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PRE-CLINICAL RESEARCH

Angiotensin-Converting Enzyme-2 Overexpression Improves Left Ventricular Remodeling and Function in a Rat Model of Diabetic Cardiomyopathy

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Objectives The aim of this study was to test the hypothesis that angiotensin (Ang)-converting enzyme-2 (ACE2) overexpression may inhibit myocardial collagen accumulation and improve left ventricular (LV) remodeling and function in diabetic cardiomyopathy.

Background Hyperglycemia activates the renin-Ang system, which promotes the accumulation of extracellular matrix and progression of cardiac remodeling and dysfunction.

Methods Ninety male Wistar rats were divided randomly into treatment (n = 80) and control (n = 10) groups. Diabetes was induced in the treatment group by a single intraperitoneal injection of streptozotocin. Twelve weeks after streptozotocin injection, rats in the treatment group were further divided into adenovirus-ACE2, adenovirus–enhanced green fluorescent protein, losartan, and mock groups (n = 20 each). LV volume; LV systolic and diastolic function; extent of myocardial fibrosis; protein expression levels of ACE2, Ang-converting enzyme, and Ang-(1-7); and matrix metalloproteinase–2 activity were evaluated. Cardiac myocyte and fibroblast culture was performed to assess Ang-II and collagen protein expression before and after ACE2 gene transfection.

Results Four weeks after ACE2 gene transfer, the adenovirus-ACE2 group showed increased ACE2 expression, matrix metalloproteinase–2 activity, and LV ejection fractions and decreased LV volumes, myocardial fibrosis, and ACE, Ang-II, and collagen expression in comparison with the adenovirus–enhanced green fluorescent protein and control groups. ACE2 was superior to losartan in improving LV remodeling and function and reducing collagen expression. The putative mechanisms may involve a shift in balance toward an inhibited fibroblast-myocyte cross-talk for collagen and transforming growth factor–beta production and enhanced collagen degradation by matrix metalloproteinase–2.

Conclusions ACE2 inhibits myocardial collagen accumulation and improves LV remodeling and function in a rat model of diabetic cardiomyopathy. Thus, ACE2 provides a promising approach to the treatment of patients with diabetic cardiomyopathy. (J Am Coll Cardiol 2012;59:739–47) © 2012 by the American College of Cardiology Foundation

The recent discovery of new family members of the renin-angiotensin (Ang) system (RAS), such as Ang-converting enzyme (ACE) 2 (ACE2), Ang-(1-7), and Ang-(1-9), has spurred new research interest in further understanding the relationship between the RAS and cardiovascular disease (1). Recently, we found that ACE2 overexpression stabilized atherosclerotic plaque at a late stage and attenuated the progression of atherosclerotic lesions at an early stage in a rabbit model of atherosclerosis (2,3). Although there is great enthusiasm for treating heart failure via ACE2 overexpression, preliminary results are controversial. ACE2-knockout mice showed severe myocardial contractile dysfunction or no changes in cardiac dimension or function (4), whereas ACE2

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Abbreviations and Acronyms

ACE = angiotensin-converting enzyme
ACE2 = angiotensin-converting enzyme 2
Ad-ACE2 = adenovirus–angiotensin-converting enzyme 2
Ad-EGFP = adenovirus–enhanced green fluorescent protein
Ang = angiotensin
ARB = angiotensin receptor blocker
DCM = diabetic cardiomyopathy
ECM = extracellular matrix
ELISA = enzyme-linked immunosorbent assay
HG = high glucose
LV = left ventricular
LVEDD = left ventricular end-diastolic diameter
LVEF = left ventricular ejection fraction
MMP = matrix metalloproteinase
RAS = renin-angiotensin system
TGF = transforming growth factor
TIMP = tissue inhibitor of metalloproteinase

overexpression for 11 weeks in the myocardium of stroke-prone spontaneously hypertensive rats resulted in marked myocardial fibrosis with reduced left ventricular (LV) ejection fraction (LVEF) (5). The mechanisms underlying these discrepancies are unclear and pending further investigation.

Diabetic cardiomyopathy (DCM) is characterized by a variety of morphological changes, including myocyte hypertrophy, myofibril depletion, interstitial fibrosis, and intramyocardial microangiopathy. Among these pathological alterations, myocardial fibrosis is a key feature of the diabetic heart, and accumulation of extracellular matrix (ECM) proteins, particularly in collagen, has been documented. Dysregulation of collagen-degrading matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) was found to play an important role in the pathogenesis of myocardial fibrosis in diabetes (6). Singh et al. (7) found that the intracellular RAS was activated by high glucose (HG), and the activated RAS promoted ECM production in cardiac myocytes and fibroblasts. Westermann et al. (6) reported that Ang type 1 receptor antagonists inhibited inflammation and matrix accumulation and decreased MMP-2 activity in DCM. On the basis of these experiments, it has been proposed that hyperglycemia may activate the RAS, which in turn promotes accumulation of ECM and progression of cardiac remodeling and dysfunction.

ACE2, a homologue of ACE, catalyzes conversion of Ang-II to a vasodilative heptapeptide Ang-(1-7) and conversion of Ang-I to the inactive nonapeptide Ang-(1-9), thereby functioning effectively as an endogenous ACE inhibitor. In the present study, we hypothesized that in the setting of DCM, ACE2 overexpression may inhibit myocardial collagen accumulation and improve LV remodeling and function by down-regulation of Ang-II, up-regulation of Ang-(1-7), and attenuation of ACE expression. A series of in vitro and in vivo experiments were designed and performed to validate this hypothesis.

Methods

See the Online Appendix for details.

Adenoviral vector construction. Murine ACE2 complementary deoxyribonucleic acid was amplified by reverse transcription polymerase chain reaction from the ribonucleic acid of mouse kidney. Recombinant adenoviruses carrying the murine ACE2 (adenovirus-ACE2 [Ad-ACE2]) or a control transgene (adenovirus–enhanced green fluorescent protein [Ad-EGFP]) were prepared with the AdMax system (Microbix Biosystems, Toronto, Ontario, Canada).

Animal model and gene transfer. Ninety male Wistar rats were first divided into treatment (n = 80) and control (n = 10) groups. Rats in the treatment group received a single intraperitoneal injection of streptozotocin to induce a diabetic status. Then rats in the treatment group were again divided into Ad-ACE2, Ad-EGFP, losartan, and mock groups (n = 20 each) (Fig. 1).

ACE2 activity assay. ACE2 fluorescence assay was based on the use of the fluorogenic peptide substrate V (7-Mca-RPPGFSAFK[Dnp]-OH, R&D Systems Inc., Minneapolis, Minnesota). Specific ACE2 activity was expressed as picomoles of substrate converted to the product per unit of time and normalized for protein content (units per milligram of protein).

Histopathology and immunohistochemistry. The extent of myocyte hypertrophy was measured on hematoxylin and eosin–stained sections. ACE2, Ang-(1-7), and collagen I and III were identified with appropriate antibodies. Masson's trichrome staining was performed to display collagen components.

Western blot analysis. The protein expression of ACE2, ACE, TIMP-1, MMP-2, MMP-9, and transforming growth factor (TGF)-β was assayed by Western blot analysis.

Echocardiography. Left ventricular end-diastolic diameter (LVEDD), LV end-systolic diameter, LV fractional shortening, LVEF, mitral peak flow velocities, and the E/A ratio were measured using echocardiography.

Hemodynamic measurement. LV systolic pressure, LV end-diastolic pressure, maximal ascending and descending rates of LV pressure, and heart rate were measured using cardiac catheterization.

Zymography. The activity of MMP-2 was evaluated by zymography.

Real-time reverse transcription polymerase chain reaction. The messenger ribonucleic acid expression of ACE2 was quantitated using reverse transcription polymerase chain reaction.

Enzyme-linked immunosorbent assay (ELISA). ELISA was performed to measure the levels of Ang-II, Ang-(1-7), and soluble collagen I and III proteins.

Co-culture of fibroblasts and myocytes. Cardiac fibroblasts were co-cultured with Ad-ACE2-transfected or non-transfected myocytes or the conditioned media of these cells, and the levels of collagen I and III and TGF-β proteins in the media were determined by ELISA.

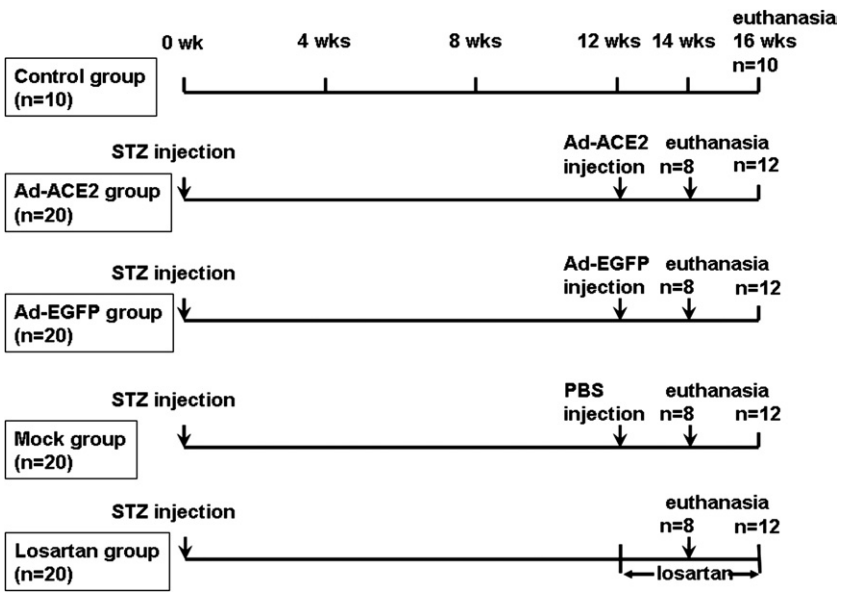

Figure 1 Flowchart Showing the Timeline of the Experiment

Ad-ACE2 = adenovirus–angiotensin-converting enzyme 2; Ad-EGFP = adenovirus–enhanced green fluorescent protein; PBS = phosphate-buffered saline; STZ = streptozotocin.

Hydrogen-3-proline incorporation assay. Hydrogen-3-proline incorporation study was performed to investigate the effects of Ad-ACE2 on collagen synthesis.

Statistical analysis. All values are expressed as mean ± SD. Data were evaluated using SPSS version 11.5 (SPSS, Inc., Chicago, Illinois). A p value <0.05 was considered significant.

Results

Efficacy of ACE2 gene transfer in vivo. ACE2 messenger ribonucleic acid and protein expression levels and ACE2 activity showed more than 4-fold increases in the Ad-ACE2 group compared with the Ad-EGFP or control group 2 weeks after ACE2 transfection. In contrast, these measurements were only slightly higher in the losartan group than in the Ad-EGFP and control groups ($p < 0.001$ to 0.01) (Figs. 2A to 2D).

Quantitation of myocyte hypertrophy in vivo. Myocyte hypertrophy with increased myocyte cross-sectional area was more prominent in the Ad-EGFP and mock groups than in the Ad-ACE2 or losartan group, with no significant difference between the Ad-EGFP and mock groups or between the Ad-ACE2 and losartan groups ($p < 0.05$) (Figs. 2E and 2F).

Echocardiographic and hemodynamic measurements. At the end of week 12, LV end-systolic diameters and LVEDDs were significantly increased, whereas LVEFs, fractional shortening, and E/A ratios were decreased in the mock, Ad-ACE2, Ad-EGFP, and losartan groups compared with the control group (all p values <0.01) (Online Table 1). At the end of week 16, LV end-systolic diameters and LVEDDs were significantly decreased, whereas LVEFs, fractional shortening, and E/A ratios were increased in the Ad-ACE2 and losartan groups in comparison with the mock and Ad-EGFP groups (all p values <0.01) (Online Table 2), although the differences between the Ad-ACE2 and control groups in LVEDD and LVEF were still significant ($p < 0.05$ for both). Importantly, LVEDD values were higher and LVEF values lower in the losartan group than in the Ad-ACE2 group ($p < 0.05$).

At the end of week 12, LV end-systolic pressures and the maximal ascending and descending rates of LV pressure were substantially decreased, whereas LV end-diastolic pressures were increased, in the mock, Ad-ACE2, and Ad-EGFP groups compared with the control group (all p values <0.05) (Online Table 3). At the end of week 16, the maximal ascending and descending rates of LV pressure were substantially increased, whereas LV end-diastolic pressures were decreased, in the Ad-ACE2 and losartan groups in comparison with the mock and Ad-EGFP groups. However, LVESP was significantly lower in the losartan group than in the Ad-ACE2, mock, and Ad-EGFP groups (all p values <0.05) (Online Table 4).

Collagen and TGF-β protein expression in vivo. Cardiac collagen deposition was lower in the Ad-ACE2 and losartan groups than in the Ad-EGFP and mock groups (Fig. 3, top row). Similarly, the protein expression levels of collagen I (Fig. 3, middle row) and collagen III (Fig. 3, bottom row) were substantially lower in the Ad-ACE2 and losartan groups than in the Ad-EGFP and mock groups. In contrast, the expression levels of total collagen, collagen I, and collagen III

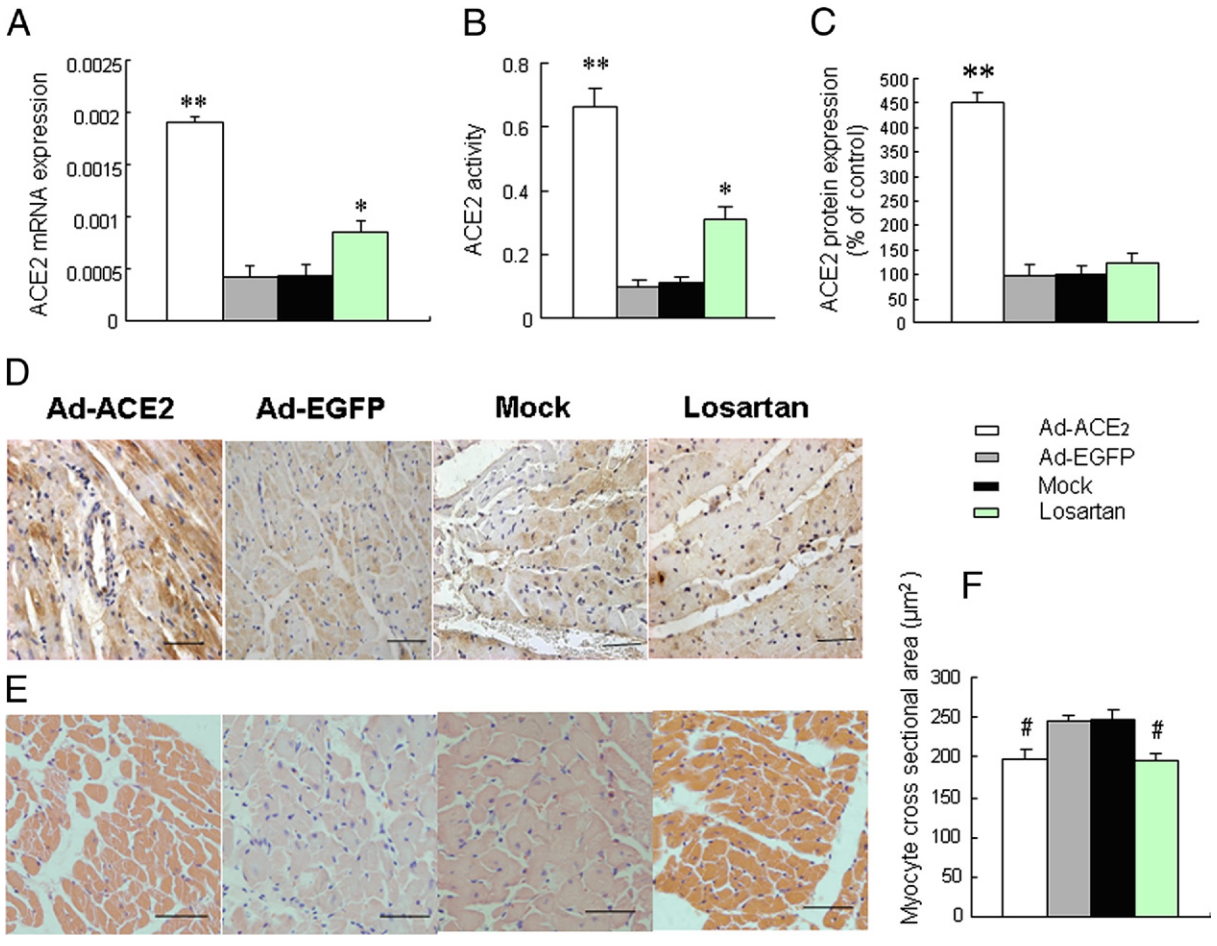

Figure 2 **ACE2 Expression and Activity After Gene Transfer in 5 Groups of Rats**

(A) Angiotensin-converting enzyme 2 (ACE2) messenger ribonucleic acid (mRNA) expression, **(B)** ACE2 activity, **(C)** representative immunostaining of ACE2 protein expression, and **(D)** quantitative analysis of ACE2 protein expression by immunostaining (scale bar = 50 μm, magnification 40×) 2 weeks after gene transfer in adenovirus–ACE2 (Ad-ACE2), adenovirus–enhanced green fluorescent protein (Ad-EGFP), mock, and losartan groups of rats; **(E)** representative hematoxylin and eosin staining of cardiomyocytes and **(F)** quantitative analysis of cardiomyocyte cross-sectional area (scale bar = 50 μm, magnification 40×) 4 weeks after gene transfer in Ad-ACE2, Ad-EGFP, mock, and losartan groups of rats. #$p < 0.05$, *$p < 0.01$, and **$p < 0.001$ versus Ad-EGFP and mock groups.

were substantially lower in the Ad-ACE2 group than in the losartan group ($p < 0.05$). Compared with the Ad-EGFP and mock groups, the protein expression levels of TGF-β were significantly reduced in the Ad-ACE2 and losartan groups (Figs. 4A1 and 4A2). However, TGF-β protein expression was still higher in the losartan group than in the Ad-ACE2 group ($p < 0.05$).

MMP-2 activity and MMP-2, MMP-9, and TIMP-1 protein expression in vivo. MMP-2 activity (Figs. 4B1 and 4B2) and MMP2 protein expression level (Fig. 4C1 and 4C2) were significantly increased in the Ad-ACE2 group in comparison with the Ad-EGFP and mock groups. MMP-2 activity was higher in the losartan group than in the Ad-EGFP and mock groups but lower than in the Ad-ACE2 group ($p < 0.01$) (Fig. 4B2). In contrast, the MMP-9 (Figs. 4D1 and 4D2) and TIMP-1 (Figs. 4E1 and 4E2) protein expression levels were similar among the 4 treatment groups.

ACE2, ACE, Ang-II, and Ang-(1-7) expression in vivo. Western blot analysis showed that ACE2 expression was increased significantly in the Ad-ACE2 group compared with the Ad-EGFP, losartan, and mock groups ($p < 0.001$) (Figs. 5A and 5B). In contrast, ACE protein expression was significantly lower in the Ad-ACE2 group than in the Ad-EGFP, losartan, and mock groups ($p < 0.01$) (Figs. 5C and 5D). As revealed by ELISA, the Ang-II protein expression level was lowest in the Ad-ACE2 group and highest in the losartan group among the 4 treatment groups ($p < 0.01$) (Fig. 5E). In contrast, the Ang-(1-7) level by ELISA was significantly higher in the Ad-ACE2 and losartan groups than in the Ad-EGFP or mock group and was higher in the Ad-ACE2 group than in the losartan group ($p < 0.01$ to 0.05) (Fig. 5F).

Ang-II and Ang-(1-7) expression in vitro. Before treatment, Ang-II expression level was higher in the HG group

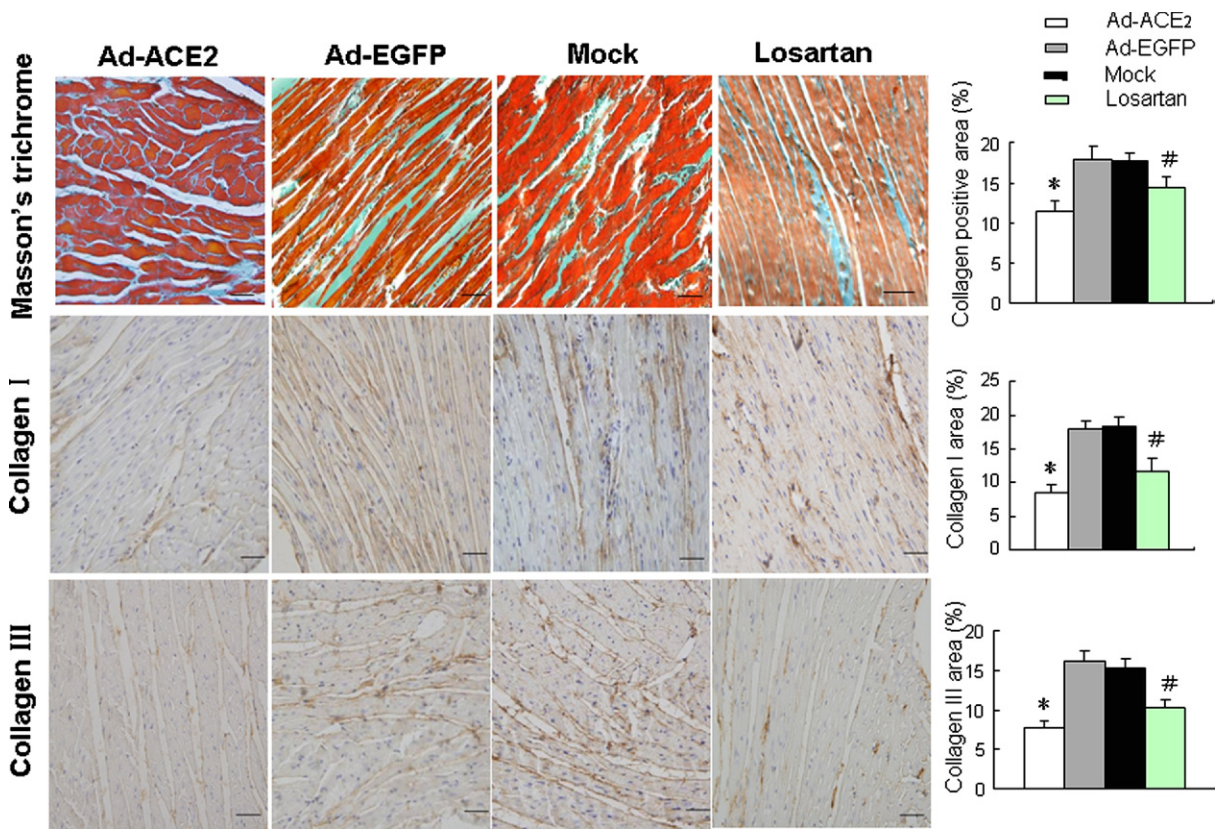

Figure 3 Masson's Trichrome Staining of Myocardium and Collagen Protein Expression in 5 Groups of Rats

(Top row) Representative Masson's trichrome staining of myocardium, **(middle row)** immunostaining of collagen I, and **(bottom row)** immunostaining of collagen III in adenovirus–angiotensin-converting enzyme 2 (Ad-ACE2), adenovirus–enhanced green fluorescent protein (Ad-EGFP), mock, and losartan groups of rats 4 weeks after gene transfer. The histograms **(right)** show quantitative analysis of the positive staining of collagen, collagen I, and collagen III. *$p < 0.01$ versus the Ad-EGFP and mock groups; #$p < 0.05$ versus the Ad-ACE2 group. Scale bars = 50 μm.

than in the normal glucose and osmotic control groups after 12-h and 48-h stimulation of the cardiac fibroblasts ($p < 0.01$ to 0.05) (Online Fig. 1A) and myocytes ($p < 0.01$) (Online Fig. 2A). The expression level of Ang-II showed a time-dependent increase with 12-h, 24-h, and 48-h stimulation after exposure to 25 mmol/l glucose ($p < 0.01$ to 0.05) (Online Figs. 1B and 2B).

After treatment, Ang-II expression level was substantially lower in the HG+Ad-ACE2 group than in the HG+Ad-EGFP and control groups with the cardiac fibroblasts (Online Fig. 1C) and myocytes (Online Fig. 2C) at 12 h ($p < 0.05$) and 48 h ($p < 0.01$). In contrast, the Ang-(1-7) expression level was significantly higher in the HG+Ad-ACE2 group than in the HG+Ad-EGFP and control groups at 24 h ($p < 0.01$) (Online Figs. 1D and 2D).

Collagen production in vitro. Collagen synthesis in fibroblasts, as evaluated by ^{3}H-proline incorporation, was significantly reduced in the HG+Ad-ACE2 group compared with the HG+Ad-EGFP or control groups. In contrast, the administration of A779 in the HG+Ad-ACE2+A779 group resulted in higher levels of collagen protein expression than those in the HG+Ad-ACE2 group ($p < 0.01$) (Online Fig. 3A).

Collagen and TGF-β protein expression in fibroblasts in vitro. ELISA showed that collagen I and III levels were significantly lower in the HG+Ad-ACE2 group than in the HG+Ad-EGFP or control group, suggesting that Ad-ACE2 transfection inhibited collagen synthesis in fibroblasts. In contrast, collagen I and III levels were significantly higher in the HG+Ad-ACE2+A779 group than in the HG+Ad-ACE2 group ($p < 0.01$) (Online Figs. 3B and 3C).

TGF-β protein expression by Western blot analysis was significantly lower in the HG+Ad-ACE2 group than in the HG+Ad-EGFP and control groups, indicating that Ad-ACE2 transfection inhibited TGF-β expression in fibroblasts ($p < 0.01$) (Online Figs. 3D1 and 3D2).

Effects of fibroblast-myocyte co-culture on collagen levels. The protein levels of collagen I and III were significantly higher in the fibroblast + nontransfected myocyte group than in the fibroblast group. In contrast, the protein levels of collagen I and III were lower in the fibroblast + ACE2-

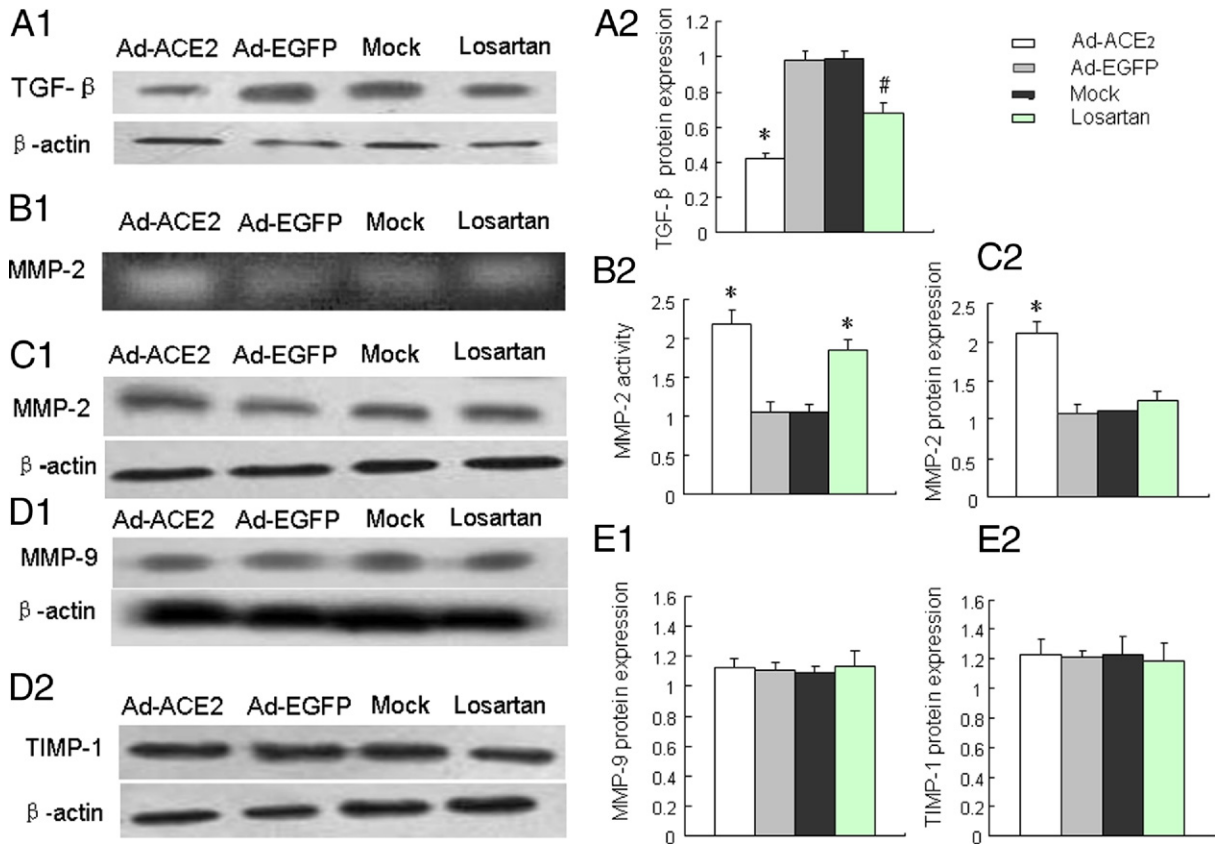

Figure 4 **TGF-β, MMP-2, MMP-9, and TIMP-1 Protein Expression in 5 Groups of Rats 4 Weeks After ACE2 Gene Transfer**

(A1) Transforming growth factor (TGF)-β protein expression in the myocardium by Western blot analysis and **(A2)** quantitative analysis of the results in **(A1)**; **(B1)** matrix metalloproteinase (MMP)–2 activity in the myocardium by zymographic analysis and **(B2)** quantitative analysis of the results in **(B1)**; **(C1)** MMP-2 protein expression in the myocardium by Western blot analysis and **(C2)** quantitative analysis of the results in **(C1)**; **(D1)** MMP-9 protein expression in the myocardium by Western blot analysis and **(D2)** quantitative analysis of the results in **(D1)**; **(E1)** tissue inhibitor of metalloproteinase (TIMP)–1 protein expression in the myocardium by Western blot analysis and **(E2)** quantitative analysis of the results in **(E1)**. *$p < 0.01$ versus adenovirus–enhanced green fluorescent protein (Ad-EGFP) and mock groups; #$p < 0.05$ versus Ad-EGFP and mock groups. ACE2 = angiotensin-converting enzyme-2; Ad-ACE2 = adenovirus angiotensin-converting enzyme-2.

transfected myocyte group than in the fibroblast + nontransfected myocyte group ($p < 0.01$) (Online Figs. 4A and 4C).

The protein levels of collagen I and III were substantially higher in the fibroblast + nontransfected myocyte media group than in the fibroblast group. In contrast, the protein levels of collagen I and III were lower in the fibroblast + ACE2-transfected myocyte media group than in the fibroblast + nontransfected myocyte media group ($p < 0.01$) (Online Figs. 4B and 4D).

Effects of fibroblast-myocyte co-culture on TGF-β levels. TGF-β levels determined by ELISA were significantly higher in the fibroblast + nontransfected myocyte group than in the fibroblast group. In contrast, TGF-β levels were lower in the fibroblast + ACE2-transfected myocyte group than in the fibroblast + nontransfected myocyte group ($p < 0.01$) (Online Fig. 4E).

TGF-β levels were substantially higher in the fibroblast + nontransfected myocyte media group than in the fibroblast group. However, TGF-β levels were lower in the fibroblast + ACE2-transfected myocyte media group than in the fibroblast + nontransfected myocyte media group ($p < 0.01$) (Online Fig. 4F).

Body weight, blood glucose, blood pressure, and heart rate. Body weight was significantly decreased in rats in the Ad-ACE2, Ad-EGFP, losartan, and mock groups from week 8 to week 16, whereas blood glucose levels were substantially increased from week 1 to week 16 and did not differ among the 4 treatment groups (Online Tables 5 and 6). At week 16, systolic and diastolic blood pressure were significantly lower in the losartan group than in the Ad-ACE2, Ad-EGFP, and mock groups, but heart rate showed no significant difference among the 4 treatment groups (Online Table 7).

Discussion

The major finding of the present study was that ACE2 overexpression in vitro decreased HG-induced Ang-II production and collagen and TGF-β expression in cardiac fibroblasts, and ACE2 overexpression in vivo attenuated

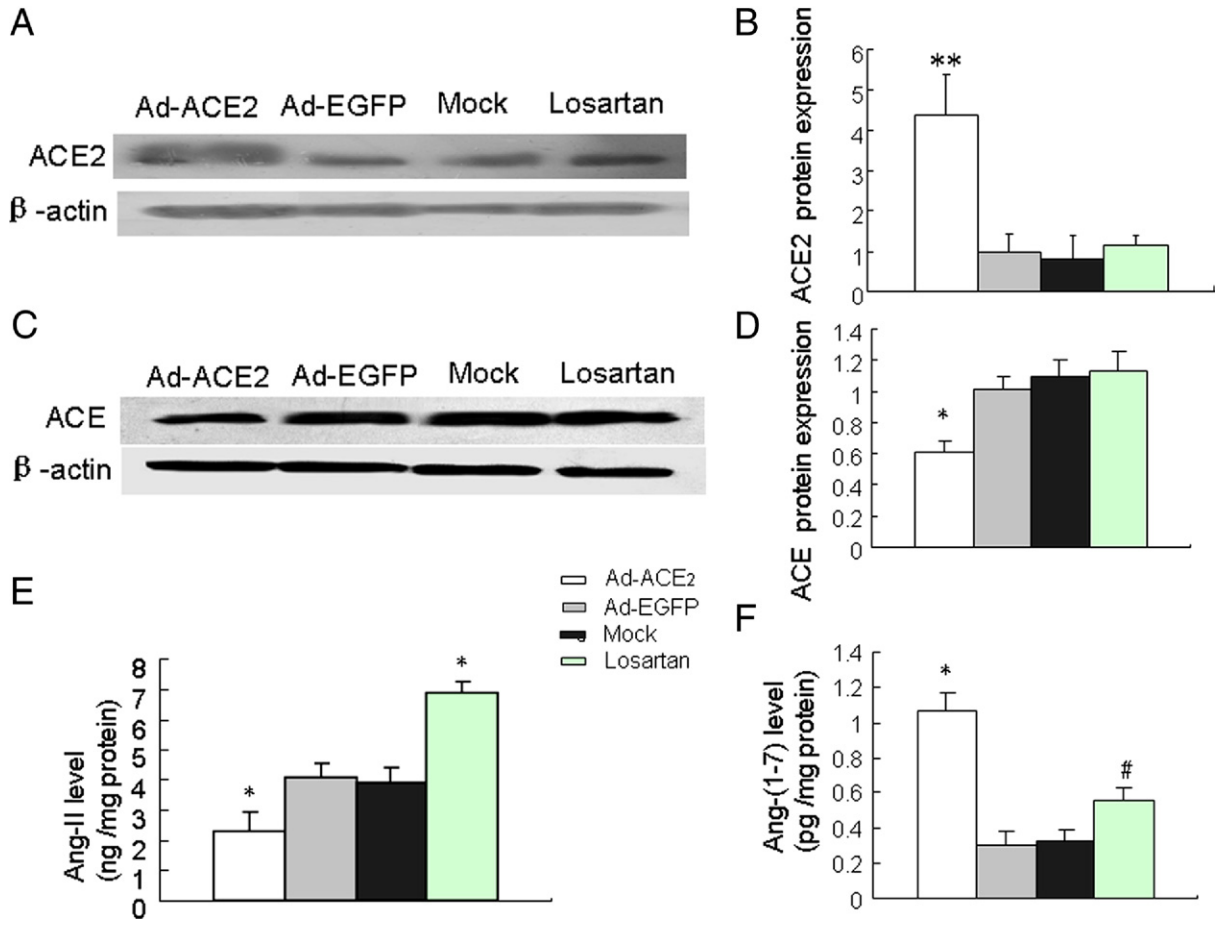

Figure 5 ACE2, ACE, Ang-(1-7), and Ang-II Protein Expression in 5 Groups of Rats 4 Weeks After ACE2 Gene Transfer

(A) Angiotensin (Ang)–converting enzyme (ACE)-2 (ACE2) protein expression in the myocardium by Western blot analysis and **(B)** quantitative analysis of the results in **(A)**; **(C)** ACE protein expression in the myocardium by Western blot analysis and **(D)** quantitative analysis of the results in **(C)**; **(E)** Ang-II levels in the myocardium by enzyme-linked immunosorbent assay (ELISA); **(F)** Ang-(1-7) levels in the myocardium by ELISA. *$p < 0.01$ versus adenovirus–enhanced green fluorescent protein (Ad-EGFP) and mock groups; **$p < 0.001$ versus Ad-EGFP and mock groups; #$p < 0.05$ versus Ad-EGFP and mock groups. Ad-ACE2 = adenovirus angiotensin-converting enzyme-2.

myocyte hypertrophy, myocardial fibrosis, and LV remodeling and improved LV systolic and diastolic function. To the best of our knowledge, this study is the first to report the therapeutic effects of ACE2 overexpression in an animal model of DCM.

The most important pathological feature of DCM is the accumulation of ECM proteins, particularly in collagen, and DCM is different from other types of cardiomyopathy in the lack of an inflammatory response (8). Available evidence indicates that accumulation of ECM is mediated by hyperglycemia, and the RAS plays an important role in collagen production in DCM (6). Several studies have demonstrated that Ang-II increases collagen production in cultured cardiac fibroblasts in vitro. Recently, Singh et al. (7) observed that inhibition of HG-induced extracellular Ang-II by candesartan only partially blocked collagen I expression, whereas inhibition of Ang-II synthesis completely prevented the HG effects, indicating that both intracellular and extracellular Ang-II levels contribute to enhanced fibrosis (7). In the present study, ACE2 overexpression lowered HG-induced Ang-II levels in cardiac fibroblasts and myocytes in vitro and inhibited collagen I and III protein production in the myocardium of rats in vivo, suggesting that the major mechanism of ACE2 overexpression in inhibiting collagen accumulation is probably the conversion of Ang-II to Ang-1-7.

Cardiac collagen content is regulated by a balance between collagen production and degradation. Extracellular degradation of collagen is the major rate limiting step in collagen metabolism and is affected by MMPs and TIMPs. Thus, accumulation of myocardial collagen has been regarded as a hallmark of myocardial fibrosis, and MMPs and TIMPs are central to the modulation of ECM composition in the diabetic heart (7,9). Among identified members of MMP family, MMP-2 and MMP-9 were found to be essential for collagen degradation. Van Linthout et al. (9)

evaluated the role of matrix MMP-2 in experimental DCM and found that MMP-2 gene expression and activity were decreased and associated with ECM accumulation. In the present study, we found that MMP-2 activity and protein expression were increased in the Ad-ACE2 group compared with the Ad-EGFP and control groups, but MMP-9 and TIMP-1 protein expression levels showed no differences among these groups, suggesting that ACE2 decreased myocardial collagen accumulation by up-regulating MMP-2. Our results agree with previous studies in which MMP-2 activity was down-regulated in experimental DCM and in cardiac fibroblasts stimulated by HG and Ang-II (10).

Another important factor regulating collagen production in DCM is TGF-β. Studies in vitro showed that TGF-β exerted a direct effect on cardiac fibroblasts by transforming these cells into more synthetic myofibroblasts (11). Westermann et al. (6) reported that TGF-β expression was significantly increased in DCM, whereas irbesartan, an Ang type 1 receptor antagonist, decreased TGF-β expression. Furthermore, in cultured human mesangial cells and cardiac fibroblasts, HG-induced increase in TGF-β production was partially blocked by the Ang type 1 receptor antagonist candesartan, which suggests that Ang-II was involved in TGF-β production. Our present study showed that TGF-β protein expression was significantly lower in the Ad-ACE2 group than that in the Ad-EGFP and mock groups, indicating that ACE2 overexpression decreased collagen production partially through inhibition of TGF-β protein expression. In view of a recent report that cardiac fibroblasts induce myocyte hypertrophy via secretion of paracrine factors (12), increased collagen and TGF-β protein expression in fibroblasts might contribute to the enhanced cardiomyocyte hypertrophy observed in the present study. In addition, Sarkar et al. (13) showed that myocytes may secrete active TGF-β in the presence of Ang-II, and TGF-β may induce interleukin-6 production in fibroblasts, thus promoting collagen synthesis. Our results confirm that myocytes promoted collagen and TGF-β production in fibroblasts, suggesting that cross-talk between myocytes and fibroblasts exists, which may play a more important role than fibroblasts alone in collagen production in DCM.

Current evidence indicates that intracellular Ang-II is an essential player in diabetes-induced organ damage (14,15). Singh et al. (7) demonstrated that intracellular Ang-II was produced in cardiac myocytes and fibroblasts in HG conditions, which up-regulates TGF-β and collagen synthesis and contributes to ECM accumulation. Although ACE inhibitors or Ang receptor blockers (ARBs) are effective in preventing diabetic complications (6,14), recent studies found that ARBs can inhibit effects of extracellular rather than intracellular Ang-II (7). Similarly, ACE inhibitors can block Ang-II synthesis catalyzed by ACE, such as in cardiac fibroblasts, but cannot block Ang-II synthesis catalyzed by chymase, such as in cardiac myocytes (7,14,15). Thus, ARBs or ACE inhibitors cannot completely inhibit RAS activation in DCM. In contrast, ACE2 catalyzes the conversion of pro-proliferative Ang-II to antiproliferative Ang-(1-7) in both fibroblasts and myocytes and may provide more benefits of cardiac protection than ARBs or ACE inhibitors in the setting of DCM. This concept is supported by the results of the present study. Although losartan treatment attenuated myocardial hypertrophy and fibrosis and improved LV remodeling and function, ACE2 gene transfer further reduced LVEDD and collagen expression and increased LVEF in comparison with the losartan group, suggesting that ACE2 is probably more effective in the treatment of DCM than ARBs. In contrast to a previous report that ACE2 deficiency exhibited diverse effects on the kidney in diabetic mice (16), our results clearly demonstrate the beneficial effects of ACE2 overexpression on DCM in rats, which is similar to the salutary effect of ACE2 in a mouse model of atherosclerosis observed by the same group of investigators (17).

The putative mechanisms underlying the therapeutic effects of ACE2 gene transfer may be 4-fold. First, ACE2 overexpression inhibits collagen accumulation by converting Ang-II to Ang-(1-7) and by enhancing MMP-2 activity. Second, the balance between ACE and ACE2 is critical for the functional state of the RAS (18). In the present study, ACE2 overexpression resulted in a shift in balance toward low ACE protein expression and hence a low Ang-II concentration. Third, the administration of A779 in the Ad-ACE2+A779 group resulted in one-third higher collagen protein expression than the Ad-ACE2 group, indicating that Ang-(1-7) may have a direct inhibitory effect on cardiac collagen synthesis. Fourth, the most important mechanism of ACE2 in inhibiting collagen accumulation may involve suppression of fibroblast-myocyte cross-talk for collagen production in the presence of HG.

Study limitations. First, the rats used in the experiment were relatively young (age 24 weeks at euthanasia), whereas diabetes commonly affects aging population in clinical practice. Thus, our results need to be validated in old diabetic mice. Second, although we found that ACE2 overexpression was associated with down-regulation of Ang-II, up-regulation of Ang-(1-7) and MMP-2 activity, attenuation of ACE expression, and inhibition of fibroblast-myocyte cross-talk, the downstream signaling pathways mediating the therapeutic effects are unclear and require further investigation.

Conclusions

Our studies in vitro and in vivo showed that ACE2 overexpression inhibited collagen accumulation and improved LV remodeling and function in a rat model of DCM. The putative mechanisms may involve a shift in balance toward an inhibited fibroblast-myocyte cross-talk for collagen and TGF-β production and enhanced collagen

degradation by MMP-2. Thus, ACE2 overexpression may provide an effective regimen for the treatment of DCM.

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Key Words: angiotensin-converting enzyme 2 ■ angiotensin II ■ diabetic cardiomyopathy ■ gene therapy ■ heart failure.

APPENDIX

For an expanded Methods section and supplemental figures and tables, please see the online version of this article.