Involvement of Angiotensin II in Progression of Renal Injury in Rats With Genetic Non-insulin-Dependent Diabetes Mellitus (Wistar Fatty Rats)

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ABSTRACT—Wistar fatty (WF) rats have a genetic predisposition to hyperglycemia, polyuria, hyperinsulinemia, hyperlipidemia, obesity and nephropathy. These phenotypic characteristics are similar to those observed in obese patients with non-insulin-dependent diabetes mellitus (NIDDM) nephropathy. In this study, the effects of two types of renin-angiotensin system inhibitors, an angiotensin II type 1-receptor antagonist (AT₁A) and an angiotensin I-converting enzyme inhibitor (ACEI), on renal injury in WF rats were studied during the progressive phase of diabetic nephropathy. An AT₁A, candesartan cilexetil (1 mg/kg), and an ACEI, enalapril (10 mg/kg), were administered orally once a day for 12 weeks, beginning when the rats were 27-week-old and already showed diabetic nephropathy and obesity. Both drugs prevented an increase in proteinuria during the experimental period. Furthermore, after 4-week intervention, the levels of proteinuria were markedly lower in drug-treated rats. At the end of the experiment, both drugs prevented the development of glomerular lesions without affecting glucose metabolism and obesity. In conclusion, the inhibition of angiotensin II activity ameliorated both existing proteinuria and the progression of proteinuria, resulting in preservation of glomerular structure. Thus angiotensin II plays important roles in the development and the progression of nephropathy in genetically obese diabetic WF rats.

Keywords: Renin-angiotensin system, Angiotensin II-receptor antagonist, Candesartan cilexetil, Non-insulin-dependent diabetes mellitus, Nephropathy

Non-insulin-dependent diabetes mellitus (NIDDM) nephropathy is the most common cause of end-stage renal disease. Angiotensin II (Ang II), which is the primary effector product of the renin-angiotensin system (RAS), not only contributes to glomerular capillary hypertension, but also has a direct influence on some functions of the mesangial cells and glomerular filtration barrier. Therefore, Ang II is thought to be a crucial factor in progressive renal failure (1, 2). The renoprotective effect of RAS inhibition by angiotensin I-converting enzyme inhibitors (ACEIs) is well established in patients with insulin-dependent diabetes mellitus (IDDM) nephropathy (3 – 5). Moreover, inhibition of RAS by ACEIs and Ang II type 1-receptor antagonists (AT₁As) has a renal protective effect in streptozotocin-induced diabetic rats, an experimental model of IDDM, which has been used extensively to clarify the pathophysiology of diabetic nephropathy (6 – 9).

Until recently, in contrast to IDDM, only a few animal studies have addressed the role of the RAS in NIDDM nephropathy. Enalapril (ENA), an ACEI, was reported to inhibit development of albuminuria and renal histopathological damage in Zucker fatty rats, an experimental model of NIDDM, during the early phase of nephropathy (10). During the progressive phase of nephropathy, however, the AT₁As, losartan and irbesartan, had no significant effect on albuminuria in Zucker fatty rats (11, 12). Both AT₁As and ACEIs ultimately block RAS, but the mechanism of the inhibition is different. AT₁As selectively inhibit the binding of Ang II to AT₁ receptors. In contrast, ACEIs inhibit angiotensin I-converting enzyme, which also degrades bradykinin, resulting not only in decreased plasma Ang II levels but also in elevated kinin activity (13, 14). Therefore, it remains unclear whether the beneficial effect of ACEIs
on proteinuria in Zucker fatty rats is solely a consequence of inhibition of Ang II activity. Otsuka Long-Evans Tokushima Fatty (OLETF) rat is also a genetic model of NIDDM and shows diabetic nephropathy (15–18). RAS inhibition was reported to delay the onset of nephropathy as well (16, 18). However, the effect of RAS inhibition in the progressive phase of nephropathy in OLETF rats, which have already showed marked proteinuria, remains to be elucidated. Thus, the renoprotective effect of RAS inhibitors during the progressive phase of nephropathy is still controversial.

Wistar fatty (WF) rats were developed by Ikeda et al., by crossbreeding Wistar Kyoto rats and Zucker fatty rats. (19). The phenotypic characteristics of WF rats, including obesity, hyperglycemia, polyuria, hyperinsulinemia and hyperlipidemia, are similar to those in obese patients with NIDDM (19, 20). Furthermore, WF rats also show proteinuria from 12 weeks of age. In our previous study, RAS inhibition was shown to prevent the onset of proteinuria in 12-week-old WF rats during the early phase of nephropathy (21), but the role of RAS after onset of nephropathy is still controversial. In this study, 27-week-old WF rats with marked obesity, diabetes and proteinuria were used to evaluate the renoprotective effects of post treatment of RAS inhibitors during the progressive phase of NIDDM nephropathy.

MATERIALS AND METHODS

Experimental design

Male WF rats (27-week-old) and age-matched male Wistar lean rats (Takeda RABICS, Ltd., Osaka) were used in this study. The WF rats were divided into three groups that were similar with respect to urinary albumin excretion, urinary total protein excretion, plasma glucose and body weight. The groups of rats were administered vehicle (0.5% methylcellulose 100 cp, n = 6), candesartan cilexetil (CC) (1 mg/kg, n = 5) or ENA (10 mg/kg, n = 5), with their lean littermates (n = 6) as the normal control rats. CC is an AT1 antagonist (22–24). These doses of CC and ENA were chosen because they have been shown to have equivalent hypotensive effects in spontaneously hypertensive rats (25). Drugs were suspended in 0.5% methylcellulose 100 cp (Wako Pure Chemical Industries, Osaka) and administered orally once a day for 12 weeks from 27 weeks of age. Animals had free access to water from a drinking nozzle and chow pellets (CLEA Rodent Diet CE-2; Clea Japan, Inc., Tokyo). All animal experiments were performed according to the guidelines of the Takeda Experimental Animal Care and Use Committee.

Measurement of plasma and urine parameters

Twenty-four-hour urine samples were collected with the aid of metabolic cages. Rats were housed individually in metabolic cages equipped with drinking bottles and food cups outside the cages. For measurement of urinary total protein and albumin concentrations, urine samples were desalted on a gel filtration column (PD-10; Pharmacia, Tokyo) equilibrated with 0.005 M (NH4)2CO3. Blood was collected from conscious rats via the tail vein using EDTA as an anti-coagulant, and plasma was prepared for measurement of plasma glucose, plasma insulin and plasma creatinine levels. Urinary total protein, urinary albumin, plasma glucose, urinary and plasma creatinine, plasma total cholesterol and plasma triglycerides were measured with commercially available kits (Wako Pure Chemical Industries, Osaka). Plasma insulin was measured by radioimmunoassay (Shionoria insulin; Shionogi Pharmaceutical Ltd., Osaka).

Measurement of blood pressure

Rats were maintained in individual chambers warmed at 37°C, and systolic blood pressure was measured with a tail-cuff (Softron BP-98A; Softron Co., Tokyo).

Histological studies

At the end of the experiment, rats were anesthetized with sodium pentobarbital, and terminal blood samples for measurement of plasma total cholesterol and plasma triglycerides levels were collected from the abdominal aorta using EDTA as an anti-coagulant. Both kidneys were removed and the wet weight was determined. The right kidney was fixed in 10% neutral buffered formalin and cut cross sectionally to include the papilla. The tissue was then embedded in paraffin for histological studies. Paraffin sections were cut and stained with periodic acid-methenamine silver for evaluation of glomerular lesions. For each rat, all glomerular cross sections present in a specimen were counted, and glomeruli exhibiting segmental (focal in a glomerulus) or global (whole glomerulus) mesangial matrix expansion were counted; then the incidence of the change was determined. The mesangial matrix expansion was considered to be present when the width of periodic acid-methenamine silver positive mesangial matrix was equal or exceeded twice the normal thickness.

Drugs

CC (TCV-116), (±)-1-(cyclohexyloxy carbonyloxy)ethyl 2-ethoxy-1-[[2’-(1H-tetrazol-5-yl)biphenyl-4-yl]methyl]-1H-benzimidazole-7-carboxylate, and ENA maleate were synthesized by Takeda Chemical Industries, Ltd. (Osaka).

Statistical analyses

All data are expressed as the mean ± S.E.M. The differences in all parameters, except glomerular mesangial matrix expansion and systolic blood pressure, between vehicle-treated WF rats and Wistar lean rats were analyzed by Student’s t-test. The effects of CC and ENA on urinary
albumin and total protein excretion, urine volume, plasma glucose, plasma insulin, creatinine clearance (Ccr) and body weight in WF rats were evaluated by repeated measure analysis of variance (ANOVA), followed by Dunnett’s test with the data for 12-week administration. Differences in plasma total cholesterol, plasma triglyceride and kidney wet weight between vehicle- and drug-treated WF rats were analyzed by Dunnett’s test. Differences in glomerular mesangial matrix expansion between vehicle-treated WF rats and Wistar lean rats were evaluated with Wilcoxon test. Differences in glomerular mesangial matrix expansion between vehicle- and drug-treated WF rats were analyzed by Dunnett’s test using ranked data. P<0.05 was considered to indicate a significant difference.

RESULTS

Figure 1 shows urinary albumin and total protein excretion. At the onset of treatment, WF rats already showed marked urinary albumin excretion (5.9 ± 0.4, 149.3 ± 10.2, 147.0 ± 12.4, 149.8 ± 13.7 mg/day for Wistar lean rats, and vehicle-, CC- and ENA-treated rats, respectively) and raised urinary total protein excretion (24.1 ± 1.4, 215.7 ± 12.4, 219.5 ± 15.9, 214.8 ± 17.1 mg/day for Wistar lean rats and vehicle-, CC- and ENA-treated rats, respectively), which increased gradually with time. Levels of urinary albumin excretion fell to 95.0 ± 18.8 mg/day and urinary total protein excretion to 133.9 ± 22.3 mg/day after 4 weeks administration of CC. ENA also reduced urinary albumin excretion to 88.3 ± 7.1 mg/day and urinary total protein excretion to 125.9 ± 10.2 mg/day after 4 weeks administration. Moreover, both drugs prevented the progression of proteinuria for up to 12 weeks. The antiproteinuric effect of these two drugs was comparable. Neither drug had any effect on Ccr (Table 1) or blood pressure (Table 2) throughout the experimental period.

The mean body weight of the WF rats was greater than that of the lean control rats throughout the experimental period (Fig. 2). At the end of the experiment, neither drug had any beneficial effect on obesity in the WF rats.

At the onset of treatment, plasma glucose and insulin levels were already higher in WF rats than in Wistar lean

| Table 1. Effects of candesartan cilexetil and enalapril on creatinine clearance (Ccr) in Wistar fatty (WF) rats |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Wistar lean rats| WF rats + Vehicle| WF rats + CC | WF rats + ENA |
| Ccr (ml/min per 100 g body wt) | Pre 0.18 ± 0.02 | 0.24 ± 0.02 | 0.21 ± 0.02 | 0.23 ± 0.02 |
|                 | 4w 0.24 ± 0.02 | 0.27 ± 0.03 | 0.27 ± 0.03 | 0.24 ± 0.01 |
|                 | 8w 0.24 ± 0.03 | 0.32 ± 0.02 | 0.23 ± 0.02 | 0.24 ± 0.01 |
|                 | 12w 0.29 ± 0.02 | 0.29 ± 0.02 | 0.26 ± 0.01 | 0.27 ± 0.02 |

Data are presented as the mean ± S.E.M. CC, candesartan cilexetil; ENA, enalapril. No significant differences between vehicle-treated and drug-treated WF rats were detected by ANOVA. Accordingly, Dunnett’s test was not performed.
Ang II and NIDDM Nephropathy in Rats

Table 2. Effects of candesartan cilexetil and enalapril on systolic blood pressure in Wistar fatty (WF) rats

|                     | Wistar lean rats | WF rats + Vehicle | WF rats + CC | WF rats + ENA |
|---------------------|------------------|-------------------|-------------|--------------|
| Systolic blood pressure (mmHg) |                  |                   |             |              |
| Pre                 | 140.5 ± 5.9      | 135.6 ± 4.3       | 128.6 ± 2.3 | 128.1 ± 5.8  |
| 11w                 | 131.8 ± 2.6      | 136.6 ± 4.5       | 112.1 ± 4.0 | 124.5 ± 4.9  |

Data are presented as the mean ± S.E.M. CC, candesartan cilexetil; ENA, enalapril. No significant difference was found between the initial and final values in each group by the paired t-test.

Table 3. Effects of candesartan cilexetil and enalapril on plasma glucose, plasma insulin and urine volume in Wistar fatty (WF) rats

|                      | Wistar lean rats | WF rats + Vehicle | WF rats + CC | WF rats + ENA |
|----------------------|------------------|-------------------|-------------|--------------|
| Plasma glucose (mg/dl) |                  |                   |             |              |
| Pre                  | 116.4 ± 1.4      | 384.0 ± 19.8a     | 397.3 ± 14.4 | 387.5 ± 20.0 |
| 4w                   | 125.8 ± 1.5      | 379.8 ± 23.8a     | 395.4 ± 14.6 | 355.3 ± 20.2 |
| 8w                   | 134.8 ± 6.6      | 385.7 ± 9.1c      | 383.2 ± 20.8 | 391.1 ± 16.1 |
| 12w                  | 148.5 ± 6.3      | 417.1 ± 15.9abc   | 395.9 ± 20.3 | 392.0 ± 24.2 |

| Plasma insulin (µU/ml) |                  |                   |             |              |
| Pre                  | 95.2 ± 12.5      | 904.7 ± 117.8b    | 1069.2 ± 92.5 | 1172.6 ± 246.4 |
| 4w                   | 162.0 ± 21.8     | 917.0 ± 132.7a   | 1412.4 ± 201.6 | 1380.6 ± 168.2 |
| 8w                   | 115.9 ± 9.0      | 1077.5 ± 126.3b  | 1589.7 ± 144.1 | 1535.2 ± 296.6 |
| 12w                  | 117.8 ± 18.4     | 1061.2 ± 111.7b  | 1603.8 ± 169.3 | 1356.4 ± 141.3 |

| Urine volume (ml/day) |                  |                   |             |              |
| Pre                  | 13.0 ± 2.3       | 45.7 ± 9.8        | 31.8 ± 5.8  | 39.8 ± 7.3   |
| 4w                   | 16.9 ± 2.6       | 31.9 ± 4.2c       | 30.9 ± 8.4  | 31.7 ± 5.8   |
| 8w                   | 13.3 ± 2.4       | 31.2 ± 10.2       | 30.6 ± 4.5  | 27.3 ± 4.6   |
| 12w                  | 16.9 ± 1.7       | 30.5 ± 6.2        | 29.0 ± 3.1  | 32.3 ± 5.2   |

Data are presented as the mean ± S.E.M. CC, candesartan cilexetil; ENA, enalapril. *P<0.05, **P<0.01 vs Wistar lean rats with Student’s t-test. No significant differences between vehicle- and drug-treated WF rats for all parameters were detected by ANOVA. Accordingly, Dunnett’s test was not performed.

Rats, and WF rats showed polyuria (Table 3). Neither CC nor ENA affected these parameters (Table 3).

The levels of plasma lipids in WF rats were higher than those in Wistar lean rats at the end of the experimental periods (Fig. 3). Plasma triglyceride was slightly lower in both drug-treated groups than in the vehicle-treated group, but the differences were not statistically significant (Fig. 3A). Both drugs caused a significant decrease in plasma total cholesterol in WF rats (Fig. 3B).

In vehicle-treated WF rats, the incidence of glomeruli with segmental and global mesangial expansion increased (Fig. 4). The number of injured glomeruli in both groups of drug-treated WF rats was significantly lower than in vehicle-treated WF rats at the end of the experiment. The mean wet weight of both kidneys from the vehicle-treated rats was higher than that for the lean control rats (Fig. 5). CC prevented enlargement of the kidneys significantly. The mean kidney wet weight was slightly lower in the ENA-treated group than in the vehicle-treated group, but the differences were not statistically significant.
DISCUSSION

The aim of this study was to investigate whether AT$_1$As as well as ACEIs, had any antiproteinuric effect during the progressive phase of renal injury in NIDDM nephropathy. Twenty-seven-week-old WF rats with obesity, hyperglycemia, polyuria, hyperinsulinemia, hyperlipidemia and diabetic nephropathy were used. Both ENA and CC were found to have a significant antiproteinuric effect in WF rats (Fig. 1). Moreover, these two types of RAS inhibitor prevented an increase in the number of injured glomeruli. These results indicate that Ang II may be involved in renal injury in WF rats. It is noteworthy that both inhibitors reduced proteinuria that had developed previously and prevented further deterioration of proteinuria. Since both inhibitors had no effect on Ccr (Table 1), it is unlikely that the significant decrease in proteinuria after 4 weeks treatment with the drugs was caused by a reduction in the glomerular filtration rate. The mechanism by which these RAS inhibitors reduced proteinuria was not addressed in this study. However, the antiproteinuric effect was not due to the improvement of the diabetes, because treatment had no effect on hyperglycemia or hyperinsulinemia (Table 3, Fig. 2). Many studies suggest that Ang II is a crucial factor in induction of glomerular dysfunction, because Ang II contributes not only to glomerular capillary hypertension but also to impairment of glomerular permselectivity (1, 2, 26). Once glomerular permselectivity is lost, proteins can...
Fig. 5. Effects of candesartan cilexetil (CC) and enalapril (ENA) on wet weight of both kidneys in Wistar fatty (WF) rats. *P<0.05 vs Wistar lean rats (Lean) with Student’s t-test. #P<0.05 vs vehicle-treated WF rats with Dunnett’s test. Data are presented as the mean ± S.E.M.

filter through the glomerular capillaries without adverse changes in glomerular hemodynamics (27, 28). It has been reported that the development of proteinuria in rats with streptozotocin-induced diabetes and patients with IDDM nephropathy was in part associated with the impairment of glomerular basement membrane permeability. Both ACEIs and AT1As have been reported to improve the impairment of permeability, resulting in a reduction in urinary protein excretion in rats with streptozotocin-induced diabetes (29–32). These evidences together with the lack of no changes in GFR in inhibitor-treated WF rats raised the possibility that RAS inhibition improved the impairment of glomerular basement membrane permeability in WF rats. As glomerular capillary pressure was not measured directly, we could not exclude the possibility that the decrease in urinary protein excretion after 4 weeks intervention was caused by fine modulations of glomerular hemodynamics. Further studies are needed to confirm our preliminary findings and elucidate the exact mechanisms behind the beneficial effects of these drugs. Micropuncture studies and the molecular analysis of the glomerular filtration barrier function are useful approaches to understand the antiproteinuric effects of RAS inhibitors in WF rats.

Both drugs caused a significant decrease in plasma total cholesterol in WF rats (Fig. 3B). Hypercholesterolemia is observed frequently in patients with nephrotic syndrome (33). This hypercholesterolemia might be caused by induction of cholesterol production in the liver in response to the loss of plasma albumin due to massive urinary albumin excretion (33). Therefore, the excessive production of plasma total cholesterol in WF rats might be caused, in part, by renal injury, and suppression of hypercholesterolemia by RAS inhibition may be a secondary effect resulting from the decrease in proteinuria. Since hypercholesterolemia could aggravate glomerular sclerosis (34), improvement of hypercholesterolemia in drug-treated WF rats might help to prevent progressive renal injury.

In conclusion, inhibition of RAS by CC and ENA improved previously developed proteinuria as well as prevented the progression of proteinuria, resulting in preservation of glomerular structure in genetically obese diabetic WF rats. Analysis of the reasons why urinary protein excretion develops in WF rats might be a useful approach to investigate the pathogenesis and mechanisms of progression of glomerular injury in patients with NIDDM nephropathy.

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