Angiotensin-(1–7) treatment mitigates right ventricular fibrosis as a distinctive feature of diabetic cardiomyopathy

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The Key Laboratory of Cardiovascular Remodeling and Function Research, Chinese Ministry of Education and Chinese Ministry of Health, Qilu Hospital, Shandong University, Jinan, Shandong, People’s Republic of China

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A WEALTH OF EPIDEMIOLOGICAL evidence has demonstrated a high incidence of cardiovascular disease among patients with diabetes mellitus. One important cardiovascular insult in diabetic patients is the development of heart failure, which is due to ill-defined cardiomyopathy, even in the absence of arterial hypertension and coronary atherosclerosis (1, 3, 13, 20). However, current diabetic therapies are not sufficient to completely prevent diabetes-induced end-organ damage even with normalized hyperglycemia. Cardiac remodeling is thought to be an important aspect of disease progression in heart failure, regardless of its cause.

Although underestimated in the past, the contribution of right ventricular (RV) function to overall myocardial contractility is considerable. RV hypertrophy was recently reported to be associated with risk of heart failure or death in a multiethnic population free of clinical cardiovascular disease at baseline (14). Several studies have demonstrated inherent differences between left ventricular (LV) and RV papillary muscle performance (4, 21). These differences in LV and RV contractile function may be important clinically in terms of the function of the right ventricle as a systemic pump and selective therapy for pump dysfunction. Adverse RV remodeling and dysfunction is a complex maladaptive process involving structural, hemodynamic, histopathological, and genetic changes. The process may be multifactorial and is frequently encountered in various disease states such as myocardial infarction, pulmonary hypertension, diabetes, and heart failure. RV dysfunction is an independent predictor of outcome in patients with myocardial infarction (17) and heart failure (5). Despite the association of RV impairment with aggravated myocardial functional outcome in distinct cardiac diseases, its role in diabetic cardiomyopathy is poorly understood.

The renin-angiotensin system (RAS), which is activated in diabetes (26), contributes to the development of LV remodeling and dysfunction, but this association has not been established for right ventricles. Inhibition of a hyperactive RAS by angiotensin-converting enzyme (ACE) inhibitors or angiotensin II type 1 (AT1) receptor blockers protects against LV remodeling, left heart failure, and mortality; however, convincing evidence for use of these therapies in RV failure is still lacking. In preliminary studies, we demonstrated that overexpression of ACE2, a zinc metalloproteinase and a new member of the RAS, attenuated LV remodeling and dysfunction induced by type 1 diabetes (7) or acute myocardial infarction (34) in rats, which was accompanied by downregulated angiotensin II (ANG-II) and upregulated angiotensin-(1–7) [ANG-(1–7)] levels. In addition, Johnson et al. (12) found that recombinant human ACE2 diminished RV hypertrophy and improved RV systolic and diastolic function in association with a normalized connexin 37 expression in a mouse RV load-stress model of early heart failure.

ANG-(1–7) is formed from ANG-I and -II by several endopeptidases and carboxypeptidases, including ACE and ACE2, and has vasoprotection, atheroprotection, and cardioprotection properties (11, 16, 24, 25). Several studies have shown that endogenous or exogenous ANG-(1–7) leads to enhanced LV functional performance in diabetic and other animal models.

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angiotesin-(1–7) is activated to mediate its effects is much debated. With the discovery of the G protein-coupled receptor Mas, which is highly expressed in several tissues, including the heart, kidney, and the vasculature, increasing evidence has shown that most effects of ANG-(1–7) are mediated through the Mas receptor (22).

Although the literature supports a beneficial role for ANG-(1–7) in LV remodeling and function, the effects of ANG-(1–7) specifically in the setting of RV dysfunction have not been evaluated. Importantly, the response of right ventricles to diabetes should not be extrapolated from left heart experiments. Notwithstanding their close vicinity, right and left ventricles are distinct from each other in embryology, structure, and function. The right ventricle is smaller, crescent shaped, and thin walled and has a much lower afterload than the left ventricle; these differences are augmented by a differing embryologic origin of the right ventricle (8, 18). Thus the right ventricle may not respond like the left ventricle to diabetes and pharmacological therapies.

Here, we aimed to investigate whether LV changes in cardiac fibrosis, hypertrophy, apoptosis, and function in diabetest are paralleled by similar RV alterations in an experimental rat model of type 1 diabetes. We also aimed to determine the effect of chronic ANG-(1–7) treatment on alterations in the right ventricle. To elucidate the possible mechanisms, we further investigated the effect of ANG-(1–7) on ACE and ACE2 expression and activities as well as protein expression of AT$_1$, AT$_2$, and Mas receptors in right ventricles of diabetic rats.

**MATERIALS AND METHODS**

**Animal model.** A total of 108 male Wistar rats (∼200 g in weight) were purchased from Vital River Laboratory Animal Technology (Beijing, China) and randomly divided into a control group and eight treatment groups (n = 12 in each group). Diabetes was induced by a single intraperitoneal injection of streptozotocin (Sigma, St. Louis, MO) in eight treatment groups, which were randomly divided at the end of 12 wk to receive vehicle (0.15 M NaCl), ANG-(1–7) (200, 400, or 800 ng·kg$^{-1}$·min$^{-1}$; Auspep, Parkville, Australia), perindopril (2 mg·kg$^{-1}$·day$^{-1}$; a gift from Servier, Melbourne, Australia) alone or with ANG-(1–7) (800 ng·kg$^{-1}$·min$^{-1}$), A779 (800 ng·kg$^{-1}$·min$^{-1}$), and Mas receptor antagonist; Auspep, Parkville, Australia) alone or with ANG-(1–7) (800 ng·kg$^{-1}$·min$^{-1}$), A779 (800 ng·kg$^{-1}$·min$^{-1}$), and Mas receptor antagonist; Auspep, Parkville, Australia) alone or with ANG-(1–7) (800 ng·kg$^{-1}$·min$^{-1}$), or PD123319 (10 mg·kg$^{-1}$·day$^{-1}$; AT$_2$ receptor antagonist; Pfizer, Groton, CT) with ANG-(1–7) (800 ng·kg$^{-1}$·min$^{-1}$) for 4 wk. ANG-(1–7), A779, and PD123319 were administered by subcutaneously implanted Alzet osmotic pumps (model 2004; Alza, Palo Alto, CA), and perindopril was given by intragastric intubation. All procedures were performed in accordance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996). The Institutional Animal Care and Use Committee at Qilu Hospital, Shandong University, reviewed and approved the protocol.

**Blood pressure measurement.** The peripheral arterial systolic and diastolic blood pressure was measured by use of a photoelectric tail-cuff device (Natsume, Tokyo, Japan) at the end of weeks 12 and 16. The rats were trained on three occasions before actual recordings, and blood pressure measurements from three readings were averaged.

**Measurement of RV pressure.** At the end of week 16, animals were anesthetized, tracheostomized, and mechanically ventilated. With a vertical incision over the abdomen, catherization was used to cut the diaphragm and expose the heart. The Millar SPR-869 microtip catheter (Millar Instruments, Houston, TX) connected to the Chart program supplied with the PowerLab system (ADInstruments, Sydney, Australia) was inserted into a surgically exposed right ventricle to directly measure heart rate, RV peak pressure, RV end-diastolic pressure, maximal rate of pressure increase, and RV relaxation time constant (12).

**Blood analysis.** After rats fasted overnight, blood samples were collected from jugular veins. The serum lipid profile and fasting blood glucose (FBG) were analyzed by use of the Bayer 1650 blood chemistry analyzer (Bayer, Tarrytown, NY).

**Preparation of paraffin-embedded tissue sections.** After physiology studies, hearts of surviving rats were arrested with use of 1 M KCl, perfusion fixed with phosphate-buffered 4% paraformaldehyde, collected in ice-cold phosphate-buffered saline (PBS), and fixed in 4% paraformaldehyde overnight at 4°C, washed with PBS, dehydrated through an ethanol series, embedded in paraffin, and cut into 4-µm sections.

**Histology.** Sections (4-µm thick) of paraffin-embedded heart tissues were stained with Masson’s trichrome and then visualized by light microscopy (Olympus, Tokyo, Japan) and photographed. The collagen components were quantified by measuring the proportion of area positively stained with Masson’s trichrome to total RV or LV free-wall area in sections by computer-assisted morphometry (ImagePro Plus 6.0; Media Cybernetics, Bethesda, MD). For each sample, all available fields (>20 fields) were measured (all fields were visualized with use of a ×40 objective lens).

To measure fibrosis, dark-green-stained collagen fibers were quantified in Masson trichrome-stained sections. The collagen volume fraction (CVF) and ratio of perivascular collagen area to luminal area (PVCA/LA) were analyzed by quantitative morphometry with ImagePro Plus 6.0. CVF was calculated as the area occupied by collagen divided by the total area in the visual field (27). Perivascular collagen was excluded from the CVF measurement. To normalize the PVCA around vessels with different sizes, perivascular collagen content was represented as PVCA/LA.

**Determination of cardiomyocyte cross-sectional area.** Sections of paraffin-embedded hearts were incubated with 5 µg/ml Alexa fluor 488-conjugated wheat germ agglutinin (Invitrogen, Carlsbad, CA) in Hank’s balanced salt solution (HBSS) in the dark for 10 min at room temperature. After being washed with PBS, cardiomyocyte membrane staining was examined by laser-scanning confocal microscopy (LSM710 Meta; Carl Zeiss, Jena, Germany). Myocyte cross-sectional area was quantified in 20 randomly chosen high-power fields in each section.

**Immunohistochemistry.** Tissue sections were prepared as described above, deparaffinized and then incubated with primary antibodies against collagen I or III (both 1:1000; both Abcam, Cambridge, UK) in Hank’s balanced salt solution (HBSS) in the dark for 10 min at room temperature. After being washed with PBS, cardiomyocyte membrane staining was examined by laser-scanning confocal microscopy (LSM710 Meta; Carl Zeiss, Jena, Germany). Myocyte cross-sectional area was quantified in 20 randomly chosen high-power fields in each section.

**RNA extraction from myocardial tissue and real-time RT-PCR.** Primer sequences for rat fibronectin-I, transforming growth factor-β1 (TGF-β1), brain natriuretic peptide (BNP), β-myosin heavy chain (β-MHC), Bax, Bcl-2, AT$_1$ receptor, AT$_2$ receptor, Mas receptor, and the housekeeping gene β-actin are in Table 1. Total RNA was extracted from freshly isolated myocardial samples by use of Trizol reagent (Invitrogen). Oligo (dT) primed cDNA synthesis was involved use of Superscript III reverse transcriptase (Invitrogen). Transcripts were amplified from reverse-transcribed cDNA by use of SYBR Green (Invitrogen). Quantitative assessment of relative gene expression levels involved the 2$^{-ΔΔCT}$ method.

**Western blot analysis.** Proteins from tissue homogenates were extracted for Western blot analysis. After quantification by use of the BCA Protein Assay Kit (Pierce, Rockford, IL), the soluble protein fraction was resuspended in Laemmli loading buffer (2% SDS, 20%
Table 1. Primer sequences for real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5'-3')</th>
<th>Reverse Primer (5'-3')</th>
<th>Product Length, bp</th>
</tr>
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<tr>
<td>β-Actin</td>
<td>CTCCTAGCTGCGACATG</td>
<td>GTATGCTAGGCTGCTATCC</td>
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<tr>
<td>BNP</td>
<td>GAGGCTGCTGCGACATG</td>
<td>GTGTCGCGCTCAGCATG</td>
<td>200</td>
</tr>
<tr>
<td>β-MHC</td>
<td>TGTGGCTGCTGCGACATG</td>
<td>GTTGTGGCTGCTGCTATCC</td>
<td>142</td>
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<tr>
<td>Fibroectin-1</td>
<td>TGCTCTGTTGCTGACACAC</td>
<td>GTTTCTGCTGCTGCTATCC</td>
<td>207</td>
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<tr>
<td>TGF-β1</td>
<td>GGTGAGGCTGCGACATG</td>
<td>AACTCGAGGCTGCTGCTACT</td>
<td>115</td>
</tr>
<tr>
<td>TGF-β2</td>
<td>GTGTTGCTGCTGACACAC</td>
<td>GTTTCTGCTGCTGCTATCC</td>
<td>125</td>
</tr>
<tr>
<td>AT1R</td>
<td>CTGCAACCTTCTGCGGAAATG</td>
<td>GTTTGCTGCTGCTGCTATCC</td>
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</tr>
<tr>
<td>AT2R</td>
<td>ACCTCGAGCTGCGACATG</td>
<td>GTTTGCTGCTGCTGCTATCC</td>
<td>160</td>
</tr>
<tr>
<td>MasR</td>
<td>TGCTGCTGCTGCGACATG</td>
<td>GTTTGCTGCTGCTGCTATCC</td>
<td>159</td>
</tr>
</tbody>
</table>

Table 2. Characteristics of 9 groups of rats 4 wk after treatment

<table>
<thead>
<tr>
<th>Control</th>
<th>Mock</th>
<th>A200</th>
<th>A400</th>
<th>A800</th>
<th>P</th>
<th>A800 + P</th>
<th>A800 + A779</th>
<th>A800 + PD123319</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, beats/min</td>
<td>385.6 ± 7.0</td>
<td>350.3 ± 7.8*</td>
<td>354.1 ± 8.2*</td>
<td>355.4 ± 8.3*</td>
<td>349.9 ± 14.5*</td>
<td>356.2 ± 6.1*</td>
<td>358.3 ± 11.9</td>
<td>356.0 ± 11.9*</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>127.8 ± 2.5</td>
<td>112.4 ± 2.2*</td>
<td>113.2 ± 4.3*</td>
<td>110.5 ± 5.6*</td>
<td>110.8 ± 5.9*</td>
<td>96.3 ± 4.7*</td>
<td>98.1 ± 4.5*</td>
<td>108.4 ± 4.9*</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>95.8 ± 3.4</td>
<td>84.5 ± 4.1*</td>
<td>86.0 ± 4.3*</td>
<td>83.8 ± 3.7*</td>
<td>84.9 ± 5.5</td>
<td>70.8 ± 4.3*</td>
<td>69.9 ± 4.2*</td>
<td>83.6 ± 6.0</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>108.5 ± 2.7</td>
<td>93.4 ± 2.2*</td>
<td>97.3 ± 3.4*</td>
<td>94.2 ± 3.6*</td>
<td>96.0 ± 4.5*</td>
<td>82.1 ± 2.6*</td>
<td>82.0 ± 2.8*</td>
<td>94.3 ± 4.4*</td>
</tr>
<tr>
<td>BNP, mg/mL</td>
<td>3.88 ± 0.37</td>
<td>23.29 ± 0.29*</td>
<td>23.33 ± 0.95*</td>
<td>22.72 ± 1.21*</td>
<td>23.35 ± 1.43*</td>
<td>22.19 ± 1.15*</td>
<td>22.67 ± 1.32*</td>
<td>23.41 ± 1.14*</td>
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<tr>
<td>TC, mmol/L</td>
<td>1.75 ± 0.09</td>
<td>2.16 ± 0.13*</td>
<td>2.17 ± 0.11*</td>
<td>2.14 ± 0.09*</td>
<td>2.17 ± 0.08*</td>
<td>2.16 ± 0.09*</td>
<td>2.12 ± 0.10*</td>
<td>2.21 ± 0.07*</td>
</tr>
<tr>
<td>TG, mg/dL</td>
<td>0.80 ± 0.09</td>
<td>1.87 ± 0.17*</td>
<td>1.82 ± 0.21*</td>
<td>1.72 ± 0.16*</td>
<td>1.84 ± 0.15*</td>
<td>1.76 ± 0.25*</td>
<td>1.88 ± 0.14*</td>
<td>1.86 ± 0.10*</td>
</tr>
<tr>
<td>ANG-(1–7), mg/mL</td>
<td>32.25 ± 1.29</td>
<td>28.86 ± 1.20</td>
<td>31.17 ± 1.52</td>
<td>32.33 ± 1.59</td>
<td>31.50 ± 1.03</td>
<td>28.33 ± 1.33</td>
<td>29.50 ± 1.09</td>
<td>29.27 ± 0.76</td>
</tr>
<tr>
<td>BNP, mg/mL</td>
<td>4.41 ± 0.64</td>
<td>6.94 ± 0.54*</td>
<td>5.88 ± 0.37*</td>
<td>5.33 ± 0.21*</td>
<td>4.92 ± 0.43*</td>
<td>5.53 ± 0.37*</td>
<td>4.72 ± 0.23*</td>
<td>6.25 ± 0.51*</td>
</tr>
</tbody>
</table>

Data are means ± SE (n = 6–12 per group). A200, ANG-1(–7) at 200 ng·kg⁻¹·min⁻¹; A400, ANG-1(–7) at 400 ng·kg⁻¹·min⁻¹; A800, ANG-1(–7) at 800 ng·kg⁻¹·min⁻¹; BW, body weight; DBP, diastolic blood pressure; dP/dtmax, maximal rate of pressure increase; dP/dtmin, maximal rate of pressure decrease; FBG, fasting blood glucose; HR, heart rate; LV, left ventricle; RV, right ventricle; MAP, mean arterial pressure; P, perindopril at 2 mg·kg⁻¹·day⁻¹; PAP, RV end-diastolic pressure; Pmax, RV peak pressure; SBP, systolic blood pressure; r, RV relaxation time constant; TC, total cholesterol; TG, triglyceride levels; TL, tibial length. *P < 0.05 vs. control group; **P < 0.05 vs. mock group; ***P < 0.05 vs. A800 group; ****P < 0.05 vs. P group.
10-µm-thick sections were obtained by use of a sliding microtome (Leica CM1900, Nussloch, Germany).

Dihydroethidium fluorescence and lucigenin-enhanced chemiluminescence. The oxidative fluorescent dye dihydroethidium was used to measure superoxide (O$_2^-$) levels in myocardial frozen sections as described previously (35). NADPH oxidase activity in myocardial sections was quantified by lucigenin-enhanced chemiluminescence as described previously (35).

Cell isolation, culture, and treatment. Primary cultures of neonatal rat RV fibroblasts and myocytes were prepared as described previously (30), with some modification. Briefly, the right ventricles of neonatal Wistar rats (1 to 2 days old) were coarsely minced and digested with 0.08% trypsin to remove erythrocytes and then digested with 0.25% trypsin and 0.05% collagenase type 2 at 37°C. The dissociated cells were placed in uncoated 100-mm culture dishes and incubated at 37°C in a 5% CO$_2$ incubator for 1 h, during which most of RV fibroblasts rapidly adhered to dishes. The attached fibroblasts were washed and further cultured in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO-BRL, Gaithersburg, MD) supplemented with 100 units/ml penicillin (GIBCO-BRL), 100 units/ml streptomycin (GIBCO-BRL), and 10% fetal bovine serum (FBS; GIBCO-BRL) at 37°C in a 5% air-5% CO$_2$ humidified atmosphere.

Fibroblasts were randomly selected for treatment with 1) 5.6 mM glucose only (normal glucose); 2) 5.6 mM glucose plus 19.4 mM mannose (osmotic control); 3) 25 mM glucose only (high glucose); 4) 25 mM glucose plus 10$^{-5}$ M ANG-(1–7) [high glucose + ANG-(1–7)]; 5) 25 mM glucose plus 10$^{-5}$ M ANG-(1–7) plus 10$^{-6}$ M A779 [high glucose + ANG-(1–7) + A779, A + A779]; or 6) 25 mM glucose plus 10$^{-5}$ M ANG-(1–7) plus 10$^{-6}$ M PD123319 [high glucose + ANG-(1–7) + PD123319, A + PD123319]. After exposure for 48 or 72 h, the cell culture supernatant was collected to measure collagen I and III content and TGF-β1 level by use of three commercial ELISA kits (collagen I and III rat ELISA kits from Chemicon, Temecula, CA; TGF-β1 rat ELISA kit from Abcam).

The unattached cells were preplated for another hour on uncoated culture discs. Fibroblasts predominantly attached to dishes, and most of the cardiomyocytes remained unattached. The cardiomyocyte-enriched cells were collected and counted. The cells were plated at 2.5 $\times$ 10$^4$ cells/well in 24-well culture dishes and maintained in DMEM/F-12 containing 100 units/ml penicillin, 100 units/ml streptomycin, 10% FBS, and 0.1 M bromodeoxyuridine under standard culture conditions. With the use of this protocol, 95% of the cells were deemed cardiomyocytes as judged by sarcomere myosin content. On the second day after plating, cells were incubated in serum-free medium containing transferrin (5 µg/ml), insulin (5 µg/ml), and 0.1 M bromodeoxyuridine for 24 h before treatment with high glucose, A, A + A779, or A + PD123319.

Enzyme-linked immunosorbent assay. Enzyme-linked immunosorbent assay (ELISA) was performed to measure the levels of ANG-II, TGF-β1, and soluble collagen I and III proteins.

Coculture of fibroblasts and myocytes. RV fibroblasts were cocultured with ANG-(1–7)-treated or nontreated RV myocytes or the conditioned media of these cells, and the levels of collagen I and III and TGF-β1 proteins in the media were determined by ELISA.

Statistical analysis. All continuous variables are expressed as means ± SE. Data were analyzed by use of SPSS v11.5 (SPSS, Chicago, IL). Analysis of intergroup differences involved one-way ANOVA. Nonparametric statistics were used for nonnormally distrib-

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Fig. 1. Diabetes in rats accelerated fibrosis of both right and left ventricles (RV and LV). A: representative Masson trichrome staining (collagen is green and myocardium red; scale bar = 100 µm) showing interstitial and perivascular fibrosis and representative immunohistochemical staining for collagen I (Coll I) and III (Coll III) content (scale bar = 100 µm). B and C: quantitative analysis of collagen volume fraction (CVF; B) and perivascular collagen area to luminal area (PVCA/LA; C). D: quantitative real-time RT-PCR analysis of mRNA expression of fibronectin I. E–G: Quantitative data of staining for Coll I (E) and Coll III (F) and their ratio (G). H: relative mRNA expression of myocardial transforming growth factor-β1 (TGF-β1). Data were relative to the level of β-actin. I: representative Western blot of TGF-β1, phosphorylated Smad3 (p-Smad3), Smad3, Smad7, and β-actin. J–L: quantification of TGF-β1 protein level (J), p-Smad3/Smad3 (K), and Smad7 protein level (L). Cropped blots are used in Fig. 1, and the blots were run under the same experimental conditions. Data are means ± SE (n = 6 per group). *P < 0.05 and **P < 0.01 vs. control group.
puted results. For comparison of two groups, unpaired Student t-test was used as appropriate. A $P < 0.05$ was considered statistically significant.

**RESULTS**

_Serum lipid profile and FBG concentrations._ One week after streptozotocin injection, FBG was markedly elevated in diabetic rats and remained elevated until the end of the experiment (Table 2). Simultaneously, serum total cholesterol and triglyceride levels were maintained at higher levels than for controls. At the end of week 16, total cholesterol and triglyceride levels or FBG did not differ among the eight treatment groups except compared with the control group (all $P > 0.05$).

_Hemodynamic measurements._ Diabetic rats showed significant systolic and diastolic dysfunction, as indicated by decreased RV maximal rates of pressure increase and decrease as well as increased RV end-diastolic pressure and relaxation time constant (Table 2). Four-week ANG-(1–7) treatment prevented RV dysfunction in part, and the effects were suppressed by coadministration of A779 or PD123319. However, ANG-(1–7) did not significantly change arterial blood pressure or heart rates of diabetic rats.

_Pathological characteristics of diabetic rats._ Consistent with our earlier observations (7, 27), diabetes induced LV remodeling, as indicated by myocardial fibrosis (Fig. 1), hypertrophy (Fig. 2, A–D, and Table 2), and apoptosis in the left ventricle (Fig. 2, E–K). In contrast, RV remodeling in diabetic rats was indicated by fibrosis of the RV free wall in the absence of hypertrophy and apoptosis.

**ANG-(1–7) suppressed diabetic RV fibrosis.** ANG-(1–7) treatment dose dependently reduced CVF and PVCA/LA in right ventricles of diabetic rats, and the effect of high-dose ANG-(1–7) was superior to perindopril (Fig. 3, A–C). The effect of ANG-(1–7) on diabetic RV fibrosis was completely reversed by A779 and partially by PD123319. With ANG-(1–7) treatment, collagen I and III content and ratio of collagen I to III were reduced (Fig. 3, A and E–G). The effect of ANG-(1–7) on collagen I and III levels and collagen I-to-III ratio was superior to perindopril, completely blocked by A779 and partially inhibited by PD123319.

Treatment with ANG-(1–7) alone or with perindopril dose dependently reduced the diabetes-induced mRNA levels of fibronectin-1 (Fig. 3D) and TGF-$\beta$-1 (Fig. 3I), and the effect of ANG-(1–7) was inhibited by A779 or PD123319. In addition, ANG-(1–7) dose dependently inhibited TGF-$\beta$-1 protein expression and Smad3 activation, as well as increased Smad7 level in diabetic rats (Fig. 3, H and J–L). The effect of ANG-(1–7) on TGF-$\beta$-1 and Smad7 level and Smad3 activation was inhibited by A779 or PD123319.

**ANG-(1–7) ameliorated RV oxidative stress of diabetic rats.** Superoxide generation and NADPH oxidase activation mediated by high glucose may be pivotal mechanisms of high-

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**Fig. 2.** Diabetes in rats accelerated hypertrophy and apoptosis of LV cardiomyocytes without altering those of RV cardiomyocytes. A: representative transverse sections stained with wheat germ agglutinin (WGA; scale bar = 20 $\mu$m). B: quantitative data for myocyte size. C–G: relative mRNA levels of brain natriuretic protein (BNP; C), $\beta$-MHC (D), $\beta$-myosin heavy chain (B–MHC; D), Bax (E), Bcl-2 (F), and ratio of Bax to Bcl-2 (G). H: representative Western blot of protein levels of Bax, Bcl-2, and $\beta$-actin. I–K: quantification of Bax (I), Bcl-2 (J), and their ratio (K). Cropped blots are used in Fig. 2, and the blots were run under the same experimental conditions. Data are means $\pm$ SE ($n = 6$ per group). *$P < 0.05$ and **$P < 0.01$ vs. control group.
Fig. 3. Treatment with angiotensin (ANG)-(1–7) alleviated diabetic RV fibrosis in rats. The effect of ANG-(1–7) on right-ventricular fibrosis was completely reversed by A779 and partially blocked by PD123319. A: representative Masson trichrome staining (collagen is green and myocardium red; scale bar = 100 μm) showing interstitial and perivascular fibrosis and representative immunohistochemical staining for Coll I and III content (scale bar = 100 μm). B–G: quantitative analysis of CVF (B), PVCA/LA (C), mRNA expression of fibronectin 1 (D), area of Coll I (E) and Coll III (F), and their ratio (G). H: representative Western blot of protein levels of TGF-β1, p-Smad3, Smad3, Smad7, and β-actin. I–L: quantification of TGF-β1 mRNA (I) and protein (J), p-Smad3/Smad3 (K), and Smad7 protein level (L). Cropped blots are used in Fig. 3, and the blots were run under the same experimental conditions. A200, low-dose ANG-(1–7) (200 ng·kg⁻¹·min⁻¹); A400, medium-dose ANG-(1–7) (400 ng·kg⁻¹·min⁻¹). Data are means ± SE (n = 6 per group). *P < 0.05 and **P < 0.01 vs. mock group; #P < 0.05 and ##P < 0.01 vs. high-dose ANG-(1–7) (800 ng·kg⁻¹·min⁻¹) (A800) group; †P < 0.05 and ††P < 0.01 vs. perindopril (P) group.
glucose-mediated injury in the cardiovascular system, such as mitochondrial damage (6). Diabetes exacerbated RV and LV oxidative stress in rats, as indicated by increased superoxide production and NADPH oxidase activation (Fig. 4, A–C). Consistent with the ANG-(1–7) normalization of pathological effects in diabetic rat hearts, the increase in superoxide production and NADPH oxidase activation was suppressed by ANG-(1–7), especially with perindopril coadministration (Fig. 4, D–F). The decreases in superoxide production and NADPH oxidase activation by ANG-(1–7) were partially restored by coadministration of A779 or PD123319.

Expression of sarcoplasmic reticulum Ca2⁺-handling proteins. We measured the expression levels of key proteins related to Ca2⁺ uptake in the sarcoplasmic reticulum, including SERCA2a and phospholamban. SERCA2a expression was decreased both in right and left ventricles of diabetic rats (Fig. 5, A and B), whereas diabetes downregulated phospholamban level only in left ventricles (Fig. 5, A and C). ANG-(1–7) alone or with perindopril dose dependently increased SERCA2a expression in diabetic right ventricles, and the effect of ANG-(1–7) was restored by coadministration with A779 (Fig. 5, D and E). However, ANG-(1–7) did not modify phospholamban expression in diabetic right ventricles (Fig. 5, D and F).

ACE and ACE2 levels and activities in vivo. Induction of diabetes increased ACE expression and activity both in right and left ventricles (Fig. 6, A, B, and D), whereas ACE2 expression and activity were increased in left ventricles but decreased in right ventricles (Fig. 6, A, C, and E). ANG-(1–7) alone or with perindopril dose dependently attenuated these diabetes-induced changes in RV enzyme levels and activities (Fig. 7, A–E).

Expression of AT1, AT2, and Mas receptors. The mRNA and protein expression of the AT1 receptor in both the right and left ventricles was higher in diabetic than control rats (Fig. 6, F, I, and J), and the increase in right ventricles was dose dependently reduced with 4-wk ANG-(1–7) treatment (Fig. 7, F, I, and J). The effect of ANG-(1–7) on AT1 receptor expression was totally blocked on coadministration of A779 or PD123319. Perindopril alone significantly increased AT1 receptor mRNA and protein expression in right ventricles, which is consistent with previously described clinical results (36), but AT1 receptor expression did not differ between rats treated with ANG-(1–7) alone at 800 ng·kg⁻¹·min⁻¹ and cotreatment with perindopril (P > 0.05).

The mRNA and protein expression of AT2 receptor both in right and left ventricles was lower in diabetic than control rats (Fig. 6, G, I, and K), and the reduced expression in right ventricles...
was dose dependently increased with ANG-(1–7) alone (Fig. 7, G, I, and K). This effect of ANG-(1–7) was completely blocked by PD123319. However, perindopril could not significantly alter AT2 receptor mRNA or protein expression (P > 0.05).

Nevertheless, diabetes did not significantly modify Mas receptor mRNA or protein level in right or left ventricles (Fig. 6, H, I, and L). As well, ANG-(1–7) did not alter Mas receptor mRNA or protein expression in right ventricles of diabetic rats (Fig. 7, H, I, and L).

ANG-(1–7) reduced the high-glucose-increased collagen content and TGF-β1 level in culture media of RV fibroblasts. High glucose alone time dependently increased collagen I and III content and TGF-β1 level in cultured media from RV fibroblasts (Fig. 8, A–C). ANG-(1–7) inhibited the effects of high glucose on collagen I and III content and TGF-β1 level at 72 h (Fig. 8, D–F), and the effects of ANG-(1–7) were totally inhibited by the addition of A779.

Fibroblast-myocyte interaction increased collagen content and TGF-β1 level. The protein levels of collagen I and III and TGF-β1 were significantly higher with coculture of fibroblasts and nontreated (high-glucose alone) myocytes than with fibroblasts alone. In contrast, the protein levels were lower with coculture of fibroblasts and ANG-(1–7)-treated myocytes than with that of fibroblasts and nontreated myocytes (P < 0.01; Fig. 9, A–C).
The protein levels of collagen I and III and TGF-β1 were substantially higher in media from fibroblasts incubated with nontreated (high-glucose alone) myocyte medium than fibroblasts incubated with high-glucose medium alone. In contrast, the protein levels were lower in media from fibroblasts cultured with ANG-(1–7)-treated than nontreated myocyte media (at least \( P < 0.05 \); Fig. 9, D–F). The inhibitory effects of ANG-(1–7) on collagen and TGF-β1 production increased by fibroblast-myocyte coculture/interaction were blunted by coadministration of A779 or PD123319.

**DISCUSSION**

By using measurements with RV catheterization and histopathological and biochemical analyses in rats with induced type 1 diabetes, we revealed that diabetic cardiomyopathy is characterized by RV fibrosis and dysfunction as well as LV fibrosis, hypertrophy, apoptosis, and dysfunction. Furthermore, ANG-(1–7) alleviated diabetic RV fibrosis and dysfunction via a complex interaction of AT2 and Mas receptors for subsequent downregulation of ACE expression and activity and AT1 re-
ceptor expression, as well as upregulation of ACE2 expression and activity and the expression of AT2 receptor and SERCA2a. High-dose ANG-(1–7) was superior to perindopril in improving RV fibrosis, which could be exploited clinically as a novel treatment for diabetic cardiomyopathy.

Diabetes seems to affect RV fibrosis and function similar to those in the left ventricle, which may be attributed to ventricular interdependence as well as the uniform effect of diabetes on both ventricles. However, remodeling of the ventricles differed. In line with a rat model of cardiac remodeling induced by deoxycorticosterone acetate (33), the cardiac hypertrophy and apoptosis response to diabetes was restricted to the left ventricle, whereas collagen deposition and other fibrosis-associated molecular analyses were indistinguishable between the left and right ventricles, which supports a humoral rather than hemodynamic etiology. LV interstitial and perivascular fibrosis can be produced by mineralocorticoids and salt without leading to substantial hypertension or cardiac hypertrophy, which further supports a humoral etiology of collagen deposition.

It has been long held that the detrimental effects of diabetes on the heart were primarily restricted to the left ventricle. However, ventricular interdependence can be expected from the geometry of the heart. Our experimental results in a rat model of diabetic cardiomyopathy showed that LV remodeling and dysfunction were paralleled by significant alterations in RV fibrosis and dysfunction. RV dysfunction is associated with a worse outcome in a variety of cardiovascular diseases, including myocardial infarction and heart failure (5, 17). The negative association of RV function with prognosis has been rarely investigated in diabetes. However, because diabetic patients often experience diabetic cardiomyopathy and myocardial ischemia, RV dysfunction might play a central role in cardiac abnormalities in this population.

In patients with heart failure, progressive RV dysfunction is associated with a high mortality and thus an accurate and reproducible technique for measuring RV function is highly warranted. Echocardiography is widely used for measuring LV systolic and diastolic function in diabetic cardiomyopathy. However, this technique is highly limited in the evaluation of RV function due to the complex RV geometry and the inability of two-dimensional echocardiography to image the entire right ventricle (9, 10). In this study, a high-fidelity Millar microtip...
A catheter was used to record RV pressure waves, which enabled highly accurate assessment of RV systolic and diastolic function.

The abnormality of sarcoplasmic reticulum Ca$^{2+}$/H$^{+}$-handling proteins plays an essential role in diabetic LV remodeling and dysfunction (15, 26). SERCA2a directly attributes to the Ca$^{2+}$/H$^{+}$ uptake and Ca$^{2+}$/H$^{+}$ release of sarcoplasmic reticulum, and phospholamban is a major modulator of SERCA2a activity (28). Our findings suggest that, in this diabetic model, SERCA2a is downregulated both in left and right ventricles, while phospho-

Fig. 8. ANG-(1–7) decreased Coll I and III and TGF-$
$1 levels after high-glucose (HG) stimulation in the culture supernatant, which was completely reversed with A779 and partially blocked with PD123319. A–C: ELISA of effect of treatment time of ANG-(1–7) on Coll I (A), Coll III (B), and TGF-$
$1 (C) levels in the culture supernatant of fibroblasts after HG stimulation. Data are means ± SE (n = 6 per group). ***P < 0.01 vs. the normal glucose (NG) group. D–F: ELISA of Coll I (D), Coll III (E), and TGF-$
$1 (F) levels in the culture supernatant of fibroblasts after stimulation with HG, with HG plus 10$^{-5}$ M ANG-(1–7) (A), HG plus 10$^{-5}$ M ANG-(1–7) plus 10$^{-6}$ M A779 (A + A779), and HG plus 10$^{-5}$ M ANG-(1–7) plus 10$^{-6}$ M PD123319 (A + PD123319). OC, osmotic control. Data are means ± SE (n = 6 per group). *P < 0.05 and **P < 0.01 vs. HG group; #P < 0.05 and ##P < 0.01 vs. A group.

Fig. 9. Fibroblast-myocyte interaction increased collagen and TGF-$
$1 production. A–C: ELISA of Coll I (A), Coll III (B), and TGF-$
$1 (C) levels in the culture supernatant of RV fibroblasts (F), cocultured fibroblasts and nontreated myocytes (F + M), cocultured fibroblasts and ANG-(1–7)-treated myocytes (F + M-A), cocultured fibroblasts and ANG-(1–7) plus A779-treated myocytes (F + M-A), and cocultured fibroblasts and ANG-(1–7) plus PD123319-treated myocytes (F + M-APD). Data are means ± SE (n = 6 per group). *P < 0.05 and **P < 0.01 vs. F group; #P < 0.05 and ##P < 0.01 vs. F + M group; †P < 0.05 and ††P < 0.01 vs. F + M-A group. D–F: ELISA of Coll I (D), Coll III (E), and TGF-$
$1 (F) levels in the culture supernatant of RV fibroblasts (F) and fibroblasts incubated with the conditioned media of nontreated myocytes (F + Me), conditioned media of ANG-(1–7)-treated myocytes (F + Me-A), conditioned media of ANG-(1–7) plus A779-treated myocytes (F + Me-A), and conditioned media of ANG-(1–7) plus PD123319-treated myocytes (F + Me-APD). Data are means ± SE (n = 6 per group). *P < 0.05 and **P < 0.01 vs. F group; #P < 0.05 and ##P < 0.01 vs. F + Me group; †P < 0.05 and ††P < 0.01 vs. F + Me-A group.
lamban is altered only in left ventricles. These results may explain in part the difference between left and right ventricles in response to diabetes. Furthermore, ANG-(1–7) treatment improved diabetic RV fibrosis and dysfunction, and the mechanism may involve preservation of SERCA2a in the right ventricle.

We found that ANG-(1–7) treatment was associated with reduced ACE protein expression and activity as well as increased ACE2 expression and activity in diabetic rat right ventricles. Paradoxically, despite reduced ACE activity in ANG-(1–7)-treated right ventricles, ANG-II level remained high (data not shown), which suggests that other enzymes or pathways besides ACE may be involved in the formation of ANG-II. One such candidate is chymase, which has been detected in cardiac and vascular tissues (29). Consistent with previous results of a model of pulmonary fibrosis induced by bleomycin (23), we first demonstrated that ANG-(1–7) treatment downregulated the level of AT1 receptor and upregulated that of AT2 receptor in diabetic RV tissues.

ANG-(1–7) can stimulate multiple receptor subtypes, although the exact mechanism is unclear. Our results demonstrated for the first time that ANG-(1–7) improved RV remodeling and function mediated through both Mas and AT2 receptors. Although ANG-(1–7) appeared to act via the Mas receptor in many in vitro/ex vivo settings (22), this selectivity was lost in vivo (19). As well, ANG-(1–7) may mediate multiple functional responses by moderate binding to the AT2 receptor. Tesanovic et al. (25) previously reported that ANG-(1–7)-evoked vasoprospection and atheroprotection were mediated by the restoration of nitric oxide bioavailability and abrogated by coadministration with PD123319 or A779. Another study demonstrated that ANG-(1–7)-stimulated nitric oxide release in bovine aortic endothelial cells was more sensitive to inhibition of AT2 receptor than Mas receptor, which also implicates multiple receptor functions (31).

In summary, we showed that ANG-(1–7) protected against RV fibrosis and dysfunction in diabetic rats without correcting hyperglycemia. The major mechanisms may involve a complex interaction of AT2 and Mas receptors for subsequent downregulation of ACE expression and activity and AT1 receptor expression, as well as upregulation of ACE2 expression and activity and the expression of AT2 receptor and SERCA2a. Thus ANG-(1–7) may provide a novel and promising therapeutic target for RV dysfunction involved in diabetic cardiomyopathy.

AUTHOR CONTRIBUTIONS


REFERENCES


