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ARTICLE

Angiotensin II downregulates ACE2-mediated enhancement of MMP-2 activity in human cardiofibroblasts

Tang-Ching Kuan, Mu-Yuan Chen, Yan-Chiou Liao, Li Ko, Yi-Han Hong, Chun-Yi Yen, Wen-Yeh Hsieh, Kun-Shan Cheng, Chien-Liang Wu, and Chih-Sheng Lin

Abstract: Angiotensin converting enzyme II (ACE2) is a component of the renin-angiotensin system (RAS) that negatively regulates angiotensin II (Ang II). Ang II, in turn, affects the expression of matrix metalloproteinases (MMPs) to induce heart remodeling. The specific mechanisms by which ACE2 regulates MMP-2, however, remain unclear. The aim of this study was to investigate the regulatory relationships between Ang II, ACE2, and MMP-2. ACE2 expression was upregulated and downregulated in human cardiofibroblasts (HCFs) by lentiviral infection. Effects on MMP-2 activity, shed ACE2 activity, extracellular signal-regulated kinase (ERK) signaling pathway, and ADAM metallopeptidase domain 17 (ADAM17) expression were assessed. ACE2 increased MMP-2 activity, and Ang II inhibited this effect through the Ang II type-1 receptor (AT1R) and ERK1/2 signaling pathway. Ang II also reduced the effect of ACE2 on ERK1/2 levels, the activity of shed ACE2, and *adam17* expression in HCFs. Additionally, these Ang II-mediated reductions could be attenuated by AT1R antagonist valsartan. In conclusion, these data help to clarify how ACE2 and Ang II interact to regulate MMP-2 and control tissue remodeling in heart disease.

Key words: angiotensin II, angiotensin-converting enzyme II, matrix metalloproteinases-2, human cardiofibroblasts.

Résumé : L'enzyme de conversion de l'angiotensine II (ECA2) est une composante du système rénine-angiotensine (SRA) qui régule négativement l'angiotensine II (Ang II). L'Ang II, elle, affecte l'expression des métalloprotéases de la matrice (MMP) pour induire un remodelage cardiaque. Les mécanismes spécifiques par lesquels l'ECA2 régule la MMP-2 demeurent cependant flous. Le but de cette étude était d'examiner les relations régulatrices entre l'Ang II, l'ECA2 et la MMP-2. L'expression d'ECA2 a été régulée à la hausse ou à la baisse par une infection lentivirale chez les fibroblastes cardiaques humains (FCH). Les effets sur l'activité de MMP-2, l'activité protéolytique d'ECA2, la voie signalétique de la kinase ERK et l'expression de ADAM17 ont été évalués. L'ECA2 stimulait l'activité de la MMP-2, et l'Ang II inhibait cet effet par l'intermédiaire du récepteur de l'Ang II de type 1 (AT1R) et de la voie signalétique de ERK1/2. L'Ang II réduisait aussi l'effet de l'ECA2 sur les niveaux de ERK1/2, l'activité protéolytique d'ECA2 et l'expression d'*adam17* chez les FCH. De plus, ces réductions provoquées par l'Ang II pouvaient être atténuées par le valsartan, un antagoniste du AT1R. En conclusion, ces données contribuent à clarifier comment l'ECA2 et l'Ang II interagissent pour réguler la MMP-2 et contrôler le remodelage tissulaire lors de maladies cardiaques. [Traduit par la Rédaction]

Mots-clés : angiotensine II, enzyme de conversion de l'angiotensine II, métalloprotéase de la matrice-2, fibroblastes cardiaques humains.

Introduction

Angiotensin II (Ang II) is a main regulator of renin-angiotensin system (RAS) and functions to repair and remodel tissues by stimulating inflammation, cell growth, apoptosis, fibrogenesis, and differentiation (Ruiz-Ortega and Ortiz 2005; Mehta and Griendling 2007). Angiotensin converting enzyme II (ACE2) is a component of RAS that negatively regulates Ang II. ACE2 efficiently cleaves a single residue phenylalanine from Ang II to generate Ang-(1-7), with about 400-fold higher catalytic efficiency than the conversion of Ang I to Ang-(1-9) by removing the C-terminal leucine residue (Vickers et al. 2002). The major function of ACE2 in RAS is to counter-regulate ACE activity by reducing Ang II bioavailability and increasing the vasoprotective/antiproliferative peptide, Ang-(1-7), formation. Hence, ACE2 plays a crucial role in maintaining the balance between the two axes ACE2/Ang-(1-7)/Mas and ACE/Ang II/AT1R of RAS. Chronic and sustained imbalance of the both axes may lead to the pathophysiology of cardiovascular, renal, and pulmonary systems (Kalea and Batlle 2010).

Ang II induces heart remodeling by creating expression imbalances between matrix metalloproteinases (MMPs) within heart tissue (Brassard et al. 2005; Yaghooti et al. 2011; Lin and Pan 2008). ACE2 deficiency increases the level of Ang II, upregulates NADPH oxidase activity, and generates superoxide. This leads to enhance MMP-mediated degradation of ECM (ECM) (Kassiri et al. 2009; Bodiga et al. 2011; Patel et al. 2012), but the specific mechanisms that regulate this process remain unclear.

The mechanisms by which ACE2 affects MMP-2 expression to attenuate heart remodeling have not been described. To characterize the relationships between Ang II, ACE2, and MMP-2, we used lentiviral infection to upregulate or knockdown ACE2 expression within human cardiofibroblasts (HCFs). In addition, the regulation of ACE2 shedding and ADAM metallopeptidase domain 17 (ADAM17) expression by Ang II was investigated in this context.

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T.-C. Kuan, M.-Y. Chen, Y.-C. Liao, L. Ko, Y.-H. Hong, C.-Y. Yen, and C.-S. Lin. Department of Biological Science and Technology, National Chiao Tung University, No.75 Po-Ai Street, Hsinchu, Taiwan.
W.-Y. Hsieh. Department of Biological Science and Technology, National Chiao Tung University, No.75 Po-Ai Street, Hsinchu, Taiwan; Division of Chest Medicine, Department of Internal Medicine, Mackay Memorial Hospital, Hsinchu, Taiwan.
K.-S. Cheng. Department of Biological Science and Technology, National Chiao Tung University, No.75 Po-Ai Street, Hsinchu, Taiwan; Division of Cardiology, Department of Pediatrics, Mackay Memorial Hospital, Hsinchu, Taiwan.
C.-L. Wu. Division of Chest Medicine, Department of Internal Medicine, Mackay Memorial Hospital, Taipei, Taiwan.
Corresponding author: Chih-Sheng Lin (e-mail: lincs@mail.nctu.edu.tw).

Materials and methods

Chemicals and reagents

Lentiviral vectors that contained human *ace2* (lenti-ACE2) and individual ACE2 shRNA (TRCN-46693 to TRCN-46697) sequences were purchased from Vectorite Biomedica Inc. (Taipei, Taiwan) and the National RNAi Core Facility Platform (Institute of Molecular Biology, Genomic Research Center, Academia Sinica, Taipei, Taiwan), respectively. DX600 and Mca-APK(Dnp) were purchased from AnaSpec (Fremont, Calif., USA). Ang II (#H1705) was obtained from Bachem (Bubendorf, Switzerland). Valsartan (#1708762) was obtained from U.S. Pharmacopeia (Rockville, Md., USA). A goat polyclonal antibody (IgG) directed against glyceraldehyde-3-phosphate dehydrogenase (GAPDH (V-18); #sc20357) and secondary antibodies (donkey anti-goat IgG, #sc2020; goat anti–rabbit IgG, #sc2004) labeled with horseradish peroxidase were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, Calif., USA). A rabbit polyclonal antibody directed against phospho-p44/42 mitogen-activated protein kinase (ERK1/2; #4370) was purchased from Cell Signaling Technology (Danvers, Mass., USA). The poly-L-lysine (0.01% solution; #P4832) and all other reagents were obtained from Sigma-Aldrich (St. Louis, Mo., USA).

Cell culture and treatment

Primary HCFs (#6300; ScienCell Research Laboratories, San Diego, Calif., USA) were cultured as described in Lin et al. (2010). Briefly, HCFs were seeded into 100 mm Petri dishes (2×10^6 cells/dish) for growth and then were seeded into 6-well plates (5×10^6 cells/well) or 24-well plates (5×10^4 cells/well) for further experiments. Petri dishes and culture plates were pre-coated with 0.01% poly-L-lysine. HCFs were cultured in fibroblast medium (#2301; ScienCell Research Laboratories) with 2% fetal bovine serum (#0010; ScienCell Research Laboratories) at 37 °C in a humidified 5% CO_2 atmosphere. The culture medium was exchanged with fresh medium every 2 days. For each experiment, cells from the passage 3 or 4 were placed in serum-free medium for 24 h prior to further experiments.

Lentivirus infection

The production of lenti-ACE2 viral particles was performed according to Huentelman et al. (2005) with slight modifications. Human *ace2* gene (Mammalian Gene Collection: cDNA clone MGC: 57146, IMAGE:5297380) were used as a template for PCR amplification. The Spe-ACE2-F and Spe-ACE2-R primers are listed in Table 1. The PCR product was cloned into the pCR II-TOPO vector (Invitrogen, Carlsbad, Calif., USA) to obtain pACE2-TOPO. The *ace2* cDNA was then excised from pACE2-TOPO and subcloned into the *Spe*I sites of the VBI-TLC vector (Vectorite Biomedica Inc.) to obtain TLC-ACE2. The lenti-ACE2 viral particles were produced by Vectorite Biomedica Inc. using TLC-ACE2 clone.

A pLKO.1-shRNA plasmid encoding a short hairpin RNA (shRNA) with sequences targeting *ace2* was introduced into HEK293T cells with lentiviral packaging vectors pMD.G and pCMV 8.91 by the National RNAi Core Facility, Taiwan. The RNAi Consortium Numbers (TRCNs) and sequences are listed in Table 2. Lenti-GFP (green fluorescent protein; Vectorite Biomedica Inc.) was used for control infections.

Overexpression and knockdown of ACE2 were performed as described in Lee et al. (2008) and Lin et al. (2012), with optimization. HCFs were infected with lentivirus for 24 h in the presence of polybrene (8 μg/mL) at different multiplicities of infection (MOI). After viral infection, the cells were cultured in fresh growth medium for 24 h prior to further experiments.

Protein extraction

Protein extraction was performed as described in Sun et al. (2008). HCFs were washed twice in D-PBS and then incubated in PRO-PREP Protein Extraction Solution (iNtRON Biotechnology, Inc., Kyungki-Do, Korea) for 10 min on ice. The cell lysis solution was then transferred into a 1.5 mL Eppendorf tube and sonicated 3 times for 5 sec each (with a 10 sec interval) on ice using an ultrasonic processor (UP950A; Hansor, Taichung, Taiwan). Finally, protein extracts were isolated by centrifugation (13 000*g*) for 5 min at 4 °C (Biofuge Primo R; Sorvall, Osterode, Germany). Total protein was measured in homogeneous extracts using the Bradford dye binding assay (Bio-Rad Laboratories, Hercules, Calif., USA), and bovine serum albumin (BSA) was used as the standard.

Table 1. Primer pairs for *ace2*, *adam17*, and *gapdh*.

Gene	Primer name	Sequence
ace2	Spe-ACE2-F	5′-GAACCCACTGCTTACTGGCTTATCG-3'
	Spe-ACE2-R	5′-GCTGGCAACTAGAAGGCACAGTCG-3'
adam17	hAdam17-F	5′-CTTTCAGCATTCTTGTCCATTGTGTG-3'
	hAdam17-R	5′-GCTCAGCATTTCGACGTTACTGGG-3'
gapdh	hGAPDH-F	5′-ACAGTCAGCCGCATCTTCTT-3'
	hGAPDH-R	5′-GTTAAAAGCAGCCCTGGTGA-3'

Note: Primers were designed based on ACE2 (cDNA clone MGC: 57146, IMAGE: 5297380), ADAM17 (GenBank ID: NM_003183.4) and GAPDH (GenBank ID: NM_002046.3).

Table 2. The sequences of shACE2 constructs.

RNAi consortium number	Sequence
TRCN0000046693	5′-GCCCTTATTTACCTGGCTGAA-3'
TRCN0000046694	5′-GCCCAAATGTATCCACTACAA-3'
TRCN0000046695	5′-GCAAAGTTGATGAATGCCTAT-3'
TRCN0000046696	5′-GCTGGACAGAAACTGTTCAAT-3'
TRCN0000046697	5′-GCCGAAGACCTGTTCTATCAA-3'

Note: Lenti-*ace2*-shRNAs were constructed by the National RNAi Core Facility Platform (Institute of Molecular Biology/Genomic Research Center, Academia Sinica, Taipei, Taiwan).

ACE2 activity assay

ACE2 activity was measured with the fluorogenic substrate Mca-APK(Dnp) as described in Hsieh et al. (2012) with slight modifications. The assay was performed in a micro-quartz cuvette with 20 μL of cellular protein, 50 μmol/L fluorogenic substrate, and a protease inhibitor cocktail (1:200; Sigma-Aldrich) in ACE2 assay buffer (75 mmol/L Tris-HCl, 1 mol/L NaCl, 5 mmol/L $ZnCl_2$, pH 6.5) in a final volume of 200 μL. All samples also contained the ACE inhibitor captopril (1 μmol/L; Sigma-Aldrich). Reaction kinetics was monitored for 1 h using a fluorescence reader at an excitation wavelength of 330 nm and an emission wavelength of 390 nm. As a control, parallel samples were analyzed in the presence of 1 μmol/L DX600, which is an ACE2 inhibitor.

Gelatin zymography

MMP-2 activity was detected by gelatin zymography as described in Chang et al. (2011). Briefly, 30 μg of cellular homogenate was mixed with 6× zymography sample buffer (0.125 mol/L Tris-HCl, pH 6.8, 50% [*v*/*v*] glycerol, 4% [*w*/*v*] SDS, and 0.005% bromophenol blue) and incubated for 10 min at room temperature (RT). Samples were then loaded into individual lanes of an 8% SDS-polyacrylamide gel that contained 1 mg/mL gelatin (Sigma-Aldrich). After electrophoresis, the gel was washed twice for 30 min in zymogram renaturing buffer (containing 2.5% Triton X-100) with gentle agitation at RT to remove the SDS. The gel was then incubated in reaction buffer (50 mmol/L Tris-HCl, pH 7.4, 200 mmol/L NaCl, 5 mmol/L $CaCl_2$, and 0.02% Brij35) overnight at 37 °C. After Coomassie brilliant blue staining, gelatinase activities were identified as clear zones against a blue background on the zymogram gels.

Western blotting

Western blots for ERK1/2 and GAPDH were performed as described in Kuan et al. (2011). Aliquots containing 30 μg of protein were electrophoresed on an 8% SDS-polyacrylamide gel and

then electrophoretically transferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore, Bedford, Mass., USA) using a TE70 semi-dry electro-blotting apparatus (Hoefer, San Francisco, Calif., USA). Membranes were blocked in 5% nonfat milk in Tris-buffered saline. Primary antibodies were diluted 1:1,000 for ACE2, ERK1/2, and GAPDH. Secondary antibodies were diluted 1:2,000. Substrates were visualized using enhanced chemiluminescence detection (Western Lightning Plus-ECL, Enhanced Chemiluminescence Substrate; PerkinElmer, Boston, Mass., USA) and exposing the membranes to X-ray film. Bands were detected at anticipated locations based on size. Band intensities were quantified by densitometric analysis using Scion Image software (Scion, Frederick, Md., USA). The amount of ACE2 and ERK1/2 was expressed relative to GAPDH.

RNA isolation and quantification

Total RNA was extracted using TRIzol Plus RNA Purification System (Invitrogen), as previously described (Pan et al. 2008). Briefly, 1 mL of TRIzol reagent was added to 5×10^6 cells and vigorously agitated for 30 sec by cell scrapers and then incubated at RT for 5 min. Next, 200 μL chloroform was added and the mixture was shaken. After centrifugation (12 000*g* for 15 min), the aqueous phase was transferred to a clean tube, precipitated with 500 μL isopropyl alcohol, and centrifuged at 12 000*g* for 15 min. The resulting RNA pellet was washed with 1 mL 75% cold ethanol and centrifuged at 12 000*g* for 5 min at 4 °C. The pellet was dried at RT and resuspended in 20 μL diethylpyrocarbonate-treated water. RNA was quantified by measuring the absorbance at 260 nm and 280 nm and was electrophoresed on a denaturing 1% agarose gel. The integrity and relative amounts of RNA were evaluated using ultraviolet visualization of ethidium bromide-stained RNA.

Reverse transcription and real-time PCR

The ReverTra Ace Set (Toyobo, Osaka, Japan) was used to synthesize cDNA. Briefly, 5 μg of total RNA was reverse transcribed in a reaction that contained 1× reverse transcription buffer, 1 mmol/L dNTPs, 0.5 pmol/L/μL oligo-dT (TOYOBO, Osaka, Japan), 0.5 U/μL RNase inhibitor (TOYOBO), and 1 μL of ReverTra Ace reverse transcriptase (TOYOBO) (total volume 20 μL). After a 60 min incubation at 42 °C and a 5 min incubation at 95 °C, semiquantitative real-time PCR was performed using SYBR Green Realtime PCR Master Mix Plus (TOYOBO) and Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, Calif., USA). Reactions contained 20 pmol/L of each primer and 5 μL cDNA in a total volume of 25 μL. PCR specificity was confirmed using agarose gel electrophoresis and melting-curve analysis. Expression of the GAPDH gene was used as an internal standard. Primer pairs for *adam17* and *gapdh* are listed in Table 1.

Statistics

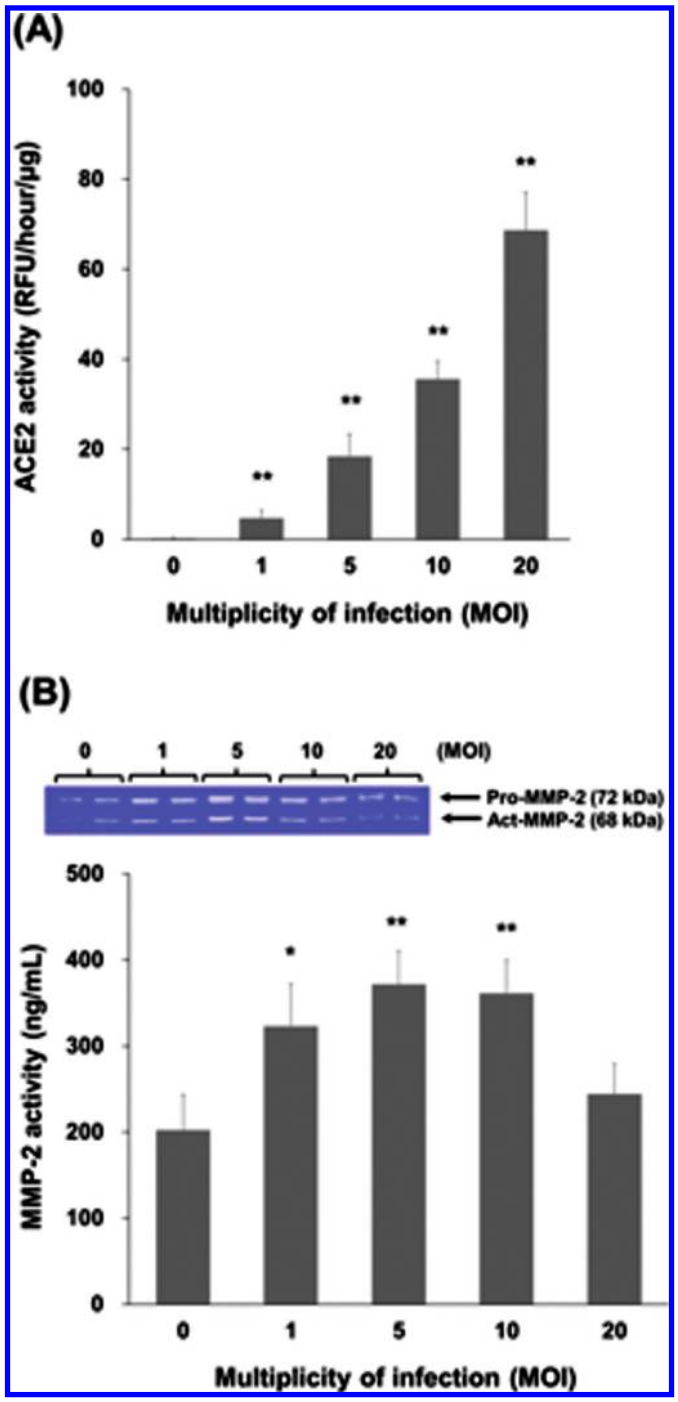
All values were expressed as the mean ± standard deviation (SD). Data were compared with a one-way analysis of variance (ANOVA) to evaluate the differences among multiple groups. A value of $p < 0.05$ was considered statistically significant.

Results

ACE2 overexpression affects MMP2 activity in HCFs

To determine the effect of ACE2 on MMP-2 activity, HCFs were infected with lenti-ACE2 at different MOI. Lenti-ACE2 effectively generated HCFs that overexpressed ACE2 (HCFs/ACE2), as the cells infected at 1, 5, 10, and 20 MOI exhibited 20-, 78-, 151-, and 292-fold greater ACE2 activity, respectively, than that of the control cells infected with lenti-GFP (Fig. 1A). Moderate increases in ACE2 elevated MMP-2 activity (Fig. 1B), in HCFs/ACE2 transfected with 5 MOI of lenti-ACE2, the MMP-2 activity level was elevated 1.8-fold compared with the controls.

Fig. 1. ACE2 and MMP-2 activities of the HCFs infected with lenti-ACE2. HCFs were infected with lenti-ACE2 at 1, 5, 10, and 20 MOI. For the lentiviral control, the HCFs were transfected with lenti-GFP (5 MOI). ACE2 (A) and MMP-2 (B) activities were measured 24 h after the lentivirus infection. ACE2 activity was measured based on the cleavage of a fluorescent substrate (37 °C for 1 h) in the presence of an ACE2-specific inhibitor. MMP-2 activity, including pro-MMP2 (72 kDa) and act-MMP2 (68 kDa), was measured using gelatin zymography. Data are from 3 independent experiments. $^*p < 0.05$ and $^{**}p < 0.01$, compared with the lentiviral control.



ACE2 knockdown downregulates MMP-2 activity in HCFs/ACE2

To determine whether ACE2 shRNAs could effectively inhibit ACE2 expression, HCFs were co-infected (at 5 MOI) with lenti-ACE2

and lenti-shACE2s (TRCN-46693 to TRCN-46697), and ACE2 activity was measured. All lenti-shACE2s suppressed ACE2 expression, but TRCN-46694 and TRCN-46697 proved most effective. As ACE2 activity levels in HCFs/ACE2 infected with TRCN-46693, -46694, -46695, -46696, and -46697 were reduced 47%, 93%, 75%, 83%, and 95%, compared with those of the lenti-GFP controls, respectively (Fig. 2A).

To confirm that ACE2 overexpression induced MMP-2 activity in HCFs, the lenti-shACE2, TRCN-46697, was used to suppress ACE2 expression in HCFs/ACE2, and MMP-2 activity was measured. MMP-2 activity was indeed reduced by TRCN-46697 in HCFs/ACE2 (Fig. 2B). As such, increasing ACE2 in HCFs could elevate MMP-2 activity in the cells.

Ang II suppresses MMP-2 activity in HCFs/ACE2

To determine whether ACE2 induced MMP-2 activity within HCFs through the angiotensin signaling pathway, HCFs/ACE2 were treated with Ang II, and MMP-2 activity levels were measured. Ang II effectively suppressed MMP-2 activity in HCFs/ACE2. In addition, Ang II was not able to affect MMP-2 activity when cells were pre-treated with valsartan, an AT1R antagonist (Fig. 3). Finally, MMP-2 activity levels in HCFs/ACE2 infected with TRCN-46697 and treated with Ang II were statistically indistinguishable from MMP-2 activity levels in HCFs/ACE2 treated with Ang II (Fig. 3). These results indicate that Ang II acts through AT1R to inhibit MMP-2 activity in HCFs/ACE2.

Ang II reduces ERK1/2 levels in HCFs/ACE2

To characterize the mechanism by which ACE2 affected MMP-2 activity in HCFs, we analyzed ERK1/2 levels in HCFs/ACE2. When HCFs/ACE2 was treated with Ang II, the effect on ERK1/2 expression mirrored the effects seen on MMP-2 activity (compare Fig. 3 and Fig. 4). ERK1/2 expression in HCFs/ACE2 infected with TRCN-46697 and then treated with Ang II was not significantly different from that measured in HCFs/ACE2 treated with Ang II alone. In addition, HCFs/ACE2 pre-treated with valsartan and then treated with Ang II exhibited levels of ERK1/2 that were similar to those of nontreated HCFs/ACE2 (Fig. 4). These results show that Ang II affects ERK1/2 expression via AT1R and suggest that an Ang II-AT1R-ERK1/2 axis inhibits MMP-2 activity in HCFs/ACE2.

Ang II suppresses the activity of shed ACE2 in HCFs/ACE2

Recent evidence suggests that ectodomain shedding of ACE2 plays an important role in RAS (Oudit et al. 2009; Clarke and Turner 2012). We therefore measured the effects of angiotensin peptides on the activity of shed ACE2 in HCFs/ACE2. When HCFs/ACE2 were treated with Ang II, the activity of shed ACE2 was reduced by 38%, compared with HCFs/ACE2 controls (Fig. 5). In addition, valsartan reversed the effect of Ang II on shed ACE2 (Fig. 5). These results imply that Ang II acts through AT1R to reduce the level of shed ACE2 activity.

Ang II downregulates *adam17* expression in HCFs/ACE2

ADAM17 has been reported as a candidate mediator of stimulated ACE2 shedding (Lambert et al. 2005). We reasoned, therefore, that changes in *adam17* expression could affect ACE2 shedding in this context. Ang II reduced *adam17* mRNA levels in HCFs/ACE2, an effect that was eliminated by valsartan pretreatment (Fig. 6).

Discussion

We investigated the effects of ACE2 and Ang II on MMP-2 activity in HCFs. ACE2 overexpression induced MMP-2 activity in HCFs. This effect on MMP-2 activity was reversed by treating HCFs/ACE2 with Ang II, presumably via an Ang II-AT1R-ERK1/2 axis. In addition, treating HCFs/ACE2 with Ang II could downregulate *adam17* expression, which has been shown to affect ACE2 shedding. In the RAS, ACE2 converts Ang II to Ang-(1-7) and improves the heart

Fig. 2. ACE2 and MMP-2 activities in the HCFs/ACE2 infected with lenti-shACE2. (A) HCFs were co-infected with lenti-ACE2 and individual lenti-shACE2 constructs (TRCN-46693 to TRCN-46697) at 5 MOI. HCFs/ACE2 was used as the control (Ct). ACE2 activity was measured 24 h after the infection procedure. (B) MMP-2 activity levels were compared among HCFs, HCFs/ACE2 and HCFs/ACE2 co-infected with lenti-shACE2 (TRCN-46697). Data are from 3 independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$, compared with Ct (A) and HCFs (B); $^{\dagger\dagger}p < 0.01$, compared with HCFs/ACE2.

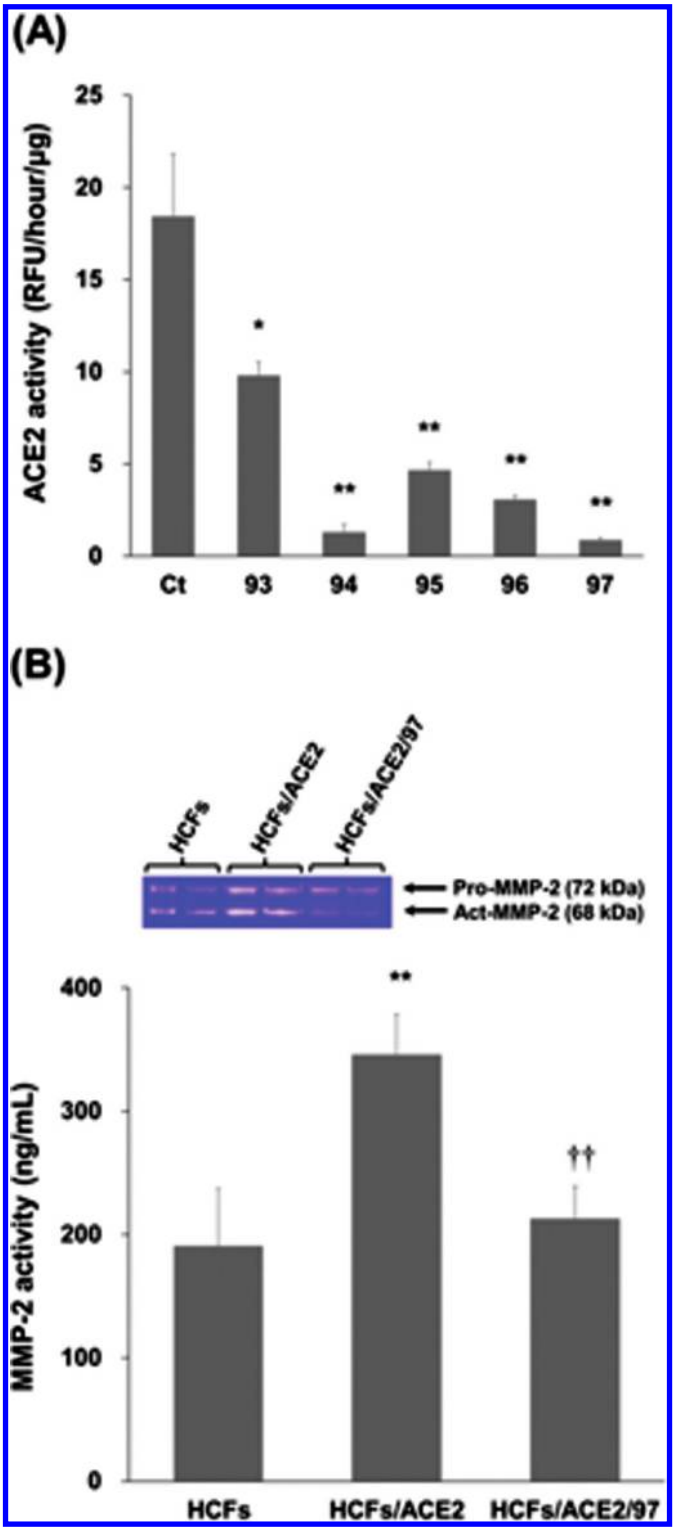
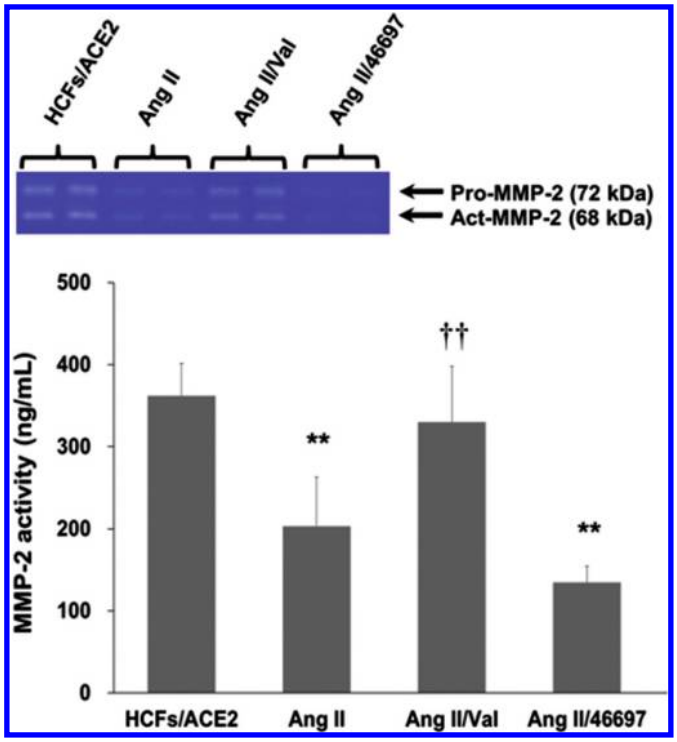


Fig. 3. MMP-2 activity in the HCFs/ACE2 treated with Ang II. Data are shown for HCFs/ACE2, HCFs/ACE2 treated with Ang II (Ang II), HCFs/ACE2 pre-treated with 1 μmol/L valsartan, an AT1R antagonist, for 1 h and then with 1 μmol/L Ang II (Ang II/Val), and HCFs/ACE2 co-infected with TRCN-46697 (lenti-shACE2) at 5 MOI and treated with 1 μmol/L Ang II (Ang II/46697). MMP-2 activity assay was performed after 24 h of Ang II exposure. Data are from 3 independent experiments. $^{**}p < 0.01$, compared with HCFs/ACE2; $^{††}p < 0.01$, compared with HCFs/ACE2 treated with Ang II.



dysfunction stimulated by disordered Ang II increase. It was reported that Ang II could downregulate MMP-2 expression and activity in cardiocytes (Pan et al. 2008). Therefore, overexpressed ACE2 can increase MMP-2 expression via the reduction of Ang II. Other regulatory pathways cannot be excluded, however, including Ang-(1-7)/Mas signaling pathway and a directly mechanism induced and (or) affected by ACE2 (Fig. 7).

Cardiac fibroblasts and myocytes play an important functional role in the heart, as cardiac myocytes constitute 30%– 40% of the total cardiac cell population and cardiac fibroblasts are the majority of other non-cardiac myocytes cells (Porter and Turner 2009). Cardiac fibroblasts and myocytes are the major cell types within heart tissue and usually investigated in heart diseases and heart remodeling. However, cardiac fibroblasts play a more significant role than cardiac myocytes in heart injury and ischemia (Martin and Blaxall 2012). Cardiac fibroblasts stimulate cytokines and growth factors expression to induce inflammation, myofibroblasts transition, ECM regulation, and fibrosis in heart injury and ischemia (Porter and Turner 2009; Krenning et al. 2010).

The role of Ang II in modulating cardiac fibroblast activity has been well characterized (Campbell and Katwa 1997; Kawano et al. 2000), but the function of ACE2 in cardiac cells is unclear. Grobe et al. (2007) used neonatal rat cardiac myocytes and fibroblasts to determine that cardiac myocytes have endogenous ACE2 activity, whereas cardiac fibroblasts do not. Cardiac fibroblasts localize to infarct zones after myocardial ischemia (MI), and HCFs isolated from patients undergoing coronary artery bypass surgery do, however, express ACE, ACE2, and AT1R (Guy et al. 2008). ACE2 overexpression in cardiac fibroblasts attenuates transforming growth factor beta (TGF-β) and hypoxia/reoxygenation-induced

Fig. 4. ERK1/2 levels in the HCFs/ACE2 treated with Ang II. Data are shown for cells treated as in Fig. 3. The cells were collected after 24 h of Ang II exposure. ERK1/2 and GAPDH (for normalization) levels were detected by Western blots. Data are from 3 independent experiments. $^{**}p < 0.01$, compared with HCFs/ACE2; $^{†}p < 0.05$, compared with HCFs/ACE2 treated with Ang II.

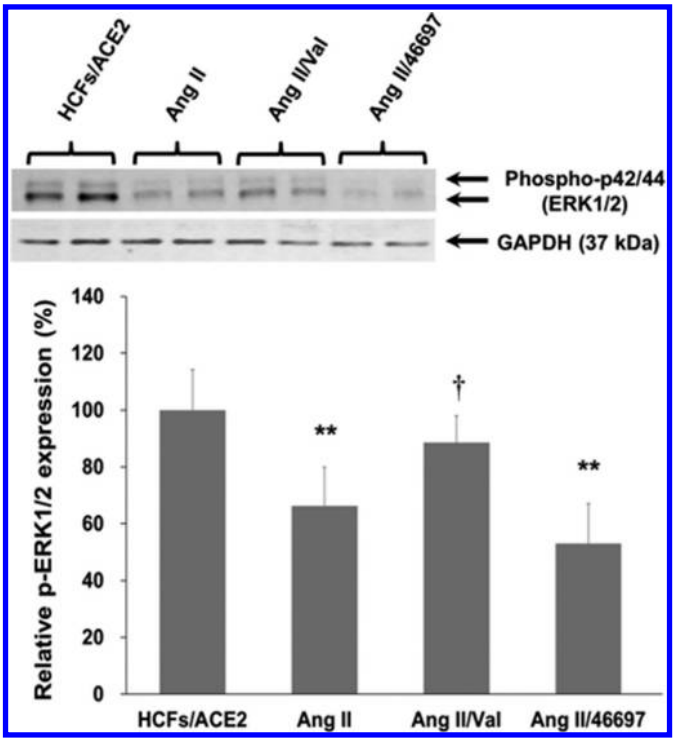
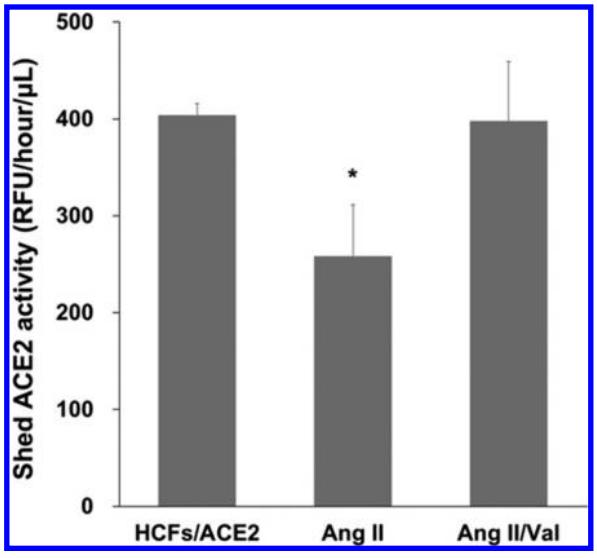


Fig. 5. Ang II suppresses the activity of shed ACE2 in HCFs/ACE2. Data are shown for the cells treated as in Fig. 3. To perform the shed ACE2 activity assay, culture medium was collected after 24 h of Ang II exposure for the determination of ACE2 activity. Data are from 3 independent experiments. $^{*}p < 0.05$, compared with HCFs/ACE2.



collagen production in these cells (Grobe et al. 2007). It has also been shown that ACE2 is released into the extracellular medium, and that ACE2 expression is not regulated by tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), or chronic hypoxia in HCFs (Turner et al. 2007). In addition, ACE2 overexpression in cardiac myocytes isolated from diabetic rats decreased high

Fig. 6. Ang II downregulates *adam17* expression in HCFs/ACE2. Data are shown for the cells treated as in Fig. 3. After 24 h of Ang II exposure, total mRNA was isolated, and *adam17* expression levels were determined using real-time RT-PCR. The *adam17* expression was normalized against *gapdh*. $^{**}p < 0.01$, compared with HCFs/ACE2; $^{\dagger}p < 0.05$, compared with HCFs/ACE2 treated with Ang II.

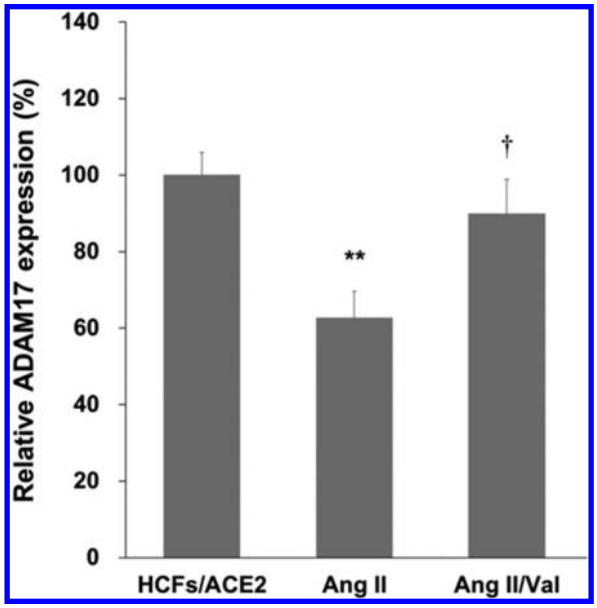
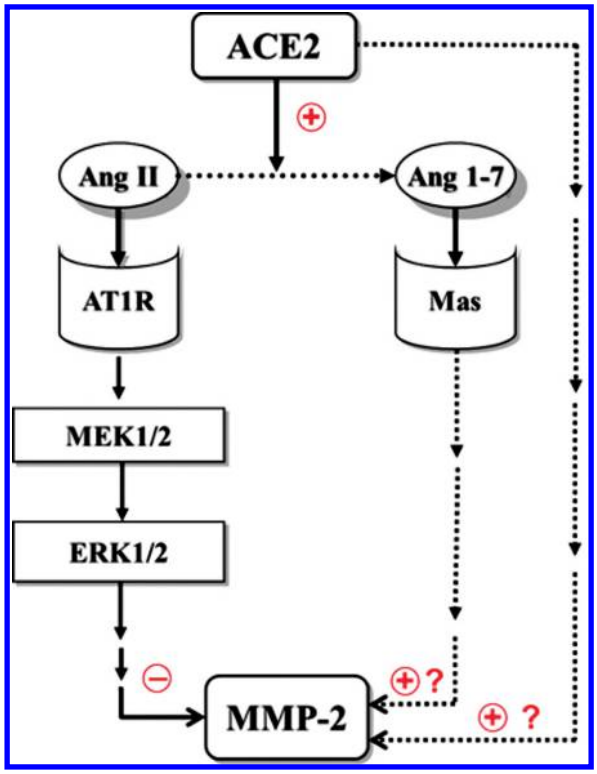


Fig. 7. Schematic diagram representing the regulation relationships between ACE2, Ang II, and MMP-2.



glucose-induced Ang II production, collagen accumulation, and TGF-β expression (Dong et al. 2012). Results from the current study indicate that ACE2 plays a protective role within cardiac fibroblasts and myocytes, opposing Ang II-induced pathophysiological changes at the molecular level. Conflicting findings may have resulted from the use of different species and different cell types (Guy et al. 2008; Lin et al. 2010).

ACE2 overexpression significantly attenuates myocardial fibrosis in mice infused with Ang II (Huentelman et al. 2005). The lenti-mACE2 approach has also been used in spontaneously hypertensive rats and rats subjected to coronary-artery ligation. Results from these experiments demonstrated that ACE2 overexpression improves high blood pressure, left ventricular wall thickness, and perivascular fibrosis (Díez-Freire et al. 2006; Der Sarkissian et al. 2008). ACE2 overexpression can reduce Ang II levels and enhance Ang-(1-7) to improve heart dysfunction (Dong et al. 2008; Guo et al. 2008). In MI and spontaneously hypertensive rats, ACE2 overexpression inhibits ACE, Ang II, and collagen expression but upregulates MMP-2 expression to attenuate left-ventricular fibrosis (Rentzsch et al. 2008; Zhao et al. 2010; Dong et al. 2012). The published data reveal that Ang II acts via AT1R to elevate MMP activity, and AT1R antagonists can inhibit MMP activation (Kassiri et al. 2009; Bodiga et al. 2011). The published record generally indicates that ACE2 regulates MMP expression (especially that of MMP-2 and MMP-9) to attenuate the pathogenesis of hypertension, heart dysfunction, and cardiac fibrosis.

In animals subjected to MI, ACE2 deficiency leads to increased phosphorylation of ERK1/2 signaling pathways and upregulated MMP-2 activity. Moreover, loss of ACE2 also increases Ang II levels, which could enhance MMP-mediated degradation of the ECM within ACE2-deficient myocardium (Bodiga et al. 2011; Patel et al. 2012). These results support our current data and suggest that the loss of ACE2 worsens post-MI ventricular remodeling that is associated with MMP-2 expression and activation (Kassiri et al. 2009). Ang II decreases cardiac contractility, suggesting that Ang II is a major factor in the development of cardiac hypertrophy and dilatation. In addition, elevated levels of Ang II activate both MAPKs and MMPs (Yamamoto et al. 2006; Patel et al. 2012). As such, some groups have infused Ang II into ACE2 knockout mice to worsen cardiac fibrosis and pathological hypertrophy (Zhong et al. 2010; Alghamri et al. 2013).

In this study, the results show ACE2 overexpression enhanced MMP-2 activity and Ang II suppressed the activation of ERK1/2 via AT1R in HCFs/ACE2. We also demonstrated the antagonistic interaction between Ang II and ACE2 in MMP-2 regulation. Although our study revealed that ACE2 regulates MMP-2 expression, the signal pathway of MMP-2 regulation by ACE2 is still unclear. Some published papers provide probable directions, as they indicated that Ang II mediated AT1R to induce NADPH oxidase and MMP activation, AT1R blocker and Ang-(1-7) supplementation inhibited NADPH oxidase and MMP activation (Kassiri et al. 2009; Bodiga et al. 2011). These results demonstrated that ACE2 overexpression regulates ACE/Ang II/AT1R axis and ACE2/Ang-(1-7)/Mas axis to affect the expression of pro-inflammatory cytokines, collagen, and MMPs. ACE2 deficiency leads to increase phosphorylation of ERK1/2 and JNK1/2 signaling pathways, up-regulate MMP-2, MMP-9, and cytokines. Moreover, loss of ACE2 is also associated with the increased Ang II level, NADPH oxidase activity, and superoxide generation that could lead to enhance MMP-mediated degradation of the ECM in ACE2-deficient myocardium, increased pathological hypertrophy, and worsening of systolic performance (Patel et al. 2012). Indeed, ACE2 may act via the NADPH oxidase and inflammatory cytokines to regulate MMP-2 expression.

Phorbol ester stimulates ADAM17 to induce ectodomain shedding of ACE2 in HEK293 and Huh7 cells, a result that has been confirmed with both ADAM17 overexpression and knockout studies (Lambert et al. 2005). ADAM17 recognizes Arg708 and Arg710 of ACE2 and cleaves the ACE2 peptide between Arg708 and Ser709 (Lai et al. 2011). Further studies indicate that ACE2 plays a protective role in severe acute respiratory syndrome (SARS), a role that involves ectodomain shedding of ACE2. The SARS coronavirus downregulates ACE2, leading to Ang II-induced cell damage (Oudit et al. 2009; Clarke and Turner 2012). The SARS coronavirus

also stimulates ADAM17 activity, which results in the shedding of the ACE2 N-terminal domain and ACE2 downregulation (Haga et al. 2008; Jia et al. 2009).

With respect to a molecular mechanism, our study revealed that Ang II inhibits ERK1/2 activation and suppresses ACE2 shedding via AT1R in HCFs/ACE2. Van Schaeybroeck et al. (2011) used isogenic Kras mutant HCT116 CRC cells to demonstrate that ERK1/2 inhibition abrogates chemotherapy-induced ADAM17 activity. This supports the result that Ang II may inhibit ERK1/2 activation to reduce ADAM17 activity and to inhibit ACE2 shedding in HCFs/ACE2. The suppression of ACE2 shedding may maintain ACE2 levels and protect the cell membrane against Ang II-induced damage.

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