Thiazolidinedione Derivatives Ameliorate Albuminuria in Streptozotocin-Induced Diabetic Spontaneous Hypertensive Rat

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Recent reports have shown that thiazolidinediones have preventive effects on urinary albumin excretion in diabetes. However, the mechanism leading to these effects has not yet been elucidated. We studied here the effects of thiazolidinediones on albuminuria and hemodynamic and morphological changes in the kidneys of streptozotocin (STZ)-induced diabetic spontaneous hypertensive rats (SHRs). Diabetes was induced in SHRs by intravenous injection of STZ (50 mg/kg). The diabetic SHRs were divided into the following 3 groups: (1) STZ-SHRs given normal chow (STZ), (2) STZ-SHRs given chow mixed with 0.1% troglitazone (STZ + tro), and (3) STZ-SHRs given chow mixed with 0.001% pioglitazone (STZ + pio). Three groups of nondiabetic SHRs were also investigated: (4) SHR, (5) tro, and (6) pio. We evaluated the urinary albumin excretion rate (AER) every 4 weeks. After 12 weeks of treatment, the animals were killed and renal morphological examinations were performed. Thiazolidinediones did not affect blood pressure or blood glucose levels. Urinary AER were markedly increased in STZ-SHRs. After 12 weeks of treatment with thiazolidinediones, the urinary AER was significantly decreased while creatinine (Cr) clearance was left unchanged. Histologically, the loss of anionic sites of glomerular basement membranes (GBM) evaluated with polyetyleneimine was suppressed significantly in the diabetic SHRs treated with thiazolidinediones. In conclusion, administration of thiazolidinediones in diabetic SHRs decreased the urinary AER and suppressed the loss of anionic sites of GBM without affecting blood pressure, blood glucose levels, or Cr clearance. These results clarify the novel therapeutic action of thiazolidinediones on diabetic nephropathy.

DIABETIC NEPHROPATHY is a serious complication in diabetic subjects. The presence of microalbuminuria in diabetic subjects predicts an increased risk for clinical proteinuria[1,2] and diabetic subjects with microalbuminuria also show a higher incidence of cardiovascular events.[1,3,4] Therefore, it is important to appropriately treat subjects with microalbuminuria as soon as they are identified.[5] As examples of appropriate treatments, it has been suggested that strict glycemic control[6,7] and the administration of angiotensin-converting enzyme (ACE) inhibitors[8,9] ameliorate microalbuminuria in diabetic subjects.

The mechanism by which diabetic nephropathy occurs is probably multifactorial based on the chronic metabolic disturbance. Chronic hyperglycemia has been postulated to cause glomerular dysfunction via various mechanisms such as the activation of the polyol-pathway,[10] increased rates of diacylglycerol synthesis with the activation of protein kinase C (PKC), and accumulation of advanced glycation end products.[11,12] A growing number of studies have shown that the pharmacological inhibition of these abnormalities can ameliorate the glomerular dysfunction.[11-13]

Troglitazone [(±)-5-[4-(6-hydroxy-2,5,7,8-tetramethylchroman-2-yl-methoxy)benzyl]-2,4-thiazolidinedione] and pioglitazone {5-[4-[2-(5-ethyl-2-pyridil)ethoxy]benzyl]-2,4-thiazolidinedione}, two thiazolidinedione derivatives (TZDs), have been shown to decrease plasma glucose levels by improving insulin resistance, a main pathophysiological feature of type 2 diabetes mellitus.[14-16] Other effects that seem to be independent of the reduced blood glucose levels have recently been reported including the inhibition of PKC-β and the retardation of microalbuminuria.[17-21] An inhibitory effect of TZDs on PKC might be one of the mechanisms of amelioration of microalbuminuria.[20,21] However, there has been a report demonstrating a retardation of microalbuminuria without an improvement in the glomerular filtration rate,[17] so it is entirely possible that the diabetes-induced glomerular dysfunctions may be ameliorated by unknown mechanisms such as a loss of negative charge of the glomerular basement membranes (GBM) induced by decreasing anionic sites, endothelial hyperpermeability, and increased synthesis of certain growth factors.

On the other hand, hypertension frequently co-exists in type 2 diabetic subjects[22] and could be another risk factor accelerating cardiovascular and renal complications.[23,24] Also, the magnitude of renal complications tends to be more progressive in subjects suffering from diabetes and hypertension concurrently than in those with either condition alone.[25,26] Therefore, the pharmacological therapy used to treat diabetic subjects with hypertension should be important.

In this study, we evaluated the effects of two TZDs (troglitazone and pioglitazone) on the development of albuminuria in streptozotocin (STZ)-induced spontaneous hypertensive rats (SHRs). Because troglitazone has not only TZD moiety but also α-tocopherol moiety, a chromane ring, in its structure. α-Tocopherol was reported to be capable of preventing the activation of the diacylglycerol-PKC pathway and to improve early glomerular dysfunction in diabetic rats.[27] Thus, we also have evaluated the effect of pioglitazone, which does not have an α-tocopherol moiety. On the other hand, We also investigated the effects of TZDs on the morphological changes of glomeruli, including the number of anionic sites of the GBM.

MATERIALS AND METHODS

Materials

Insulinopenic diabetes was induced in male SHRs (Funabashi Farm, Chiba, Japan) (8 weeks old, body weight 200 to 220 g, n = 30) by

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intravenous injection of STZ (50 mg/kg; Sigma, St Louis, MO). One week after STZ injection, we confirmed that the blood glucose levels of the diabetic rats were over 300 mg/dL and then started this study. The diabetic SHRs were divided into the following 3 groups (n = 5): (1) STZ-SHRs given normal chow (Oriental Yeast Co, Tokyo, Japan) (STZ), (2) STZ-SHRs given chow mixed with 0.1% troglitazone (Sankyo Pharmaceutical, Tokyo, Japan) (STZ + tro), and (3) STZ-SHR given chow mixed with 0.001% pioglitazone (Takeda Chemical Industries, Osaka, Japan) (STZ + pio). Three groups (n = 5) of nondiabetic SHRs were also investigated: (4) SHR, (5) tro, and (6) pio.

Blood Sampling and Analysis

Blood samples were obtained from the tail vein before and every 4 weeks after induction of diabetes, and body weights were monitored. Blood glucose levels were measured by the glucose oxidase method. The residual blood samples were centrifuged, and serum was frozen at –70°C for subsequent measurement of creatinine (Cr) and insulin levels. Serum Cr levels were measured by a quantitative colorimetric assay (BML, Tokyo, Japan). Serum insulin levels were measured by an enzyme-linked immunosorbent assay using an anti-rat insulin antibody (Rat Insulin ELISA, Mercodia, Uppsala, Sweden).

Blood Pressure Measurement

Systolic blood pressure (sBP) was measured by indirect tail-cuff plethysmography (BP-98A, Softron, Tokyo, Japan) in unanesthetized rats before and every 4 weeks after the induction of diabetes.

Urinary Albumin Excretion Rate

Urinary albumin excretion rate (AER) was assessed before and every 4 weeks after the induction of diabetes. After housing the animals in individual metabolic cages for 24 hours, their 24-hour urine samples were collected, quantified, and frozen at –70°C for later analysis of albumin and Cr levels. Urinary albumin levels were determined by an enzyme-linked immunosorbent assay using an anti-rat albumin antibody (Nephrat, Exocell Inc, Philadelphia, PA). Urinary Cr levels were measured by a quantitative colorimetric assay (BML).

Morphological Analysis

Mesangial expansion. After 12 weeks of administration of TZDs, animals were killed. Mesangial expansion was evaluated by determining the ratio of mesangial area (MA) to glomerular tuft area (GTA). Right kidneys were fixed in 10% buffered formalin and embedded in paraffin. Light microscopy was performed on periodic acid–Schiff (PAS)-stained specimens in 6-μm serial sections containing 40 to 60 glomeruli. MA and GTA were quantitatively analyzed by using NIH image 1.58. We randomly examined 10 glomeruli and determined the average MA to GTA ratio (MA/GTA) in each rat.

Anionic sites on the lamina rara externa. To evaluate the effects of TZDs on the number of anionic sites of GBM, the animals were injected in the tail vein with 2 mL/kg of 0.5% polyetyleneimine (PEI; molecular weight 70,000; Wako Pure Chemical Ind, Osaka, Japan) solution adjusted to pH 7.3 with HCl and 400 mOsm with NaC.[28] Fifteen minutes after injection, the bilateral kidneys were removed and weighed. Then small tissue blocks of the left renal cortex (<1 mm^3) were taken and immersed in 0.1% glutaraldehyde-2% phosphotungstic acid solution. After 1 hour, the tissue blocks were washed in cacodylate buffer (pH 7.3, 400 mOsm) for 10 minutes 3 times consecutively, and then postfixed in 1% OsO_4 at 4°C for 2 hours. After the tissues were embedded in Epon 812, ultrathin sections were prepared and stained with 10% uranyl acetate and lead citrate for 10 minutes. Once stained, the ultrathin sections were examined in a Hitachi H-600 transmission electron microscope (Hitachi, Tokyo, Japan). The anionic sites were identified as particles intensively stained with PEI. The number of anionic sites per 1,000 nm of lamina rara externa (LRE) of GBM was determined from electron micrographs (an average of 10 random visual fields) in each rat at a final magnification of 100,000×. The magnification of electron micrograph was 40,000× and the negatives were enlarged 2.5 times. The counting of anionic sites was performed by 2 observers blinded for the treatment groups.

Statistical Analysis

All data were expressed as the mean or the mean ± SEM. Statistical analyses were performed using analyis of variance (ANOVA) and Scheff's *F*-test. *P* values less than .05 were considered statistically significant.

RESULTS

Nondiabetic SHRs increased in body weight after 4 weeks of treatment, only the diabetic SHRs had lower body weights than the nondiabetic SHRs, but there were no significant differences among the 3 diabetic groups (Table 1). The sBP significantly increased after 4 weeks of treatment in all groups. Diabetic SHRs had significantly lower sBP than nondiabetic SHRs. The administration of TZDs did not affect sBP (Table 1).

Blood glucose levels increased in STZ-treated SHRs after 4 weeks of treatment, while there were no significant differences among the groups (Table 1). We confirmed that the STZ-injected SHRs to be in an insulinopenic state because serum insulin levels were decreased to less than 0.1 ng/mL at 12 weeks of treatment. In nondiabetic SHRs, troglitazone and pioglitazone did not affect the serum insulin levels at 12 weeks of treatment.

During the observation period, mean creatinine clearance (Ccr) values were increased in diabetic SHRs (no significant difference). TZDs did not affect Ccr levels in diabetic SHRs (Table 1). The results of AER are shown in Fig 1. AER levels were slightly increased in the nondiabetic SHRs, but there were no significant differences among the 3 groups. AER levels were significantly higher in the diabetic SHRs than in the nondiabetic SHRs after 4 weeks of the treatment ($P < .05$). At 12 weeks of treatment, both TZDs significantly suppressed AER levels of the diabetic SHRs. No significant difference was shown between troglitazone and pioglitazone in inhibitory effects on AER.

Morphological Evaluation

Mesangial expansion. The values of MA/GTA are listed in Table 2. MA/GTA was significantly higher in diabetic SHRs than in nondiabetic rats, while TZDs did not suppress the increase of MA/GTA in the former.

Anionic sites on LRE of GBM. The representative anionic sites on LRE of GBM are shown in Fig 2. In untreated diabetic SHRs, anionic sites were scanty and their arrangement was irregular compared with those in nondiabetic SHRs. On the other hand, the anionic sites in diabetic SHRs treated by TZDs were plentiful and arranged regularly, almost like those in nondiabetic SHRs. To evaluate the change of anionic sites quantitatively, we counted the number of anionic sites per 1,000 nm of LRE of GBM (Table 3). In untreated diabetic SHRs, the number of anionic sites was significantly decreased as compared with that in nondiabetic SHRs. On the other hand,

Table 1. Changes in Body Weight, sBP, Blood Glucose, and Ccr in Nondiabetic, Untreated Diabetic, and Treated Diabetic SHRs

	Weeks of Treatment			
	0	4	8	12
BW (g)				
SHR	212 ± 3.34	297 ± 2.81	350 ± 2.73	383 ± 4.23
STZ	209 ± 3.37	216 ± 7.90*	236 ± 12.4*	244 ± 14.1*
STZ + tro	212 ± 2.11	229 ± 8.90*	247 ± 12.9*	280 ± 8.05*
STZ + pio	207 ± 2.79	194 ± 13.2*	207 ± 20.1*	257 ± 8.05*
sBP (mm Hg)				
SHR	143 ± 4.08	197 ± 4.39	176 ± 6.61	184 ± 3.87
STZ	137 ± 3.96	163 ± 4.69*	163 ± 3.93	170 ± 1.67
STZ + tro	138 ± 6.95	160 ± 3.86*	166 ± 3.96	170 ± 1.96
STZ + pio	131 ± 7.08	160 ± 4.38*	162 ± 3.08	168 ± 2.00
Blood glucose (mg/dL)				
SHR	156 ± 7.73	134 ± 8.53	124 ± 6.85	115 ± 5.70
STZ	144 ± 5.34	500 ± 26.7*	482 ± 38.5*	491 ± 32.1*
STZ + tro	139 ± 6.49	425 ± 37.1*	386 ± 25.9*	423 ± 56.7*
STZ + pio	145 ± 6.00	473 ± 25.7*	403 ± 26.1*	398 ± 51.9*
Ccr (mL/min/kg)				
SHR	4.40 ± 0.24	4.43 ± 0.24	3.64 ± 0.21	4.77 ± 0.26
STZ	4.65 ± 0.36	5.31 ± 0.25	4.75 ± 0.13	5.73 ± 0.30
STZ + tro	4.52 ± 0.36	6.52 ± 0.43	5.86 ± 1.31	5.43 ± 0.32
STZ + pio	4.44 ± 0.58	5.72 ± 0.61	6.12 ± 0.58	5.76 ± 0.35

NOTE. Data are the mean ± SEM.

Abbreviations: sBP, systolic blood pressure; Ccr, creatinine clearance.

*$P < .05$ *v* nondiabetic.

TZDs prevented the decreases of anionic sites. There was no significant difference between troglitazone and pioglitazone.

DISCUSSION

Troglitazone and pioglitazone, ligands for peroxisome proliferator–activated receptor-γ (PPAR-γ), are classified as TZDs and newly developed insulin sensitivity-enhancing agents. Besides lowering blood glucose levels, beneficial effects for the lipid profile and blood pressure have been reported.[14,15] Thus far, we have reported that TZDs suppressed the cytokine-induced production of monocyte chemoattractant protein-1 from cultured mesangial cells, and assumed that TZDs might ameliorate the progression of the diabetic nephropathy.[29] In this study, we demonstrated that the administration of TZDs (troglitazone and pioglitazone) prevented the increase of urinary albumin excretion and the loss of anionic sites on GBM in STZ-induced diabetic SHRs after 12 weeks of treatment. Two different TZDs were used, because troglitazone has not only TZD moiety but also α-tocopherol moiety, a chromane ring, in its structure. α-Tocopherol has been reported to have a beneficial effect on diabetic nephropathy.[27] Thus, we also have evaluated the effect of pioglitazone, which does not have an α-tocopherol moiety. Our results that both TZDs have the same effects indicate that TZD itself has an inhibitory potential on the development of diabetic nephropathy.

Although several recent reports have shown that TZDs may ameliorate albuminuria induced by hyperglycemia in vivo,[17-20] the mechanism of this effect remains unclear. Since the STZ-

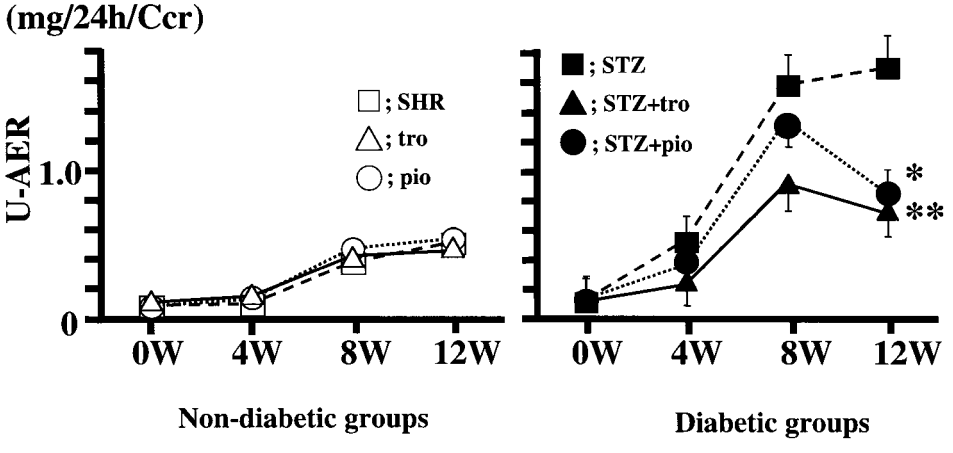

Fig 1. Changes in urinary AER in each group. There were no significant differences among nondiabetic rats at any point. The AER levels were significantly higher in diabetic *v* nondiabetic rats after 4 weeks of treatment. At 12 weeks of treatment, AER of treated diabetic rats was suppressed to significantly lower levels than that of untreated diabetic rats. There was no significant difference between the 2 treated diabetic groups. Data are mean ± SEM. (*$P < .05$ *v* STZ, **$P < .01$ *v* STZ).

Table 2. Ratio Between MA and GTA in Each Group

Rat Group	No. of Animals	MA/GTA
SHR	5	0.165 ± 0.004
tro	5	0.174 ± 0.008
pio	5	0.151 ± 0.012
STZ	5	0.237 ± 0.004*
STZ + tro	5	0.238 ± 0.009*
STZ + pio	5	0.245 ± 0.006*

NOTE. Data are the mean ± SEM.
Abbreviations: MA, mesangial area; GTA, glomerular tuft area.
*$P < .05$ *v* SHR.

Table 3. Number of Anionic Sites on the Lamina Rara Externa in Each Group

Rat Group	No. of Animals	No. of Anionic Sites/1,000 nm
SHR	5	20.8 ± 1.23
tro	5	22.6 ± 0.66
pio	5	20.9 ± 0.58
STZ	5	16.4 ± 0.61*
STZ + tro	5	22.1 ± 0.77**
STZ + pio	5	22.3 ± 0.71**

NOTE. Data are the mean ± SEM.
Abbreviation: LRE, lamina rara externa.
*$P < .05$ *v* SHR.
†$P < .01$ *v* STZ.

induced diabetic SHRs used in this study were in an insulinopenic state, TZDs neither affected their blood glucose nor insulin levels. Moreover, since TZDs were also shown not to change their blood pressures, these factors were not thought to affect our results. Progressing hyperfiltration is well recognized as one of the functional changes in the early stage of diabetic nephropathy. Findings on Ccr in this study suggested that TZDs did not prevent the progression of hyperfiltration. We evaluated MA/GTA ratios as the marker of mesangial expansion. The ratios were significantly higher in diabetic groups, while TZDs did not suppress the mesangial expansion. It is well established that mesangial expansion is the important structural change in diabetic nephropathy.[30,31] These results show that the inhibitory effects of TZDs on albuminuria may not have been due to the improvement of renal hemodynamics.

This study also showed that TZDs inhibited the loss of anionic sites of GBM. This is the first report to describe an effect of TZDs on the anionic sites. We assume that the preventive effect of TZDs on the loss of anionic sites is one of the major factors of the amelioration of albuminuria in diabetic rats. Anionic sites consist of glycosaminoglycans and are rich in heparan sulfate proteoglycans (HSPG).[32-34] These anionic sites of the GBM regulate the transudation of circulating macromolecules across the GBM as a charge-selective filtration barrier; hence, their loss leads to an increase in the permeation of anionic proteins such as ferritin and albumin.[35,36] Many reports on rats and human kidneys suggest that the content of HSPG in GBM is decreased in diabetic nephropathy.[37,38] It has been suggested that the diminution of HSPG on GBM under a hyperglycemic state is due to a suppression of HSPG synthesis on the LRE of GBM and insufficient sulphation of HSPG. In par-

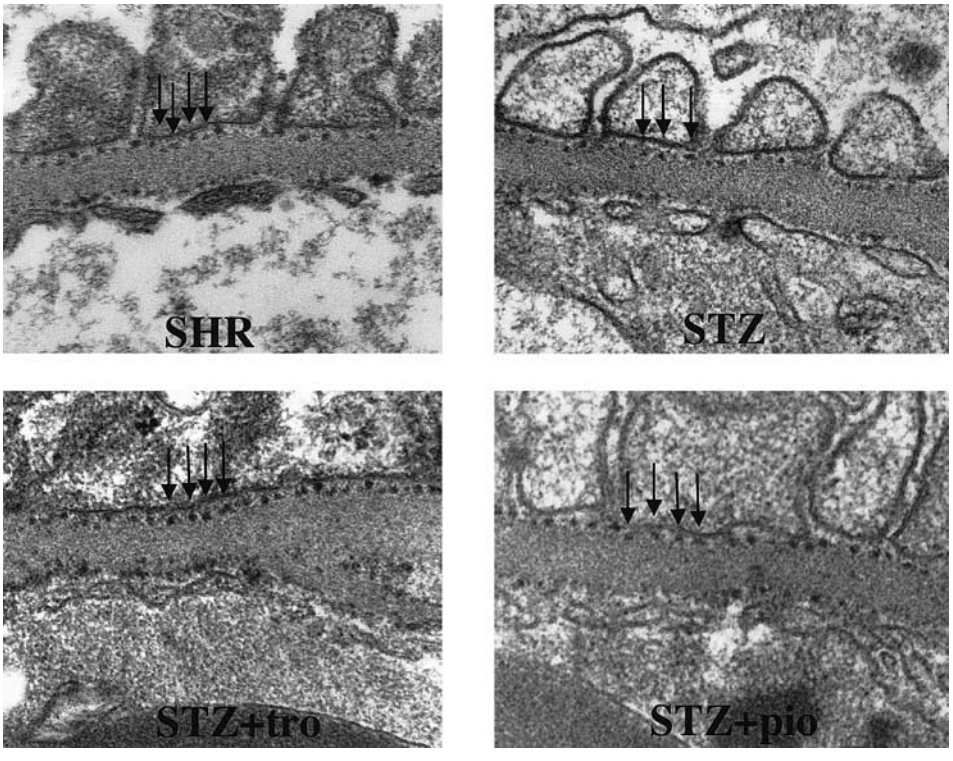

Fig 2. Electron micrographs of GBM at 12 weeks of treatment. Arrows show anionic sites on the LRE of GBM. In the STZ group, anionic sites were scanty and their arrangements were more irregular than those in SHRs. In the STZ + tro and STZ + pio groups, anionic sites were plentiful and arranged regularly, resembling to those of SHRs. (Original magnification × 100,000).

ticular, the hyperglycemia-induced inhibition of glucosaminyl *N*-deacetylase, a key enzyme in HSPG synthesis, has been shown to lead to low-sulfated HSPG molecules.[39-41] HSPG molecules on the LRE of GBM were synthesized in the mesangial cells as well as in the epithelial cells of glomeruli. Therefore, it is possible that the functional changes of mesangial cells by hyperglycemia may induce the diminution of HSPG. On the other hand, the glomerular expansion, the primary morphological change of glomeruli induced by hyperglycemia, makes the distribution of HSPG sparse. Since there was no significant difference in the MA/GTA ratios among diabetic treatment groups in this study, it is unlikely that the inhibitory effects of TZDs on loss of anionic sites were caused by morphological changes of glomeluri. Thus, TZDs may inhibit the loss of anionic sites via improvements in HSPG synthesis, HSPG sulfation, or mesangial cell function. The mechanism by which TZDs preserve the anionic sites remains to be investigated.

Hypertension is another risk factor accelerating cardiovascular and renal complications, and hypertension frequently coexists with diabetes in type 2 diabetic subjects .[22] The magnitude of renal dysfunction in diabetic subjects with hypertension tends to be more progressive.[23-26] Therefore, pharmacological treatment for patients of this type will likely prove to be important. In this study, we showed that TZDs ameliorate albuminuria in diabetic hypertensive models.

In conclusion, we have revealed that the administration of troglitazone and pioglitazone reduced albuminuria and inhibited the loss of anionic sites on the LRE of GBM in STZ-induced diabetic SHRs. These results suggest that TZDs may be useful to ameliorate diabetic nephropathy via direct effects on the anionic sites of GBM.

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