**Full Paper**

**Long-Term Angiotensin II Blockade May Improve Not Only Hyperglycemia but Also Age-Associated Cardiac Fibrosis**

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**Abstract.** In the present study, the effects of long-term angiotensin (Ang) II antagonism on the development of cardiac and endothelial disorders were examined in Spontaneously Diabetic Torii (SDT) rats. Blood glucose concentration started to increase markedly in the untreated SDT rats from 20 weeks of age, while the blood glucose concentrations of candesartan cilexetil–treated SDT rats were significantly lower until 30 weeks of age. Cardiac function deteriorated in SDT rats and was accompanied by severe cardiac fibrosis, cardiac hypertrophy, and microstructural pathologic change in cardiomyocytes. Cardiac function was very well preserved in the age-matched Sprague Dawley (SD) rats, but cardiac fibrosis developed with aging. Candesartan cilexetil treatment improved cardiac structural remodeling and cardiac function in SDT rats. Surprisingly, the degree of cardiac fibrosis in candesartan cilexetil–treated SDT rats was less than that of SD rats. Immunohistological staining confirmed that in addition to collagen deposition, fibroblasts and myofibroblasts were the main cellular components in the cardiac fibrotic areas. The diabetic hearts showed positive staining for ACE, Ang II, and AT₁ receptors. SDT rats also showed decreased endothelial function, which was improved with candesartan cilexetil treatment. These findings indicate that Ang II is involved in the development of cardiac dysfunction by accelerating cardiac remodeling and cardiomyocyte damage in the presence of hyperglycemia. On the other hand, although the mechanisms responsible for the cardiac fibrosis that occurs under normal conditions may differ greatly from those responsible for cardiac fibrosis with hyperglycemia, Ang II seems to play an important role in both.

**Keywords:** angiotensin II, intervention, insulin resistance, heart failure, fibrosis

**Introduction**

Diabetes mellitus (DM) is a syndrome characterized by disordered metabolism and inappropriately high glucose levels resulting from either low insulin levels or abnormal resistance to insulin’s effects. DM can cause many serious complications such as heart failure (HF), renal failure, and nerve damage. Epidemiological studies have demonstrated that the baseline prevalence and subsequent incidence of HF in DM patients were more than twice those in non-diabetic patients (1, 2). In addition, DM is a risk factor for HF progression. For example, diabetic patients with asymptomatic ischemic cardiomyopathy have a significantly increased incidence of HF symptoms, HF-related hospitalization, and sudden death (3). In diabetic patients, hyperglycemia itself appears to play an important role in the pathogenesis of HF. For example, it was reported that for each 1% elevation of hemoglobin A₁c (HbA₁c), the risk of developing HF increases by about 8% (4). These deleterious effects of hyperglycemia have been attributed to the increased advanced glycosylation end products (AGEs) that occur following increased blood glucose levels. AGEs have been reported to accelerate the generation of reactive oxygen species, and these, in turn, cause myocardial dysfunction and apoptosis (5). Therefore, strict control of the blood glucose level is now currently thought to be associated with a better
prognosis in DM patients. On the other hand, activation of the renin-angiotensin system (RAS) is also thought to play an important role in the pathophysiology of DM. In high-risk individuals, such as those with hypertension or chronic HF, large clinical trials have shown that RAS inhibition can delay and/or prevent the onset of DM (6). Moreover, DM is usually associated with RAS activation, and pharmacological blockade of angiotensin (Ang) II action has been confirmed to improve the prognosis of diabetic patients (7). Thus, Ang II plays an important role not only in the developing stage of DM, but also in the progression of DM-related pathology. To date, several diabetic animal models, such as obese Zucker rats (8) and Otsuka Long-Evans Tokushima Fatty (OLETF) rats (9), have been developed to explore the pathophysiological mechanisms of human type 2 DM. The effects of RAS inhibition in the pathology of these DM models have been often studied, and these experiments have generated powerful, persuasive evidence of the advantages of RAS inhibition (10 – 13). However, little information concerning the effects of long-term Ang II blockade on HF, as well as the distribution of RAS components, such as ACE, Ang II, and Ang II receptors, in failing hearts at a later phase of DM has been acquired. Therefore, the present study was designed to investigate whether long-term blockade of Ang II type 1 (AT1) receptors with candesartan cilexetil provides functional and structural benefits with respect to HF at a later phase of DM in the Spontaneously Diabetic Torii (SDT) rat, which has been established as a rat model of non-obese type 2 diabetes (14, 15). Meanwhile, the distribution of cardiac ACE, Ang II, and Ang II receptors, as well as microstructural pathologic changes in the myocardium, were examined using immunohistological staining and electron microscopy.

Material and Methods

Animals

Twelve male, 10-week-old, SDT rats were studied. Six age-matched Sprague–Dawley (SD) rats were used as controls. The animals were housed in a climate-controlled room (temperature 22 ± 2°C, humidity 45 ± 10%, and a 12-h lighting cycle) with free access to food and water. Twelve male SDT rats were randomized to treatment with candesartan cilexetil (2.5 mg/kg per day in drinking water) or placebo vehicle (0.12 v/v% ethanol/polyethylene glycol). The experimental procedures used were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (Animal Research Laboratory, Osaka Medical College).

Measurement of body weight (BW), mean arterial blood pressure (MABP), and blood glucose concentration

The blood glucose levels were determined using Glutest Neo (Sanwa Kagaku Kenkyusho Co., Ltd., Nagoya), as previously described (16). MABP was measured using an automatic sphygmomanometer (BP Monitor for Rats and Mice Model MK-2000; Muromachi Kikai Co., Ltd., Tokyo). Measurements, including BW, were taken at 10, 20, 30, 40, and 50 weeks of age.

Echocardiographic study and preparation of tissue samples

Echocardiographic studies were performed for the three groups at 50 weeks of age using an echocardiographic system (Nemio 30; Toshiba, Tokyo) according to previously described methods (17). In brief, after intraperitoneal injection of ketamine HCl (25 – 50 mg/kg) and xylazine (5 – 10 mg/kg), M-mode tracings and pulse-wave Doppler spectra (E and A waves) of mitral inflow were recorded for each group. After this procedure, both SD and SDT rats, treated with candesartan cilexetil or untreated, were sacrificed with an overdose of sodium pentobarbital. The hearts were harvested and fixed in methanol–Carnoy’s fixative and embedded in paraffin. To evaluate endothelial function, the rats’ carotid arteries were immediately removed.

Histological examination

To evaluate the areas of cardiac fibrosis and to determine the cellular characteristics of cardiac fibrosis, as well as the distribution of RAS components in the hearts, the following general histological and immunohistological examinations were performed after 3-μm-thick sections were obtained from the paraffin-embedded cardiac samples, using previously described methods (18).

Azan Mallory staining was used to determine collagen distribution.

Expression of angiotensin-converting enzyme (ACE), AT1 receptors, and AT2 receptors, as well as the distribution of Ang II, was determined by using mouse anti-human ACE monoclonal antibody (Chemicon, Temecula, CA, USA) and anti-human AT1 (sc-1173; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), AT2 (sc-9040; Santa Cruz Biotechnology, Inc.), and anti-human Ang II (IgG Corporation, Nashville, TN, USA) polyclonal antibodies.

Anti-human alpha-smooth muscle actin (α-SMA; Dako, Glostrup, Denmark) and anti-bovine vimentin (Wako, Osaka) antibodies were used to identify fibroblasts and myofibroblasts in the areas of cardiac fibrosis.
Electron microscopic study

Electron microscopic studies were performed according to the methods described previously by Hayashi et al. (19). In brief, for the transmission electron microscopic study, the specimens were fixed in 4% paraformaldehyde containing 0.25% glutaraldehyde and 4.5% sucrose, and ultrathin sections obtained from the embedded blocks were examined using an electron microscope (H-7650; Hitachi, Tokyo). For the scanning electron microscopic study, the specimens were fixed in 3% glutaraldehyde and macerated in sodium hydroxide solution to eliminate the cellular components.

Assessment of endothelial function

Endothelial function was assessed using carotid ring segments according to previously described protocols (20). In brief, the ring segments were mounted in a 5-ml tissue bath containing Tyrode’s solution composed of 137 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl₂, 1.1 mM MgCl₂, 0.42 mM NaH₂PO₄, 12 mM NaHCO₃, and 5.7 mM glucose. The Tyrode’s solution was kept at 37°C, pH 7.4, and bubbled continuously with 5% CO₂ in oxygen. After maximal contraction of the ring segments was obtained with KCl (50 mM), the segments were pre-contracted with 0.1 μM noradrenaline. When the noradrenaline-induced contraction reached its peak, the segments were challenged with 3 μM acetylcholine (ACh) to induce maximal relaxation. Then, the segments were further challenged with 0.1 mM papaverine to obtain maximum relaxation when the ACh-induced relaxation had reached bottom. The degree of ACh-induced maximal relaxation in these segments is expressed as a percentage of the relaxation elicited by 0.1 mM papaverine.

Statistical analyses

All numerical data shown in the text are expressed as the mean ± S.E.M. Significant differences among the mean values of multiple groups were evaluated using one-way ANOVA followed by a post hoc analysis (Fisher’s test). P<0.05 was the threshold for significance.

Results

BW increased in a time-dependent manner in SD rats (Fig. 1). However, in the untreated SDT rats, BW stopped increasing during the entire experimental period. Candesartan cilexetil treatment significantly decreased the BW loss in SDT rats. MABP at 10 weeks of age did not differ significantly between SD and untreated SDT rats (Fig. 1). However, MABP was significantly higher in the rats that were randomly assigned to receive candesartan cilexetil. In the untreated SDT rats, MABP tended to increase with aging. Candesartan cilexetil treatment significantly suppressed the MABP increase in SDT rats. Blood glucose concentrations did not exceed 100 mg/dl in the SD rats throughout the experimental period (Fig. 1). However, blood glucose concentration started to increase markedly in the untreated SDT rats from 20 weeks of age and
remained elevated until the end of the experiments. In comparison with the untreated SDT rats, the blood glucose concentrations of candesartan cilexetil–treated SDT rats were significantly lower until 30 weeks of age, after which they began to increase; however the blood glucose levels were still lower in the candesartan cilexetil–treated SDT rats than in the untreated SDT rats.

Figure 2 shows representative echocardiograms of the SD rats and the SDT rats treated with and without candesartan cilexetil. As can be seen in the bar graphs of Fig. 2, the chronic DM resulted in a substantial reduction in ejection fraction (EF) and tended to decrease the ratio of the E-wave to A-wave velocity (E/A). Candesartan cilexetil treatment significantly increased EF and E/A compared with the untreated SDT rats.

Figure 3 shows the changes in the ratio of whole heart weight (HW) to BW and in the areas of cardiac fibrosis of the three experimental groups at 50 weeks of age. In comparison with SD rats, the ratio of HW to BW was significantly increased in the untreated SDT rats, suggesting that the SDT rats had cardiac hypertrophy. On the other hand, candesartan cilexetil treatment significantly suppressed the development of cardiac hypertrophy in the diabetic rats. Representative Azan Mallory staining patterns of cardiac fibrosis are shown in Fig. 4. As can be seen in the bar graphs of Fig. 3, there was no significant difference in the areas of cardiac fibrosis between SD rats and untreated SDT rats. However, candesartan cilexetil treatment suppressed cardiac fibrosis significantly in the SDT rats compared with both the SD rats and the untreated SDT rats. Increases in areas of cardiac fibrosis appeared to result from increased deposition of collagen in the myocardial interstitium. As shown by scanning electron microscopy in Fig. 5, the thick interstitial collagen fiber deposits in the SD and untreated SDT rats were remarkable. However, the interstitial collagen fibers in the candesartan cilexetil–treated SDT rats were very thin compared to the other two groups.

To identify cellular features in the cardiac fibrotic areas, immunohistological examinations were performed using antibodies derived from vimentin and \(\alpha\)-SMA. Vimentin intermediate filaments are major structural components of the cytoskeleton and are mainly expressed in mesenchymal cells such as fibroblasts and myofibroblasts (21). \(\alpha\)-SMA is not simply a contractile protein that is widely expressed in contractile vascular smooth muscle cells (VSMCs), but it is also expressed during the time when the phenotypic change from fibroblast to myofibroblast occurs (22). Therefore, the
Fig. 3. Bar graph shows changes in the ratio of the heart weight to the body weight and the area of fibrosis in the cardiac sections obtained from SD rats, untreated SDT rats, and candesartan cilexetil–treated rats at 50 weeks of age. *$P<0.05$, **$P<0.01$, ***$P<0.001$. Each group contained 6 animals.

Fig. 4. Representative Azan Mallory staining and immunostaining for vimentin and $\alpha$-SMA in the serial sections obtained from SD rats, untreated SDT rats, and candesartan cilexetil–treated rats at 50 weeks of age.
proportions of fibroblasts and myofibroblasts can be calculated by vimentin and α-SMA staining. For example, cells in the fibrotic area that are only positive for vimentin are considered to be fibroblasts. Cells that are positive for both vimentin and α-SMA are considered to be myofibroblasts. Figure 4 shows representative Azan Mallory staining and immunohistological staining for vimentin and α-SMA of three serial cardiac sections obtained from SD, untreated SDT, and candesartan cilexetil–treated SDT rats. As can be seen in these photographs, the vimentin-positive cells are always scattered along the fibrotic areas, and some of these cells are also positively stained with α-SMA, indicating that in addition to collagen deposition, fibroblasts and myofibroblasts are the main cellular components in cardiac fibrotic areas. As can be seen in the transmission electron micrographs (Fig. 5), myocardial cells appeared normal in SD rats. However, a variety of degenerative changes was observed in untreated SDT rats, including nuclear invagination, deformed mitochondria, disheveled alignment of myofibrils, and increased glycogen granules in the cardiomyocytes. In particular, marked thickening of the capillary basement membrane was present. All of the above degenerative changes were suppressed by candesartan cilexetil treatment.

Figure 6 shows representative immunohistological staining for ACE, Ang II, and Ang II receptors from the SDT failing heart. ACE was expressed primarily in the interstitial cellular constituents of the myocardium and arterial endothelial cells. From their cellular shape and distribution pattern, these cellular constituents look very much like fibroblasts and myofibroblasts. Ang II–positive staining was also found in the fibroblast or myofibroblast-like cells, as well as in the arterial smooth muscle cells. Very strong positive staining for AT₁ receptors was found in the cardiomyocytes. AT₁ receptors were also expressed in the arterial smooth muscle cells. On the other hand, no positive staining for AT₂ receptors was found in the failing hearts. Similar staining patterns for these RAS components were also observed in cardiac sections obtained from SD rats and from candesartan cilexetil–treated rats (data not shown).

In comparison with the SD rats, ACh-induced relaxation tended to be less in the diabetic untreated SDT rats, although it was restored with candesartan cilexetil treatment in SDT rats (Fig. 7).

Discussion

The present study showed that long-standing diabetes was accompanied with deposition of collagen in the
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myocardial interstitium, microstructural changes in cardiomyocytes, and attenuated endothelial function, all of which may contribute to the development and progression of HF in SDT rats. To our surprise, cardiac fibrosis also developed in the non-diabetic normal SD rats with aging, and the degree of these changes did not differ significantly from that which occurred in the diabetic hearts, although cardiac function was preserved in the normal SD rats. On the other hand, long-term AT1-receptor blockade with candesartan cilexetil was very beneficial, not only with respect to cardiac remodeling, but also with respect to microstructural pathological changes in the cardiomyocytes and endothelial function. ACE, Ang II, and AT1 receptors were distributed in both normal and diabetic hearts, and the present study indicated that Ang II, via AT1-receptor stimulation, plays an important role in the development and progression of HF in SDT rats. In addition, the present study suggests that the cardiac fibrosis that develops with aging may be delayed by long-term AT1-receptor blockade because candesartan cilexetil treatment reduced the cardiac fibrosis to a very small extent in the SDT rats, and, as shown in Fig. 3, the degree of cardiac fibrosis was less in the candesartan cilexetil-treated SDT rats than in the normal SD rats.

Clinical studies have confirmed that diabetic patients are at increased risk of cardiomyopathy, and HF is a
major cause of death in these patients (1, 2). Histological studies of biopsies taken from diabetic hearts have demonstrated that interstitial fibrosis and myocyte hypertrophy were the major pathologic changes (23, 24). Moreover, ultrastructural changes in myocytes, such as mitochondrial degeneration and derangement of myofibrils, have also been reported in diabetic hearts (25). In addition to these morphological changes, endothelial dysfunction commonly occurs with chronic DM (26). The present study also confirmed the presence of marked cardiac hypertrophy in untreated SDT rats. In untreated SDT rats, the thick deposits of interstitial collagen fibers among the cardiomyocytes as observed by scanning electron microscopy, as well as the intense fibrotic region observed by Azan Mallory staining, were similar to those described in previous reports that examined diabetic hearts. Thus, SDT rats reflect diabetic pathology and the SDT rat model is suitable for investigating the pathophysiology of DM.

In the present study, long-term AT$_1$-receptor blockade with candesartan cilexetil in the SDT rats provided major benefits by improving cardiac remodeling and preventing ultrastructural changes in the myocytes, which indicates that Ang II, through AT$_1$-receptor stimulation, plays an important role in the pathogenesis of end organ damage following persistent hyperglycemia. How AT$_1$-receptor blockade could significantly improve diabetic end organ damage may be explained by the following divergent mechanisms. Since the prognosis of diabetes has been shown to be significantly improved by tight control of blood glucose levels, the hyperglycemia itself is believed to cause glucotoxicity in several organs, including the heart. Therefore, improvement of insulin sensitivity and protection of the pancreatic islets with AT$_1$-receptor blockade may play a significant role. AT$_1$-receptor blockade usually increases skeletal muscle blood flow, and this vasodilatory effect is commonly associated with improvement of insulin resistance (27). Increases in adiponectin with Ang II blockade (28) and activation of PPAR gamma-receptors by some ARBs (29) are also involved in increased insulin sensitivity. Glucose-stimulated insulin secretion may also be inhibited by the increase of locally produced Ang II (30) and the blockade of Ang II actions, resulting in an increased circulating insulin level in diabetic subjects. Therefore, AT$_1$-receptor blockade by an ARB decreases blood glucose concentrations, which, in turn, may spontaneously attenuate diabetic end organ damage, such as pathologic cardiac remodeling, caused by persistent hyper-glucotoxicity associated with hyperglycemia. In fact, the blood glucose lowering effect with Ang II blockade has been reported in several animal models of type 2 diabetes; for example, it has been reported previously that the elevation of blood glucose level was suppressed markedly by the treatment with losartan in db/db mice, which develop type 2 diabetes in association with highly reproducible obesity due to mutation in the leptin receptor (31). On the other hand, although it was not in diabetic patients, Kitamura et al. have noted that the blood glucose lowering effect could be observed by the treatment of ARB in newly diagnosed hypertensive patients within 6 months of ARB therapy (32). In the present study, we also found that the blood glucose concentrations in the candesartan cilexetil-treated SDT rats were significantly lower than in the untreated SDT rats throughout the experimental period, indicating that this effect may relate to some extent to the cardio-protective mechanism of candesartan cilexetil treatment.

It has been convincingly demonstrated that Ang II, by accelerating the production of reactive oxygen species (ROS), plays an important role in the pathogenesis of cardiac hypertrophy, fibrosis, and ventricular dysfunction (33). Ang II receptors have been characterized in cardiomyocytes (34) and cardiac fibroblasts (35), as well as in the endothelial lining of coronary arteries (36). The present study also confirmed that AT$_1$ receptors are present in cardiomyocytes and coronary arterial smooth muscles. In addition, ACE was expressed in the cardiac fibroblast and myofibroblast-like cells, as well as in the endothelial cells of the coronary arteries. Importantly, Ang II itself, as shown by immunostaining, could be clearly observed in the cardiac fibroblast and myofibroblast-like cells, as well as in the coronary arterial smooth muscles. However, there was no positive staining for the AT$_2$ receptor in the diabetic hearts. Given that cardiac hypertrophy and fibrosis were improved significantly by the blockade of AT$_1$ receptors in the present study, it is clear that the RAS components examined in the present study are involved in the pathogenesis of cardiac remodeling in diabetic hearts. Our findings also provided further evidence showing that the Ang II blockade can improve cardiac diastolic dysfunction in patients suffering from diabetic mellitus. As observed in a previous clinical trial (37), Kawasaki et al. found that the mitral E/A ratio was increased significantly with chronic candesartan treatments in patients with diabetes mellitus. More interestingly, in serum, the carboxy-terminal propeptide of procollagen type I (PIP), an index of collagen type I synthesis, was decreased and the carboxy-terminal telopeptide of collagen type I (CITP), an index of collagen type I degradation, was increased following the treatment with candesartan. These findings indicate that the improvement of diastolic dysfunction by the chronic candesartan treatments in diabetic patients is resulted
partly from the attenuation of myocardial fibrosis through the modification of collagen biosynthesis.

An interesting finding in the present study is that age-related impairments of various organ functions may be improved with long-term AT\(_1\)-receptor blockade. As shown in Fig. 3, in normal SD rats, although cardiac hypertrophy did not appear with aging, cardiac fibrosis was evident at 50 weeks of age, and the degree of fibrosis evaluated on Azan Mallory staining was very similar to that seen in the diabetic hearts obtained from the untreated SDT rats. Although cardiac function was preserved in the normal SD rats, the discrepancy between well-preserved cardiac function and severe cardiac fibrosis may be explained by the differences in the microstructural pathological changes between diabetic and non-diabetic hearts. As can be seen in Fig. 6, microstructural changes, such as mitochondrial degeneration and the disheveled alignment of myofibrils, in the cardiomyocytes of SD were less than those observed in the diabetic cardiomyocytes obtained from untreated SDT rats. This indicates that in diabetic subjects, not only is deposition of collagen contents but there are also changes in the energy-related microstructures and the contractile myofibril, which may also play an important role in the impairment of cardiac function. Although cardiac fibrosis seen in the SD rats did not significantly affect their cardiac function, as evaluated on echocardiography at 50 weeks of age, it should be pointed out that further progression in the cardiac fibrosis would result not only in increased cardiac stiffness but also in destruction of the cardiac conductive system, which would ultimately lead to cardiac dysfunction. Therefore, the prevention of diabetic end organ damage, which usually occurs in several vital organs, including the heart, and advances with the fibrogenetic aging process, is a strategy to prolong the life-span and improve quality of life. The present study indicates that attenuation of the action of Ang II may achieve such a strategy.

In conclusion, under conditions of chronic hyperglycemia, cardiac function may be reduced, with the development of cardiac pathological remodeling and microstructural changes in the cardiomyocytes, as well as attenuation of endothelial function. Since these functional and structural changes were significantly improved with AT\(_1\)-receptor blockade, and given that components of the RAS system such as ACE, Ang II, and AT\(_1\) receptors were also all present in the diabetic hearts, AT\(_1\) receptors appear to play an important role in the promotion of end organ damage in diabetes. Additionally, although the mechanisms of the cardiac fibrosis that occurs with normal aging may differ greatly from those operative under hyperglycemic conditions, Ang II appears to play an important role in these processes.

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