Subdepressor dose of benidipine ameliorates diabetic cardiac remodeling accompanied by normalization of upregulated endothelin system in rats

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CALCIUM CHANNEL BLOCKERS (CCBs) are widely used clinically for the management of hypertension, angina pectoris, and cardiac arrhythmias (45). Their pharmacological and therapeutic properties are attributable to the block of the influx of Ca2+ through L-type Ca2+ channels in vascular smooth muscle and/or cardiac muscle (7). CCBs have been shown to inhibit collagen deposition in the extracellular matrix formed by cultured human fibroblasts (46), to prevent renal fibrosis (12, 29), and to reduce cardiac fibrosis in spontaneously hypertensive rats (6, 52). Furthermore, long-acting CCBs, amlodipine and lacidipine, can reduce cardiac hypertrophy and cardiac hypertrophy-related gene programming in stroke-prone spontaneously hypertensive rats (30, 32), suggesting the preventive effect of CCBs on hypertensive cardiac remodeling.

Cardiac remodeling is characterized by increased cardiomyocyte volume and collagen deposition (54, 57). Endothelin (ET)-1 promotes growth of cardiomyocytes in vitro (22, 38), induces collagen synthesis by cardiac fibroblasts (17, 38), and stimulates the development of left ventricular (LV) hypertrophy (27, 51). Thus ET-1 may be of significant importance in the development of cardiac remodeling. Indeed, the involvement of ET-1 in hypertensive cardiac remodeling has been suggested by the findings that ET-1 blockade therapy can prevent cardiac fibrosis and fibrosis-associated molecular events in hypertensive animal models such as deoxycorticosterone acetate-salt hypertension (1, 2).

Circulating plasma ET-1 levels have been shown to be higher in diabetic subjects than in nondiabetic controls (11, 53), and the plasma concentration of Big ET-1, a precursor of ET-1, has been found to be elevated in patients with diabetes mellitus (55). ET-1 has been implicated in the pathogenesis of insulin resistance-induced cardiovascular complications in human diabetes (15, 42). Notably, cardiac remodeling, including cardiac fibrosis, is promoted from the early stage of developing diabetes in Otsuka Long-Evans Tokushima Fatty (OLETF) rats (37). This strain displays stable clinical and pathological features that resemble human Type 2 diabetes, and one of its characteristics is an early manifestation of existence of insulin resistance. Our group (25) previously showed that benidipine, a long-acting dihydropyridine CCB, improved cardiac remodeling in OLETF rats at the insulin-resistance stage. In the present study, we examined the effects of long-term treatment with benidipine at dosage insufficient on blood pressure on cardiac expression levels of prepro-ET-1, ET-1, ETA and ETB receptors, and ET-1 signaling molecules, including p38MAPK and JNK, in this insulin-resistant diabetic model to assess whether the beneficial actions of benidipine involve suppression of the ET-1 signaling pathways, which may be exaggerated in insulin-resistant diabetes independently of the reduction in blood pressure.

MATERIALS AND METHODS

Animals and drug treatment. 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 (Tokushima, Japan) and maintained under constant temperature and lighting conditions with free access to food and water. At 8 wk of age, male OLETF rats were randomly divided into two groups. Only one group of 12 animals was given daily benidipine (3 mg/kg po) in 0.3% carboxymethylcellulose solution. This treatment period was 12 wk, and the animals were used at 20 wk of age, a time when they were in the early stage of Type 2 diabetes with modest hyperglycemia and prominent insulin resistance (see RESULTS). Twelve male Long-Evans Tokushima Otsuka (LETO)
rats, which developed from the same colony by selective mating but did not develop diabetes, served as controls. On the day of the experiments, rats were euthanized under gaseous ether anesthesia. The hearts were rapidly excised, and the whole heart and LV weights were measured. Some portion of the LV was dipped into optimum cutting temperature (OCT) compound (Sakura Finetech, Tokyo, Japan) and immediately frozen in liquid nitrogen. The remaining portion was preserved at −80°C without OCT compound.

Histopathology examination. For histopathological analysis, the tissues were fixed in 4% formalin solution, dehydrated, embedded in paraffin, and then sliced into 5-μm-thick sections. After being deparaffinized, in addition to the routine hematoxylin and eosin staining, the sections were stained for collagen using van Geison’s trichrome method.

Enzyme immunoassay for ET-1. Concentrations of ET-1 in sera and tissue extracts were determined using an ET-1 Enzyme Immunoassay Kit (Immuno-Biological, Fujioka, Japan). The reported cross-reactivity of the antibody for the former was <0.1% for all Big ETs, <0.1% for ET-3, and 3.3% for ET-2. The assay procedure was carried out according to the manufacturer’s instructions.

Western blot analysis. Immunoblotting was performed as described in our previous report (35). Samples of tissue homogenate were subjected to electrophoresis on polyacrylamide gels, and proteins were transferred to polyvinylidene difluoride filter membrane. After being blocked with 5% nonfat milk in phosphate-buffered saline, the membranes were incubated with specific antibody recognizing ET receptor, ETA receptor, ETB receptor, p38MAPK, JNK, or phospho-JNK. For the recognition of p38MAPK, JNK, and phospho-JNK, we used the following commercially available antibodies: rabbit anti-p38 MAPK polyclonal antibody (Stressgen, Victoria, Canada), rabbit anti-JNK polyclonal IgG (Upstate Biotechnology, Lake Placid, NY), and rabbit anti-phospho-JNK polyclonal antibody (Cell Signaling Technology, Beverly, MA). After being extensively washed 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-rabbit IgG (Jackson Immunoresearch, West Grove, PA) 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).

Statistical analysis. Values are means ± SE. Statistical assessment of the data was made by one-way analysis of variance with multiple comparisons by Fisher’s protected least significant difference test. Nonparametric data were analyzed by the Mann-Whitney’s t-test or Wilcoxon’s signed rank test. A P value <0.05 was considered statistically significant.

RESULTS

Characteristics of experimental animals. The general features of OLETF rats at 20 wk of age have been well described in our group’s previous report (25), along with those of age-matched LETO rats. In brief, after 24 h of fasting, OLETF rats were modestly hyperglycemic (197 ± 10 vs. 164 ± 4 mg/dl, n = 12 animals for each, P < 0.01), and their plasma insulin level was much higher than in LETO rats (4.31 ± 0.07 vs. 0.47 mg/dl, P < 0.01). Treatment of OLETF rats with benidipine for 8 wk altered neither hyperglycemia (201 ± 6 mg/dl, n = 12 animals) nor hyperinsulinemia (4.34 ± 0.41 mg/dl). Heart and LV weights were 1.4-fold higher in OLETF than in LETO rats. Benidipine treatment had no effect on the increases in heart and LV weights. Systolic blood pressure of OLETF rats was slightly but significantly higher than that of

Table 1. Primers and conditions for real-time quantitative PCR analyses

<table>
<thead>
<tr>
<th>Primers (5’-3’)</th>
<th>Gene</th>
<th>Sense</th>
<th>Antisense</th>
<th>Product Size, bp</th>
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<tbody>
<tr>
<td>Prepro-ET-1</td>
<td>ACGTTCGTTTTGCTGGATCC</td>
<td>GCTTGGACTGAGAACAGGAAA</td>
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<tr>
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<td>CCGTGGGATTAGGCTGAGGTCG</td>
<td>CCGTGGGATTAGGCTGAGGTCG</td>
<td>209</td>
<td></td>
</tr>
<tr>
<td>ECE</td>
<td>CACGATTGAGGACGAGAATC</td>
<td>CACGATTGAGGACGAGAATC</td>
<td>191</td>
<td></td>
</tr>
<tr>
<td>p38</td>
<td>CCAGATGGCCAGGAGATGAACT</td>
<td>CCAGATGGCCAGGAGATGAACT</td>
<td>202</td>
<td></td>
</tr>
</tbody>
</table>

ET-1, endothelin-1; ETα and ETβ, ET receptors A and B, respectively; ECE, ET-converting enzyme.

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Fig. 1. Photomicrographs of hematoxylin- and eosin-stained (A) and van Gieson’s stained (B) left ventricular (LV) sections from Long-Evans Tokushima Otsuka (LETO), Otsuka Long-Evans Tokushima Fatty (OLETF), and benidipine (Ben)-treated OLETF rats. Original magnification is ×400.

Fig. 2. Endothelin-1 (ET-1) levels in plasma (A) and LV tissues (B) and gene expression levels of prepro-ET-1 (C) and endothelin-converting enzyme (ECE; D) in LV tissues of LETO, OLETF, and Ben-treated OLETF rats. Plasma and tissue ET-1 levels were determined by ELISA. Expression of prepro-ET-1 mRNA and ECE mRNA was quantitatively evaluated by real-time PCR. Data are means ± SE; n = 12 animals. **P < 0.01 and ***P < 0.001, compared with corresponding values obtained in LETO rats; ##P < 0.01, compared with corresponding values in OLETF rats without any treatment.
LETO rats (140 ± 3 vs. 129 ± 3 mmHg, P < 0.001). Benidipine therapy lowered the blood pressure (132 ± 4 mmHg) in OLETF rats, but this effect was statistically insignificant.

**Manifestation of cardiac remodeling.** In Fig. 1A, a general histological architecture of LETO, OLETF, and benidipine-treated OLETF rat hearts is shown. OLETF rats showed significant cardiomyocyte hypertrophy, which was restored by benidipine treatment. Thus the values for the myocyte scan area were 521 ± 43, 626 ± 34, and 503 ± 35 μm² in LETO, OLETF, and benidipine-treated OLETF rats, respectively (P < 0.01). The van Gieson’s method of staining for collagen was used to assess the degree of perivascular fibrosis (Fig. 1B). The area of perivascular fibrosis in coronary arterioles, which was corrected for total vessel area, was much larger in OLETF than in LETO rats (74 ± 13% vs. 26 ± 3%, P < 0.01). The development of fibrosis in the perivascular area in OLETF rats was prevented by benidipine treatment (28 ± 4%, P < 0.01). The wall-to-lumen ratio of coronary arterioles in OLETF rats (2.8 ± 0.6, P < 0.01) was approximately threefold greater than that in LETO rats (1.0 ± 0.2). Benidipine treatment significantly reduced this ratio (1.3 ± 0.2, P < 0.01).

**Cardiac expression of ET-1 system.** As shown in Fig. 2A, the plasma ET-1 level, as determined by ELISA, was significantly elevated in OLETF compared with LETO rats. Furthermore, the immunoreactive ET-1 level in LV tissues was significantly higher in OLETF than in LETO rats (Fig. 2B). In LV sections from both LETO and OLETF rats, ET-1 was immunocytochemically demonstrable in coronary vessels and cardiomyocytes, but increased immunoreactivity was noted in the LV sections from OLETF rats (Fig. 3A). Benidipine treatment of OLETF rats reduced immunoreactive ET-1 in the plasma and tissues to the levels obtained in LETO rats (Figs. 2, A and B, and 3A). When gene expression of prepro-ET-1, a precursor of ET-1, and endothelin-converting enzyme (ECE), which specifically cleaves Big ET-1, was assessed by using real-time PCR, the levels of prepro-ET-1 mRNA and ECE mRNA in LV tissues were significantly greater in OLETF than in LETO rats (Fig. 2, C and D). The increases in their gene expression levels were completely blocked by benidipine treatment.

On Western blots, the ETₐ and ETₐ receptor levels were determined by measuring the density of a single band migrating at 48 and 49.5 kDa, respectively. As revealed by the cumulative data for quantitative immunoblotting (Fig. 4, A and B), both the ETₐ and ETₐ receptor protein levels in LV tissues were significantly higher in OLETF than in LETO rats. Immunofluorescence studies showed that positive staining for the ETₐ receptor was detected not only in coronary vessels but also in cardiomyocytes, whereas the ETₐ receptor was mainly localized to coronary vessels in nondiabetic LETO rats (Fig. 3, B and C). In LV sections from OLETF rats, however, diffuse and strong immunoreactivity of both the ETₐ and ETₐ receptors was observed. In OLETF rats treated with benidipine, the expression levels and patterns of the two ET receptors were returned to those levels seen in LETO rats (Figs. 3, B and C, and 4, A an B). The increases in the ETₐ and ETₐ receptor proteins in LV tissues of OLETF rats correlated with increases in their mRNAs, which were obtained from real-time PCR (Fig. 4, C and D). Benidipine treatment of OLETF rats reduced their gene expression levels to those of nondiabetic controls.

The expression level of JNK, a subfamily of MAPK that ET-1 can activate in smooth muscle cells (13) and in cardiomyocytes (9), was significantly enhanced in LV tissues of OLETF rats compared with those of LETO rats (Fig. 5A). The increase in ET-1 activity was prevented by benidipine at the plasma and tissue levels. ET-1 immunoreactivity was also present in the myocardium of LETO rats (Fig. 1B). In OLETF rats, ET-1 was present in the vessel wall and the myocardium, and in OLETF rats treated with benidipine, ET-1 immunoreactivity was reduced to levels similar to those in LETO rats (Fig. 4A). The ET-1 receptor (ET₁) and ET-2 receptor (ET₂) are expressed in smooth muscle cells, cardiomyocytes, and endothelial cells. ET-1 can activate both ET₁ and ET₂ receptors in smooth muscle cells and cardiomyocytes. ET-1 stimulates the expression of ET₁ and ET₂ receptors, which can be blocked by benidipine treatment. Benidipine treatment of OLETF rats reduced their gene expression levels to those of nondiabetic controls.
results of Western blot analysis also revealed that LV expression of JNK dually phosphorylated at threonine-183 and tyrosine-185 was increased in OLETF rats (Fig. 5B). The increases in LV expression of JNK and phospho-JNK seen in OLETF rats were reversed by benidipine treatment. As shown in Fig. 5C, Western blot analysis showed a significant increase in the expression of p38MAPK, which is also involved in the signal transduction pathway associated with ET-1 stimulation in cardiomyocytes (10), in LV tissues of OLETF rats. The increase in p38MAPK transcripts in OLETF rat LV tissues was quantitatively confirmed by analysis using the real-time PCR method (Fig. 5D). The increases in protein and mRNA expression levels of p38MAPK were blocked by benidipine treatment.

DISCUSSION

In the present study, we used the OLETF strain of rats, which has been established as an animal model of congenital diabetes by selective mating (28). This strain has an early manifestation of glucose metabolic disorders. Our group’s previous work revealed that OLETF rats exhibited significant insulin resistance and impaired glucose tolerance with hyperglycemia at 20 wk of age. At this age, OLETF rats were found to show modest hyperglycemia and prominent hyperinsulinemia after 24 h of fasting. Thus our subjects were in the stage of insulin resistance of Type 2 diabetes seen early in this strain.

Plasma ET-1 level was significantly higher in OLETF than in age-matched nondiabetic LETO rats. In addition, a significant increase in ET-1 levels was found in LV tissues of OLETF rats. This was further confirmed by our immunofluorescent study showing strongly increased ET-1 labeling of coronary vessels and cardiomyocytes in LV sections from OLETF rats. Possible reasons for increased ET-1 levels may include hyperinsulinemia seen in our model, because several studies in insulin-resistant humans have reported increased ET-1 serum concentrations that directly correlated with the levels of hyperinsulinemia (15, 42). It also should be noted that insulin stimulates ET-1 production in endothelial cells in vitro (40). ET-1 is produced primarily in endothelial cells by gene activation of prepro-ET-1 and subsequent processing by proteolytic enzymes into the biologically active peptide (18). The increase in ET-1 peptide level in LV tissues of rats with heart failure has been shown to originate from upregulation of prepro-ET-1 mRNA (31). We found that gene expression of prepro-ET-1 and ECE was upregulated in LV tissues of OLETF rats. The increase in prepro-ET-1 expression may be associated with an increase in expression of ETA and ETB.
receptor mRNA. This has been suggested by the previous report (43) showing coordinated increases in prepro-ET-1 and ETA and ETB receptor mRNA levels in LV tissues of rats with heart failure induced by coronary artery ligation. In this study, expression of ETA and ETB receptors was evidently upregulated in LV tissues of OLETF rats at both gene and protein levels. These results suggest that cardiac ET-1 system is greatly accelerated in this animal model at the early insulin-resistant stage of diabetes.

We also found that the two MAPKs, JNK and p38MAPK, were more abundantly expressed in LV tissues of OLETF than in those of LETO rats. JNK is activated by its dual phosphorylation motif, which results in phosphorylating serine residues in the NH2-terminal region of c-jun, thereby increasing in transcriptional activating activity of c-jun (44). Our finding that LV expression of phospho-JNK was significantly higher in OLETF than in LETO rats indicates an increase in activated cardiac ET-1 in insulin-resistant diabetes. ET-1 can activate the JNK pathway in smooth muscle cells and cardiomyocytes (9, 13). The existence of the signaling pathway linking the ET<sub>A</sub> receptor to JNK has been documented (36). ET-1 has been also shown to activate p38MAPK via ET<sub>A</sub> receptors in rat myocardial cells (56). However, studies of COS cells transfected with ET<sub>B</sub> receptors have indicated that this receptor subtype can stimulate activation of JNK and p38MAPK (3). Thus it remains probable that both MAPKs may be necessary for the signal transduction pathways associated with activation of each ET receptor subtype, but upregulation of the two MAPK signaling pathways could lead to further enhancement of ET-1-induced biological responses of the cell. Interestingly, both JNK and p38MAPK have been implicated in hypertrophy of cardiomyocytes, although the exact roles of each of these pathways have yet to be determined (47, 59).

ET-1 is powerfully hypertrophic in cardiac myocytes (23, 49). ET-1 also activates the procollagen I promoter (8) and collagen synthesis in fibroblasts (19); it can modulate collagenase activity, although it is unclear which ET receptor subtype mediates these effects. Moreover, ET-1 potently stimulates, via ET<sub>B</sub> receptors, aldosterone release (4) and therefore might increase collagen synthesis via enhanced aldosterone secretion. In this study, our histopathology showed evident cardiomyocyte hypertrophy and prominent perivascular fibrosis in the
heart of OLETF rats. Furthermore, our group (25) previously demonstrated a significant increase in cardiac collagen deposition in this animal model. We thus suggest that the profound histopathological changes in the heart (referred to as cardiac remodeling), found in OLETF rats, partly resulted from overproduction of ET-1 and overexpression of ET\(_A\) and ET\(_B\) receptors and their signal transduction pathways. Our group’s (25, 26) previous studies showed that transforming growth factor-\(\beta_1\) (TGF-\(\beta_1\)) was highly expressed in the OLETF rat heart at both protein and mRNA levels. TGF-\(\beta_1\) is known to be a powerful stimulant of cardiomyocyte hypertrophy (48) and collagen synthesis in the heart (5). This implies that superinduction of TGF-\(\beta_1\) transcription could be causally involved in the development of cardiac remodeling at the early insulin-resistant stage of diabetes seen in this study. ET-1 has been reported to stimulate production of TGF-\(\beta_1\) in rat liver stellate cells (17). Furthermore, ET\(_A\) receptor antagonism can prevent the elevation of cardiac TGF-\(\beta_1\) mRNA and protein levels in deoxycorticosterone acetate-salt hypertensive rats (2). We thus assume that TGF-\(\beta_1\) may play an adjuvant role in the ET-1-related cardiac remodeling process in OLETF rats.

The key finding in this study is the demonstration that treatment of OLETF rats with benidipine, a long-acting CCB, at the subdepressor dose greatly inhibited the increased plasma and tissue levels of ET-1 and suppressed overexpression of prepro-ET-1, ECE, ET\(_A\), and ET\(_B\) receptors, and two ET-1 signal transduction pathways (JNK and p38MAPK) without affecting the plasma glucose and insulin concentrations. The ability of diltiazem and verapamil to decrease ET release from cultured coronary smooth muscle and endothelial cells has been previously demonstrated (20). Furthermore, accumulating evidence suggests that CCB therapy can result in a marked inhibition of overexpression of ET-1 genes in different rat models of hypertension, and this effect plays an important role in the prevention of cardiac remodeling in hypertensive rats (14, 31, 33). Benidipine treatment strongly prevented cardiomyocyte hypertrophy and perivascular fibrosis in the OLETF rat heart. This amelioration may be, at least in part, attributed to reductions in the expression levels of ET-1 and its related molecules in the heart. Potential mechanisms for the regulation of ET-1 gene by CCBs may be considered on the basis of the existence of two putative binding sites identified in the promoter sequence of this gene (41). Moreover, the calcium influx through L-type calcium channels can trigger c-fos activation, and, therefore, calcium channel blockers may modulate indirectly the interaction of the product of this immediate early gene with the activator protein-1 (AP-1) binding site present in the promoter sequence of ET-1 gene (14, 39).

The blood pressure in OLETF rats was modestly reduced, although this reduction was statistically insignificant. The increases in ET-1 and ET\(_A\) receptor levels in the heart are suggested to be secondary to hypertension (50). Thus the effect of benidipine therapy to normalize the upregulated ET-1 system in OLETF rat hearts may be partly due to the modestly lowering action on blood pressure. Furthermore, we have previously found that angiotensin II is upregulated in coronary vessels of the OLETF rat heart, and the blockade of angiotensin II type 1 receptors with candesartan can greatly reverse diabetes-induced cardiac remodeling (24). In this regard, angiotensin II can induce ET-1 gene expression in rat aortic smooth muscle cells (21), and angiotensin-converting enzyme inhibition can decrease circulatory ET-1 levels in lean noninsulin-dependent diabetic patients (16). Thus the possible mutual cross talk between angiotensin II and ET-1 may exist in our diabetic rat model. Although not addressed in this work, whether angiotensin II type 1 receptor blockers and ET-1 receptor antagonists can prevent the upregulation of the ET-1 system and of angiotensin II in OLETF rat hearts, respectively, would well deserve further study. Finally, the question remains as to whether the beneficial effects of benidipine observed in OLETF rats resulted from its nonspecific actions in addition to its specific action on L-type calcium channels. Further study to examine whether other long-lasting CCBs can also exhibit the same effects on cardiac remodeling and upregulation of cardiac ET-1 system in OLETF rats, because benidipine may clarify this issue.

In conclusion, OLETF rats showed marked increases in local generation of ET-1 and expression of the ET-1 cellular signaling system (ET\(_A\) and ET\(_B\) receptors, JNK, and p38MAPK) in the heart at the insulin-resistant stage of Type 2 diabetes. This change in the ET-1 system was completely reversed by treatment with benidipine at the subdepressor dose. Furthermore, benidipine therapy ameliorated cardiac remodeling in OLETF rats. This amelioration may be partly due to the suppressive effect on increased expression of the ET-1 system in the OLETF rat heart. We thus represent the first study that provides a new understanding of the roles of the ET-1 system in the mechanisms for the blood pressure-independent cardioprotective effects of CCBs in insulin-resistant diabetes.

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BENIDIPINE ON THE ET SYSTEM IN DIABETES

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