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# Spironolactone prevents early renal injury in streptozotocin-induced diabetic rats

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**Spironolactone prevents early renal injury in streptozotocin-induced diabetic rats.**

*Background.* Glomerular and tubulointerstitial injury leads to chronic impairment of renal function, and thus, reversal of the injury may improve renal function and survival. The present study investigated whether and how mineralocorticoid receptor antagonist spironolactone ameliorates early renal injury in streptozotocin-induced diabetic rats.

*Methods.* Streptozotocin (65 mg/kg, single intraperitoneal injection)- or vehicle-administered rats were used as diabetic or control rats, respectively. The streptozotocin-administered rats were treated with spironolactone (50 mg/kg/day sc) for 3 weeks. Among the 3 groups of rats, we compared renal fibrosis and renal hypertrophy, using picro-sirius red staining and immunohistochemistry of ED-1 macrophage marker, plasminogen activator inhibitor-1 (PAI-1), and transforming growth factor (TGF)-β1.

*Results.* Three weeks after administration of streptozotocin, rats exhibited increased collagen deposition in glomerular, tubulointerstitial, and perivascular areas in the kidney, which was completely attenuated by spironolactone treatment. In rats given streptozotocin alone, there were increases in ED-1–positive cell, PAI-1 expression, and TGF-β1 expression in glomeruli and tubulointerstitiums, which were also suppressed by spironolactone treatment. Maximal glomerular and proximal tubular areas were not significantly different among the 3 groups. Rats given streptozotocin alone revealed an increase in proximal tubule wall-to-lumen ratio that was not influenced by treatment with spironolactone.

*Conclusion.* Streptozotocin-induced renal fibrosis, PAI-1 expression, TGF-β1 expression, and macrophage infiltration occur via mineralocorticoid receptor, and spironolactone ameliorates renal fibrosis presumably via the inhibition of macrophage infiltration, PAI-1 expression, and TGF-β1 expression in streptozotocin-induced early diabetic injury.

**Key words:** mineralocorticoid antagonist, diabetes mellitus, renal fibrosis, macrophage infiltration, plasminogen activator inhibitor-1, transforming growth factor-β1.

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Diabetic nephropathy, a common complication in patients with either type 1 or type 2 diabetes mellitus, has long been recognized to cause severe morbidity and mortality. The renal structural alterations in susceptible patients are characterized by the early appearance of renal hypertrophy, the progressive accumulation of extracellular matrix in the glomerulus and tubulointerstitium, and consequently, glomerulosclerosis and tubulointerstitial fibrosis [1, 2]. Because glomerulosclerosis and tubulointerstitial fibrosis cause renal dysfunction, reversal of these changes may improve kidney function and survival. Current therapeutic approaches to the reversal of progressive renal fibrosis in diabetes have been tried, but have been relatively ineffective. On the other hand, a mineralocorticoid receptor (MR) antagonist, spironolactone, has been shown to prevent cardiovascular injury [3–5], including diabetic renal injury [6]. Miric et al [6] reported that 8 weeks after single streptozotocin (STZ) administration, rats revealed increases in tubulointerstitial collagen volume fraction and kidney fibronectin concentrations, which were reversed by treatment with spironolactone for 4 weeks starting 4 weeks after STZ administration. However, the mechanisms of action of spironolactone are lacking.

Transforming growth factor (TGF)- β1 is known to increase extracellular matrix synthesis, inhibit matrix degradation, up-regulate adhesion molecules, and enhance chemoattraction [7, 8]. Increased TGF-β1 expression has been reported in experimental diabetic kidneys and human diabetic patients [9–13], and TGF-β1 is an important mediator in the diabetic renal injury [11–13]. The renoprotective effects of angiotensin I converting enzyme (ACE) inhibition have been associated with reductions in renal TGF-β1 expression in experimental diabetic nephropathy [14, 15]. However, it is not known whether spironolactone affects TGF-β1 expression in diabetic kidneys.

Plasminogen activator inhibitor-1 (PAI-1) is the major physiologic inhibitor of plasminogen activators (tissue-type plasminogen activator and urokinase-type

plasminogen activator) in vivo [16], and has been implicated in extracellular matrix accumulation by its effects to inhibit matrix degradation [17, 18]. PAI-1 synthesis is stimulated by TGF-β in normal and nephritic glomeruli [19]. Angiotensin II and aldosterone stimulate expression of PAI-1 in vitro in a number of cell types [20–23]. Inhibition of ACE prevented PAI-1 expression and perivascular fibrosis in a rat model with cardiovascular remodeling induced by chronic inhibition of nitric oxide synthesis [24]. Angiotensin II type 1 (AT1) receptor antagonist inhibited angiotensin II–induced PAI-1 expression in the kidney [25]. However, it is not known whether spironolactone can also act by these mechanisms in diabetic nephropathy.

Experimental evidence has consistently demonstrated an important role for macrophage in the development of both glomerular and tubulointerstitial injury [26]. Infiltrated macrophages release lysosomal enzymes, nitric oxide, reactive oxygen intermediates, and TGF-β, which have been reported to play an essential role in renal damage [27–29]. However, while the presence of macrophage has been noted in both the glomerulus and tubulointerstitium in human diabetic nephropathy [30], the mechanisms which lead to their infiltration in diabetes are not well understood. Recently, Kelly et al [31] used transgenic diabetic (mRen-2)27 rats treated with STZ, and found that the tubulointerstitial injury in diabetic nephropathy is implicated in osteopontin expression and macrophage accumulation, which were inhibited by treatment with a blockade of the ACE. However, whether the renoprotective effects of spironolactone in diabetes may be related to a reduction in macrophage infiltration has not yet been explored.

Short term-exposure to moderate and severe hyperglycemia can cause the pathologic alteration in resistance artery. Vranes et al [32] reported that vascular hypertrophy with collagen deposition was developed in superior mesenteric artery from rats 3 weeks after single STZ injection, and that this increase in blood vessel size is due to an increase in the medial smooth muscle layer of the vessel. This finding of vascular hypertrophy has been extended to include the renal vasculature [33]. Therefore, the present study used a diabetic rat model 3 weeks after single STZ administration, and examined whether and how spironolactone suppresses renal injury, including renal fibrosis and renal hypertrophy, using picro-sirius red staining and immunohistochemistry of ED-1, PAI-1, and TGF-β1.

## METHODS

### Animals

Experimental protocol for this study was approved by Animal Ethics Committee of Jichi Medical School. Male Sprague-Dawley rats (Japan SLC, Tokyo, Japan) weighing 180 to 200 g were divided into 3 groups ($N = 6$/group) after measuring of body weight. Group 1 was given 65 mg/kg of STZ dissolved with 0.01 mol/L citrate buffer (pH 4.5) (single intraperitoneal injection). Group 2 was similarly given with an equal volume of citrate buffer. Group 3 was subcutaneously treated with 50 mg/kg of spironolactone (Pharmacia Corp., Peapack, NJ, USA) dissolved in sesame oil for 21 days after STZ administration. Groups 1 and 2 were also given an equal volume of sesame oil in a similar manner.

Systolic blood pressures were measured by tail-cuff plethysmography (Softran, Tokyo, Japan) before and 7, 14, and 20 days after vehicle or STZ administration. On study day 20, individual rats were placed in metabolic cages to obtain 24-hour urine collections for measurements of urine protein and albumin concentrations. The urinary protein and albumin concentrations were recorded with urinary creatinine concentration. On study day 21, after determination of the body weight, animals were decapitated. Collected trunk blood was used for determination of plasma glucose, creatinine, and aldosterone concentrations. Plasma aldosterone concentrations were measured by a radioimmunoassay kit (Abbott Japan, Tokyo, Japan). The right kidney was then removed and weighed. Upper third portion of the kidney was transversely cut and was stocked in 3.7% formaldehyde solution. Specimens were embedded in paraffin and cut into 5-μm thick slices transversely on a microtome (Leica Corp., Nussloch, Germany).

### Histology and immunohistochemistry

The kidney tissues were used for hematoxylin and eosin, picro-sirius red staining, and immunohistochemistry of ED-1 macrophage marker, PAI-1 protein, and TGF-β1 protein (avidin-biotin peroxidase complex method; Vectastain Elite ABC kit, Vector Laboratory Inc., Burlingame, CA, USA). For the 3 immunohistochemistries, sections were incubated for 16 hours with rabbit polyclonal antibodies against ED-1 (1:200; Serotec, Oxford, UK), PAI-1 (1:200; American Diagnostica, Inc., Greenwich, CT, USA), and TGF-β1 (1:50; Santa Cruz, CA, USA). Normal rabbit IgG was used as a negative control in the staining. The number of ED-1–positive cells in tubulointerstitium was determined according to the method described by Mai et al [34]. A semiquantitative score of PAI-1 immunostaining was used to evaluate the degree of PAI-1 protein expression in glomerulus and tubulointerstitium according to the method described by Brown et al [35] and Zang et al [36], respectively. The TGF-β1 immunostaining was also scored to analyze the degree of TGF-β1 protein expression in glomerulus and tubulointerstitium according to the methods described by Shankland et al [37] and Toblli et al [38], respectively. For the counting of ED-1–positive cells and the

**Table 1.** Pathophysiologic parameters in control rats, rats treated with streptozotocin (STZ) alone, and rats treated with STZ plus spironolactone (SPL)

| Parameter | P-Glu *mg/dL* | P-Cr *mg/dL* | P-Na *mmol/L* | P-K *mmol/L* | ΔBW *g* | Rt-KW/BW $\times 10^{-3}$ | U-V *mL/day* | U-Prot (mg/mg Cr) | P-Aldo (ng/dL) |
|---|---|---|---|---|---|---|---|---|---|
| Control | 122 ± 6 | 0.16 ± 0.02 | 127.3 ± 0.8 | 5.56 ± 0.25 | 76 ± 3 | 3.38 ± 0.08 | 11.3 ± 0.9 | 1.29 ± 0.31 | 27.7 ± 6.4 |
| STZ | 488 ± 52[b] | 0.14 ± 0.02 | 125.3 ± 3.2 | 5.67 ± 0.18 | 22 ± 11[b] | 5.16 ± 0.14[b] | 18.2 ± 2.4[a] | 1.05 ± 0.36 | 24.8 ± 7.4 |
| STZ+SPL | 587 ± 43[b] | 0.17 ± 0.02 | 124.0 ± 2.4 | 6.00 ± 0.38 | –16 ± 11[b,d] | 5.12 ± 0.17[b] | 24.9 ± 1.5[b,c] | 0.88 ± 0.16 | 90.0 ± 15.3[b,d] |

Abbreviations are: P-Glu, plasma glucose concentration; P-Cr, plasma creatinine; P-Na, plasma sodium; P-K, plasma potassium; ΔBW, change in body weight (+ΔBW and −ΔBW represent gain and loss of body weight, respectively); Rt-KW/BW, ratio of right kidney weight to body weight; U-V, urinary volume; U-Prot, urinary protein; P-Aldo, plasma aldosterone concentration. Values are mean ± SEM. The number of animals in each group was 6.
[a] $P < 0.05$; [b] $P < 0.01$ vs. control by ANOVA; [c] $P < 0.05$; [d] $P < 0.01$ vs. STZ by ANOVA.

scoring of PAI-1 and TGF-β1 immunostaining, 100 glomeruli (magnification, ×400) and 40 tubulointerstitial areas of the stained tissue within a given field without vessels or glomeruli (magnification, ×200) were selected at random and were determined by computerized image analysis system (Mac Scope, Fukui, Japan).

### Morphometric analysis

Sections stained with picro-sirius red were analyzed for collagen volume fraction in glomerular, tubulointerstitial, and perivascular areas with an image analysis system. For analyses of the glomerular and tubulointerstitial collagen volume fractions, 20 glomerular capsules and 20 fields without vessels or glomeruli were randomly selected from each kidney section, respectively. The glomerular and tubulointerstitial collagen volume fractions were then calculated as percentage of stained area within traced glomerular capsules and as percentage of total area within a field, respectively. Perivascular collagen volume fraction was measured as an area for a sample of the vessels in the section in order to correct differences in vessel size. Data were expressed as an area of perivascular collagen per media area.

Glomerular and tubular cross-sectional areas were decided in sections stained with hematoxylin and eosin. Thirty glomerular Bowman's capsules including tuft and outer surfaces of proximal tubules including brush border were traced, and the maximum cross-sectional areas were determined. Then, the ratio of the cross-sectional area of the outer surface of the proximal tubule to the cross-sectional area of the inner surface of the tubule was determined as a parameter of tubular hypertrophy.

### Statistics

Results are presented as mean ± SEM, and data were analyzed by Student unpaired *t* test or analysis of variance (ANOVA) for repeated measures, following by post-hoc analysis with Fisher test, where appropriate. $P < 0.05$ was considered statistically significant.

## RESULTS

Table 1 shows pathophysiologic parameters in the 3 groups of rats. Plasma glucose levels in rats given STZ alone (488 ± 52 mg/dL) were markedly increased as compared with control rats (122 ± 6 mg/dL). In rats given STZ plus spironolactone, they were 587 ± 43 mg/dL, values that were not significantly different from those in rats given STZ alone. Plasma creatinine levels were not significantly different among the 3 groups. Plasma sodium concentrations were without significant changes among the 3 groups. Plasma potassium concentrations in the rats given STZ plus spironolactone (6.00 ± 0.38 mmol/L) were slightly greater than those in control rats (5.56 ± 0.25 mmol/L) and in the rats given STZ alone (5.67 ± 0.19 mmol/L), but did not arrive at statistical significance. Table 2 shows systolic blood pressure in the 3 groups of rats for up to 20 days after the treatment with vehicle or STZ. Systolic blood pressure on study day 0, 7, 14, or 20 was not significantly different among the 3 groups. On the other hand, in rats given STZ alone, systolic blood pressure on study day 20 (126 ± 1 mm Hg) was slightly but significantly greater than that on study day 0 (116 ± 2 mm Hg). Body weight increase in rats given STZ alone (22 ± 11 g) was significantly smaller than in control rats (76 ± 3 g). In sharp contrast, body weight decrease (16 ± 11 g) was observed in rats given STZ plus spironolactone. In rats given STZ alone, kidney weight as a parameter of kidney hypertrophy was 5.16 ± 0.14 mg/g body weight, the value that was significantly greater than in control rats (3.38 ± 0.08 mg/g body weight). Treatment with spironolactone (5.12 ± 0.17 mg/g body weight) had no effect on STZ-induced increase in kidney weight. Urine volume in rats given STZ alone (18.2 ± 2.4 mL/day) was significantly greater than in control rats (11.3 ± 0.9 mL/day), whereas

**Table 2.** Systolic blood pressure in control rats, rats treated with streptozotocin (STZ) alone, and rats treated with STZ plus spironolactone (SPL) on study day 0, 7, 14, and 20

| Day | 0 | 7 | 14 | 20 |
|---|---|---|---|---|
| Control | 120 ± 3 | 118 ± 1 | 117 ± 2 | 120 ± 3 |
| STZ | 116 ± 2 | 119 ± 3 | 119 ± 3 | 126 ± 1[a] |
| STZ + SPL | 119 ± 2 | 117 ± 2.4 | 123 ± 2 | 118 ± 4 |

Values (mm Hg) are mean ± SEM; the number of animals in each group was 6.
[a] $P < 0.01$ vs. systolic blood pressure in rats given STZ alone on day 0 by ANOVA.

in rats given STZ plus spironolactone (24.9 ± 1.5 mL/day) it was significantly greater than in rats given STZ alone and control rats. Urinary protein excretion was without significant changes among the 3 groups. Urinary albumin concentrations in 2 rats given STZ alone were 0.815 and 0.787 mg/mg creatinine, but urine albumin was not detected in the remaining 4 rats given STZ alone. On the other hand, urine albumin was not detected in control rats or rats given STZ plus spironolactone. Plasma aldosterone concentrations in rats given STZ alone (24.8 ± 7.4 ng/dL) were not significantly different from those in control rats (27.7 ± 6.4 ng/dL), whereas in rats given STZ plus spironolactone, they were markedly increased (90.0 ± 15.3 ng/dL).

Figure 1 shows histologic findings by picro-sirius red staining of transverse kidney sections in control rats (Fig. 1A), rats given STZ alone (Fig. 1B), and rats given STZ plus spironolactone (Fig. 1C). In rats given STZ alone, periarteriolar fibrosis, tubulointerstitial fibrosis, and intraglomerular fibrosis were found. In marked contrast, in rats given STZ plus spironolactone, the STZ-induced periarteriolar fibrosis, tubulointerstitial fibrosis, and intraglomerular fibrosis were normalized. Figure 2 summarizes the alteration of collagen volume fraction in glomerular area (Fig. 2A), tubulointerstitial area (Fig. 2B), and perivascular area (Fig. 2C) of the right kidney in the 3 groups of rats, respectively. Glomerular collagen volume fraction in rats given STZ alone (4.43 ± 1.76%) was markedly increased as compared with control rats (0.71 ± 0.24%). In rats given STZ plus spironolactone, glomerular collagen volume fraction was 0.79 ± 0.12%, a value that was significantly smaller than in rats given STZ alone, but was not significantly different from that in control rats. Alteration of tubulointerstitial collagen volume fraction was markedly similar to that of the glomerular collagen volume fraction (Fig. 2B; control rats: 0.15 ± 0.02%; rats given STZ alone: 0.40 ± 0.07%; rats given STZ plus spironolactone: 0.18 ± 0.01%). Perivascular collagen volume fraction in rats given STZ alone was 1.48 ± 0.19%, significantly greater than in control rats (0.97 ± 0.07%). In rats given STZ plus spironolactone, perivascular collagen volume fraction was reduced to the control level by the treatment with spironolactone (0.99 ± 0.11%).

We next examined the number of ED-1–staining cells, and PAI-1 and TGF-β1 immunostaining in the 3 groups of rat kidneys. Figure 3A to C shows histologic findings of the ED-1–positive cells as a marker of macrophage in kidneys from control rats, rats given STZ alone, and rats given STZ plus spironolactone, respectively. In control kidney sections, very few macrophages were detected in glomeruli. In rats given STZ alone, macrophage infiltration was facilitated in glomeruli and tubulointerstitium. Treatment with spironolactone ameliorated the STZ-induced macrophage infiltration in both areas.

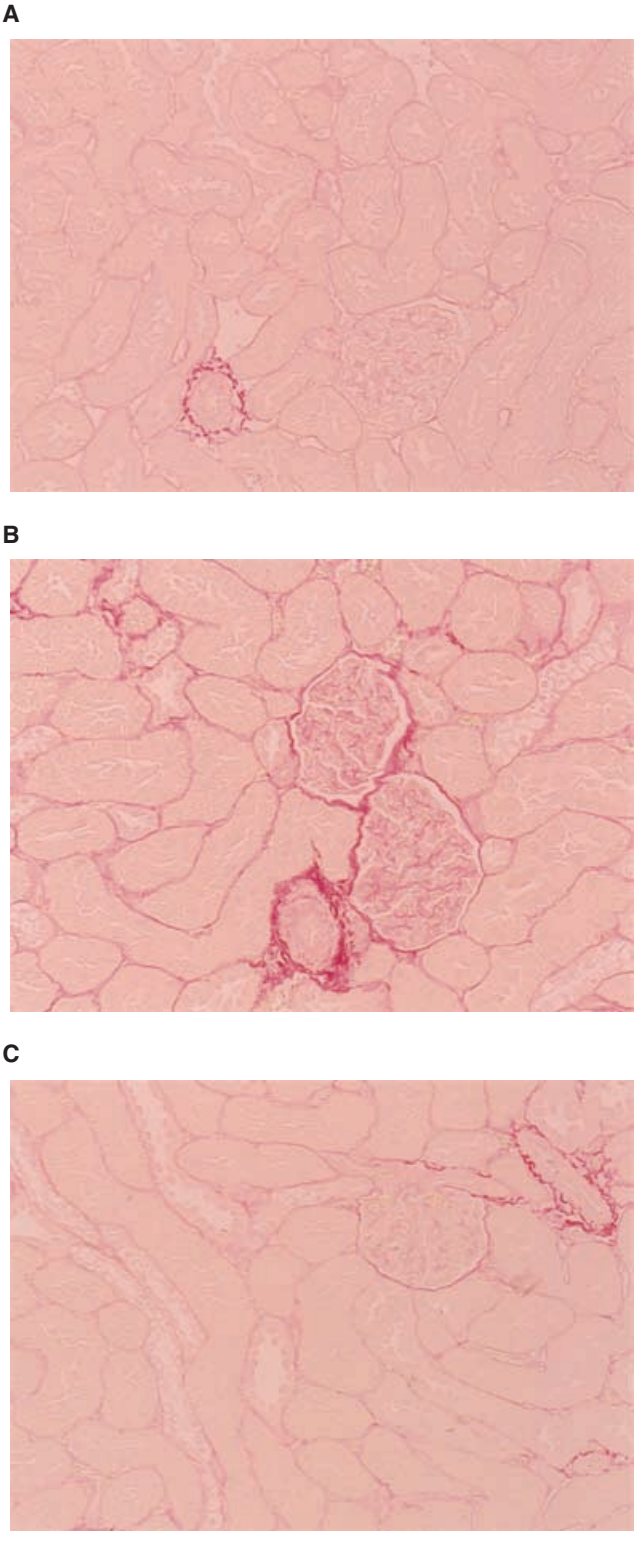


**Fig. 1. Histologic findings by picro-sirius red staining of transverse kidney sections in the control rats (*A*), rats given streptozotocin (STZ) alone (*B*), and rats given STZ plus spironolactone (SPL) (*C*).** Magnification, ×200.

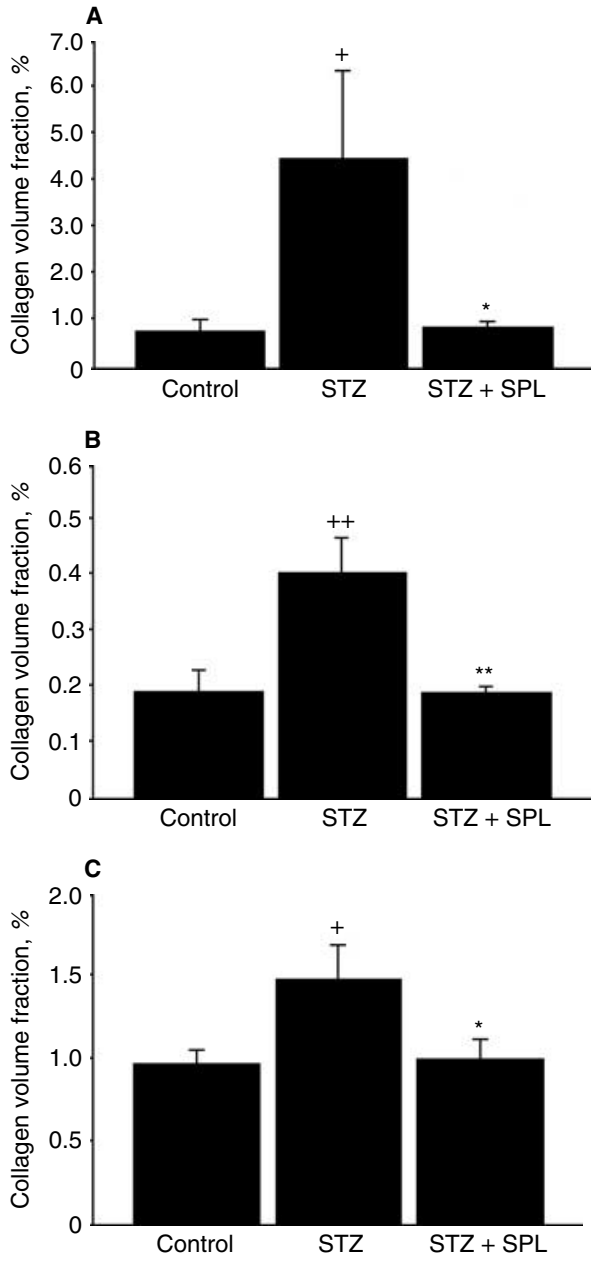


**Fig. 2. Collagen volume fraction determined by picro-sirius red staining in the three groups of rat kidneys.** (*A*) glomerulus; (*B*) tubulointerstitium; (*C*) perivascular area. Values are mean ± SEM. $^{+}P < 0.05$; $^{++}P < 0.01$vs. control; $^{*}P < 0.05$; $^{**}P < 0.01$ vs. STZ alone by ANOVA.

Figure 4A and B shows quantitation of macrophage number in glomeruli and tubulointerstitium from 3 groups of rat kidneys, respectively. In glomeruli of rats given STZ alone, the number of ED-1–positive cells was 3.57 ± 0.13/glomerulus, significantly greater than in control rats (1.21 ± 0.15/glomerulus). Treatment with spironolactone completely suppressed the STZ-induced increase in the number of ED-1–positive cells (1.67 ± 0.16/glomerulus). In tubulointerstitium of rats given STZ alone, the number of ED-1–positive cells was 1.42 ± 0.13/mm$^2$, significantly greater than in control rats (0.74 ± 0.13/mm$^2$). On the other hand, in tubulointerstitium of rats given STZ plus spironolactone, the number of ED-1–positive cells was 0.64 ± 0.10/mm$^2$, a value that was significantly smaller than that in rats given STZ alone, but was not significantly different from that in control rats. Figure 3D to F shows PAI-1 immunostaining in kidneys from control rats, rats given STZ alone, and rats given STZ plus spironolactone, respectively. In control kidney sections, PAI-1 immunostaining was detected in some tubulointerstitiums, whereas very few PAI-1 immunostainings were observed in glomeruli. In sharp contrast, in rats given STZ alone, PAI-1 immunostaining was increased diffusely in the tubulointerstitium and glomerulus. Treatment with spironolactone markedly suppressed the STZ-induced PAI-1 immunostaining in both areas; the suppression of PAI-1 immunostaining was especially more prominent in the glomerulus than in the tubulointerstitium. Figure 4C and D shows the alteration of the score of PAI-1 immunostaining in glomeruli and tubulointerstitium of each group, respectively. In glomeruli, the score in rats given STZ alone was increased to 1.90 ± 0.14 as compared with the control rats (0.72 ± 0.03). Spironolactone completely suppressed the STZ-induced increase in the score (0.96 ± 0.16). In tubulointerstitium of rats given STZ alone, the PAI-1 immunostainning area was significantly increased to 20.47 ± 1.79 (%/mm$^2$) as compared with that in control rats (5.93 ± 0.34%/mm$^2$). The PAI-1 immunostaining area in rats given STZ plus spironolactone (10.83 ± 1.46%/mm$^2$) was significantly smaller than that in rats given STZ alone, but was still significantly greater than that in the control rats. Figure 3G to I shows TGF-β1 immunostaining in kidneys from control rats, rats given STZ alone, and rats given STZ plus spironolactone, respectively. In control kidney sections, TGF-β1 immunostaining was detected in some glomeruli and tubulointerstitiums. The rats given STZ alone exhibited greater TGF-β1 immunostaining in glomeruli and tubulointerstitiums, whereas treatment with STZ plus spironolactone showed smaller TGF-β1 immunostaining areas in glomeruli and tubulointerstitiums. Figure 4E and F shows alteration of the score of TGF-β1 immunostaining in glomeruli and tubulointerstitium, respectively. In glomeruli, the score in rats given STZ alone was significantly increased to 1.42 ± 0.13 as compared with control rats (0.74 ± 0.13). Spironolactone completely suppressed the STZ-induced increase in the score (0.64 ± 0.10). In tubulointerstitium, the score of TGF-β1 immunostaining in rats given STZ alone was 1.63 ± 0.17, a value that was significantly greater than in control rats (0.60 ± 0.08). In rats given STZ plus spironolactone, the score was 0.97 ± 0.02, a value that was significantly smaller than in rats given STZ alone, but was still significantly greater than in control rats.

To clarify the cause of increase in kidney weight in rats given STZ alone and in rats given STZ plus spironolactone, we finally determined the maximal glomerular

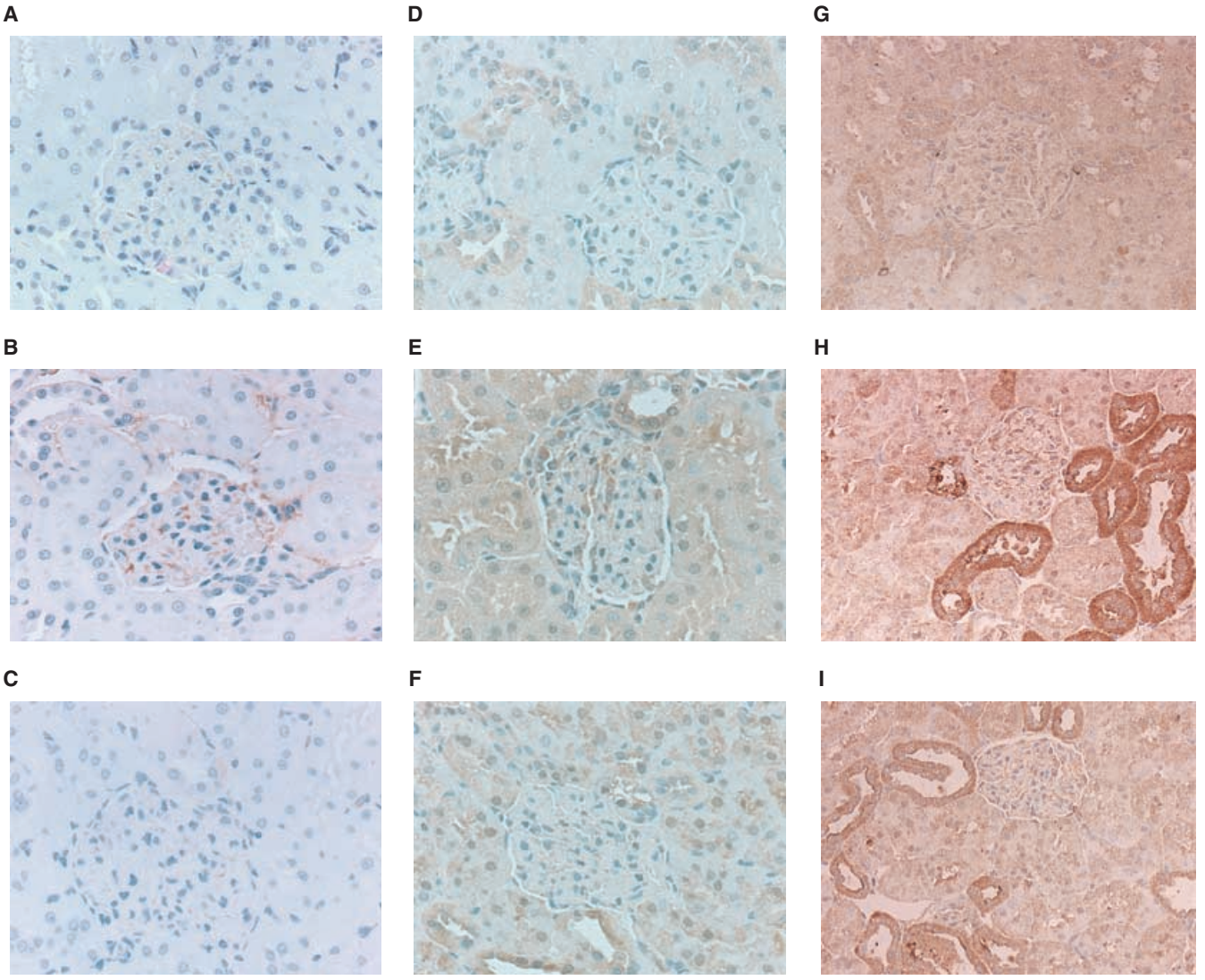


**Fig. 3. Immunohistochemical findings by anti-ED-1 antibody (*A-C*), anti-plasminogen activator inhibitor-1 (PAI-1) antibody (*D-F*), and anti-transforming growth factor-β1 (TGF-β1) antibody (*G-I*) in the three groups of rat kidneys.** (A), (D), and (G), control rats; (B), (E), and (H), rats given STZ alone; (C), (F), and (I), rats given STZ plus spironolactone. Magnification, ×400.

Bowman's capsular area and proximal tubular cross-sectional area, and the proximal tubule wall-to-lumen ratio, as shown in Figure 5. There were no significant changes of maximal glomerular Bowman's capsular area or proximal tubular cross-sectional area among the 3 groups. In marked contrast, the proximal tubule wall-to-lumen ratio as a parameter of tubular hypertrophy in rats given STZ alone was increased to 23.8 ± 0.2 as compared with the control rats (12.6 ± 0.9). Spironolactone had no effect on STZ-induced increase in the ratio (24.7 ± 3.5).

## DISCUSSION

Eight weeks after single STZ injection, rats have been reported to exhibit hyperglycemia and renal fibrosis [6]. In this model, spironolactone had an antifibrotic effect on the kidney [6], a finding attributed to blockade of tissue MRs. The goal of the present study was to determine the mechanisms by which hyperglycemic state induces renal injury via MRs, especially the involvement of macrophage infiltration, PAI-1 expression, and TGF-β1 expression in the detrimental effects of the exposure to hyperglycemia on kidney. To do this, we used the MR antagonist spironolactone to examine the effects of the drug on hyperglycemia-induced hypertension, renal fibrosis, tubular hypertrophy, macrophage infiltration, PAI-1 expression, and TGF-β1 expression.

We observed that 3 weeks after the single STZ injection, there were significant increases in rats in blood glucose levels, kidney weight, as well as intraglomerular fibrosis, tubulointerstitial fibrosis, and periarteriolar fibrosis, and tubular hypertrophy. In rats given STZ alone, there were no significant increases in plasma creatinine levels or urinary protein excretion when compared with

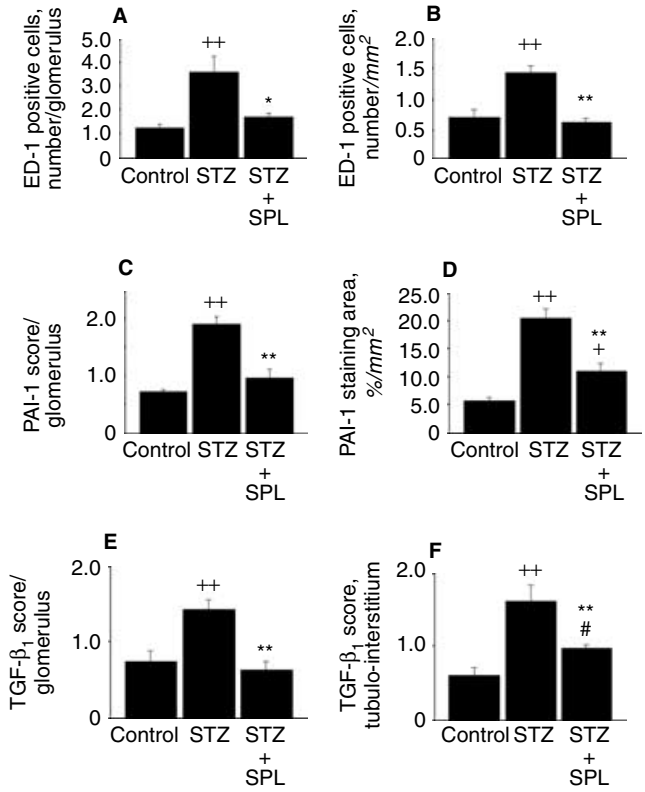


**Fig. 4. The number of ED-1–positive cells in glomerulus (*A*) and tubulointerstitium (*B*), the score of PAI-1 immunostaining area in glomerulus (*C*), the PAI-1 immunostaining area in tubulointerstitium (*D*), and the score of TGF-β1 immunostaining area in glomeruli (*E*) and tubulointerstitium (*F*) in the three groups of rat kidneys.** Values are mean ± SEM. $^{+}P < 0.05$; $^{++}P < 0.01$ vs. control; $^{*}P < 0.05$; $^{**}P < 0.01$ vs. STZ alone by ANOVA, $^{\#}P < 0.05$ vs. STZ alone by unpaired Student *t* test.

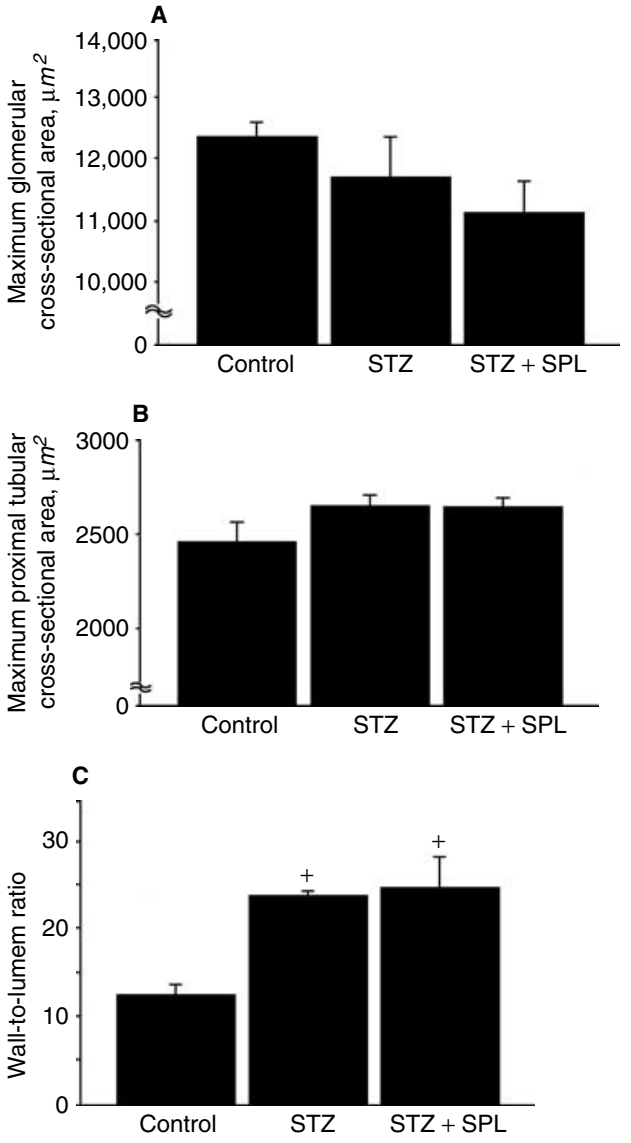


**Fig. 5. The maximal glomerular Bowman's capsule area (*A*), maximal proximal tubular area (*B*), and proximal tubule wall-to-lumen ratio (*C*) in the three groups of rat kidneys.** Values are mean ± SEM. $^{+}P < 0.01$ vs. control by ANOVA.

the control rats. In 2 of 6 rats given STZ alone, urine albumin was detected. Therefore, 3 weeks after the single STZ injection the rats exhibited early stage of diabetic nephropathy [39, 40]. Interestingly, we also found that PAI-1 protein expression, TGF-β1 protein expression, and macrophage infiltration were markedly increased in the kidney from rats given STZ alone. Furthermore, we observed that treatment with spironolactone completely inhibited the STZ-induced increase in intraglomerular fibrosis, tubulointerstitial fibrosis, and periarteriolar fibrosis. We also demonstrated that spironolactone had no effects on STZ-induced increases in kidney weight or tubular hypertrophy. These findings indicate that the STZ-induced tubulointerstitial fibrosis, intraglomerular fibrosis, and periarteriolar fibrosis occur via MRs, whereas the STZ-induced increase in kidney weight or tubular hypertrophy does not. The present study also observed that the STZ-induced PAI-1 protein expression, TGF-β1 protein expression, and macrophage infiltration in the glomeruli and tubulointerstitium were inhibited by treatment with spironolactone, consistent with the notion that the STZ-induced PAI-1 protein expression, TGF-β1 protein expression, and macrophage infiltration in the glomeruli and tubulointerstitium occur also via MRs. This is the first demonstration of a link between the MRs and PAI-1 expression, TGF-β1 expression, and macrophage infiltration in diabetic nephropathy.

The mechanisms whereby STZ-induced proximal tubule hypertrophy occurs via spironolactone-insensitive processes are not clear at present. Rats given STZ alone and rats given STZ plus spironolactone exhibited elevated blood glucose levels with similar magnitude. Therefore, it is possible that the elevated blood glucose levels may contribute to the spironolactone-insensitive tubular hypertrophy. This possibility is supported by the report of Ziyadeh et al [41], who showed that in proximal tubule cells in culture, elevated glucose concentrations induced cell hypertrophy. In glomerulus and

tubulointerstitium of rats given STZ alone, PAI-1 expression, TGF-β1 expression, and macrophage infiltration were increased. Spironolactone partially inhibited the STZ-induced PAI-1 expression and TGF-β1 expression in tubulointerstitium, whereas it completely inhibited the STZ-induced macrophage infiltration in glomerulus and tubulointerstitium, as well as the STZ-induced PAI-1 expression and TGF-β1 expression in glomerulus. These findings suggest that, in the kidneys of rats given STZ with and without spironolactone, the PAI-1 expression may be parallel with the TGF-β1 expression, and that the effect of spironolactone on STZ-induced renal PAI-1 expression and TGF-β1 expression differs between glomerulus and tubulointerstitium. In other words, in tubulointerstitium, the STZ-induced PAI-1 and TGF-β1 expression is mediated by spironolactone-sensitive and -insensitive processes, whereas, in glomerulus, the STZ-induced PAI-1 and TGF-β1 expression is exclusively mediated by spironolactone-sensitive MRs. Further studies will be required to clarify the spironolactone-insensitive processes.

Recent studies indicate that aldosterone contributes to renal injury in animal models of hypertension [3, 42, 43]. MR antagonism with spironolactone decreases glomerular damage and arteriopathy in the stroke-prone spontaneously hypertensive rats [3] and in the 5/6 nephrectomy model of hypertension [42]. In spontaneously hypertensive rats, aldosterone induces and spironolactone attenuates renal injury in the absence of blood pressure effects [3, 43], suggesting that aldosterone exerts direct nephrotoxic effects. In the present study, we indeed observed that rats given STZ alone exhibited renal fibrosis, which was completely inhibited by treatment with spironolactone. In rats given STZ alone, on study day 20, there was a slight elevation of systolic blood pressure compared with that on study day 0, although in control rats or rats given STZ plus spironolactone, on study day 20 there was no change in the blood pressure compared with that on study 0. On the other hand, the blood pressure on study day 0, 7, 14, or 20 was not significantly different among the 3 groups of rats. Therefore, the effect of spironolactone on STZ-induced renal fibrosis should possibly occur independently of blood pressure effect, although we cannot completely exclude the blood pressure effect. More importantly, this study is the first to demonstrate that aldosterone contributes to hyperglycemia-induced increase in PAI-1 expression, TGF-β1 expression, and macrophage infiltration in the kidney via MRs. Alternatively, the aldosterone-induced renal injury may occur via endothelin (ET)-1 activation, because in aldosterone-infused rats, cardiac and aortic interstitial and perivascular collagen depositions were increased, and all of them were prevented by BMS 182874, the selective ET-1 A receptor antagonist [44]. Furthermore, Pu et al [45] reported that aldosterone infusion in rats raised blood pressure, increased plasma ET-1 levels, induced oxidative stress, and collagen and fibronectin deposition in the media of small mesenteric arteries and increased abundance of intercellular adhesion molecule-1 in the vascular wall, and that these changes were prevented by treatment with spironolactone and BMS 182874. Therefore, the aldosterone-induced effects may be mediated by ET-1–sensitive processes that involve oxidative stress.

Numerous other studies have demonstrated a renoprotective effect of ACE inhibitors and AT1 receptor antagonists in both hypertensive and normotensive settings [46–48]. In addition, ACE inhibitors and AT1 receptor antagonists have previously been shown to suppress PAI-1 expression in a balloon-injured aorta model and in radiation nephropathy [49–51]. Diabetic rats given STZ showed elevated expression of monocyte chemoattractant protein-1 (MCP-1), a key macrophage chemoattractant and activator, macrophage number in glomeruli, and proteinuria, which were suppressed by the treatment with the ACE inhibitor enalapril and the AT1 receptor antagonist candesartan [52]. Furthermore, Kelly et al [31] reported that ACE inhibition with perindopril was associated with attenuation of osteopontin expression, macrophage infiltration, and tubulointerstitial injury in transgenic diabetic rats treated with STZ. However, they have not yet addressed the inhibitory effects of the MR antagonism with spironolactone on PAI-1 expression, TGF-β1 expression, and macrophage infiltration in their animal models.

The mechanism by which aldosterone contributes to the development of renal fibrosis in animal models of diabetes is not known at present; however, several lines of evidence support the hypothesis that aldosterone causes thrombosis and accumulation of extracellular matrix, leading to fibrosis by increasing PAI-1 expression. First, aldosterone interacts with angiotensin II to increase PAI-1 expression in both vascular smooth muscle cells and endothelial cells [23]. Second, serum aldosterone concentrations correlate with PAI-1 antigen concentrations in humans [23, 53], supporting a role of aldosterone in vivo in regulation of PAI-1 as well. In support of a role for PAI-1 in the regulation of fibrosis, Eitzman et al [17] have reported that bleomycin-induced pulmonary fibrosis is increased in transgenic mice overexpressing PAI-1 and decreased in PAI-1 knockout mice relative to control rats. Alternatively, aldosterone may induce an increase in TGF-β1 expression, and consequently increase extracellular matrix synthesis, inhibit matrix degradation, up-regulate adhesion molecules, enhance chemoattraction [7, 8], and increase PAI-1 expression [19], leading to renal fibrosis. It is also possible that aldosterone may increase MCP-1 expression, which is therefore involved in progressive glomerular and tubulointerstitial damage via macrophage recruitment and activation. This is supported by the recent report of Blasi et al [54], who showed that aldosterone/salt-induced renal injury and fibrosis were associated with macrophage infiltration and up-regulation of cytokines, including MCP-1,

and that all of them were attenuated by treatment with eplerenone (a selective MR antagonist). Future studies will be required to clarify how PAI-1, TGF-β1, and macrophage infiltration are implicated in diabetic kidney fibrosis.

In the present study, we found that plasma aldosterone concentrations in rats given STZ alone were similar to those in the control rats, whereas, in rats given STZ plus spironolactone, they were significantly greater than those in rats given STZ alone and control rats. This finding is in good agreement with those of Miric et al [6]. They reported that 8 weeks after single STZ administration, plasma aldosterone concentrations in rats were not different from those in control rats, whereas in rats given STZ plus spironolactone, plasma aldosterone concentrations were markedly elevated, despite the fact that spironolactone reversed the STZ-induced increase in tubulointerstitial collagen volume faction and fibronectin concentration [6]. Similarly, Brown et al [35] reported that spironolactone suppressed radiation-induced nephrosclerosis via inhibition of PAI-1 expression, in spite of normal serum aldosterone levels. Taken together with the present finding that spironolactone inhibited the development of fibrosis, PAI-1 protein expression, TGF-β1 protein expression, and macrophage infiltration in the early stage of diabetic kidney, this suggests that circulating concentrations of aldosterone do not reflect local tissue levels of aldosterone. In support of this hypothesis, extra-adrenal synthesis of aldosterone has been documented in vascular smooth muscle cells [55] and heart [56]. For these reasons, the demonstration that aldosterone antagonism attenuates the development of fibrosis, PAI-1 protein expression, TGF-β1 protein expression, and macrophage infiltration in the early stage of diabetic nephropathy may provide the better evidence for a role of endogenous aldosterone in this model of diabetic nephropathy. Whether aldosterone is actually produced in the diabetic kidney will have to wait further investigation.

Spironolactone is known to cause hyperkalemia. However, in rats given STZ plus spironolactone (50 mg/kg/day sc) for 21 days, plasma potassium concentrations were not significantly elevated compared with those in control rats or rats given STZ alone (see Table 1). This may result from the normal renal function in rats given STZ plus spironolactone. Similarly, Miric et al [6] reported that there were no significant differences of plasma potassium concentrations among rats 8 weeks after single STZ administration, rats given spironolactone at the same doses for 4 weeks starting 4 weeks after STZ administration, control rats, and control rats given spironolactone alone for 4 weeks. The result that plasma potassium concentrations did not significantly differ among the 3 groups of rats excludes the possibility that plasma potassium is implicated in early diabetic renal injury.

In rats given STZ alone, the body weight gain was less than that in the control rats. On the other hand, rats given STZ plus spironolactone exhibited a body weight loss. The reasons for this are not known at present. The increase in urine volume was greater in rats given STZ plus spironolactone than in given STZ alone, whereas the increase in blood glucose levels was not significantly different between the 2 groups of rats. Thus, the body weight loss in rats given STZ plus spironolactone may be due to a greater increase of urine output, although we did not measure water intake or food intake in the rats.

## CONCLUSION

STZ-induced renal fibrosis, PAI-1 expression, TGF-β1 expression, and macrophage infiltration occur via MRs, and spironolactone ameliorates renal fibrosis presumably via the inhibition of PAI-1 expression, TGF-β1 expression, and macrophage infiltration in STZ-induced early diabetic kidney. Spironolactone is generally used as a diuretic to reduce the extracellular fluid volume without causing potassium depletion or hypokalemia in advanced renal injury in diabetes. However, the present study strongly indicates that inhibition of the MRs by spironolactone might provide a novel therapeutic strategy in early diabetic injury as an antifibrotic agent as well.

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## REFERENCES

1. Mauer SM, Steffes MW, Ellis EN, *et al*: Structural-functional relationships in diabetic nephropathy. *J Clin Invest* 74:1143–1155, 1984
2. Ziyadeh FN, Goldfarb S: The renal tubulo-interstitium in diabetes mellitus. *Kidney Int* 39:464–475, 1991
3. Rocha R, Chander PN, Khanna K, *et al*: Mineralocorticoid blockade reduces vascular injury in stroke-prone hypertensive rats. *Hypertension* 31:451–458, 1998
4. Brilla CG, Matsubara LS, Weber KT: Anti-aldosterone treatment and the prevention of myocardial fibrosis in primary and secondary hyperaldosteronism. *J Mol Cell Cardiol* 25:563–575, 1993
5. Fujisawa G, Okada K, Muto S, *et al*: Na/H exchange isoform 1 is involved in mineralocorticoid induced cardiac injury. *Hypertension* 41:493–498, 2003
6. Miric G, Dallemagne C, Endre Z, *et al*: Reversal of cardiac and renal fibrosis by pirfenidine and spironolactone in streptozotocin-diabetic rats. *Br J Pharmacol* 133:687–694, 2001
7. Sharma K, Ziyadeh FN: Renal hypertrophy is associated with up-regulation of TGF-β1 gene expression in diabetic BB rat and NOD mouse. *Am J Physiol* 267:F1094–F1098, 1994
8. Sharma K, Jin Y, Guo J, *et al*: Neutralization of TGF-β by anti-TGF-β antibody attenuates kidney hypertrophy and the enhanced extracellular matrix gene expression in STZ-induced diabetic mice. *Diabetes* 45:522–530, 1996
9. Shankland SJ, Scholey JW, Ly H, *et al*: Expression of transforming growth factor-β1 during diabetic renal hypertrophy. *Kidney Int* 46:430–442, 1994

10. Pfeiffer A, Middleberg-Bispring K, Drewes C, *et al*: Elevated plasma levels of transforming growth factor-β1 in NIDDM. *Diabetes Care* 19:1113–1117, 1996
11. Park I-S, Kiyomoto H, Abboud SL, *et al*: Expression of transforming growth factor-β and Type IV collagen in early streptozotocin-induced diabetes. *Diabetes* 46:473–480, 1997
12. Nakamura T, Fukui M, Ebihara I, *et al*: mRNA expression of growth factors in glomeruli from diabetic rats. *Diabetes* 42:450–456, 1993
13. Young BA, Johnson RJ, Alpers CE, *et al*: Cellular events in the evolution of experimental diabetic nephropathy. *Kidney Int* 47:935–944, 1995
14. Gilbert RE, Cox A, Wu LL, *et al*: Expression of transforming growth factor-β1 and type IV collagen in the renal tubulointerstitium in experimental diabetes. *Diabetes* 47:414–422, 1998
15. Hill C, Logan A, Smith C, *et al*: Angiotensin converting enzyme inhibitor suppresses glomerular transforming growth factor beta receptor expression in experimental diabetes in rats. *Diabetologia* 44:495–500, 2001
16. Saksela O, Rifkin DB: Cell-associated plasminogen activation: Regulation and physiologic functions. *Annu Rev Cell Biol* 4:93–126, 1988
17. Eitzman DT, McCoy RD, Zheng X, *et al*: Bleomycin-induced pulmonary fibrosis in transgenic mice that either lack or overexpress the murine plasminogen activator inhibitor-1 gene. *J Clin Invest* 97:232–237, 1996
18. Eddy SS: Plasminogen activator inhibitor-1 and the kidney. *Am J Physiol Renal Physiol* 283:F209–F220, 2002
19. Tomooka S, Border WA, Marshall BC, *et al*: Glomerular matrix accumulation is linked to inhibition of the plasmin protease system. *Kidney Int* 42:1462–1469, 1992
20. Vaughan DE, Lazos SA, Tong K: Angiotensin II regulates the expression of plasminogen activator inhibitor-1 in cultured endothelial cells. *J Clin Invest* 95:995–1001, 1995
21. Van Leeuwen RT, Kol A, Andreotti F, *et al*: Angiotensin II increases plasminogen activator inhibitor type 1 and tissue-type plasminogen activator messenger RNA in cultured rat aortic smooth muscle cells. *Circulation* 90:362–368, 1994
22. Kagami S, Kuhara T, Okada K, *et al*: Dual effects of angiotensin II on the plasminogen/plasmin system in rat mesangial cells. *Kidney Int* 51:664–671, 1997
23. Brown NJ, Kin KS, Chen YQ, *et al*: Synergistic effect of adrenal steroids and angiotensin II on plasminogen activator inhibitor-1 production. *J Clin Endocrinol Metab* 85:336–344, 2000
24. Katoh M, Egashira K, Mitsui T, *et al*: Angiotensin-converting enzyme inhibitor prevents plasminogen activator inhibitor-1 expression in a rat model with cardiovascular remodeling induced by chronic inhibition of nitric oxide synthesis. *J Mol Cell Cardiol* 32:73–83, 2000
25. Nakamura S, Nakamura I, Ma L, *et al*: Plasminogen activator inhibitor-1 expression is regulated by the angiotensin type 1 receptor in vivo. *Kidney Int* 58:251–259, 2000
26. Nikolic-Paterson DJ, Lan HY, Hill PA, *et al*: Macrophages in renal injury. *Kidney Int* 45(Suppl):S79–S82, 1994
27. Cooper ME: Pathogenesis, prevention, and treatment of diabetic nephropathy. *Lancet* 352:213–219, 1998
28. Baud L, Hagege J, Sraer L, *et al*: Reactive oxygen production by cultured rat glomerular mesangial cells during phagocytosis is associated with stimulation of lipoxygenase activity. *J Exp Med* 158:1836–1842, 1983
29. Young BA, Johnson RJ, Alpers CE, *et al*: Cellular events in the evolution of experimental diabetic nephropathy. *Kidney Int* 47:335–342, 1995
30. Wada T, Furuichi K, Sakai N, *et al*: Up-regulation of monocyte chemoattractant protein-1 in tubulointerstitial lesion of human diabetic nephropathy. *Kidney Int* 58:1492–1499, 2000
31. Kelly DJ, Wilkinson-Berka JL, Richardo SD, *et al*: Progression of tubulointerstitial injury by osteopontin-induced macrophage recruitment in advanced diabetic nephropathy of transgenic (mRen-2)27 rats. *Nephrol Dial Transplant* 17:985–991, 2002
32. Vranes D, Cooper ME, Dilley RJ: Cellular mechanism of diabetic vascular hypertrophy. *Microvasc Res* 57:8–18, 1999
33. Vranes D, Dilley RJ, Cooper ME: Vascular changes in diabetic kidney: Effects of ACE inhibition. *J Diabetes Complication* 9:296–300, 1995
34. Mai M, Geiger H, Hilgers KF, *et al*: Early interstitial changes in hypertension-induced renal injury. *Hypertension* 22:754–765, 1993
35. Brown NJ, Nakamura S, Ma L-J, *et al*: Aldosterone modulates plasminogen activator inhibitor-1 and glomerulosclerosis. *Kidney Int* 58:1219–1227, 2000
36. Zhang G, Kim H, Cai X, *et al*: Urokinase receptor modulatescellular and angiogenic responses in obstructive nephropathy. *J Am Soc Nephrol* 14:1234–1253, 2003
37. Shankland SS, Scholey JW, Thai K, *et al*: Expression of transforming growth factor-β1 during diabetic renal hypertrophy. *Kidney Int* 46:430–442, 1994
38. Toblli JE, Ferder L, Stella I, *et al*: Protective role of enalapril for chronic tubulointerstitial lesion of hyperoxaluria. *J Urology* 166:275–280, 2001
39. Mogensen CE, Christensen CK, Vittinghus F: The stages in diabetic renal disease. With emphasis on the stage of incipient diabetic nephropathy. *Diabetes* 32(Suppl 2):64–78, 1983
40. Rasch R, Dorup J: Quantitative morphology of the rat kidney during diabetes mellitus and insulin treatment. *Diabetologia* 40:802–809, 1997
41. Ziyadeh FN, Snipes ER, Watanabe M, *et al*: High glucose induces cell hypertrophy and stimulates collagen gene transcription in proximal tubule. *Am J Physiol* 259:F704–F714, 1990
42. Greene EL, Kren S, Hostetter TH: Role of aldosterone in the remnant kidney model in the rat. *J Clin Invest* 98:1063–1068, 1996
43. Rocha R, Chander PN, Zuckerman A, *et al*: Role of aldosterone in renal vascular injury in stroke-prone hypertensive rats. *Hypertension* 33:232–237, 1999
44. Park JB, Schiffrin EL: Cardiac and vascular fibrosis and hypertrophy in aldosterone-infused rats: Role of endothelin-1. *Am J Hypertens* 15:164–169, 2002
45. Pu Q, Neves F, Virdis A, *et al*: Endothelin antagonism on aldosterone-induced oxidative stress and vascular remodelling. *Hypertension* 42:49–55, 2003
46. Anderson SG, Rennke HG, Brenner BM: Therapeutic advantage of converting enzyme inhibitors in arresting progressive renal disease associated with systemic hypertension in rat. *J Clin Invest* 77:1993–2000, 1986
47. Tanaka R, Sugihara K, Takematsu A, *et al*: Internephron heterogeneity of growth factors and sclerosis-modulation of platelet derived growth factor by angiotensin II. *Kidney Int* 47:131–139, 1995
48. Kaneto H, Morrissey J, McCracken R, *et al*: Enalapril reduces collagen type IV synthesis and expansion of the interstitium in the obstructed rat kidney. *Kidney Int* 45:1637–1647, 1994
49. Oikawa T, Freeman M, Lo W, *et al*: Modulation of plasminogen activator inhibitor-1 in vivo: A new mechanism for the anti-fibrotic effect of renin-angiotensin inhibitors. *Kidney Int* 51:164–172, 1997
50. Cohen EP, Fish BL, Moulder JE: Treatment of radiation nephropathy with captopril. *Radiat Res* 132:346–350, 1992
51. Hamdan AD, Quist WC, Gagne JB, *et al*: Angiotensin-converting enzyme inhibition suppresses plasminogen activator inhibitor-1 expression in the neointima of balloon-injured rat aorta. *Circulation* 93:1073–1078, 1996
52. Kato S, Luyckx VA, Ots M, *et al*: Renin-angiotensin blockade lowers MCP-1 expression in diabetic rats. *Kidney Int* 56:1037–1048, 1999
53. Brown NJ, Agirbasli MA, Williams GH, *et al*: Effect of activation and inhibition of the renin angiotensin system on plasma PAI-1 in humans. *Hypertension* 32:965–971, 1998
54. Blasi ER, Rocha R, Rudolph AE, *et al*: Aldosterone/salt induced renal inflammation and fibrosis in hypertensive rats. *Kidney Int* 63:1791–1800, 2003
55. Hatakeyama H, Miyamori I, Fujita T, *et al*: Vascular aldosterone. Biosynthesis and a link to angiotensin II-induced hypertrophy of vascular smooth muscle cells. *J Biol Chem* 269:24316–24320, 1994
56. Silvestre J-S, Heymes C, Oubenaissa A, *et al*: Activation of cardiac aldosterone production in rat myocardial infarction. Effect of angiotensin II receptor blockade and role in cardiac fibrosis. *Circulation* 99:2694–2701, 1999