Renal-Protective Effect of Nondepressor Dose of Cicletanine in Diabetic Rats With Hypertension

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We assessed the renal and cardiac benefits of cicletanine (CIC), a furopyridine derivative drug with diuretic and antihypertensive properties, in diabetic spontaneously hypertensive rats with renal impairment. Uninephrectomized streptozotocin (STZ)-diabetic spontaneously hypertensive Izm rats (SHRIzm) (10 weeks old) were randomly assigned to receive vehicle or CIC (100 mg/kg/day, orally), and age-matched, uninephrectomized STZ diabetic Wistar-Kyoto Izm rats (WKYIzm) were assigned to receive vehicle for up to 12 weeks. Blood pressure increased progressively in diabetic SHRIzm but not in diabetic WKYIzm. Urinary albumin excretion increased significantly in both diabetic SHRIzm and diabetic WKYIzm throughout the experiment. The antihypertensive effect of CIC was not significantly observed in diabetic SHRIzm. However, the subdepressor doses of CIC significantly decreased urinary albumin excretion, serum creatinine, and blood urea nitrogen in diabetic SHRIzm. These results were confirmed by morphological analysis of kidneys in each group of rats. The index of focal glomerular sclerosis (FGS) in diabetic SHRIzm was significantly higher than that in diabetic WKYIzm. The CIC treatment significantly and effectively protected against an increase in the index of FGS in diabetic SHRIzm. Moreover, CIC treatment significantly attenuated the increase in the heart weight to body weight ratio in diabetic SHRIzm. Treatment with CIC did not affect urinary and blood glucose concentrations at this dose. These results suggest that CIC has a renal-protective action, which is not related to improvement of diabetes or improvement of high blood pressure in diabetic rats with hypertension. The action might be due to the reduction of intraglomerular capillary pressure or protection of the renal glomerular vascular endothelial cell injury and mesangial cell injury through stimulation of PGI2 generation or elimination of free radicals, although the mechanism remains to be further investigated. Am J Hypertens 2000; 13:298–306 © 2000 American Journal of Hypertension, Ltd.

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Cicletanine [1,3-dihydro-6-methyl-7-hydroxy-3-(4-chloro-phenyl)furo(3,4-c)pyridine] (CIC), a furopyridine derivative with diuretic and antihypertensive properties, lowers blood pressure in hypertensive animals and humans.1 The drug does not belong to a typical class of antihypertensive drugs (ie, adrenoceptor agents, diuretics, ACE inhibitors, AI1 receptor antagonists, and calcium channel blockers). This drug is reported to stimulate the synthesis of PGI2, which has vasodilating and natriuretic actions and inhibits platelet aggregation. Cicletanine acts directly on vascular smooth muscle by increasing PGI2 synthesis and interacting with various agents, which mobilize intracellular Ca2+ ion.3

Urinary PGI2, measured as 6-keto-PGF1α and thromboxane B2 were reported to be increase in diabetic rats.4,5 Zola et al reported6 that thromboxane synthesis inhibition increases renal PGI2 and prevents renal disease progression in rats with remnant kidney. Because CIC supports PGI2 production, CIC might have a renal-protective effect in diabetic rats with hypertension. We have recently reported the renal-protective effect of a nondepressor dose of cicletanine in diabetic normotensive Wistar-Kyoto rats.7

Hypertension is frequently associated with diabetes mellitus. It is known that a model combining genetic hypertension with streptozotocin-induced diabetes has accelerated nephropathy, as determined by both functional and structural parameters, compared with a normotensive diabetic model.8 Treatment with CIC also is also reported to have stimulatory effects on the kallikrein-kinin system.9 In addition, CIC showed reversing effect on angiotensin II–precontracted rat glomeruli by inactivating the glibenclamide-sensitive potassium channels.10 CIC also reduced the vascular reactivity to angiotensin II in rats,11 although the effect of increased synthesis of PGI2, caused by CIC, may stimulate renin release from the kidneys.12 Therefore, CIC may have a renal-protective effect, not only by reducing systemic hypertension, but also by reducing glomerular hyperfiltration in diabetic nephropathy. We have been shown that angiotensin converting enzyme (ACE) inhibitors and angiotensin II receptor antagonists are beneficial against hyperfiltration damage in the nephrons of the remnant kidney in diabetic nephropathy.13–16 However, the renal protective effects of CIC on diabetes with hypertension have not been studied. The present study was undertaken to determine whether treatment of streptozotocin diabetic hypertensive rats with CIC influences urinary albumin excretion.

METHODS

Eight-week-old spontaneously hypertensive Izmo rats (SHRIzm) and Wistar-Kyoto Izmo rats (WKYIzm) were studied (Charles River Japan, Atsugi, Japan). Under light ether anesthesia, the right kidney was removed through a flank incision. One week after the operation, the rats were injected intraperitoneally with streptozotocin (Sigma Chemical Co, St Louis, MO). Because SHRIzm are known to be more susceptible to the diabetogenic effects of streptozotocin (STZ) than WKYIzm, and because feeding affects the diabetogenic effect of STZ, the doses of STZ were adjusted to 35 mg/kg in SHRIzm and 45 mg/kg in WKYIzm.13–15,17 The injection time was 12 pm for SHRIzm and 6 pm for WKYIzm. Urine was tested for glucose content to document glycosuria 3 to 4 days after the streptozotocin (STZ) administration. Only those rats with urine glucose concentration > 500 mg/dL were used in the present study.14–16 The rats were fed a regular diet (CE-2 [0.19 wt% sodium, 0.25 wt% potassium, and 20.8 wt% protein]; Nippon Crea, Chiba, Japan) and had free access to tap water. Each rat was housed throughout the study in a metabolic cage designed to prevent feces–urine contact (Sugiyamagen, Tokyo, Japan) for collection of 24-h urine samples.

One week after the STZ administration, when the rats were 10 weeks old, baseline measurements of body weight, systolic blood pressure (SBP), urine volume, and urinary excretion of albumin were made. The diabetic WKYIzm were then assigned to receive vehicle only (n = 17), and the diabetic SHRIzm were assigned to receive vehicle only (n = 13) or CIC 100 mg/kg/day (n = 17) orally, in 0.5% carboxymethyl-cellulose sodium (Kanto Kagaku Co, Japan) solution (5 mL/kg) daily between 9 am and 10 am for 12 weeks. The rats did not receive insulin treatment for the duration of the study. Blood glucose was measured every 4 weeks using the blood from rat tail vein. At the end of the experiment, the rats were killed by decapitation and the trunk blood was collected in polyethylene tubes for the determination of serum creatinine, blood urea nitrogen, and blood glucose.

Kidneys, which were removed when the rats were killed, were measured for weight gravimetrically, and portions were fixed in 10% buffered formalin. Paraffin sections 3 μm thick were cut, and were stained with hematoxylin and eosin, periodic acid-Schiff reagent, Masson’s trichrome, and Azan Mallory. High-power fields were used to examine for evidence of focal sclerosis.13,18–20 For calculating focal glomerular sclerosis, 150 to 200 glomeruli from each stained section were examined. Markedly deformed or globally sclerotic glomeruli were excluded from the calculations. The degree of sclerosis in each glomerulus was subjectively graded on a scale of 0 to 4: grade 0, no change; grade 1, sclerotic area ≤ 1/4 of the glomerulus or distinct adhesion present between capillary tuft and Bowman’s capsule; grade 2, sclerosis of 1/4 to 1/2 of the total glomerular area; grade 3, sclerosis of > 1/2 the glomerulus but not global; and grade 4, global...
sclerosis. An Index of Sclerosis (IS) was calculated using the following formula:

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IS = (1 \times N_1 + 2 \times N_2 + 3 \times N_3 + 4 \times N_4)/(N_0 + N_1 + N_2 + N_3 + N_4),
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where \( N \) is the number of glomeruli in each grade of sclerosis.20

All of the rats were maintained in a humidity- and temperature-controlled room (55% ± 10% and 22° ± 2°C, respectively) during the study. The STZ was dissolved in 10 mmol/L citrate buffer (pH 4.5) immediately before use. The SBP was recorded in conscious rats, using the developed indirect tail-cuff method (UEDA UR 5000, Ueda Industries Co, Tokyo, Japan), without anesthesia, between 1 pm and 3 pm.21 The SBP measured using this method correlates well with that measured using a direct method.21 Direct blood pressure measurement was performed in six rats from each group 1 or 2 days before decapitation. Their mean SBP, measured by the tail-cuff method, was the same as that of the rest of the rats from each group measured by the same method. The rats were anesthetized with intraperitoneal urethane (1 g/kg, Wako Chemicals, Osaka, Japan) and intraperitoneal \( \alpha \)-chlo-ralose (50 mg/kg, Wako Chemicals, Japan). Body temperature was maintained at 37°C with an electric heater during the experimental procedure. The left femoral artery was cannulated using a PE-50 cannula for arterial blood pressure measurement between 1 pm and 3 pm. The femoral arterial catheter was connected to a pressure transducer (DX-360, Nihon-Kohden Co, Tokyo, Japan) and a recorder (6172A, San-Ei, Japan) for monitoring arterial pressure.

Urine volume and body weight was measured gravimetrically. Urinary albumin was measured using an enzyme-linked immunosorbent assay (Nephrat; Exocell Inc, Philadelphia, PA). Blood glucose, serum creatinine, and urine glucose were measured using a standard auto-analysis technique.18

Statistical Analysis Values are expressed as means ± SEM. With respect to blood urea nitrogen, blood glucose, serum creatinine, heart weight to body weight ratio, and kidney weight to body weight ratio, comparisons between the different groups of rats were performed using the unpaired Student \( t \) test. With respect to glomerular sclerosis index, comparisons between the different groups of rats were performed using the nonparametric Kruskal-Wallis test. For body weight, SBP, and urinary albumin, comparisons between the different groups of rats were performed by analysis of variance (ANOVA) with repeated measures over the duration of the study. Statistically significant differences on each day were assessed between groups by the unpaired Student \( t \) test, with \( P \) < .05 being considered statistically significant. The statistical analysis in the present study was performed using the Macintosh statistical package software Statview 4.02 (Abacus Concepts Inc, Berkeley, CA).

RESULTS

Body weight was significantly more in diabetic WKYIzm than that in diabetic SHRlzm groups before the administration of CIC (Table 1). All of the three diabetic groups gained weight over the period of experiment (\( P < .001 \)). Furthermore, CIC treatment did not affect BW compared with the control group. Heart rate in each treated group was not significantly differ-
ent from that in the control group during the experimental period (Table 1).

Diabetes in STZ-treated rats was characterized by marked polyuria, glycosuria, and hyperglycemia. Diabetes in STZ-WKYIzm was more pronounced than that in STZ-SHRIzm and was characterized by hyperglycemia during the study period (Table 1). The CIC treatment did not affect blood glucose compared with that in the control group.

The SBP was significantly higher in diabetic SHRIzm groups than that in diabetic WKYIzm before the administration of CIC (Figure 1). In vehicle-treated diabetic SHRIzm, the SBP was 221 ± 6 mm Hg at age 10 weeks and rose slightly and significantly to 253 ± 6 mm Hg at age 22 weeks (Figure 1). The antihypertensive effect of CIC was not significantly observed. At the end of the study, we measured systolic blood pressure of six anesthetized rats in each group directly. It was confirmed that the dose in this experiment was nonhypotensive in diabetic SHRIzm (control: 219 ± 10 mm Hg, CIC 100 mg/kg; 209 ± 7 mm Hg [not significantly]), although the antihypertensive effect of urethane on SBP in both groups of rats was not completely absent.56 In vehicle-treated diabetic WKYIzm, the SBP was 146 ± 2 mm Hg at age 10 weeks and did not rise significantly (Figure 1).

Albuminuria progressively increased in both diabetic SHRIzm and diabetic WKYIzm over the experimental period (Figure 2). In vehicle-treated diabetic SHRIzm, urinary albumin excretion was 2.8 ± 1.2 mg/day at age 10 weeks and increased remarkably during the experimental period to 89.0 ± 9.5 mg/day (Figure 2). In addition, albuminuria in diabetic SHRIzm was more severe than that in diabetic WKYIzm (P < .01, by ANOVA; Figure 2). The CIC treatment significantly reduced urinary albumin excretion compared with that in vehicle-treated diabetic SHRIzm (P < .01, by ANOVA; Figure 2) to the level that was similar to the vehicle-treated diabetic WKYIzm (CIC treated SHRIzm-DM: 49.2 ± 14.0 mg/day, vehicle-treated diabetic WKYIzm: 31.9 ± 6.1 mg/day [not significant]) at age 22 weeks. In contrast, CIC treatment did not affect urine volume, urinary sodium excretion, or urinary potassium excretion compared with that in the control group throughout the experimental period. Blood urea nitrogen and serum creatinine in the CIC-treated diabetic SHRIzm were lower than those in the vehicle-treated diabetic SHRIzm (Figure 3).
These results were confirmed by morphological analysis of kidneys in each group of rats. CIC treatment significantly protected against an increase in the index of focal glomerular sclerosis (Figure 3). Marked deformed glomeruli were <2% in each section, and they were excluded from the calculation. No globally sclerotic glomeruli (grade 4) were seen in any section. We also examined whether the pharmacological manipulation reduced the interstitial and vascular damage usually associated with glomerulosclerosis. However, we could not find any typical interstitial or vascular damage in diabetic SHR groups. Apparently, 12 weeks was insufficient time to cause significant interstitial or vascular damage in diabetic SHR, although some interstitial and vascular damage was found in the nontreated diabetic SHR.

The heart weight to body weight ratio in the CIC-treated diabetic SHRzm was lower than that in the vehicle-treated diabetic SHRzm (Figure 4).

**DISCUSSION**

The STZ-diabetic rat has been extensively used for study of hemodynamic, metabolic, and other factors contributory to the development of diabetic nephrop-
Changes in glomerular and basement membrane biochemistry and metabolism, glomerular function, filtration fraction, capillary pressure, and single nephron filtration rate have been identified in this experimental model. The ability of pharmacological intervention to reduce elevated urinary albumin excretion, the established parameter of diabetic renal dysfunction, has been interpreted as reflecting a salutary influence on the pathogenesis of diabetic nephropathy. Therefore, reduction or prevention of increased albumin excretion is being used in clinical and experimental studies to assess the efficacy of therapeutic interventions on the course of diabetic nephropathy in human subjects and in animal models, and to evaluate pathogenic influences relevant to diabetic nephropathy.

This is the first report to demonstrate the effects of CIC on urinary albumin excretion and renal histology in diabetic SHR. The antihypertensive effect of CIC was not significantly observed in our experiment. This was confirmed by the direct measurement of blood pressure in rats examined. The subdepressor doses of CIC significantly decreased urinary albumin excretion, serum creatinine, and blood urea nitrogen. These results were confirmed by morphological analysis of kidneys. The CIC treatment significantly and effectively protected against an increase in the index of focal glomerular sclerosis in diabetic SHR. Treatment with CIC did not affect urinary or blood glucose concentrations at the dose we used; therefore, CIC did not affect the condition of diabetes mellitus in the rats examined in this experiment. It is interesting that CIC has a renal-protective action, which is not related to improvement of diabetes or improvement of high blood pressure in this model. Therefore, there is a possibility that the kidney-protective effects of CIC could be dissociated from the effects on systemic blood pressure reduction. It was recently found that CIC, but not trichloromethiazide diuretic, improves glomerular injuries in Dahl salt-sensitive rats, although the fact that both drugs lowered SBP similarly may support this notion.

Mechanisms of the renal-protective action of CIC that are not related to improvement of diabetes and improvement of high blood pressure in this model are not fully elucidated in the present study. It has been suggested that glomerular hypertension contributes to the initiation and progression of diabetic renal disease. Amelioration of glomerular hypertension by ACE inhibitors prevents the development of glomerular injury and proteinuria in this model. The ACE is identical to kininase II, an enzyme that degrades bradykinin; such a biological action of ACE inhibitors could be due to bradykinin potentiation. We and others have reported that urinary active kallikrein is reduced in diabetic rats compared with nondiabetic control rats. Treatment with CIC has protective effects on renal kallikrein-kinin systems. It has been reported that kinins cause selective efferent arteriolar dilation during ACE inhibitor administration in rats with severe volume depletion. Therefore, the kidney protecting effects of CIC might be due to the reduction of intraglomerular capillary pressure through the activation of the kallikrein-kinin system.

Although plasma renin activity tended to rise after the administration of CIC in man, the rise was small and not statistically significant except at 24 h. CIC is reported to have inhibitory effects on angiotensin II. Angiotensin II mainly constricts efferent arterioles in the kidney, thus contributing to the maintenance of glomerular capillary pressure and glomerular filtration. Therefore, kidney-protecting effects of CIC might be due to the reduction of intraglomerular capillary pressure by blocking the action of angiotensin II.

In view of the action of PGI2 on glomerular filtration, CIC might worsen the renal outcome because PGI2 may increase glomerular capillary pressure through an action to dilate the afferent arteriole. Urinary PGI2, measured as 6-keto-PGF1α, and thromboxane B2 are reported to be increased in diabetic rats. Zola et al reported that thromboxane synthesis inhibition increases renal PGI2 and prevents renal disease progression in rats with remnant kidney. Therefore, stimulated PGI2 production does not necessarily worsen the renal outcome. Stimulated PGI2 generation or elimination of free radicals by CIC may protect the renal glomerular vascular endothelial cell injury and mesangial cells, although the mechanism remains to be further investigated.

The heart weight to body weight ratio in the CIC treated diabetic SHRIzm was lower than that in the vehicle-treated diabetic SHRIzm. Treatment with CIC is reported to prevent cardiac hypertrophy and athrosclerosis, not only through attenuating high blood pressure but also through direct inhibitory effects on actin formation and protein synthesis in cultured vascular smooth muscle cells from Dahl hypertensive rats. Also, CIC may have a cardiac-protective action that is not related to improvement of diabetes and improvement of high blood pressure in diabetic rats with hypertension, although we cannot completely deny that the decrease in heart weight to body weight ratio in CIC-treated diabetic SHRIzm is most likely the consequence of lower body weight in the vehicle-treated diabetic SHRIzm. Whether CIC has a direct cardioprotective effect should be further elucidated by histological analysis or by a molecular technique in this model.

The dose of streptozotocin used in the present study was same as that in our previous studies, and was lower than that in other workers’ studies. In the
present rat model of diabetes, to enable rats to survive during the long study periods, the administration of larger doses of STZ than those used in the present experiment and the supplementation of exogenous insulin to prevent extreme hyperglycemia is required. In our preliminary study, approximately 40% of the rats that were given 60 mg/kg of STZ without the administration of exogenous insulin died in the subsequent 9-week observation period. It is known that insulin has various actions besides controlling blood glucose. To avoid such actions of exogenously administered insulin, we administered a lower dose of STZ than were used in other workers’ studies. In the present study, diabetes in STZ-treated WKYIzm was more pronounced than that in STZ-treated SHRIzm during the study period. At the moment, we have no definite explanation for the discrepancy between the present results. In addition, previous data on similar level of diabetes were observed both in STZ-treated (35 mg/kg) SHR and STZ-treated (45 mg/kg) WKY. These may be due to the differences between species of animals or to experimental design, including the timing of STZ injection, inasmuch as feeding affects the diabetogenic effect of STZ and STZ injection time was changed to 12 pm for SHRIzm and to 6 pm for WKYIzm according to the preliminary experiment.

The study period of 12 weeks may be too short for the assessment of glomerulosclerosis in two-kidney diabetic rats. In our study, the diabetic rats were heminephrectomized and genetically hypertensive. Unilateral nephrectomy in rats with diabetes mellitus has been shown to accelerate markedly the development of diabetic glomerulopathy. In O’Donnell et al reported that focal glomerulosclerosis was evident (13.2%) in glomeruli from heminephrectomized diabetic rats and 0.5% in glomeruli from the nephrectomized controls and 0% in glomeruli from two-kidney diabetic rats in 8 to 12 week observation period. A significant decrease in urinary albumin excretion and in the IGS was achieved in our rats treated by CIC, which also suggest that the study period of 12 weeks may not be too short for the assessment of glomerulosclerosis in the rats we used.

In summary, we have clearly demonstrated that the subdepressor dose of CIC significantly decreased urinary albumin excretion, serum creatinine and blood urea nitrogen, and heart weight to body weight ratio in diabetic SHRIzm. It appears that CIC has a renoprotective action that is not related to improvement of diabetes or to improvement of high blood pressure in this model. These results were confirmed by morphological analysis of kidneys in each group of rats. The actions might be due to the reduction of intraglomerular capillary pressure or to the protection against renal glomerular vascular endothelial cell injury and mesangial cell injury through stimulation of PGL generation or elimination of free radicals, although the mechanism remains to be further investigated.

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