Changes of endothelin in streptozotocin-induced diabetic rats: effects of an angiotensin converting enzyme inhibitor, enalapril maleate

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Abstract

Endothelin-1 (ET-1) concentrations are increased in patients with diabetes mellitus, particularly those with diabetic retinopathy, or essential hypertension. We hypothesized that ET-1 might participate in the development and progression of diabetic microangiopathy. In this study, the effects of the angiotensin converting enzyme (ACE) inhibitor, enalapril maleate, on diabetic angiopathy were examined in streptozotocin (STZ)-induced diabetic (STZ-DM) rats by monitoring variations in renal function and ET-1 concentrations in blood and organ tissues.

Significant increases in kidney weight and in concentrations of urinary albumin, N-acetyl-D-glucosaminidase (NAG) and serum ET-1 were observed in the STZ-DM rats as compared with the non-diabetic rats, and the concentration of ET-1 in the kidneys tended to be increased. Microscopic and electron microscopic analyses showed increased mesangial cell proliferation, matrix expansion and enlarged mesangial area in the kidney of the diabetic rats. After administration of the ACE inhibitor, increased concentrations of urinary albumin and NAG in the STZ-DM rats were reduced to the control values with a slight improvement in the electron microscopic changes.

These data suggest that ET-1 may be involved in the development and progression of diabetic nephropathy and may explain, in part, why diabetes is liable to complicate hypertension. ACE inhibitor may help to restore diabetic nephropathy in the STZ-induced diabetic rats.

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Introduction

Diabetic vascular complications are important factors which influence the prognosis of diabetic patients. The development and progression of vascular endothelial cell damage is the basis of diabetic microangiopathy. In addition, hypertension may potentiate diabetic vascular complications.

Endothelin-1 (ET-1), a potent vasoconstricting factor that causes proliferation of vascular smooth muscle cells (Itoh et al. 1988, Yanagisawa et al. 1988, Inoue et al. 1989), is a peptide produced and secreted from vascular endothelial cells. It has been reported that ET-1 concentrations are increased in the sera of diabetic patients (Takahashi et al. 1990), particularly those with diabetic retinopathy (Kawamura et al. 1993), and that in diabetic rats endothelin A receptor antagonist and angiotensin converting enzyme (ACE) inhibitor could prevent the diabetic microangiopathy (Cooper et al. 1994, Dhein et al. 2000). This suggests that ET-1 may participate in the development and progression of diabetic microangiopathy. Serum concentrations of ET-1, which has a potent vasoconstrictive action, are also increased in essential hypertension (Saito et al. 1990, Naruse et al. 1991).

Therefore, we have studied the involvement of ET-1 in the development and progression of diabetic vascular complications, especially diabetic nephropathy, the effects of an ACE inhibitor on renal function, and the ET-1 concentrations in sera and various organ tissues of streptozotocin–induced diabetic rats.

Materials and Methods

Preparation of diabetic rats

Diabetic rats were prepared by intraperitoneal administration of streptozotocin (STZ) at 60 mg/kg body weight to 10-week-old male Wistar rats (STZ-induced diabetic rats, STZ-DM). Those with a fasting blood glucose level greater than 200 mg/dl in the early morning were used for the experiments.
Treatment with ACE inhibitor

The STZ-DM rats were kept under diabetic conditions for 4 weeks, after which 5 mg/kg body weight of ACE inhibitor enalapril maleate (provided by Merck-Banyu Pharmaceutical Co., Ltd, Tokyo, Japan) dissolved in a 5% glucose solution was given orally, daily, for 4 weeks.

Methods of collecting blood samples and organs

Each blood sample was collected using cardiocentesis under anesthesia during fasting. The blood sample was then immediately centrifuged to obtain the serum which was stored at −20°C until assayed. At the same time, the removed organs (brain, heart, kidney and liver) were also immediately frozen and stored at −80°C.

Measurements

The animals were given regular laboratory chow and were randomly divided into the following four groups (8 rats in each group) and then treated as described in the parentheses: 1, control group (treated with saline instead of STZ, non-diabetic rats); 2, diabetic group (STZ-induced DM rats treated with saline instead of the ACE inhibitor); 3, ACE inhibitor-treated control group (non-diabetic rats treated with the ACE inhibitor); and 4, ACE inhibitor-treated diabetic group (STZ-induced DM rats treated with the ACE inhibitor).

The systolic and diastolic blood pressures (BP, mmHg) measured by noninvasive tail cuff plethysmography before death were 149 ± 3 (mean ± s.d., systolic) and 100 ± 7 (mean ± s.d., diastolic) in the control group, 134 ± 18 and 83 ± 20 respectively in the diabetic group, 113 ± 8 and 61 ± 16 respectively in the ACE inhibitor-treated control group, and 122 ± 4 and 73 ± 13 respectively in the ACE inhibitor-treated diabetic group.

Regarding the systolic BP, the difference between the ACE inhibitor-treated control group and the ACE inhibitor-treated diabetic group was statistically significant (P=0.04), while the others showed no significant difference.

Regarding the diastolic BP, a statistically significant difference was observed between the control group and the ACE inhibitor-treated diabetic group (P=0.01), while the others showed no significant difference.

The following data were obtained from each rat: body weight, weights of brain, heart, kidney and liver, fasting blood glucose (FBG) level measured by Glutest senser (Sanwakagaku Laboratory, Nagoya, Japan), serum creatinine level (Perrone et al. 1992), amounts of urinary albumin (measured by enzyme immunoassay (Fielding et al. 1983)) and N-acetyl-fl-L-glucosamidase (NAG) (measured by the method reported previously by Noto et al. (1983)), and ET-1 concentrations in sera and tissues, brain, heart, kidney and liver. In addition, histological examination was performed using electron microscopy for each specimen. Serum and urinary enalapril maleate (ACE inhibitor) levels were measured by the method reported previously (Ulm et al. 1982).

Assay for ET-1

Serum ET-1 concentrations were measured using an RIA (Ando et al. 1989). The ET-1 concentrations in the respective organ tissues were measured using the following method. After homogenization with 1·0 M acetic acid containing peptatin (10 µg/ml) for 1 min, the homogenates were heated in boiling water for 10 min, and centrifuged at 55 000 g for 40 min at 4°C to obtain the supernatant. From the supernatant, the ET-1 was extracted with a Sep-pak C-8 column (Ando et al. 1989) and measured using an RIA kit (Sumikin Bioscience, Sagamihara, Japan). Intra- and interassay coefficients of variation were 10·0 and 12·4%. The recovery of ET-1 in this assay system was 97%.

Statistical analysis

Data are expressed as mean ± s.d. (standard deviation). Significant differences between the groups were determined by Kruskal–Wallis test or two-factor factorial ANOVA and/or repeated measures ANOVA, and differences were considered significant at P<0·05.

All experiments were performed in accordance with the guidelines on the handling of laboratory animals of our institution.

Results

Body weights

Body weights of the control group (non-diabetic rats) were increased at 4 weeks and at 8 weeks after the saline treatment, whereas those of the diabetic and the ACE inhibitor-treated diabetic group were significantly decreased after the STZ treatment in contrast to those of the non-diabetic groups (Table 1).

Organ weights (brain, heart, kidney and liver)

The weights of the kidneys in the diabetic group and the ACE inhibitor-treated diabetic group were significantly increased compared with those of the non-diabetic controls. However, there was no significant difference in the weights between the diabetic group and the ACE inhibitor-treated diabetic group (Table 1). The weights of brain, heart and liver were similar in the four groups (data not shown).

Fasting blood glucose (FBG) and serum creatinine levels

The FBG level was measured in blood samples drawn from the tail vein of each rat in the early morning after
overnight fasting. FBG levels in the diabetic group and the ACE inhibitor-treated diabetic group were significantly increased, but there was no significant difference between the two diabetic groups (Table 1). Serum creatinine levels in each group varied from 0·8 to 1·4 mg/dl respectively and they showed no statistically significant changes (data not shown).

NAG concentrations

The amount of urinary NAG was significantly \( P=0.04 \) increased in the diabetic group compared with the control group and this increase was restored to normal by treatment with the ACE inhibitor. Urinary NAG levels were: control (non-diabetic) group 10·1 U/l, diabetic group 25·7 ± 19·5 U/l, ACE-inhibitor-treated control group 9·6 ± 5·8 U/l and ACE-inhibitor-treated diabetic group 10·5 ± 8·7 U/l.

Urinary albumin levels

The concentration of urinary albumin was also increased significantly \( P=0.04 \) in the diabetic group compared with the control group. The treatment of the STZ-DM rats with the ACE inhibitor for 4 weeks partly restored albumin excretion. Urinary albumin levels were: control group \( (2·2 ± 1·2) \times 10^{-2} \) mmol/l, diabetic group \( (9·6 ± 6·0) \times 10^{-2} \) mmol/l, ACE inhibitor-treated control group \( (2·0 ± 1·7) \times 10^{-2} \) mmol/l and ACE inhibitor-treated diabetic group \( (5·4 ± 4·7) \times 10^{-2} \) mmol/l.

Serum ET-1 concentrations

There was a significant increase in the serum ET-1 concentrations in the diabetic group as compared with controls, and treatment with the ACE inhibitor significantly reduced the ET-1 serum levels (Table 2).

ET-1 concentrations in organ tissues

The ET-1 concentrations in the whole kidneys of the diabetic group increased significantly as compared with the non-diabetic rats (Table 2). The ET-1 contents in the kidneys were compared as a function of the amounts of protein and DNA in the kidney tissues. There were no differences in the amounts of protein and DNA between the rats of each group. The ET-1 concentrations revised by protein or DNA concentrations tended to increase in the diabetic group as compared with that in the control group, but those in the ACE inhibitor-treated diabetic group did not differ from those in the control and diabetic groups (Table 2).

### Table 1: Body and kidney weights and fasting blood glucose (FBG) levels in control (non-diabetic), diabetic and ACE inhibitor-treated control and diabetic rats. The values are means ± S.D. \((n=8 \text{ in each group})\)

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>FBG (mmol/l)</th>
<th>Kidney weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial 4 weeks</td>
<td>8 weeks</td>
<td>Initial 4 weeks</td>
</tr>
<tr>
<td>Control (non-diabetic)</td>
<td>319 ± 5</td>
<td>346 ± 6c</td>
<td>392 ± 8c</td>
</tr>
<tr>
<td>Diabetic</td>
<td>320 ± 4</td>
<td>309 ± 9a</td>
<td>297 ± 14a</td>
</tr>
<tr>
<td>ACE inhibitor-treated control</td>
<td>317 ± 6</td>
<td>348 ± 9c</td>
<td>394 ± 9c</td>
</tr>
<tr>
<td>ACE inhibitor-treated diabetic</td>
<td>320 ± 5</td>
<td>309 ± 10ab</td>
<td>295 ± 17ab</td>
</tr>
</tbody>
</table>

\*\( P=0.04 \), compared with those in control non-diabetic rats; \^{*}P=0.04 \), compared with those in ACE inhibitor-treated control rats; \,**P=0.04 \), compared with those in the initial period.

### Table 2: Serum and renal tissue ET-1 levels in control, diabetic, ACE inhibitor-treated control and diabetic rats. The values are means ± S.D. \((n=8 \text{ in each group})\)

<table>
<thead>
<tr>
<th>Serum ET-1 level (pg/ml)</th>
<th>Renal tissue ET-1 level</th>
<th>Control (non-diabetic)</th>
<th>Diabetic</th>
<th>4 weeks after the treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum ET-1 level (pg/ml)</td>
<td>Renal tissue ET-1 level</td>
<td>Control (non-diabetic)</td>
<td>Diabetic</td>
<td>ACE inhibitor-treated control</td>
</tr>
<tr>
<td>pg in whole kidney</td>
<td>pg/mg protein</td>
<td>pg/mg DNA</td>
<td>pg in whole kidney</td>
<td>pg/mg protein</td>
</tr>
<tr>
<td>5·02 ± 3·01</td>
<td>66·9 ± 39·2</td>
<td>0·86 ± 0·28</td>
<td>8·2 ± 2·2</td>
<td>8·2 ± 2·2</td>
</tr>
</tbody>
</table>

\*\( P=0.04 \), statistically significant, compared with that of control rats; \,**P=0.04 \), statistically significant, compared with that of diabetic rats.

The protein and DNA concentrations were determined by Bio-Rad protein assay kit (Bradford 1976) and the method reported by Labarca and Paigen (1980).
There were no significant differences in the ET-1 concentrations in the heart, liver and brain among the four groups (Table 3).

**Table 3** Cardiac, hepatic and cerebral ET-1 levels in control, diabetic, ACE inhibitor-treated control and diabetic rats. The values are means ± s.d. (*n*= 8 in each group)

<table>
<thead>
<tr>
<th></th>
<th>Control (non-diabetic)</th>
<th>Diabetic</th>
<th>4 weeks after the treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>ACE inhibitor-treated control</td>
</tr>
<tr>
<td>Cardiac ET-1 (pg/g wet weight)</td>
<td>27·6 ± 9·3</td>
<td>38·8 ± 14·9</td>
<td>30·8 ± 13·9</td>
</tr>
<tr>
<td>Hepatic ET-1 (pg/g wet weight)</td>
<td>6·9 ± 4·5</td>
<td>6·2 ± 3·8</td>
<td>5·9 ± 3·7</td>
</tr>
<tr>
<td>Cerebral ET-1 (pg/g wet weight)</td>
<td>25·1 ± 4·3</td>
<td>27·6 ± 5·1</td>
<td>23·2 ± 5·9</td>
</tr>
</tbody>
</table>

Microscopic and electron microscopic findings in the kidney tissue specimens

Increased mesangial cell proliferation, matrix expansion and enlarged mesangial area were observed in the diabetic rat (middle panel), differing from the non-diabetic rat (control) (left panel). The right panel shows the situation in the ACE inhibitor-treated diabetic rats, which is slightly improved compared with that of the diabetic rats.

**Figure 1** Histological examination of the renal tissue from the STZ-induced diabetic rats by electron microscopic images (× 8300). Mesangial cell proliferation and matrix expansion were observed in the diabetic rat (middle panel), differing from the non-diabetic rat (control) (left panel). The right panel shows the situation in the ACE inhibitor-treated diabetic rats, which is slightly improved compared with that of the diabetic rats.

Enalapril maleate (ACE inhibitor) levels in serum and urine

Serum and urinary enalapril maleate levels in control (non-diabetic) and diabetic rats were undetectable. Serum enalapril maleate level in ACE inhibitor-treated control rats was 12·1 ± 2·5 µg/l (mean ± s.d.), and that in ACE inhibitor-treated diabetic rats was 13·2 ± 2·1 µg/l. Urinary enalapril maleate level in ACE inhibitor-treated control rats was 10·1 ± 8·9 µg/l, and that in ACE inhibitor-treated diabetic rats was 10·5 ± 7·6 µg/l.

There were no statistically significant differences in enalapril levels which were enough to inhibit ACE activity (Cohen & Kurz 1982, Cohen et al. 1983) in serum and in urine between ACE inhibitor-treated normal and diabetic rats.

**Discussion**

Diabetic nephropathy is an important factor determining the prognosis of diabetic patients. In diabetic nephropathy, increased intraglomerular pressure due to renal glomerular vascular lesions induces microalbuminuria. When this lesion advances further apparent and persistent proteinuria...
due to glomerular sclerosis occurs leading to deterioration of renal function and chronic renal failure. ACE inhibitors have specific actions (Heeg et al. 1987, Remuzzi et al. 1991, Gansevoort et al. 1993) in reducing the progression of diabetic nephropathy.

The findings in our animal model, namely the increases in kidney weight, urinary albumin and NAG concentrations, mesangial cell proliferation, matrix expansion and enlarged mesangial area, are associated with diabetic nephropathy.

In the STZ-DM rats, treatment with enalapril maleate effectively reduced the levels of urinary NAG and albumin, and there were slight improvements in histological abnormalities. These data suggest that ACE inhibitors reduce the progress of diabetic nephropathic dysfunction (Hocher et al. 1998) and its associated morphology.

Brenner, Hostetter and colleagues have proposed the glomerular hyperfiltration theory (Hostetter et al. 1981, Brenner et al. 1982). This suggests that the rise in blood osmotic pressure with hyperglycemia and the increase in circulating blood volume promotes the hypersecretion of atrial natriuretic peptide (ANP) which causes the dilatation of glomerular afferent arterioles. Hyperglycemia also causes the production of angiotensin II, which stimulates arteriolar constriction but acts more strongly on the glomerular efferent arterioles. These mechanisms induce an imbalance between the constriction and dilatation of the afferent and efferent arterioles, resulting in increased intraglomerular pressure, and the subsequent development and progression of diabetic nephropathy (Hostetter et al. 1981, Brenner et al. 1982).

ACE inhibitors cause dilatation of the efferent arterioles more selectively than the afferent arterioles (Anderson et al. 1986, Fujiwara et al. 1991) and inhibit degradation of bradykinin, which also dilates the efferent arterioles (Kon et al. 1993). This may decrease intraglomerular pressure and improve glomerular hyperfiltration. It has also been demonstrated using cultured mesangial cells that angiotensin II acts directly on mesangial cell proliferation and matrix expansion which constitute glomerular sclerosis (Ichikawa & Harris 1991, Ray et al. 1991).

In the ACE inhibitor-treated diabetic group, the urinary NAG concentration was decreased to the normal level. NAG is an enzyme located in renal glomeruli and proximal tubular cells and is released into the urine when renal parenchymal cells are damaged.

The reduction in urinary NAG levels in STZ-DM rats by the ACE inhibitor may be due to decreased intraglomerular pressure (Anderson et al. 1986, Fujiwara et al. 1991) and the slight increase in the permeability of glomerular basement membranes (Wiegmann et al. 1992). As the ACE inhibitor did not affect blood glucose in the diabetic rats, the reduction of urinary albumin and NAG secretion was not attributable to improvements in glycemic control.

In the diabetic group, the ET-1 concentration tended to increase in the kidney. This change may be due to ET-1 in the renal cells because ET-1 exists not only in the vascular endothelial cells but also in the mesangial cells or tubular cells (Kosaka et al. 1989, Simonson & Dunn 1990, Kohan 1991). Fukui et al. (1994) reported that increases in ET-1 mRNA (2, 3 kilobases (kb)) in isolated glomeruli from diabetic rat kidney were partly reversed by enalapril treatment. However, MacCumber et al. (1989) detected two forms of ET mRNA, 3.7 and 2.5 kb, in rat tissues and assumed that the 2.5-kb transcript represents ET-1 and the 3.7-kb transcript reflects ET-3, so that kidney, eye and brain predominantly express ET-3 while lung expresses both ET-1 and ET-3. Moreover, employing in situ hybridization, they confirmed that renal ET mRNA was prominently localized to the medulla (MacCumber et al. 1989). We have tried to conduct Northern analysis of ET-1 mRNA in our experimental rat kidney. However, it was unsuccessful, probably because we used an unsuitable probe for renal ET mRNA or did not extract the microorganelle from the tissue specimens. Serum ET-1 concentration which was augmented in diabetic rats was significantly decreased by the ACE inhibitor treatment. This mechanism is not clear, but it might be caused by a decrease in angiotensin II, since angiotensin II has been reported to promote the production and secretion of ET in vivo (Moreau et al. 1997) and in vitro (Emori et al. 1989).

The ET-1 concentration in the kidney was not significantly changed by the administration of the ACE inhibitor nor was it correlated with serum ET-1 concentration. The renin–angiotensin system within the vascular wall (Unger

**Table 4.** The maximum and minimum widenings of mesangial matrix in the electron microscopic image (×8300) of control, diabetic and ACE inhibitor-treated rats. The values are means ± S.D. Numbers in parentheses are the number of the electron microscopic images of the rat kidney

<table>
<thead>
<tr>
<th>Group</th>
<th>Maximum widening (μm)</th>
<th>Minimum widening (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (non-diabetic) (5)</td>
<td>1·75 ± 0·22</td>
<td>0·69 ± 0·04</td>
</tr>
<tr>
<td>Diabetic (5)</td>
<td>3·31 ± 0·09*</td>
<td>2·01 ± 0·20*</td>
</tr>
<tr>
<td>ACE inhibitor-treated diabetic (5)</td>
<td>2·20 ± 0·47**</td>
<td>0·90 ± 0·05**</td>
</tr>
</tbody>
</table>

*p=0·01, statistically significant, compared with that in control non-diabetic rats; **p=0·03, statistically significant, compared with that in diabetic rats.
& Gohlke 1990) may be affected by ACE inhibition. ET-1 induces the constriction of glomerular interlobular arcuate arteries, and afferent and efferent arterioles (Simonson et al. 1989) and also mesangial cell proliferation, causing a reduction in renal blood volume and glomerular filtration and a decrease in the urinary Na excretion (Goetz et al. 1988, Miller et al. 1989). Olbrich et al. (1996) observed that ACE inhibitor treatment could prevent an impairment of NO-dependent regulation in small resistance vessels. These data, including the changes of ET-1 concentration due to ACE inhibition, may at least partly cause the reduction of urinary albumin and NAG secretion.

In conclusion, the increased urinary albumin and NAG secretion in STZ-induced diabetic nephropathy was reduced by ACE inhibition. ACE inhibitors may, in part, exert their effects in diabetic microangiopathy by reducing ET-1 levels.

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