Original Article

Angiotensin II Type 1 Receptor Blocker Attenuates Myocardial Remodeling and Preserves Diastolic Function in Diabetic Heart

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Blockade of the renin-angiotensin system reduces cardiovascular morbidity and mortality in diabetic patients. Angiotensin II (Ang II) plays an important role in the structural and functional abnormalities of the diabetic heart. We investigated whether or not Ang II type 1 receptor blocker (ARB) could attenuate left ventricular (LV) remodeling in male mice with diabetes mellitus (DM) induced by the injection of streptozotocin (200 mg/kg, i.p.). Diabetic mice were treated with candesartan (1 mg/kg/day; DM+Candesartan, *n*=7) or vehicle (DM+Vehicle, *n*=7) for 8 weeks. Heart rate and aortic blood pressure were comparable between the groups. Normal systolic function was preserved in diabetic mice. In contrast, diastolic function was impaired in DM+Vehicle and was improved in DM+Candesartan, as assessed by the deceleration time of the peak velocity of transmitral diastolic flow (40.3±0.3 *vs.* 37.3±0.5 ms, $p<0.01$) and the time needed for relaxation of 50% maximal LV pressure to baseline value (τ; 10.6±0.7 *vs.* 8.7±0.6 ms, $p<0.05$) without significant changes in heart rate and aortic blood pressure. Improvement of LV diastolic function was accompanied by the attenuation of myocyte hypertrophy, interstitial fibrosis and apoptosis in association with the expression of connective tissue growth factor (CTGF) and myocardial oxidative stress. Moreover, candesartan directly inhibited Ang II–mediated induction of CTGF in cultured cardiac fibroblasts. ARB might be beneficial to prevent cardiac abnormalities in DM. (*Hypertens Res* 2007; 30: 439–449)

***Key Words*: diabetes mellitus, apoptosis, remodeling, extracellular matrix, oxidative stress**

Introduction

Diabetes mellitus (DM) often leads to congestive heart failure even in the absence of any other risk factors such as coronary artery disease or hypertension, suggesting that DM itself causes a specific form of cardiomyopathic state independent of vascular complications (*1*). Specifically, diastolic dysfunction has been recognized as a hemodynamic hallmark seen in DM and ultimately contributes to the development of heart failure (*2*). DM causes myocardial structural remodeling characterized by myocyte hypertrophy and apoptosis as well as interstitial fibrosis (*3*), which increases cardiac muscle stiffness and may contribute to impaired diastolic function. Although the features of diabetic heart disease have been well identified, its pathogenesis underlying the myocardial remod-

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Table 1. Characteristics of Animal Models

	Control+Vehicle (n=7)	Control+Candesartan (n=7)	DM+Vehicle (n=7)	DM+Candesartan (n=7)
Body weight (g)	36.7±0.9	37.5±1.0	30.9±2.1**	30.8±2.5**
Plasma glucose (mg/dL)	124±5	116±5	509±38**	514±22**
Echocardiographic data				
Heart rate (bpm)	424±11	421±10	422±6	419±13
LV EDD (mm)	3.56±0.08	3.54±0.09	3.39±0.13	3.30±0.04
LV ESD (mm)	2.13±0.04	2.10±0.10	2.14±0.09	2.01±0.05
Fractional shortening (%)	39.6±0.9	36.4±0.8	36.9±0.9	37.3±1.2
IVS thickness (mm)	0.66±0.02	0.69±0.03	0.64±0.02	0.66±0.02
PW thickness (mm)	0.72±0.02	0.71±0.03	0.67±0.03	0.66±0.02
E/A	1.61±0.08	1.73±0.20	1.25±0.07	1.60±0.07
Dct (ms)	33.3±0.6	35.6±0.6	40.3±0.3**	37.3±0.5**††
Hemodynamic data				
Heart rate (bpm)	427±10	427±11	416±15	426±11
Mean aortic pressure (mmHg)	81±2	77±2	83±3	80±2
LV EDP (mmHg)	1.1±0.2	1.8±0.7	3.0±0.6*	1.8±0.5
LV dP/dt_{max} (mmHg/s)	11,415±733	10,262±933	8,928±1,297	11,018±155
LV dP/dt_{min} (mmHg/s)	7,252±302	6,749±364	4,343±662**	6,394±558†
τ (ms)	8.1±0.7	7.9±0.8	10.6±0.7*	8.7±0.6†
Organ weight data				
LV wt (mg)	100±3	101±4	84±7	82±7
LV wt/BW (mg/g)	2.7±0.1	2.7±0.1	2.7±0.1	2.7±0.1

DM, diabetes mellitus; LV, left ventricular; EDD, end-diastolic dimension; ESD, end-systolic dimension; IVS, interventricular septum; PW, posterior wall; Dct, the deceleration time of peak velocity of transmitral diastolic flow; EDP, end-diastolic pressure; τ, the time needed for relaxation of 50% maximal LV pressure to baseline value; wt, weight; BW, body weight. Values are means±SEM. *$p<0.05$, **$p<0.01$ *vs*. Control+Vehicle. †$p<0.05$, ††$p<0.01$ *vs*. DM+Vehicle.

eling process and, in particular, an effective treatment strategy for this condition have not been elucidated.

Hyperglycemia stimulates the production of angiotensin II (Ang II) in cardiac myocytes *via* the upregulation of most components of the renin-angiotensin system from angiotensinogen to Ang II type 1 (AT_1) receptors (*3*). By binding to and activating AT_1 receptors, Ang II exerts a variety of nonhemodynamic effects that are linked to cardiovascular pathophysiology (*4*). The fibrogenic effects of Ang II are attributable to the induction of mediators including transforming growth factor-β (TGF-β). TGF-β induces the synthesis of extracellular matrix (ECM) proteins and prevents their degradation, thus leading to the excess deposition of ECM and fibrosis in various organs, including the heart and kidney. Connective tissue growth factor (CTGF) is a downstream mediator of TGF-β, mediating the profibrotic actions of TGF-β. In fact, elevated levels of both TGF-β and CTGF have been found in the diabetic heart (*5*).

The present study was performed to determine whether the Ang II receptor blocker (ARB) could attenuate myocardial remodeling and preserve diastolic function in DM. Furthermore, the cellular mechanisms were evaluated to further elucidate critical pathways implicated in DM-associated cardiac remodeling and in mediating cardiac protection by ARB in this disease state.

Methods

Animals

DM was induced in male CD-1 mice, 5–7 weeks old and 25–35 g in body weight, by intraperitoneal injection of 200 mg/kg body weight of streptozotocin (STZ, Sigma Chemical, St. Louis, USA) dissolved in sodium citrate saline buffer (pH 4.5) (*6*). Tail vein blood glucose was measured 5 days after injection to insure induction of diabetes. As a control, vehicle (0.1 mol/L citrate buffer, pH 4.5) was injected in another group of mice. These animals were further randomized for treatment with an ARB, candesartan (1 mg/kg body weight/day p.o.; DM+Candesartan group), or to receive a vehicle (DM+Vehicle group). Control animals were also randomized for treatment with candesartan (Control+Candesartan group) or to receive vehicle (Control+Vehicle group). All four groups (Control+Vehicle, n=7; Control+Candesartan, n=7; DM+Vehicle, n=7; DM+Candesartan, n=7) were followed for further 8 weeks. This assignment procedure was performed using numeric codes to identify the animals.

The study was approved by our Institutional Animal

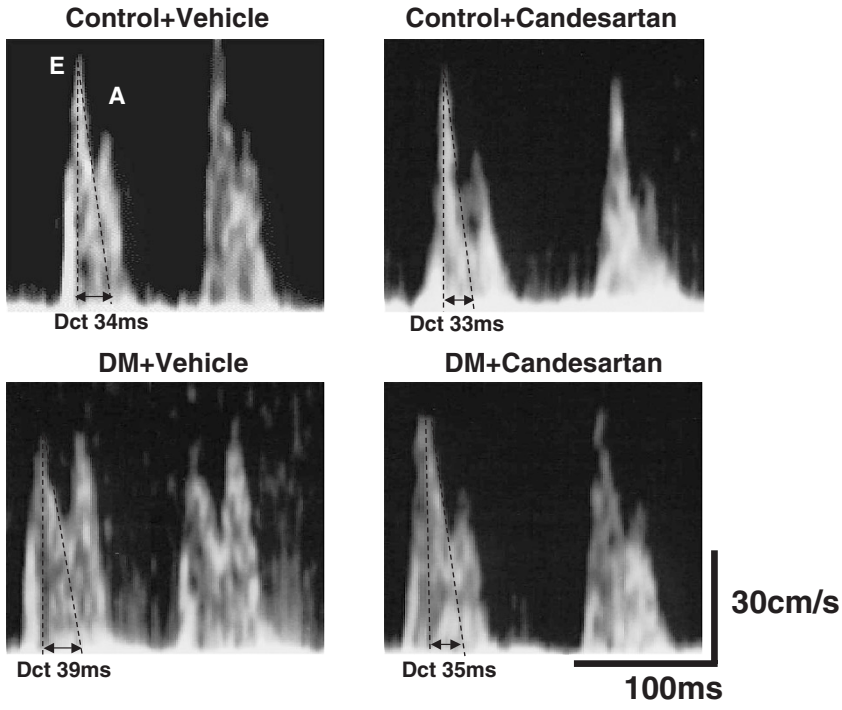

Fig. 1. *Representative Doppler flow measurements of mitral inflow obtained from four groups: Control+Vehicle, Control+Candesartan, DM+Vehicle and DM+Candesartan mice. Early and late mitral inflow velocity (E-wave and A-wave, respectively) and E-wave deceleration time (Dct) were measured from the Doppler recordings in the standard fashion.*

Research Committee and conformed to the animal care guidelines of the American Physiological Society.

Echocardiographic and Hemodynamic Measurements

After 8 weeks of treatment, echocardiographic studies were performed under light anesthesia with tribromoethanol/amylene hydrate (Avertin; 2.5% w/v, 8 μL/g, i.p.) and spontaneous respiration. Two-dimensional targeted M-mode tracings were recorded at a paper speed of 50 mm/s as described previously (*6*, *7*). To assess the diastolic cardiac function, two-dimensional guided Doppler flow measurements of mitral inflow were obtained. Mitral inflow velocities were recorded only after extensive scanning from multiple vantage points to ensure that the maximal velocity was obtained. In most situations, this was an apical window that corresponds to an "off-axis" apical window (displaced toward the parasternal window). Early and late mitral inflow velocity (E-wave and A-wave, respectively), and E-wave deceleration time (Dct) were measured from the Doppler recordings in the standard fashion, and the E-to-A ratio was calculated (*8*).

Under the same anesthesia with Avertin, a 1.4 Fr micromanometer–tipped catheter (Millar Instruments, Houston, USA) was inserted into the right carotid artery and then advanced into the left ventricle (LV) to measure LV pressures. The following indices of cardiac performance were measured and averaged from three consecutive beats; LV systolic pressure, LV end-diastolic pressure (EDP), the maximum and minimum values of the first derivative of LV pressure (LV dP/dt_{max} and LV dP/dt_{min}, respectively), and the time needed for relaxation of 50% maximal LV pressure to baseline value (τ) (*6*).

Plasma Glucose

After completion of the cardiac function measurements, blood samples were collected for the determination of plasma glucose.

Myocardial Histopathology

After the *in vivo* studies, the heart was excised and dissected into the right and left ventricles, including the septum. The LV was cut into three transverse sections; apex, middle ring and base. From the middle ring, 5 μm sections were cut and stained with Masson's trichrome. The myocyte cross-sectional area was determined by quantitative morphometry of tissue sections from the mid-LV. Collagen distribution was also determined by using picrosirius red (0.1% Sirius Red F3BA in picric acid)–stained sections. Slides were left in 0.2% phosphomolybdic acid for 5 min and washed. They were then left in picrosirius red for 90 min, in 1 mmol/L HCl for 2 min and in 70% ethanol for 45 s (*6*).

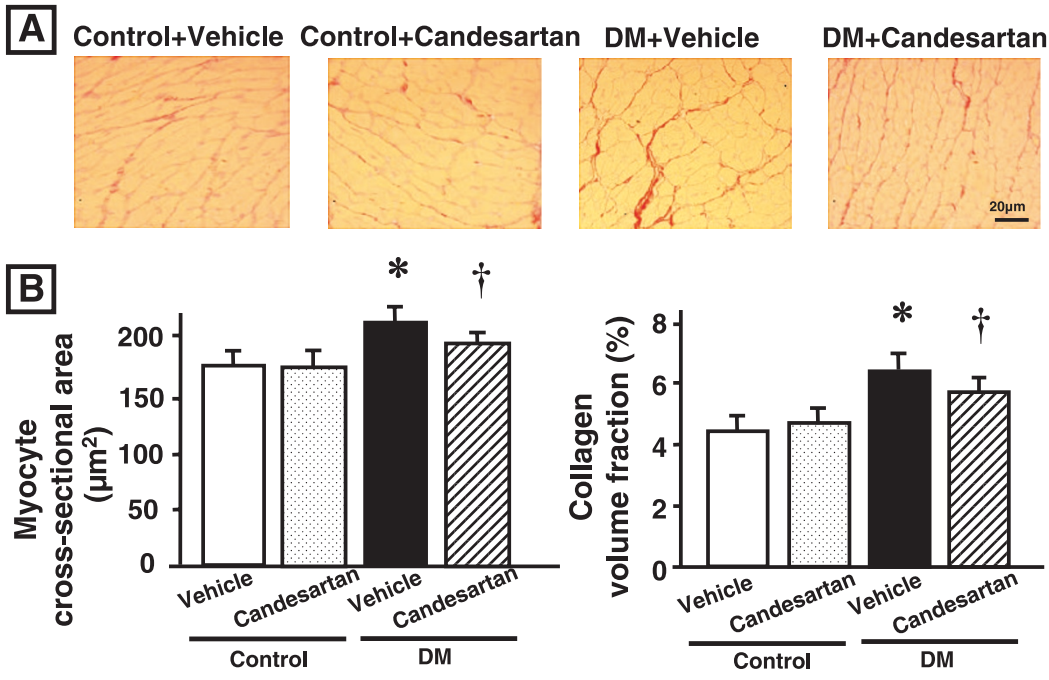

Fig. 2. *A: Representative photomicrographs of Masson-trichrome–stained LV cross section obtained from four groups: Control+Vehicle, Control+Candesartan, DM+Vehicle and DM+Candesartan mice. B: Summary data for myocyte cross-sectional area and collagen volume fraction in Control+Vehicle, Control+Candesartan, DM+Vehicle and DM+Candesartan mice (n=7 for each). Values are means±SEM. *p<0.05 for the difference from the Control+Vehicle value. †p<0.05 for the difference from the DM+Vehicle value.*

To detect apoptosis, tissue sections from the mid-LV were stained with terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) staining. The TUNEL-positive nuclei were counted, and the data were normalized per 10^5 total nuclei identified by hematoxylin-positive staining in the same sections. We further examined whether or not apoptosis is present in the non-infarcted LV by the more sensitive ligation-mediated PCR fragmentation assays (Maxim Biotech, South San Francisco, USA) (*6*, *7*).

TGF-β, CTGF and Matrix Metalloproteinases

The alterations of profibrotic mediators including TGF-β, CTGF and matrix metalloproteinases (MMPs) were determined in this model. TGF-β gene expression level was assessed by ribonuclease protection assay. The value of each hybridized probe was normalized to that of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) in each template set as an internal control. CTGF protein levels were quantified by Western blot analysis using a specific antibody against recombinant mouse CTGF. To confirm the amount of loaded proteins, total proteins were also visualized by Coomassie Brilliant Blue (CCB) staining. Within a given experiment, the densitometric values were normalized by using standards concurrently run within the same gel, and each value was calculated as a ratio to Control+Vehicle.

Myocardial MMP levels including MMP-2 and MMP-9 were determined in the LV using gelatin zymography. The LV myocardial samples were homogenized (~30-s bursts) in 1 mL of an ice-cold extraction buffer containing cacodylic acid (10 mmol/L), NaCl (0.15 mol/L), $ZnCl_2$ (20 mmol/L), NaN_3 (1.5 mmol/L) and 0.01% Triton X-100 (pH 5.0). The homogenate was then centrifuged (4°C, 10 min, 10,000 × *g*) and the supernatant decanted and saved on ice. The pH levels of the samples were adjusted to 7.5 using Tris (1 mol/L). The final protein concentration of the myocardial extracts was determined using a standardized colorimetric assay. The extracted samples were then aliquoted and stored at −80°C until the time of assay. The myocardial extracts were then directly loaded onto electrophoretic gels (SDS-PAGE) containing 1 mg/mL of gelatin under non-reducing conditions. The myocardial extracts at a final protein content of 5 μg were loaded onto the gels using a 3:1 sample buffer (10% SDS, 4% sucrose, 0.25 mol/L Tris-HCl and 0.1% bromophenol blue, pH 6.8). The gels were run at 15 mA/gel through the stacking phase (4%) and at 20 mA/gel for the separating phase (10%), while the running buffer temperature was maintained at 4 °C. After SDS-PAGE, the gels were washed twice in 2.5% Triton X-100 for 30 min each, rinsed in water and incubated for 24 h in a substrate buffer at 37°C (50 mmol/L Tris-HCl, 5 mmol/L, $CaCl_2$ and 0.02% NaN_3, pH 7.5). After incubation, the gels were stained with Coomassie brilliant blue R-250. The zymo-

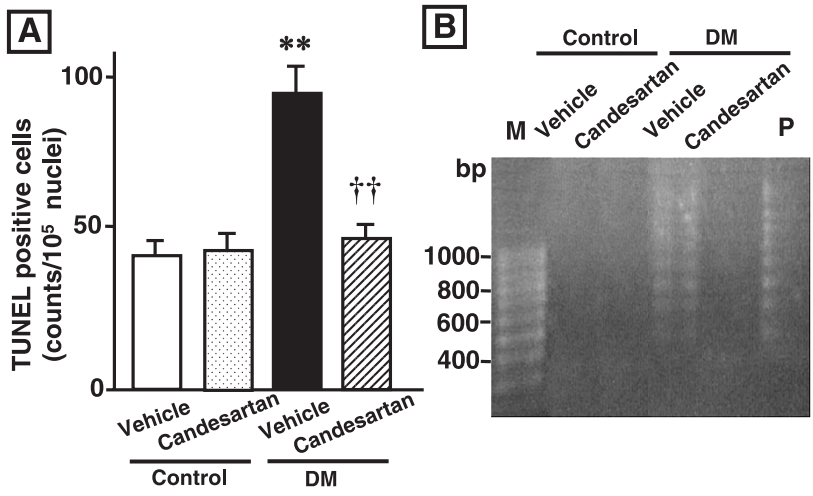

Fig. 3. *A: The number of TUNEL-positive cells in the LV from four groups: Control+Vehicle, Control+Candesartan, DM+Vehicle and DM+Candesartan mice (n=5 for each). Values are means±SEM. **p<0.01 for the difference from the Control+Vehicle value. ††p<0.01 for the difference from the DM+Vehicle value. B: DNA ladder indicative of apoptosis in the genomic DNA from the LV. M, marker; P, positive control.*

grams were digitized, and the size-fractionated bands, which indicated the MMP proteolytic levels, were measured by the integrated optical density in a rectangular region of interest.

Lipid Peroxidation

Lipid peroxidation is a major biochemical consequence of oxidative stress on biological tissue. We therefore determined the degree of lipid peroxidation in the myocardial tissue through biochemical assay of thiobarbituric acid–reactive substances (TBARS) (*6*, *7*). In brief, LV myocardial tissue was homogenized (10% w/v) in 1.15% KCl solution (pH 7.4). The homogenate was mixed with 0.4% sodium dodecyl sulfate, 7.5% acetic acid adjusted to pH 3.5 with NaOH and 0.3% thiobarbituric acid. Butylated hydroxytoluene (0.01%) was added to the assay mixture to prevent autoxidation of the sample. The mixture was kept at 5°C for 60 min and was heated at 100°C for 60 min. After cooling, the mixture was extracted with distilled water and *n*-butanol:pyridine (15:1, v/v) and centrifuged at 1,600 × *g* for 10 min. The absorbance of the organic phase was measured at 532 nm. The amount of TBARS was determined by the absorbance with a molecular extinction coefficient of 156,000 and expressed as μmol/g wet weight.

CTGF in Cultured Cardiac Fibroblasts

Cardiac fibroblasts from neonatal Wistar Kyoto rats (1–3 days old) were isolated by modified versions of previously described methods (*9*). Briefly, 20–30 neonatal hearts were rapidly excised from anesthetized animals, minced, and added to Hank's balanced salt solution (HBSS). Two sequential digestions were performed using HBSS with trypsin-EDTA, 10% fetal bovine serum (FBS) and 1 mg/mL collagenase. Dissociated cells and debris from the first digestion were discarded. Supernatants from the remaining digestion were added to tubes containing plating medium and Dulbecco's modified Eagle's medium (DMEM), and were centrifuged at 100 × *g* for 5 min (0°C). Cells were resuspended in plating medium and placed in a humidified incubator (5% CO_2) for 70 min in a 10 cm dish to allow for the selective adhesion of fibroblasts. Newly cultured fibroblasts were maintained in plating medium for 48 h. The medium was then switched to DMEM with 10% FBS and 1% penicillin-streptomycin liquid. Six days after the initial dispersion, fibroblasts were passaged using 0.1% trypsin and seeded at a density of 3–5 × 10^3/cm^2 on 6 cm plates. After 72 h, the medium was changed to serum-free DMEM, and experiments were performed 1 day later. At the time of the experiments, cultures were subconfluent.

CTGF protein levels were quantified by Western blot analysis using a specific antibody against recombinant mouse CTGF as described above.

Statistical Analysis

Data are expressed as means±SEM. Between-group comparison of means was performed by one-way ANOVA, followed by *t*-tests. Bonferroni's correction was done for multiple comparisons of means. $p<0.05$ was considered statistically significant.

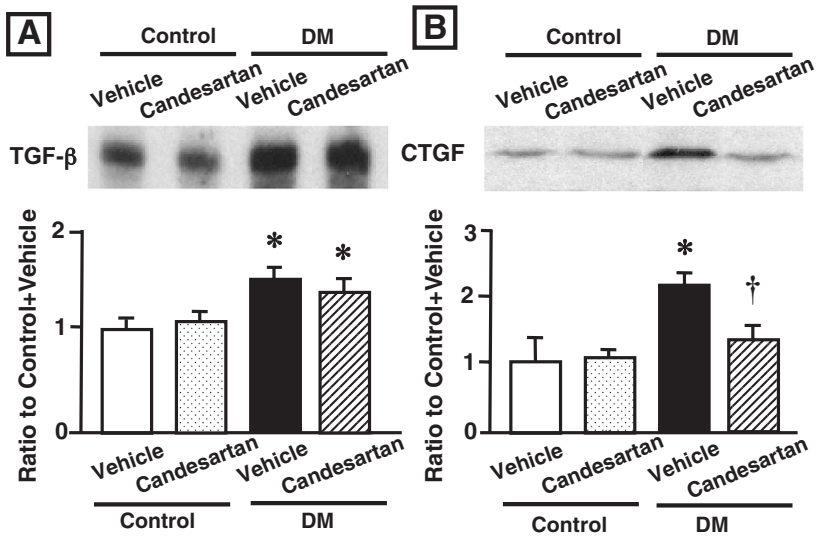

Fig. 4. *Myocardial TGF-β (A) and CTGF (B) levels in the LV from four groups: Control+Vehicle, Control+Candesartan, DM+Vehicle and DM+Candesartan mice (n=6 for each). Data are expressed as the ratio to Control+Vehicle values concurrently run on the same gel. Values are means±SEM. *p<0.05 for the difference from the Control+Vehicle value. †p<0.05 for the difference from the DM+Vehicle value.*

Results

Body Weight and Plasma Glucose

There were no deaths in any of the groups. At 8 weeks, DM mice had elevated plasma glucose levels compared with control animals, which candesartan did not alter (Table 1). DM animals gained less body weight than control mice, though body weight did not differ between DM+Vehicle and DM+Candesartan (Table 1).

Echocardiography and Hemodynamics

The echocardiographic and hemodynamic data of mice at 8 weeks are shown in Table 1. There was no significant difference in heart rate or aortic blood pressure among the four groups. Normal systolic function, as assessed by echocardiographic fractional shortening and LV dP/dt_{max}, was preserved in both DM+Vehicle and DM+Candesartan mice. In contrast, diastolic function, as assessed by the deceleration time of the peak velocity of transmitral diastolic flow (Dct) and the time needed for relaxation of 50% maximal LV pressure to baseline value (τ), was impaired in DM; this impairment was significantly attenuated by candesartan (Table 1 and Fig. 1). In addition, LV dP/dt_{min} was decreased in DM+Vehicle compared to Control+Vehicle and significantly increased in DM+Candesartan. LV EDP was elevated in DM+Vehicle to a level barely above statistical significance and lowered in DM+Candesartan, though not significantly ($p=0.09$).

Myocardial Histopathology

Increased cardiac myocyte size, indicating hypertrophy, was evident in DM+Vehicle, and this increase was significantly attenuated in DM+Candesartan (Fig. 2). Collagen volume fraction, indicating interstitial fibrosis, was greater in the DM+Vehicle group than in the control groups. These fibrotic changes in the heart were significantly reduced in the DM+Candesartan group (Fig. 2).

The number of TUNEL-positive cells in the LV was increased in DM+Vehicle and was significantly decreased by candesartan (Fig. 3A). In addition, the intensity of the DNA ladder indicated that the apoptosis in DM+Candesartan was lower than that in DM+Vehicle (Fig. 3B).

Myocardial TGF-β, CTGF and MMPs

To further assess the alterations of profibrotic mediators for their involvement in interstitial fibrosis, myocardial levels of TGF-β, CTGF and MMPs were determined. Myocardial TGF-β and CTGF expression was increased in DM+Vehicle (Fig. 4). Increased myocardial TGF-β expression was not affected by candesartan, whereas CTGF protein levels were significantly decreased in DM+Candesartan. Myocardial MMP-2 zymographic levels were also increased in DM, and this increase was not altered by candesartan (Fig. 5). MMP-9 zymographic levels were not altered in either group.

Lipid Peroxidation

Myocardial TBARS measured in the LV were significantly

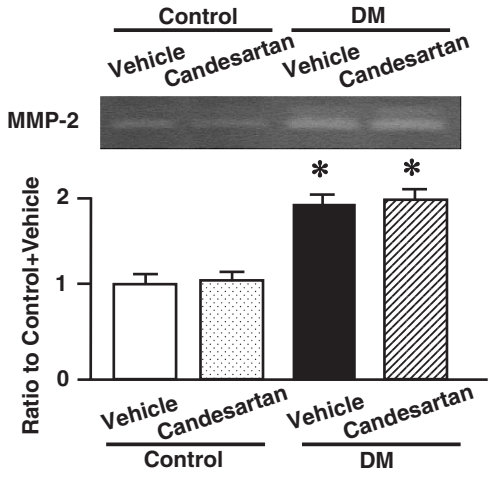

Fig. 5. *Representative gelation zymography indicating myocardial MMP-2 activity and its summary data in the LV from four groups: Control+Vehicle, Control+Candesartan, DM+Vehicle and DM+Candesartan mice (n=6 for each). Data were expressed as the ratio to Control+Vehicle values concurrently run on the same gel. Values are means±SEM. *p<0.05 for the difference from the Control+Vehicle value.*

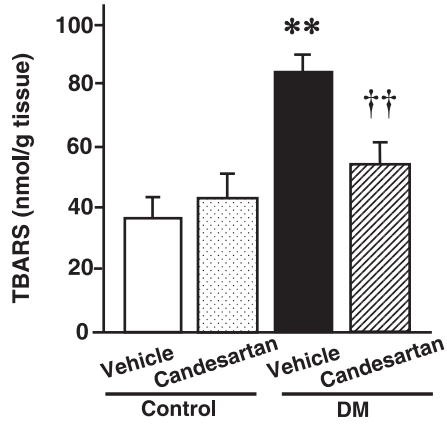

Fig. 6. *Lipid peroxidation as indicated by TBARS in the LV from four groups: Control+Vehicle, Control+Candesartan, DM+Vehicle and DM+Candesartan mice (n=5 for each). Each assay was performed in triplicate. Values are means±SEM. **p<0.01 for the difference from the Control+Vehicle value. ††p<0.01 for the difference from the DM+Vehicle value.*

greater in the DM+Vehicle group than in the control groups and were significantly lower in the DM+Candesartan group (Fig. 6).

CTGF in Cultured Cardiac Fibroblasts

CTGF protein levels were increased by the incubation of cardiac fibroblasts with Ang II (0.1 μmol/L, 1 h). This increase of CTGF was significantly inhibited by the incubation of cells with CV-11974 (0.1 μmol/L), an active metabolite of candesartan and a specific inhibitor of the AT_1 receptor, but not with PD123319 (0.1 μmol/L), a specific inhibitor of the Ang II type 2 receptor (Fig. 7). This indicated that Ang II can directly induce CTGF expression *via* AT_1 receptors in cardiac fibroblasts.

Discussion

The present study demonstrated that candesartan protected the diabetic heart against myocardial remodeling and dysfunction. It concurrently improved LV diastolic function and also reduced myocyte hypertrophy, interstitial fibrosis and apoptosis in association with myocardial CTGF expression without affecting blood pressure. Moreover, candesartan directly inhibited Ang II–mediated induction of CTGF in cultured cardiac fibroblasts. Candesartan also reduced oxidative stress in the diabetic heart. Even though previous studies have already demonstrated that the renin-angiotensin system is activated in the diabetic heart (*3*, *10*), the present study specifically provided direct evidence for the protective role of ARB against myocardial remodeling and dysfunction in this disease state by attenuating the induction of CTGF and oxidative stress. Therefore, the novel finding of the present study, from the viewpoint of the pathophysiology and treatment of maladaptive myocardial remodeling in response to DM, is that Ang II is critically involved in this disease process and may be a potential therapeutic target.

DM causes both diastolic and systolic cardiac dysfunction (*11*, *12*), but the former usually becomes impaired before the latter develops. The impairment of diastolic function despite normal systolic function is thought to result from increased myocardial stiffness. The present study has demonstrated that the improvement of diastolic function in DM+Candesartan mice (Table 1) is associated with the attenuation of myocyte hypertrophy and interstitial fibrosis (Fig. 2). Previous studies have demonstrated that diabetes induced by STZ leads to an increased collagen deposition (*13*), thus increasing myocardial stiffness and decreasing LV compliance (*14*, *15*). Even though we did not directly assess diastolic function by using the stress-strain relations and therefore could not comment on the contribution of myocardial stiffness to LV function in the present study, the decrease in myocardial fibrosis by candesartan can well be considered to contribute to the physiological improvement of diastolic properties in DM mice. In fact, prior studies have generally shown an association between increased cardiac fibrosis and diastolic chamber stiffening (*16*).

The beneficial effects of candesartan shown in the present study were not due to its effects on diabetes itself, since body weight and plasma glucose levels were comparable between DM+Vehicle and DM+Candesartan groups (Table 1). More importantly, the effects might not be attributable to those of

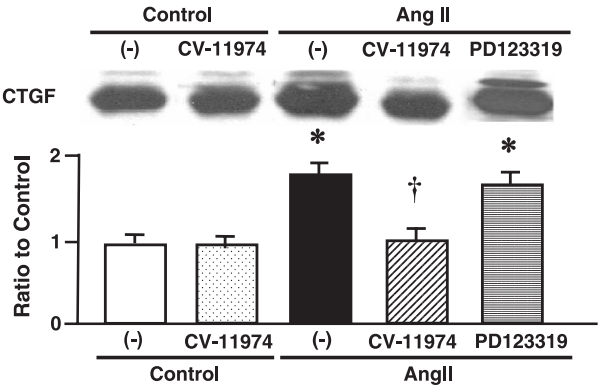

Fig. 7. *Representative Western blot analysis of CTGF protein expression in cultured cardiac fibroblasts induced by Ang II. Fibroblasts were incubated with or without Ang II (0.1 μmol/L, 1 h) in the presence or absence of the pretreatment with CV-11974 (0.1 μmol/L) or PD123319 (0.1 μmol/L).*

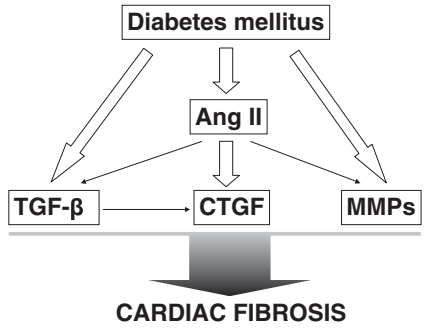

Fig. 8. *Potential mediators involved in cardiac fibrosis in DM.*

candesartan on hemodynamics, since blood pressure and heart rate were not altered in our model treated by candesartan at a dose of 1 mg/kg/day (Table 1). However, the present study could not completely exclude the possibility that candesartan might lower blood pressure during the conscious state.

There may be several factors whereby Ang II contributes to myocardial remodeling in diabetes *via* a variety of nonhemodynamic effects that are linked to the activation of AT_1 receptors apart from its direct hemodynamic effects (*4*, *17*). A possible role for growth factors in diabetes-related end-organ complications is increasingly being recognized. TGF-β and CTGF can induce the production of collagen and fibronectin from cardiac fibroblasts and myocytes, and increased expression of both factors has been documented in the diabetic heart (*5*, *18*, *19*). TGF-β is a locally generated cytokine that has a major influence on fibroblast proliferation and ECM production, particularly of collagen and fibronectin, while reducing the degradation of these components. Moreover, Ang II is a well-known mediator to induce and activate TGF-β. In addition, CTGF has a unique TGF-β response element in its promoter region (*20*), acts downstream of TGF-β, and thus can induce the production of collagen and fibronectin from cardiac fibroblasts and myocytes (*21*). Therefore, one proposed mechanism by which candesartan might inhibit the ECM alterations in DM is related to decreases in both TGF-β and CTGF expression and the resultant amelioration of cardiac fibrosis. In fact, the present study demonstrated that both TGF-β and CTGF were increased in the diabetic heart. However, the attenuation of cardiac fibrosis by candesartan was associated with the decrease in the expression of CTGF, but not with that of TGF-β, suggesting that CTGF rather than TGF-β mainly contributed to cardiac fibrosis in this model. Consistent with our findings, CTGF was reduced in diabetic nephropathy when the animals were treated with ARB (*22*).

To further prove that Ang II can be a direct inducer of CTGF expression by the activation of AT_1 receptors, we determined the effects of Ang II on CTGF induction in cultured cardiac fibroblasts *in vitro* (Fig. 7). The present results are in line with a previous study showing the induction of CTGF in a rat fibroblast cell line that overexpressed the AT_1 receptor (*23*). In addition, the direct induction of CTGF by Ang II has been shown to play an important role in cardiac fibrosis in a 2-kidney, 1-clip model of renovascular hypertension (*24*). Therefore, even though CTGF was characterized as a downstream mediator of TGF-β, other factors such as bioactive lipids might also have been implicated in the subsequent induction of CTGF (*25*). In a number of previous studies using different models of cardiac fibrosis, TGF-β mediated the modulation of ECM protein synthesis by Ang II, and TGF-β itself was induced by Ang II (*26*). Therefore, we could not completely exclude the contribution of TGF-β as a mediator of CTGF induction at later time points in the diabetic heart.

Another possible mechanism is the contribution of myocardial MMP-2 expression in this model, since it has been reported that MMP activation plays an important role in the pathophysiology of LV remodeling (*27*, *28*) and, moreover, since myocardial MMPs can be activated by Ang II (*29*). Based on these findings, the activation of the renin-angiotensin system might contribute to the activation of MMP-2 and thus to the development of LV remodeling in the diabetic heart. However, this possibility is less likely in the present study because the increased MMP-2 gelatinolytic activity was not altered by the treatment of diabetic animals with candesartan (Fig. 4).

Even though Ang II has been well documented to increase several proteins involved in cell growth and matrix regulation—1) growth factors such as TGF-β and CTGF, 2) cytokines and chemokines including MCP-1 and 3) MMPs (Fig. 8) (*4*, *17*)—the present study has indicated that CTGF induction by Ang II is critically involved in cardiac fibrosis associated with DM.

Finally, recent studies have demonstrated that apoptosis is thought to contribute to the progressive deterioration of LV function in the diabetic heart (*3*). This is an intriguing obser-

vation in light of the remodeling process known to occur within DM, which is characterized by a loss of myocytes, hypertrophy of remaining myocytes, and interstitial replacement fibrosis. Moreover, Ang II can mediate myocyte apoptosis, which may lead to myocardial remodeling in diabetes. Therefore, in DM mice, candesartan could directly ameliorate apoptosis and eventual diabetic cardiac remodeling. The decrease of myocyte hypertrophy and interstitial fibrosis by candesartan in DM mice (Fig. 2) did not affect LV weight (Table 1), possibly due to the inhibition of apoptosis and the possible increase in the number of viable myocytes.

A growing body of evidence suggests that oxidative stress is increased in the diabetic heart (*30*, *31*). Further, antioxidants have been shown to prevent structural and functional alterations of the diabetic heart (*15*, *32*). Ang II can activate NAD(P)H oxidase, the most important vascular enzymatic source of superoxide production (*33*). Therefore, the activation of the renin-angiotensin system may contribute to increased oxidative stress through the expression of an NAD(P)H-dependent oxidase in these settings (*34*, *35*). We have recently demonstrated that overexpression of glutathione peroxidase (GSHPx), an antioxidant enzyme, improved LV diastolic function and also reduced myocyte hypertrophy, apoptosis and interstitial fibrosis in DM (*6*). Therefore, the present study has further confirmed the involvement of oxidative stress in the pathophysiology of cardiac remodeling associated with diabetes and the role of Ang II in oxidative stress in this disease state (Fig. 6). Both experimental and clinical studies have shown that DM causes a specific form of myocardial damage independent of coronary atherosclerosis and manifests itself as LV dysfunction. The findings of the present study may further draw attention to the early and intensive treatment of heart failure by using ACE inhibitors or ARBs in diabetic patients. ACE inhibitors and ARBs could inhibit the occurrence of diabetes itself (*36*). Moreover, recent clinical trials have consistently shown that the pharmacological blockade of Ang II reduces cardiovascular mortality and morbidity in diabetic patients (*37*, *38*). Approximately 20–40% of patients with heart failure have preserved systolic function and are thought to have an impairment of diastolic function as the primary mechanism leading to symptomatic heart failure (*39*). Diabetes is recognized as one of the major risk factors associated with diastolic heart failure (*40*). Despite the high prevalence of diabetes among patients with this type of heart failure, the treatment of diastolic heart failure remains empirical (*41*). There are currently few clinical data to support the efficacy of any particular class of drugs for diastolic heart failure. The CHARM-Preserved trial has demonstrated that candesartan can significantly reduce the need for hospitalization due to heart failure with preserved ejection fraction (*42*). Therefore, the present study should help clarify the potential that candesartan may play in the treatment of diastolic heart failure.

There are several limitations to be acknowledged in this study. First, the echocardiographic assessment of LV diastolic function in mice is somewhat difficult. However, the intra- and interobserver variabilities of our echocardiographic measurements were small and the measurements were highly reproducible (*43*). Therefore, our technique was capable of noninvasively assessing the LV structure and function in mice. Second, the relationships between cardiac function and structural alterations were analyzed only at 8 weeks after the induction of diabetes. A longer follow-up must be performed to establish whether or not myocardial dysfunction due to hyperglycemia may eventually lead to clinical heart failure in diabetes. Third, although the present study suggests the association of Ang II, oxidative stress and myocardial remodeling in the diabetic heart, a cause-and-effect relationship has not been established and further studies are clearly needed. Fourth, the present study could not determine the role of oxidative stress in the induction of TGF-β and CTGF and the effects of ARB on these responses in cultured cardiac fibroblasts.

In conclusion, candesartan inhibited the development of LV remodeling and diastolic dysfunction associated with diabetes. These beneficial effects of candesartan were associated with the attenuation of myocyte hypertrophy, apoptosis and interstitial fibrosis in association with the decrease in myocardial CTGF expression as well as oxidative stress. ARBs could be beneficial in the prevention of diabetic heart disease.

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