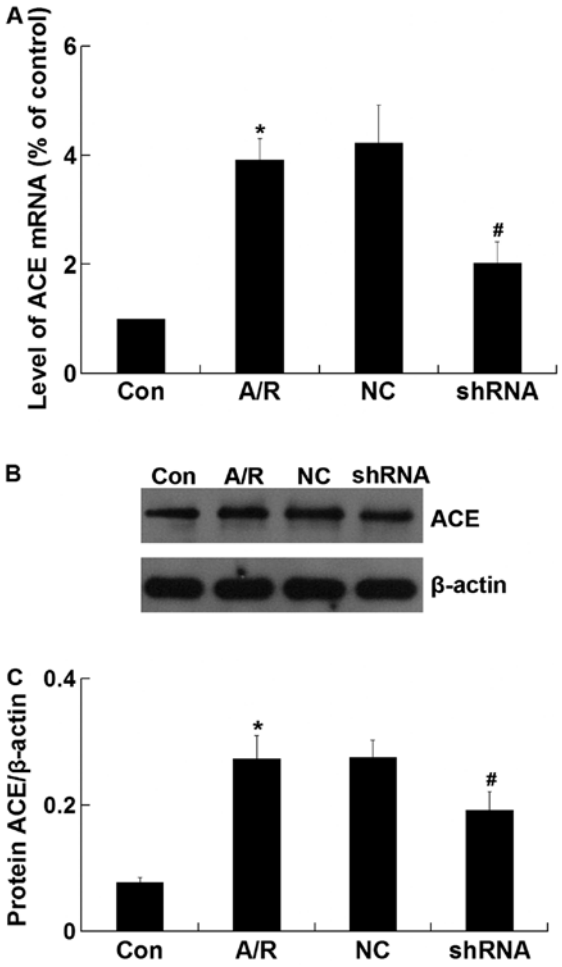

Figure 3. Angiotensin-converting enzyme (ACE) expression in the different experimental groups. (A) ACE mRNA expression was examined by reverse transcription PCR. The results were expressed as the relative expression to GAPDH and plotted as the ratio of the control (Con) group. (B) Representative western blot analyses of ACE and β-actin expression. (C) Bands were analyzed and quantified by densitometry and the ACE/β-actin ratio was evaluated. Data are expressed as means ± SD (n=9). *P<0.05 vs. the Con group; #P<0.05 vs. the anoxia/reoxygenation (A/R) group.

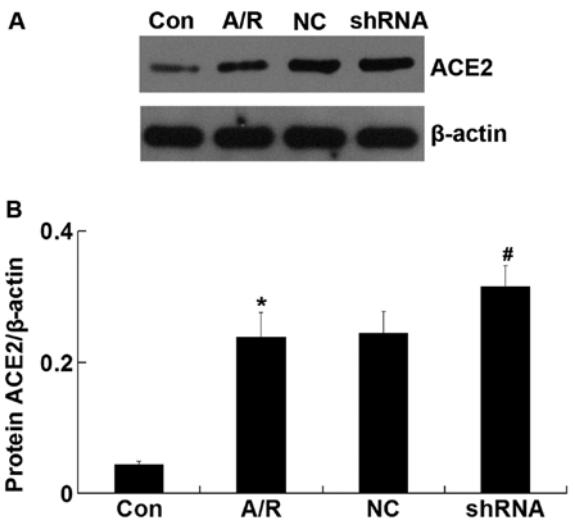

Figure 4. Angiotensin-converting enzyme 2 (ACE2) protein level in the different experimental groups. (A) Representative western blot analyses of ACE2 and β-actin expression. (B) Bands were analyzed and quantified by densitometry and the ACE2/β-actin ratio was evaluated. Data are expressed as means ± SD (n=9). *P<0.05 vs. the control (Con) group; #P<0.05 vs. the anoxia/reoxygenation (A/R) group.

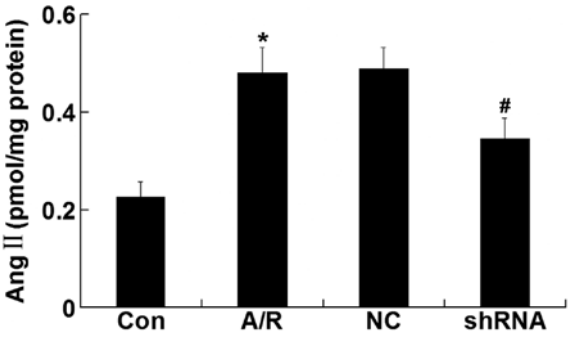

Figure 5. Angiotensin II (Ang II) level in the culture in different experimental groups. Data are expressed as means ± SD (n=9). *P<0.05 vs. the control (Con) group; #P<0.05 vs. the anoxia/reoxygenation (A/R) group.

(P<0.05) but remained unchanged by transfection of the negative control ACE-shRNA plasmids (Fig 4).

Ang II level. The Ang II level was elevated in H9c2 cardiomyocytes undergoing A/R (P<0.05). However, ACE-shRNA plasmid transfection significantly inhibited the elevation of Ang II level induced by A/R (P<0.05) although it remained unchanged by transfection of the negative control ACE-shRNA plasmids (Fig. 5).

Apoptosis. Flow cytometry was used to quantify the rate of cell apoptosis. Apoptosis was promoted in H9c2 cardiomyocytes undergoing A/R (P<0.05). Transfection ACE-shRNA plasmids showed a significant resistance in apoptosis in H9c2 cardiomyocytes undergoing A/R (P<0.05). The negative control ACE-shRNA plasmid treatment was without effect on A/R-induced apoptosis (Fig. 6).

Activity of caspase-3. The activity of caspase-3 was enhanced in H9c2 cardiomyocytes after A/R (P<0.05). However, transfection of the ACE-shRNA plasmids significantly inhibited the activation of caspase-3 induced by A/R (P<0.05) but remained unchanged by treatment with the negative control ACE-shRNA plasmids (Fig. 7).

Bcl-2 and Bax protein expression. Bax, Bcl-2 and the Bax/Bcl-2 ratio were significantly increased in H9c2 cardiomyocytes subjected to A/R (P<0.05). However, transfection of the ACE-shRNA plasmids significantly inhibited the elevation of BAX and Bax/Bcl-2 ratio and promoted the upregulation of Bcl-2 induced by A/R (P<0.05) but remained unchanged by transfection of the negative control ACE-shRNA plasmids (Fig. 8).

Discussion

To the best of our knowledge, the present study has demonstrated for the first time that pretreatment with ACE-shRNA plasmids markedly suppressed the increase of intracellular ACE expression and Ang-II level induced by A/R in H9c2

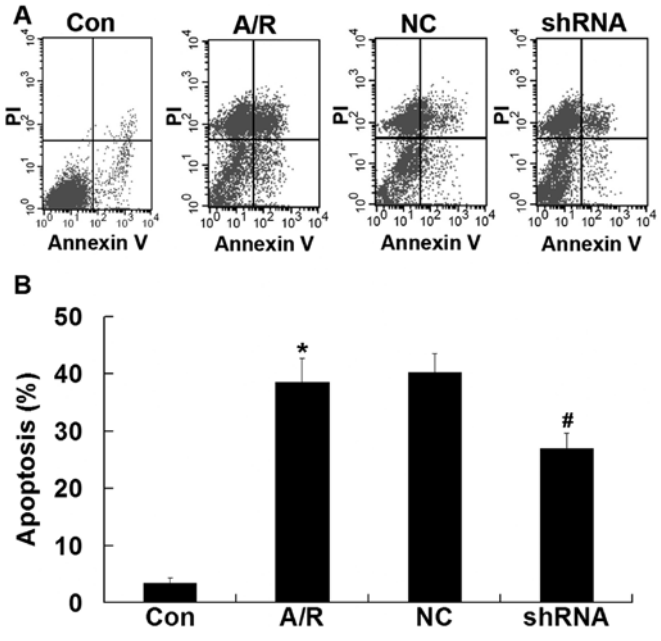

Figure 6. Effect of angiotensin-converting enzyme (ACE)-shRNA plasmid transfection on the apoptosis in H9c2 cardiomyocytes subjected to anoxia/reoxygenation (A/R). (A) Detection of apoptotic cells by Annexin V and propidium iodide (PI) double staining. Cardiomyocytes were treated with ACE-shRNA plasmids, stained with Annexin V and PI labeling and analyzed by flow cytometry. (B) Column bar graph of apoptosis. Data are expressed as means ± SD (n=9). *P<0.05 vs. the control (Con) group; #P<0.05 vs. the A/R group.

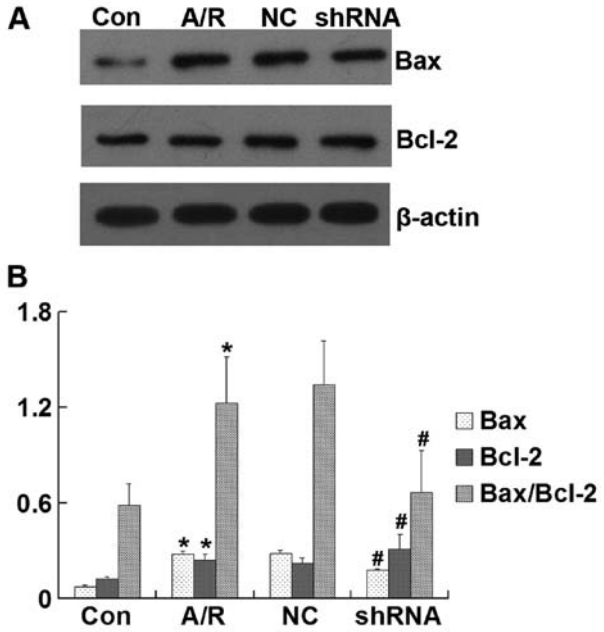

Figure 8. The levels of Bax and Bcl-2 protein and the Bax/Bcl-2 ratio in mitochondria of H9c2 cardiomyocyte in the different experimental groups. (A) Representative western blot analyses of Bax, Bcl-2 and β-actin expression. (B) Bands were analyzed and quantified by densitometry and the Bax/β-actin, Bcl-2/β-actin and Bax/Bcl-2 ratios were evaluated. Data are expressed as means ± SD (n=9). *P<0.05 vs. the control (Con) group; #P<0.05 vs. the anoxia/reoxygenation (A/R) group.

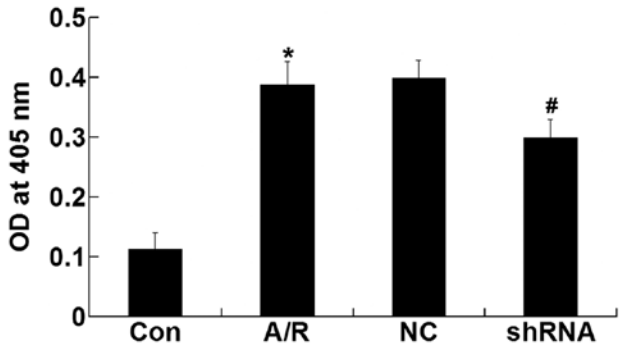

Figure 7. Effect of angiotensin-converting enzyme (ACE)-shRNA plasmid transfection on caspase-3 activity in H9c2 cardiomyocytes exposed to anoxia/reoxygenation (A/R). Caspase-3 activity was measured as described in Materials and methods. Data are expressed as means ± SD (n=9). *P<0.05 vs. the control (Con) group; #P<0.05 vs. the A/R group.

cardiomyocytes. Gene silencing of intracellular ACE significantly inhibited the decrease of cell viability and increase of apoptotic H9c2 cardiomyocytes undergoing A/R. At the same time, we demonstrated that the gene silencing of intracellular ACE significantly promoted the expression of ACE2, decreased caspase-3 activity and Bax levels, and enhanced the expression of Bcl-2 in H9c2 cardiomyocytes subjected to A/R. The results suggest that the gene silencing of intracellular ACE has great potential in the treatment of cardiomyocyte apoptosis after I/R injury by regulating the intracellular RAS, and regulating the mitochondrial pathway of apoptosis.

The measure of cell viability is usually used as indicator of cell damage. In this study, we demonstrated that the cell viability was decreased in H9c2 cardiomyocytes subjected to A/R. However, inhibition of the intracellular ACE gene expression significantly attenuated cell injury induced by A/R in H9c2 cardiomyocytes, suggesting that the gene silencing of intracellular ACE is capable of protecting H9c2 cardiomyocytes against A/R injury.

Following MI, the apoptotic process of cardiomyocytes is initiated and becomes markedly accelerated during early reperfusion (23,24). Extracellular stimuli trigger apoptosis by the death receptor- and mitochondrial-mediated pathways (25). Caspase-3 activity, a key terminal effector of apoptosis, was found to increase subsequent to I/R injury (26). Consistent with previous results (4,23,24), the results of the present study demonstrated that apoptosis and caspase-3 activity were significantly increased in H9c2 cardiomyocytes subjected to A/R. Transfection of the ACE-shRNA plasmids for 48 h prior to the onset of A/R significantly inhibited the apoptosis and caspase-3 activity by decreasing the levels of ACE and Ang II, suggesting that intracellular RAS is important in cardiomyocyte apoptosis induced by A/R. Gene silencing of intracellular ACE protects cardiomyocytes against A/R injury by anti-apoptosis. ACE gene silencing and ACE inhibitors are capable of reducing Ang II levels, whereas ACE inhibitors increase ACE expression in cardiomyocytes by a feedback mechanism (13). Additionally, ACE gene silencing decreased ACE expression during A/R, which constitute the essential differences in the underlying mechanism of anti-apoptosis between ACE gene silencing and ACE inhibitors during I/R. In addition to systemic ACE inhibition, the gene silencing of intracellular ACE may be another important way to protect cardiomyocytes against I/R injury.

The Bcl-2 family proteins are significant regulators in the intrinsic pathway of apoptosis (27,28). Bcl-2, one of the anti-apoptotic members of this family, is able to decrease apoptosis by preventing the release of cytochrome *c* (an apoptosis-inducing factor) (29,30). Conversely, Bax, one of the pro-apoptotic members of this family, is able to induce apoptosis by promoting cytochrome *c* release (31). The ratio of BCL-2/Bax plays a pivotal role in the mitochondrial pathway of apoptosis (32,33). Findings of previous studies showed that the infarct size and apoptosis were reduced by the overexpression of Bcl-2 or knockout of Bax in mice after I/R injury (34,35). Hypoxia is capable of inducing the p53 expression followed by the upregulation of the Bax level in cardiomyocytes (36,37). Similar to those results, our results demonstrated that, with the increase of Bax/Bcl-2 ratio in H9c2 cardiomyocytes subjected to A/R, apoptosis was significantly increased. However, the gene silencing of intracellular ACE significantly inhibited the increase of Bax/Bcl-2 ratio induced by A/R in H9c2 cardiomyocytes, thereby inhibiting caspase-3 activation and resulting in the lower apoptosis. This finding suggests that the gene silencing of intracellular ACE, with the inhibition of apoptosis induced by A/R, may be related to the regulation of the mitochondrial pathway of apoptosis in cardiomyocytes.

In addition to the classical circulating RAS, previous studies have demonstrated the existence of an intracellular RAS in several cell types, such as cardiomyocytes, fibroblasts and vascular smooth muscle cells (7,38). Moreover, the production of intracellular Ang II generally includes ACE-dependent and/or ACE-independent (cathepsin D and chymase) mechanisms in various pathological conditions (39,40). Singh *et al* demonstrated that the chymase is responsible for Ang II synthesis in cardiomyocytes in high-glucose conditions (41). However, both ACE and chymase are responsible for Ang II synthesis in cardiomyocytes subjected to I/R (39). Our data revealed that the synthesis of Ang II was significantly reduced in H9c2 cardiomyocytes after A/R by ACE gene silencing. This suggested that, at least in part, the ACE is responsible for Ang II synthesis in H9c2 cells under A/R conditions.

ACE2, an enzyme sharing some homology with ACE, was identified in 2000 (9,10). However, unlike ACE, the major substrate of ACE2 appears to be Ang II, rather than Ang I (11). In addition, ACE2 activity cannot be inhibited by ACE inhibitors (9,10). ACE2 is able to catalyze the conversion of Ang II to Ang1-7, which opposes the actions of Ang II (42,43). It has been suggested that ACE2 may be crucial in regulating the local levels of Ang II and Ang1-7 (44). Previous studies showed that ACE2 gene silencing increased the level of Ang II in the heart of mice (45,46). In addition, the expression of ACE2 was increased in patients with heart failure induced by ischemia (47). In their study, Ferrario *et al* found that treatment with ACE inhibitors increased ACE2 activity (48). Consistent with that result, our study has shown that ACE gene silencing significantly upregulated the expression of ACE2 in H9c2 cardiomyocytes undergoing A/R. These results suggest that gene silencing of intracellular ACE, with upregulation of Ang II level induced by A/R inhibited, may be associated with the increase of ACE2. Previously, it was demonstrated that aldosterone inhibited ACE2 gene expression by a minarelocorticoid receptor and Ang II did not reduce ACE2 expression in cultured neonatal rat cardiomyocytes (49,50). However, Ang II might indirectly decrease the ACE2 gene expression by increasing the aldosterone level *in vivo* (50). The underlying mechanism of the elevation of ACE2 expression in H9c2 cardiomyocytes after A/R by ACE gene silencing, however, remains to be elucidated.

Taken together, the present study has shown that the inhibition of apoptosis induced by A/R in H9c2 cardiomyocytes by ACE gene silencing is associated with the downregulation of ACE and upregulation of ACE2, resulting in the decrease of Ang II level, and consequently the regulation of the intrinsic pathway of apoptosis, suggesting that the gene silencing of intracellular ACE has great potential in the treatment of cardiomyocyte apoptosis after I/R injury by regulation of intracellular RAS. In the future, *in vivo* studies should be carried out to determine the potentially protective role of intracellular ACE gene silencing for the treatment of myocardial I/R injury.

Acknowledgements

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