Conclusions—In addition to upregulation of PAI-1, downregulation of MMP-2 and MT1-MMP might play a crucial role in coronary matrix remodeling in insulin-resistant diabetic rats. These molecules appear to be regulated by angiotensin II via stimulation of TGF-β1. (Arterioscler Thromb Vasc Biol. 2003;23:2021-2026.)

Key Words: matrix remodeling ■ matrix metalloproteinases ■ diabetes ■ plasminogen activator inhibitor-1 ■ angiotensin II

Collagen in the normal adult heart serves several important functions, which include providing a supportive structural lattice for cardiomyocytes and coronary vessels and connecting individual myocytes and myofibrillar bundles to integrate individual cardiac contractions. However, a disproportionate increase in collagen accretion or collagen resorption from normal levels can cause defects in the function and supporting structural lattice of the heart. It has been widely known that diabetes is associated with alterations in extracellular matrix (ECM) turnover and regulation.1 Pathologic remodeling characterized by ECM deposition might contribute to cardiovascular complications that are the leading cause of morbidity and mortality in diabetic patients.2 Alteration in diastolic filling of the left ventricle (LV) associated with reciprocal changes in the LV collagen gene and accumulation of cardiac collagen in diabetic rats3,4 suggest that increased interstitial cardiac collagen might cause cardiac fibrosis and result in greater LV stiffness and decreased LV wall compliance, thus leading to diastolic dysfunction and eventual heart failure in diabetes.

Clearly, an imbalance between ECM production and degradation must underlie the process of ECM expansion, but these dynamics in the diabetic heart are poorly understood. Matrix metalloproteinases (MMPs) are primarily responsible for the breakdown of ECM proteins such as collagen and elastin, and their activity is tightly regulated by tissue inhibitors of MMPs (TIMPs).5,6 Recent experimental evidence indicates that expression of MMP-2 and MMP-9 is altered in renal tissues and vasculature from diabetic animals and patients.7–10 However, whether and to what degree diabetes affects the cardiac expression profile of the MMP system remains unknown.

Some molecular interactions between the MMP and fibrinolytic systems might play a role in the regulation of ECM components. The fibrinolytic system contains plasminogen, which is converted to plasmin by tissue-type (t-PA) or urokinase-type (u-PA) plasminogen activator (PA). Plasmin degrades fibrin into soluble degradation products, which can be prevented by specific PA inhibitors (PAI-1 and PAI-2). Recent observations in mice with inactivation of the main
components of the fibrinolytic and MMP systems have suggested that the 2 systems, in concert, contribute to vascular remodeling in the setting of cardiovascular disease. Interestingly, fibrinolytic system activity is known to be diminished in diabetic and insulin-resistant, nondiabetic human subjects, which might be attributable to several mechanisms, including increased PAI-1 production.

The renin-angiotensin system is a major determinant in the development of cardiac remodeling. Angiotensin II is known to stimulate fibrous tissue formation by promoting transforming growth factor-\(\beta_1\) (TGF-\(\beta_1\)) synthesis via angiotensin II type 1 (AT\(_1\)) receptors. It has been shown that AT\(_1\) receptor blockade reduces myocardial hypertrophy, decreases myocardial fibrosis, and attenuates cardiac remodeling to the same degree as angiotensin-converting enzyme inhibition in the rat ischemic heart failure model. Our recent work found that angiotensin II labeling and AT\(_1\) receptor expression in coronary vessels were profoundly increased in Otsuka Long-Evans Tokushima Fatty (OLETF) rats, a model of human non-insulin-dependent diabetes mellitus (NIDDM), compared with nondiabetic Long-Evans Tokushima Otsuka (LETO) rats. However, it is not known whether AT\(_1\) receptor blockade can produce a favorable effect on altered expression of molecules associated with ECM turnover and regulation that might contribute to the development of coronary remodeling seen in diabetes. This study aimed to gain insight into the molecular mechanisms underlying coronary remodeling of ECM in the heart observed at the insulin-resistant stage of NIDDM in OLETF rats, and we sought to evaluate the therapeutic effect of candesartan, an AT\(_1\) receptor blocker, on the altered expression of molecules involved in coronary matrix remodeling in diabetic animals.

Methods

The Methods section can be accessed online (please see http://atvb.ahajournals.org).

Results

General Features of Animals

The characteristics of OLETF rats at 20 weeks of age have been well shown in our previous reports, along with those of age-matched LETO rats. In brief, OLETF rats were modestly hyperglycemic (\(157 \pm 6\) [mean \(\pm\) SD] vs 108 \(\pm\) 3 mg/dL, \(P < 0.05\)), and their plasma insulin level was much higher than in LETO rats (1.12 \(\pm\) 0.31 vs 0.39 \(\pm\) 0.07 ng/mL, \(P < 0.05\)). Treatment of OLETF rats with candesartan for 8 weeks did not alter hyperglycemia (\(173 \pm 4\) mg/dL) and hyperinsulinemia (\(1.53 \pm 0.29\) ng/mL). Heart and LV weights were 1.2-fold higher in OLETF than in LETO rats. Candesartan treatment significantly lowered mean blood pressure, from 119 \(\pm\) 4 to 103 \(\pm\) 5 mm Hg (\(P < 0.05\)), but had no influence on the increase in heart and LV weights in OLETF rats.

Expression of AT\(_1\) and AT\(_2\) Receptors

As demonstrated in our previous report, strongly increased immunofluorescence staining for the AT\(_1\) receptor was detected in LV cross sections of OLETF rats compared with those of LETO rats (please refer to online Figure IA, which can be accessed at http://atvb.ahajournals.org). Positive staining for the AT\(_1\) receptor was diversely observed not only in coronary vessels but also in cardiomyocytes. In contrast, immunofluorescence staining for the AT\(_2\) receptor showed that its expression was mainly localized to coronary vessels and their surroundings and was apparently similar in OLETF and LETO rats.

On Western blots, immunodetectable AT\(_1\) receptor was found at higher levels in LV tissues from OLETF rats, whereas LV expression level of the AT\(_2\) receptor was unchanged by diabetes (see online Figure IB). Densitometric quantification of the signal revealed that the LV expression levels of AT\(_1\) and AT\(_2\) receptors in OLETF rats were 215 \(\pm\) 25% and 103 \(\pm\) 15% of controls, respectively.

Expression of TGF-\(\beta_1\)

In situ hybridization studies indicated that coronary expression of TGF-\(\beta_1\) mRNA was evidently higher in OLETF than in LETO rats (Figure 1A). When the numbers of mean mRNA grains per section of coronary vessel were calculated (15 fields \(\times\) 10 samples), TGF-\(\beta_1\) mRNA in OLETF rats was increased 2.9-fold compared with LETO rats. Treatment of OLETF rats with candesartan reduced its mRNA expression nearly to that of nondiabetic controls. Immunofluorescence staining for TGF-\(\beta_1\) protein showed that it was more strongly expressed in LV sections from OLETF than in those from LETO rats. The increased levels of TGF-\(\beta_1\) protein were found in the coronary vessel and its perivascular area (Figure 1B). Furthermore, cardiomyocytes also abundantly expressed TGF-\(\beta_1\) protein in OLETF rats (see online Figure II). Immunoblot obtained with TGF-\(\beta_1\) antiserum showed a single band that migrated with an apparent molecular mass of 44 kDa (Figure 1C). The relative amount of immunodetectable TGF-\(\beta_1\), as determined by densitometric scanning, was increased in OLETF rats to 245 \(\pm\) 15% of nondiabetic controls. Candesartan resulted in significant improvement in the...
diabetes-induced increase in the TGF-β1 protein level (90±6%).

Activity and Expression Levels of MMPs, MT1-MMP, and MMP Inhibitor

The activities of pro-MMP-2 and active MMP-2 in LV tissues from the 3 groups were evaluated by gelatin zymography. Two gelatinolytic bands were found in the gel, representing pro-MMP-2 and active MMP-2 (Figure 2A). A 92-kDa band of pro-MMP-9 was undetectable in any samples of myocardial extract. Activities of both pro-MMP-2 and active MMP-2 were significantly reduced in OLETF compared with LETO rats (Figure 2B and 2C). Candesartan treatment of OLETF rats increased the activities nearly to the levels obtained in LETO rats.

Total (latent + active) MMP-2 levels as determined by ELISA were significantly reduced in LV tissues from OLETF (0.72±0.05 ng/mL) compared with LETO (1.04±0.12 ng/mL) rats. OLETF rats treated with candesartan did not show any reduction in total MMP-2 tissue levels (1.16±0.15 ng/mL). Active MMP-2 levels measured in LV tissues from OLETF rats were 98±10% and 99±13%, respectively, of those in LETO rats. The MMP-2 decrease in OLETF rats was associated with a decrease in membrane type-1 MMP (MT1-MMP), which activates pro-MMP-2. A 39±7% reduction in MT1-MMP was observed in the coronary vascular area of OLETF compared with LETO rats, and the reduced MT1-MMP expression was recovered by candesartan treatment (Figure 3B). No difference was found in TIMP-2, which forms a complex with MT-MMP species and thereby facilitates activation of MMP-2, between the LETO and OLETF rat groups (see online Figure III).

Expression of Fibrinolytic Regulators

PAI-1 mRNA was evidently increased in coronary vessels of LV sections from OLETF rats compared with the findings from LETO rats (Figure 4A). Quantification of the mRNA signal showed a 1.9-fold increase in OLETF compared with LETO rats. Immunofluorescence studies showed more abundant PAI-1 protein expression in coronary vessels of LV sections from OLETF rats compared with LETO rats. Immunofluorescence staining for MMP-2 was detected in coronary vessels and the perivascular area of LV sections from OLETF compared with those from LETO rats (Figure 3B). Western blot analysis showed that MMP-2 protein was decreased to 62±10% of LETO rats (Figure 3C). This marked reduction in MMP-2 protein expression was blocked by candesartan treatment (91±9%).

The expression levels of MMP-9 and MMP-13, other members of the MMP family, did not differ between OLETF and LETO rats (see online Figure III). Immunoblot analysis revealed that the LV tissue MMP-9 and MMP-13 protein levels in OLETF rats were 98±10% and 99±13%, respectively, of those in LETO rats. The MMP-2 decrease in OLETF rats was associated with a decrease in membrane type-1 MMP (MT1-MMP), which activates pro-MMP-2. A 39±7% reduction in MT1-MMP was observed in the coronary vascular area of OLETF compared with LETO rats, and the reduced MT1-MMP expression was recovered by candesartan treatment (Figure 3B). No difference was found in TIMP-2, which forms a complex with MT-MMP species and thereby facilitates activation of MMP-2, between the LETO and OLETF rat groups (see online Figure III).

Figure 2. A, Representative gelatin zymogram of LV protein extracts from LETO, OLETF, and candesartan-treated (CAN) OLETF rats. The lytic band at 62 kDa corresponds to the active form of MMP-2, the lytic band at 72 kDa is consistent with pro-MMP-2, and the lytic band at 92 kDa corresponds to pro-MMP-9. B and C, Densitography of zymographic gels. Data are mean±SD (n=4). *P<0.01 vs LETO rats. Statistical assessment of the data was made by ANOVA with repeated measurements, followed by the Fisher’s protected least significance t test.

Figure 3. A, In situ hybridization analysis showing gene expression for MMP-2 in LV sections from LETO, OLETF, and candesartan-treated (CAN) rats. B, Confocal images showing immunofluorescence labeling for MMP-2 and MT1-MMP in LV sections from the 3 groups of rats. Immunostaining was mainly focused on coronary vessels (inner diameter <100 μm). Original magnification ×400. C, Western blot indicating a decrease in expression of 62-kDa band (MMP-2) in diabetic LV tissues.
sections from OLETF than in those from LETO rats (Figure 4B). In OLETF rats, positive staining was also marked in the perivascular area. The results of Western blot analysis (Figure 4C) revealed that PAI-1 protein expression was increased to 180±20% of that of LETO rats. The increases in protein and mRNA expression levels of PAI-1 seen in OLETF rats were reversed by candesartan treatment. On immunoblots, PAI-1 protein expression in candesartan-treated OLETF rats was 115±10% of control. In contrast to PAI-1, the 2 PAs, t-PA and u-PA, were expressed equally in coronary vessels of LV sections from LETO and OLETF rats (see online Figure IV).

Expression of Collagen Types I and III and Fibrin
In LV sections from OLETF rats, abnormal, diffuse immunoreactivity of collagen types I and III was observed (Figure 5A and 5B). Thus, their expression in OLETF rats was distributed not only to the coronary vessels but also to the perivascular area. Immunofluorescence staining also revealed that diabetic coronary vessels exhibited both intravascular and extravascular fibrin staining (Figure 5C). In contrast, these immunostainings were weak in LV sections from nondiabetic rats. In OLETF rats treated with candesartan, the expression patterns of collagen types I and III and fibrin were returned to those seen in control nondiabetic LETO rats. Immunoblot analysis (Figure 5D) showed increases to 190±15%, 230±20%, and 200±18% of controls for collagen type I, collagen type III, and fibrin, respectively, in LV tissues from OLETF rats. These values were reversed to 110±10%, 115±15%, and 112±12%, respectively, by candesartan treatment.

Perivascular Fibrosis and Cardiac Collagen Deposition
Figure 6 shows representative photomicrographs of Masson’s trichrome-stained LV sections from LETO, OLETF, and candesartan-treated (CAN) OLETF rats. The wall-to-lumen ratio of coronary arterioles in OLETF rats (2.75±0.42, P<0.01) was ~3-fold greater than that in LETO rats (0.90±0.11). Candesartan treatment reduced this ratio (1.30±0.19, P<0.01). The area of perivascular fibrosis in coronary arterioles, which was corrected for total vessel area, was larger in OLETF than in LETO rats (85±14% vs 27 ±3%, P<0.01). The development of fibrosis in the perivascular area in OLETF rats was prevented by candesartan treatment (32±4%, P<0.01). The ratio of collagen to noncollagen protein in LV tissues of OLETF rats (7.0±0.8%) was higher (P<0.01) that that in LETO rats (4.6±0.5%), and this change was strongly blocked by candesartan treatment (5.1±0.5%).

Discussion
In the present investigation, OLETF rats were used at 20 weeks of age, which were at the stage of NIDDM with modest hyperglycemia and prominent insulin resistance. At this age, OLETF rats exhibited cardiac structural remodeling with the development of perivascular fibrosis. We also showed a significant increase in cardiac collagen deposition in these diabetic rats, as reported in previous studies from this laboratory.18,19 Support for our findings is also provided by the results of other investigators who demonstrated that cardiac fibrosis could be promoted from the prediabetic state in OLETF rats.4 We found that TGF-β1 was highly expressed in coronary vessels and the perivascular area, as well as in cardiomyocytes in the OLETF rat heart. TGF-β1 has been shown to stimulate collagen production in vitro20 and activate a wide array of processes that collectively increase ECM production.21 Accordingly, the increased production of collagen types I and III, which represent the primary collagen phenotypes expressed in the heart ECM,22 as seen in the OLETF rat heart, appears to be causally related to TGF-β1 overexpression. Intensive investigation into the pathogenesis
of ECM expansion in diabetes has consistently implicated this proclerotic cytokine as a key mediator.\textsuperscript{23,24} We thus suggest that superinduction of TGF-\(\beta\), transcription plays a crucial role in the cardiac remodeling process, particularly in cardiac fibrosis, seen at the early insulin-resistant stage of diabetes.

Reduced degradation of ECM could lead to accumulation of ECM components, including collagen. MMPs are a family of proteolytic enzymes that degrade ECM proteins. Several studies have documented downregulation of 2 gelatinases, MMP-2 and MMP-9, in diabetic renal tissues.\textsuperscript{7,8} We demonstrated that in coronary vessels from OLETF rats, there is a significant decrease in MMP-2 gene and protein levels compared with vessels from LETO rats that do not have diabetes. Furthermore, MMP-2 activity was significantly attenuated in OLETF rat hearts. No significant difference between diabetic and nondiabetic coronary vessels was seen for expression of another gelatinase, MMP-9. Also, coronary expression of MMP-13, referred to as collagenase-3, which degrades connective tissue collagens, remained unchanged in diabetes.

A raised level of TIMP proteins, by acting as an endogenous system for the deactivation of MMPs, might be responsible for a net reduction in ECM breakdown. Increased renal expression of TIMP-1, which inhibits activity of MMPs, including MMP-9, has been shown before structural changes in ECM architecture in diabetic rats.\textsuperscript{25} However, TIMP-1 has been found to be downregulated in renal tissues from diabetic patients.\textsuperscript{26} We estimated coronary expression of TIMP-2, which can form a complex with MT-MMP species, and this complex can facilitate activation of MMP-2, but there was no significant between-group difference.

Activation of pro-MMP-2 occurs primarily on the cell surface by membrane-bound MT1-MMP, which can degrade ECM proteins directly. To date, 6 MT-MMP genes have been identified,\textsuperscript{27} but both smooth muscle and endothelial cells possess MT1-MMP and provide a localized MMP activation system.\textsuperscript{28,29} The current study thus investigated MT1-MMP levels in coronary vessels. In agreement with previous results from the internal mammary artery of diabetic patients,\textsuperscript{19} a significant decrease in the MT1-MMP protein level was observed in coronary vessels and the perivascular area of OLETF rats. This reduction in MT1-MMP, an MMP activator protein, in the diabetic heart could provide a mechanistic explanation regarding the regulation of ECM turnover in coronary remodeling in diabetes.

There are observations that hyperinsulinemic NIDDM patients often display reduced fibrinolysis, possibly because of elevated plasma PAI-1.\textsuperscript{30} Changes in plasma PAI-1 appear to be dependent on the conditions that affect endogenous plasma insulin levels.\textsuperscript{31} Thus, increased insulin due to the compensatory hyperinsulinemia might contribute to the increased level of PAI-1 gene expression in NIDDM. Consistently, we showed that protein and gene expression of PAI-1 was markedly increased in coronary vessels of OLETF rats with prominent hyperinsulinemia. Because plasmin not only degrades fibrin but also converts latent pro-MMPs to active MMPs, which in turn degrade the ECM, the increase in expression of PAI-1, by inhibiting the fibrinolytic system at the PA level, could contribute to decreased ECM degradation and hence, accumulation. On the other hand, increased fibrin deposition seen in OLETF rats would be a result of reduced fibrinolysis due to the increased PAI-1 expression. Although the 2 types of PAs (t-PA and u-PA) play an important role in the conversion of plasminogen to the active enzyme plasmin, OLETF rats showed no change in expression levels of t-PA and u-PA in coronary vessels.

In the present investigation, we found that treatment of OLETF rats with the AT\(_1\) receptor blocker candesartan prevented perivascular fibrosis in the heart, reduced the protein and gene expression levels of TGF-\(\beta\), in coronary vessels to those of nondiabetic controls, and blocked collagen type I and III overproduction. Treatment with candesartan inhibited the increased expression of PAI-1 in coronary vessels of OLETF rats. Candesartan also recovered the reduced activity and expression of MMP-2 and the decreased expression of MT1-MMP in the diabetic rat heart. Although candesartan significantly lowered blood pressure in OLETF rats, the therapeutic effect of candesartan in the diabetic heart is unlikely to be due to its lowering effect on blood pressure, because treatment with hydralazine (3 mg \(\cdot\) kg\(^{-1}\) \(\cdot\) d\(^{-1}\)), which also lowered blood pressure to the same extent as candesartan, had no effect on cardiac expression of TGF-\(\beta\), and perivascular fibrosis in OLETF rats (authors’ unpublished observations). These results suggest a potentially central role for AT\(_1\) receptor activation in the molecular changes associated with coronary matrix remodeling in diabetes. Angiotensin II has been shown to stimulate TGF-\(\beta\) production in cardiac fibroblasts.\textsuperscript{31,32} Because TGF-\(\beta\) has been implicated as a key mediator for ECM expansion in diabetes,\textsuperscript{23,24} it would be logical to suspect that angiotensin II stimulates cardiac collagen production by promoting TGF-\(\beta\) synthesis via AT\(_1\) receptor activation. Furthermore, it is known that TGF-\(\beta\) stimulates PAI-1 biosynthesis in cultured cells.\textsuperscript{33} TGF-\(\beta\) is therefore thought to have the potential to be a major regulator of PAI-1 in our experimental diabetes. Although the present experiments cannot determine whether angiotensin II regulates MMP-2 and MT1-MMP expression directly or indirectly through TGF-\(\beta\), there is a recent report showing the negative regulation of MMP-1 and MMP-2 by TGF-\(\beta\) in human gingival fibroblasts.\textsuperscript{34}

In conclusion, increases in local generation of angiotensin II and expression of AT\(_1\) receptors in coronary vessels observed at the insulin-resistant stage of NIDDM in OLETF rats\textsuperscript{18} appear to modulate coronary expression of ECM-related molecules and lead to coronary matrix remodeling, possibly associated with overproduction of TGF-\(\beta\). This
study especially represents the first report that, in addition to the increased expression of PAI-1, the reduced activity and expression of MMP-2 and the decreased expression of MT1-MMP also play a role in coronary remodeling characterized by ECM deposition in diabetes. Our results provide important information regarding the molecular and cellular bases of cardiac collagen deposition and matrix remodeling in diabetes and contribute to our understanding of a rational basis for therapeutic strategies for diabetic cardiovascular complications.

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References

Role of Angiotensin II in Altered Expression of Molecules Responsible for Coronary Matrix Remodeling in Insulin-Resistant Diabetic Rats
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Online Figure I

A

LETO  OLET

AT$_1$

AT$_2$

B

LETO  OLET

AT$_1$

40 kDa

AT$_2$

41 kDa

Online Figure I
Online Figure II
Online Figure III
Online Figure IV
Methods

Experimental Animals

The experimental design was approved by the Hokkaido University School of Medicine Animal Care and Use Committee. Animals were obtained from the Tokushima Research Institute, Otsuka Pharmaceutical and maintained under constant temperature and lighting conditions with free access to food and water. At age 8 weeks, male OLETF rats were randomly divided into two groups. One group of 10 animals was kept on a standard diet, whereas the other group of 10 animals was supplemented with candesartan (1 mg/kg/day). This treatment period was 12 weeks, and the animals were used at age 20 weeks, a time when they were in the early stage of NIDDM with modest hyperglycemia and prominent insulin resistance. Ten male LETO rats served as controls. On the day of the experiments, rats were euthanized under 3% halothane in room air, followed by 1% halothane in 21% O₂-79% N₂ mixture through a gas mask connected to a Harvard respirator. The hearts were rapidly excised, and the whole heart and LV weights were measured. Some portion of LV was dipped into optimum cutting temperature (OCT) compound and immediately frozen in liquid nitrogen. The remaining portion was preserved at –80°C without OCT compound.

In Situ Hybridization

Frozen cryostat tissue sections, 10-15 µm in thickness, were prepared and mounted on glass slides precoated with 3-amino-propyltriethoxysilane. The sections were then fixed by 4% paraformaldehyde with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0). The oligonucleotide probes were labeled with 35S-dATP, using terminal deoxypiribonucleotidyl transferase (Promega Corp.). The radiolabeled probes were hybridized to the tissue in a prehybridization buffer for 10 h at 42°C. The
sections were either exposed to Hyperfilm-βmax (Amersham) for 4 weeks or dipped in Kodak NTB2 nuclear track emulsion and exposed for 4 to 8 weeks. The specificity of in situ hybridization was confirmed by the disappearance of signals when excessive doses of the corresponding nonlabeled antisense oligonucleotides (cold) were added to the labeled antisense oligonucleotides (hot) hybridization fluid. The mRNA grains per blood vessel were quantified using an image-analyzing software (Microcomputer Imaging Device; Imaging Research).

**Immunohistochemistry and Immunofluorescent Labeling**

For immunohistochemical determination of targeted molecules, the following commercially available antibodies were used: anti-human AT$_1$ receptor rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-human AT$_2$ receptor goat polyclonal antibody (Santa Cruz Biotechnology), anti-human TGF-β$_1$ rabbit polyclonal antibody (R and D System), anti-human MMP-2 rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-rat MMP-9 rabbit polyclonal antibody (Abcam), anti-rat MMP-13 mouse monoclonal antibody (Chemicon International), anti-human membrane-type-1-MMP mouse monoclonal antibody (Fuji Chemical), anti-human TIMP-2 goat polyclonal antibody (Santa Cruz Biotechnology), anti-rat PAI-1 rabbit polyclonal antibody (American Diagnostica), anti-human melanoma t-PA goat polyclonal antibody (American Diagnostica), anti-rat u-PA goat polyclonal antibody (Santa Cruz Biotechnology), anti-human collagen type I rabbit polyclonal antibody (Rockland), anti-human collagen type III rabbit polyclonal antibody (Rockland), and anti-human fibrin monoclonal antibody (Chemicon International).

Frozen cryostat sections (8 μm thick) were fixed in acetone and air-dried. Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide
for 15 min. After being blocked by normal goat serum to prevent nonspecific staining by the secondary antibody, the sections were incubated with primary antibodies overnight at 4°C, followed by exposure to a suitable secondary antibody coupled to horseradish peroxidase. Immunostains were visualized by light microscopy with diaminobenzidine. The specificity of the immunoreaction was evaluated in comparison with the negative control specimen in which non-immune IgG was used instead of the primary antibodies. Quantification of immunoreactivity by pixel intensity was analyzed using an image-analyzing software (Microcomputer Imaging Device).

After overnight incubation with each primary antibody as mentioned above, the sections were exposed to the fluorescence secondary antibody, Cy3-conjugated AffiniPure anti-rabbit IgG or fluorescein-conjugated AffiniPure goat anti-rabbit, anti-goat, or anti-mouse IgG (Jackson Immunoresearch Laboratories), for 2 h according to the manufacturer’s instructions. The samples processed without primary antibodies served as negative controls. Immunofluorescent images were observed under the Laser Scanning Confocal Imaging System (MRC-1024, Bio-Rad).

**Western Blot Analysis**

Samples of tissue homogenate were subjected to electrophoresis on polyacrylamide gels, and proteins were transferred to polyvinylidene difluoride filter membrane. After blocking with 5% non-fat milk in phosphate-buffered saline, the membranes were incubated with specific antibody recognizing each of the targeted molecules. After extensive washing with phosphate-buffered saline containing 0.1% Tween 20 to remove any nonspecifically bound primary antibodies, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse, anti-rabbit or anti-goat antibody. The blots were visualized with the enhanced chemiluminescence detection system
Gelatin Zymography

A gelatin zymography kit (Yagai Corp.) was used to demonstrate the presence of total MMP-2 activity in the tissue samples. Twice-crystallized gelatin (1 mg/ml) was incorporated into an 8% SDS-PAGE gel. Each tissue sample (20 µl) was mixed with the same amount of sample buffer containing a final concentration of 2.5% SDS without reducing agent. After electrophoresis, the gels were soaked in 2.5% Triton X-100 to remove SDS and then incubated in activating buffer for 30 h. Subsequently, gels were stained with Coomassie brilliant blue and de-stained. Gelatinolytic activity was visualized as clear white bands of 72 and 62 kDa, corresponding to proMMP-2 and active MMP-2, against a blue background. The relative activities of proMMP-2 and active MMP-2 were analyzed using an image analyzer (Northern Light; Image Research Inc.).

Enzyme Immunoassay for MMP-2

Concentrations of MMP-2 in sera and tissue extracts were determined using an ELISA-based MMP-2 activity assay kit (Amersham Pharmacia Biotech) which employs a specific anti-MMP-2 antibody with no cross-reactivity reported to other MMPs. Samples were incubated with p-aminophenylmercuric acetate during assay procedures to activate latent MMP-2.

Morphometric Analysis

Frozen cryostat sections (8 µm thick) were fixed with acetone. The sections were then stained with Masson’s trichrome stain. The transsectional images of coronary
arterioles with inner diameter less than 100 μm were analyzed using an image-analyzing software. The wall-to-lumen ratio was calculated as the area of the vessel wall divided by the total area of vessel lumen. The area of perivascular fibrosis was estimated as the percent fibrosis surrounding the blood vessel for each visual field area of the blood vessel.

Collagen in tissue samples was detected using a Collagen staining kit (Collagen Research Center). Then, the ratio of collagen to non-collagen protein was calculated.

**Statistical Analysis**

Data are shown as mean ± SD. Means were compared by ANOVA, followed by the Fisher protected least significance t test for multiple comparisons. Differences were considered significant at a value of P<0.05.
Figure I-online. Protein expression of AT$_1$ and AT$_2$ receptors in LV tissues of LETO and OLETF rats. A, Confocal images showing immunofluorescence for AT$_1$ and AT$_2$ receptor proteins. Positive staining is focused on coronary vessels (inner diameter: <100 µm) and cardiomyocytes. Magnification ×400. B, Western blot indicating a marked increase in expression of 40-kDa band (AT$_1$) and no change in expression of 41-kDa band (AT$_2$) in diabetic LV tissues.

Figure II-online. Confocal images showing immunofluorescence labeling for TGF-β$_1$ in LV sections from LETO, OLETF, and candesartan-treated OLETF (CAN) rats. Immunostaining was focused on cardiomyocytes. Original magnification ×400.

Figure III-online. Confocal images showing immunofluorescence labeling for MMP-9, MMP-13, and TIMP-2 in LV sections from LETO, OLETF, and candesartan-treated OLETF (CAN) rats. Immunostaining was mainly focused on coronary vessels (inner diameter <100 µm). Original magnification ×400.

Figure IV-online. Confocal images showing immunofluorescence labeling for t-PA, and u-PA in LV sections from LETO, OLETF, and candesartan-treated OLETF (CAN) rats. Immunostaining was mainly focused on coronary vessels (inner diameter <100 µm). Original magnification ×400.
Online Figure II

LETO  OLETF  CAN
Online Figure III
Online Figure IV