Effects of Angiotensin Receptor Blocker on Oxidative Stress and Cardio-Renal Function in Streptozotocin-Induced Diabetic Rats

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It is becoming increasingly evident that cumulative oxidative stress has a very close relationship with the progression of many human diseases such as diabetes mellitus (DM), cardiovascular diseases, and cancer.1 It has also been shown that oxidative stress may play a role in the pathogenesis of diabetic complications such as cardiomyopathy and nephropathy.2,3 Hyperglycemia can induce increased production of reactive oxygen metabolites and species. Elevated glucose concentrations may also increase the levels of oxygen radicals-scavenging enzymes in cultured endothelial cells4 and the kidney of rats with streptozotocin (STZ)-induced diabetes.5 An imbalance between reactive oxygen species (ROS) generation and antioxidant capacity favoring the former leads to oxidative stress and oxidative damage. Most vascular ROS are produced by nicotinamide adenine dinucleotide phosphate (NAPDH) oxidase, a multisubunit enzyme that catalyzes O2− production. Activation of NADPH oxidase, auto-oxidation of glucose, and the formation of advanced glyca tion end products seem to be relevant to the elevated oxidative stress in diabetes. NADPH oxidase consists of membrane-associated subunits (gp91phox and p22phox) and cytosolic subunits (p47phox, p67phox, and Rac).6 Nox4 was identified initially as a kidney NADPH oxidase and recent studies have shown that Nox4 is also abundant in vascular cells, especially endothelial cells, and is implicated in vascular pathologies. An elevation in angiotensin II (Ang II) levels is a frequent occurrence in a diverse number of cardio- and reno-vascular diseases. An important effect of Ang II is the activation of NADPH oxidase, a major source of ROS production by vascular cells.7 Inhibition of the Ang II type I receptor (AT-1R) by angiotensin receptor blockers (ARB) in some studies of experimental animals showed that this ARB reduces activities of oxidases and completely prevents the increase in NADPH oxidase activity caused by Ang II.8–11 Oxidative stress also plays a key role in the development of hypertrophy in diabetic conditions. It has been studied extensively that transforming growth factor-β1 (TGF-β1) as a mediator of a hypertrophic and prosclerotic changes in diabetic diseases.12,13 Ang II may induce myocardial fibrosis and thickening of the vessel wall in hypertension by increasing the production of TGF-β1. The circulating levels of TGF-β1 are increased in patients with diabetic nephropathy and reduced progression of this complication during treatment with angiotensin-converting enzyme inhibitors (ACEI) is associated with reduced levels of TGF-β1.14 Although the understanding of how hyperglycemia-induced oxidative stress ultimately leads to tissue damage has advanced considerably in recent years,15 effective therapeutic strategies to prevent or delay the development of this damage remain limited.16 The present study investigated the effect of an ARB, losartan, on cardio-renal function, fibrosis, and oxidative stress-related factors such as protein expression of NADPH oxidase subunits p22phox and Nox4, and oxidative stress marker, superoxide dismutase (SOD) activity and malondialdehyde (MDA) level in the heart and kidney of STZ-diabetic rats.

Key words diabetes mellitus; oxidative stress; angiotensin receptor blocker; heart; kidney

MATERIALS AND METHODS

Materials Unless otherwise stated all reagents were analytical grade and were purchased from Sigma (Tokyo, Japan). Losartan was kindly donated by Banyu Pharmaceuticals, Tokyo, Japan.

Experimental Design Male Sprague-Dawley rats (8

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weeks old) weighing 250—280 g were purchased from Charles River Japan Inc. (Kanagawa, Japan). Diabetes was induced by a single intraperitoneal injection of STZ (55 mg/kg body weight; Sigma-Aldrich, Tokyo, Japan) diluted in citrate buffer, pH 4.5 (n = 12). Age-matched non-diabetic control rats (group N; n = 5) were each injected with an equal volume of citrate buffer. Animals' blood glucose levels were determined using Medi-safe chips (Terumo Inc., Tokyo, Japan). Rats were considered diabetic when the non-fasted blood glucose levels were ≥300 mg/dl. One week after the induction of DM, rats were divided randomly into two groups and treated over a period of 4 weeks treatment with losartan (group DL; n = 6) at a dose of 30 mg/kg/d, and a diabetic group (group D; n = 6) and a non-diabetic group (group N) received only the vehicle. Drug and vehicle were administered daily by gavage. On study day 27, individual rats were placed in metabolic cages to obtain 24-h urine collections for the measurement of urine creatinine and protein concentrations, and the body weight (BW) was measured. Throughout the study, all animals were cared for in accordance with the guidelines of our institute and Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Hemodynamic and Echocardiographic Study Rats were anesthetized with 2% halothane in O2 and subjected to surgical procedures to measure hemodynamic parameters on day 28. After instrumentation, the concentration of halothane was reduced to 0.5% to record steady state hemodynamic data. Hemodynamic parameters, such as mean blood pressure (MBP), peak left ventricular (LV) pressure (LVP), central venous pressure (CVP), LV end-diastolic pressure (LVEDP), and the rate of intra-ventricular pressure rise and decline (±dp/dt), were recorded as described previously.17 Two-dimensional echocardiographic studies were performed under 0.5% halothane anesthesia using an echocardiographic machine equipped with a 7.5-MHz transducer (SSD-5500; Aloka, Tokyo Japan). M-mode tracings were recorded from the epicardial surface of the right ventricle; the short axis view of the left ventricle was recorded to measure the LV dimension in diastole (LVDd) and LV dimension in systole (LVDs). LV fractional shortening (FS) and ejection fraction (EF) were calculated as diastolic dimensions and expressed as a percentage. The study was performed in a blinded manner.

Estimation of Biochemical Parameters Blood samples were collected in heparinized syringes by heart puncture immediately after echocardiographic measurements. The collected blood was utilized for the subsequent determination of creatinine and blood urea nitrogen (BUN) and was stored at −80°C. Urinary protein excretion was determined by the Bradford method.18 Serum and urinary creatinine levels were determined by the Jaffe method.19 The creatinine clearance rate (Ccr) was calculated and expressed as milliliters per minute. BUN was determined by the diacetylmonoxime method.20

Histopathological Analysis After the measurement of echocardiographic parameters, hearts and kidneys were excised and weighed immediately (HW and KW), and the heart and kidney weight to BW ratio (HW/BW and KW/BW) was calculated. Kidney tissue was decapsulated. Half of each heart and kidney was immediately snap-frozen in liquid nitrogen for subsequent protein extraction and enzymatic assays. The remaining excised hearts and kidneys were cut into about 2-mm transverse slices and fixed in 10% formalin. After being embedded in paraffin, several transverse sections were obtained from the ventricle and kidney and stained by Azan–Mallory staining. Using the specimens stained with Azan–Mallory at the middle level of the left ventricle, the area of fibrosis was quantified by a color image analyzer (CIA-102; Olympus, Tokyo, Japan), using the differences in color (blue fibrotic area opposed to red myocardium or kidney) of the photomicrographs of Azan–Mallory stained slides. The results are presented as the ratio of fibrotic area to the whole area of myocardium or kidney.17

Western Blotting LV and whole kidney tissues were prepared from rats treated as described above for 28 d and age-matched untreated normal control rats. For the determination of protein levels of TGF-β1, AT-1R, and NADPH oxidase subunits p22phox and Nox4, equal amounts of protein extracts (30 μg) were separated by 12.5% (for TGF-β1 and p22phox) and 10% (AT-1R and Nox4) sodium dodecyl sulfate polyacrylamide gel electrophoresis (Bio-Rad, CA, U.S.A.) and transferred electrophoretically to nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline TWEEN (20 mM Tris, pH 7.6, 137 mM NaCl, and 0.1% Tween 20). All antibodies were purchased from Santa Cruz Biotechnology Inc. (CA, U.S.A.) apart from anti-TGF-β1 (Promega Corporation, Madison, WI, U.S.A.) and used at a dilution of 1 : 1000. After overnight incubation in 4°C with primary antibody, the bound antibody was visualized using the respective horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology Inc.) and chemiluminescence developing agents (Amersham Biosciences, Buckinghamshire, U.K.). The level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was estimated in every sample. Films were scanned, and band densities were quantified with densitometric analysis using Scion Image program (Epson GT-X700, Tokyo, Japan). Finally, western blotting data were normalized by those for cardiac and renal GAPDH.

Measurement of SOD Activity and MDA Content Heart and renal tissues were rinsed, weighed, resuspended at 50 mg/ml in normal saline, and homogenized. After centrifugation at 3000 r/min for 10 min at 4°C, the supernatants were collected and analyzed with corresponding assay kits (R&D Systems Inc., Minneapolis, U.S.A. for SOD and Oxitex, ZeptoMetrix Corporation, New York, U.S.A. for MDA) according to manufacturer’s instructions.

Statistical Analysis Data were presented as mean±S.E. and were analyzed using one-way analysis of variance (ANOVA) followed by Tukey or Bonferroni methods for post hoc analysis and two-tailed t-test when appropriate. A value of p<0.05 was considered statistically significant. For statistical analysis GraphPad Prism 5 software (San Diego, CA, U.S.A.) was used.

RESULTS

Effects of Losartan on Myocardial Functions Although heart rate (HR), CVP, and LVP were not different among the three groups of rats, LVEDP was significantly higher and MBP, LVP, and ±dp/dt were significantly lower in
group D in comparison with group N, indicating LV dysfunction in vehicle-treated rats. Losartan treatment reduced LVEDP significantly in comparison with group D (9.24 ± 0.6 vs. 5.4 ± 0.5 mmHg, p < 0.05, Table 1). Echocardiographic data revealed that both LVDd and LVDs were increased significantly in group D compared with group N. In addition, LV systolic function, as assessed by FS and EF, were also reduced in group D compared with those in group N (Table 1). Losartan treatment improved the LV systolic function by increasing both FS and EF significantly compared with those in group D (FS; 30 ± 0.5 vs. 44 ± 1.4%, EF; 62 ± 3.8 vs. 80 ± 1.4%, p < 0.05).

Histopathology No changes were found in HW/BW in group D or group DL compared with group N. KW/BW in group D was significantly larger than and group N; however, losartan treatment did not reduce this parameter (Table 1). Hearts and kidneys from group D rats showed massive fibrosis compared with those from group N rats (Figs. 1A, B). The percentage areas of cardiac and renal fibrosis were significantly lower in losartan-treated rats compared to vehicle-treated rats (Figs. 1C, D).

Assessment of Biochemical Parameters Blood glucose levels and urine volumes were significantly higher in group D and group DL relative to that in group N (Table 1). Urinary protein concentration and BUN were significantly elevated in group D compared with group N. Losartan treatment significantly reduced the urinary protein concentration but not BUN levels (Figs. 2A, B). In addition, Ccr (measured from 24-h urine collections) was similar in all three groups (Fig. 2C).

Effects of Losartan on the Levels of SOD and MDA in Heart and Renal Tissues Significant increases in MDA level and decreases in SOD activity in the heart and kidney tissues were found in group D compared with group N. Treatment with losartan significantly decreased MDA levels (heart; 0.56 ± 0.8 vs. 0.08 ± 0.02 nmol/mg tissue, kidney; 0.8 ± 0.05 vs. 0.07 nmol/mg tissue, kidney).

<table>
<thead>
<tr>
<th>Blood glucose (mg/dl)</th>
<th>Day 28</th>
<th>Group N</th>
<th>Group D</th>
<th>Group DL</th>
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<tbody>
<tr>
<td>172 ± 44</td>
<td>359 ± 6.0*</td>
<td>548 ± 36*</td>
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</table>

<table>
<thead>
<tr>
<th>Urine volume (ml/kg/BW/24 h)</th>
<th>Day 28</th>
<th>Group N</th>
<th>Group D</th>
<th>Group DL</th>
</tr>
</thead>
<tbody>
<tr>
<td>896 ± 57.5*</td>
<td>1052 ± 67.5*</td>
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</tbody>
</table>

Results are presented as the mean ± SE. BW, body weight; HW, heart weight; HW/BW, ratio of heart weight to body weight; KW, kidney weight; KW/BW, ratio of kidney weight to body weight; CVP, central venous pressure; MBP, mean blood pressure; LVP, left ventricular pressure; LVEDP, left ventricular end-diastolic pressure; +dp/dt, rate of intra-ventricular pressure rise and decline; HR, heart rate; LVDd, left ventricular dimension in diastole; LVDs, left ventricular dimension in systole; LVPWd, left ventricular posterior wall diameter; FS, fractional shortening; EF, ejection fraction; group N, age-matched non-diabetic rats; group D, diabetic rats treated with vehicle; group DL, diabetic rats treated with losartan (30 mg/kg/d); #p < 0.05 vs. group N and *p < 0.05 vs. group.

Table 1. Changes in Histopathological, Hemodynamic, Echocardiographic and Biochemical Parameters after 4 Weeks of Treatment with Losartan in Diabetic Rats Induced by Streptozotocin

Fig. 1. Azan–Mallory Staining for Fibrosis of Cross-Sectional Tissue Slices (A) Hearts; Magnification ×100, (B) Kidneys; Magnification, ×200 and Quantitative Analysis of Fibrosis in (C) Heart and (D) Kidney

Fibrosis is indicated by the blue area as opposed to the red area. Each bar represents mean ± S.E. *p < 0.05 vs. group N; #p < 0.05 vs. group DL. Group N, age-matched non-diabetic rats; Group D, diabetic rats treated with vehicle; Group DL, diabetic rats treated with losartan (30 mg/kg/d).
0.52 ± 0.09 vs. 0.29 ± 0.03 nmol/mg tissue, \( p < 0.05 \) and slightly increased SOD activity (heart; 1.75 ± 0.6 vs. 2.21 ± 0.2 U/mg protein, kidney; 1.6 ± 0.2 vs. 1.95 ± 0.3 U/mg protein) in the heart and kidney tissues (Figs. 3A—D).

**DISCUSSION**

The present study examined the effect of the ARB losartan on oxidative stress and cardio-renal function in diabetic rats induced by STZ. The present data demonstrated that losartan (30 mg/kg/d).
treatment improved myocardial function and ameliorated proteinuria. It was also demonstrated that losartan treatment down-regulated TGF-β1 expression in myocardial and renal tissues. Treatment with losartan also attenuated the increased expression of NADPH oxidase subunits p22phox and Nox4 in the heart and kidney of STZ-induced diabetic rats.

Numerous reports have demonstrated that oxidative stress induced by diabetes plays an important role in the development and progression of diabetic vascular complications including cardiomyopathy and nephropathy. Indeed, there is emerging evidence that the formation of ROS is a direct consequence of hyperglycemia. Biomarkers for oxidative damage to DNA, lipids, and proteins also supported the concept of increased oxidative stress in diabetes.21,22) In the present study, there were a significant increase in MDA level and decrease in SOD activity both in heart and kidney tissue, which were consistent with other reports.23) These results showed that hyperglycemia caused an imbalance between the production of free radicals and the antioxidant defense system in diabetic conditions. Strategies to reduce oxidative stress in diabetes mellitus may exert favorable effects on the progression of diabetes.

Hyperglycemia activates the local renin-angiotensin system, resulting in the formation of Ang II,24) and it has been shown in both clinical and experimental studies that Ang II induces oxidative damage by producing ROS through the nicotinamide adenine dinucleotide (NADH)/NADPH oxidase system.25) Most importantly, a number of clinical studies have showed that blockade of Ang II with either ACEI or ARB can prevent or delay the progression of diabetic conditions. Treatment with either an ACEI or an ARB resulted in lower levels of vascular superoxide anions.25–27) Consistent with previous reports, in the present study, expression of AT-1R increased after STZ injection both in cardiac and renal tissue, and losartan treatment attenuated the elevated expression of AT-1R as well as the reduction in oxidative stress level in these two organs, especially by decreased MDA levels, whereas for SOD activity, losartan treatment could increase the activity but the results were not significant.

DM causes myocardial structural remodeling characterized by myocyte hypertrophy and apoptosis as well as interstitial fibrosis,28) which increases cardiac muscle stiffness and may contribute to impaired diastolic function. In the present study, losartan treatment improved myocardial function by increasing both EF and FS, reducing LVEDP, and attenuating the massive fibrosis. Tsutsui et al. (2007) reported that candesartan, another ARB, also improved LV diastolic function and attenuated myocyte hypertrophy in diabetic mice.29)

TGF-β1 is known to be up-regulated in diabetic kidney, which contributes to the proliferation of mesangial cells and extracellular matrix (ECM) production, which are the major pathological changes in early diabetic nephropathy.24) TGF-β1 induced plasminogen activator inhibitor type-1 (PAI-1) expression could decrease ECM degradation and contribute to the fibrotic process. Both TGF-β1 and oxidative stress can induce over expression of PAI-1 in the glomeruli, leading to renal fibrosis. Previous study also showed that losartan could suppress the expression of TGF-β1 in the kidney of diabetic rats and attenuate the renal lesions caused by diabetic rats.30) TGF-β1 is also up-regulated in failing human hearts and various experimental models of cardiac hypertrophy and fibrosis, and elevated oxidative stress plays a critical role in the development and progression of cardiac remodeling associated with heart failure.31) The present study also found up-regulation of TGF-β1 in diabetic rats both in heart and renal tissues. In this study, losartan decreased oxidative stress levels in these tissues and lessened the up-regulation of TGF-β1. All of these might contribute to the improvement of renal function and cardiac function, as shown by the decrease in urinary protein levels and improved LV systolic function by increased FS and EF and improved LV diastolic function by decreased LVEDP. In addition, the area of fibrosis in both myocardium and kidney cortex also decreased by the treatment.

In this study, it was shown that the expression of essential subunits of NADPH oxidase, Nox4 and p22phox, was increased both in heart and kidney of diabetic rats, and these increases were normalized by losartan treatment. Although we did not measure the level of mRNA expression, these levels might be paralleled with their protein expressions.32–35) Nox4 was initially identified as a kidney NADPH oxidase that might be involved in oxygen sensing and cellular senescence.36) Recent studies have shown that Nox4 is also abundant in vascular cells, especially endothelial cells,37) and implicated in vascular pathologies. Cucuraru et al. (2005) reported that Nox4 may play a critical role in the pathologic activation of cardiac fibroblasts in cardiac fibrosis associated with human heart failure.31) Induction of Nox4 in LV tissue was also reported in chronic pressure overload model in mice.39) At the nucleus of human umbilical vein endothelial cells, Nox4 is likely to form a functional complex with p22phox and constitutively produces superoxide, thereby regulating gene expression via a mechanism for oxidative stress response.39) Cooperation between Nox4 and p22phox is also suggested by a finding that Nox4 and p22phox are increased concomitantly at the protein level in kidney of diabetic rats.35)

Gorin et al. (2005) reported that NADPH oxidase Nox4 protein expression was increased in diabetic kidney cortex compared with non-diabetic controls and was down-regulated in phosphorothioated antisense oligonucleotides for Nox4, indicating that Nox4 is the major source of ROS in the kidneys during the early stages of diabetes.40) Consistent with the other reports, the present study also showed that the activity of NADPH oxidase, in parallel with the levels of its constituent proteins (Nox4 and p22phox) are increased in heart and kidney in an animal model of diabetes, and these results suggested that NADPH oxidase might play a crucial role in the development of diabetic vascular complications. Another study using NADPH oxidase inhibitor, apocynin, showed that blockade of NADPH oxidase in diabetic rats prevents the membrane translocation of p47phox and the increased expression of gp91phox in the kidney, and prevents oxidative stress, proteinuria, and glomerulopathy, while enhancing NO generation.41)

This 4-week ARB treatment did not cause significant pathological changes in the kidneys of diabetic rats. This result was inconsistent with another report that chronic treatment of another ARB, irbesartan, in the same animal model attenuated renal dysfunction by increased creatinine and urea clearance along with reduced albumin excretion rate as compared with untreated diabetic rats.26) This might be because
in the present study ARB treatment was only performed for 4 weeks. However, losartan treatment resulted in a significant reduction of proteinuria that confirmed the present results. It is possible that this effect was the result of improvement of endothelial function. Recently, the usefulness of microalbuminuria in predicting increased risk of cardiovascular and renal diseases has become well established in diabetic patients, as well as in essential hypertensive patients and in the general population. Thus, the reduction of microalbuminuria could be a relevant therapeutic strategy for reducing or preventing cardiovascular events in patients with diabetes.

In conclusion, the present study provided evidence that fibrosis and oxidative stress occur in vivo in the heart and kidney in diabetic rats, and that ARB treatment could improve cardio-renal function by attenuating fibrosis and reversing oxidative stress, suggesting the beneficial effect of ARB treatment in the prevention of development and progression of diabetic disease.

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