We have previously reported that N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP), which is a tetrapeptide hydrolyzed by ACE, inhibits the transforming growth factor-β (TGF-β)-induced expression of extracellular matrix proteins via inhibition of the Smad signaling in human mesangial cells. To test in vivo the antifibrotic efficacy of Ac-SDKP, we examined whether long-term Ac-SDKP treatment can prevent renal insufficiency and glomerulosclerosis in diabetic db/db mice. Diabetic db/db mice or nondiabetic db/m mice were treated with Ac-SDKP for 8 weeks using osmotic minipumps. The treatment with Ac-SDKP increased plasma Ac-SDKP concentrations by approximately threefold in both groups but did not affect the blood glucose levels. Histologically, the increased glomerular surface area, mesangial matrix expansion, and overproduction of extracellular matrix proteins in db/db mice were significantly inhibited by Ac-SDKP. Furthermore, Ac-SDKP treatment normalized the increased plasma creatinine value in db/db mice, whereas the albuminuria in Ac-SDKP–treated db/db mice was somewhat decreased as compared with nontreated db/db mice, although the difference was not statistically significant. In addition, the nuclear translocation of Smad3 was inhibited by Ac-SDKP. These results demonstrate that long-term Ac-SDKP treatment ameliorates renal insufficiency and glomerulosclerosis in db/db mice via inhibition of TGF-β/Smad pathway, suggesting that Ac-SDKP could be useful in the treatment of diabetic nephropathy. *Diabetes* 54:838–845, 2005

**Diabetic nephropathy** is a leading cause of end-stage renal disease, accounting for ~40% of all new patients requiring renal replacement therapy in many countries. Therapeutic strategies for diabetic kidney disease are thus urgently needed worldwide. In the diabetic state, multiple biochemical mechanisms, such as those involving growth factors and cytokines (1), activation of protein kinase C extracellular-regulated protein kinase pathway (2,3), enhanced polyol pathway (4,5), and altered redox state and oxidative stress (6), have been proposed to be involved in the development of diabetic nephropathy.

Transforming growth factor-β (TGF-β) is a key cytokine that regulates development, cell proliferation, and matrix protein synthesis (7,8). During the glomerular scarring process, TGF-β plays a major role in extracellular matrix protein accumulation (9). TGF-β is upregulated in the kidneys of diabetic animal models (10–12) and patients with diabetic nephropathy (13,14). Indeed, the administration of neutralizing anti–TGF-β antibody ameliorated functional and morphological abnormalities in the kidneys of diabetic animals (10,12). Therefore, downregulation of TGF-β signaling provides a useful therapeutic strategy for inhibiting diabetic kidney disease.

Recent large clinical trials (15–17) clearly demonstrated that treatment with ACE inhibitors improves clinical outcome in patients with progressive renal disease, and it is widely appreciated that ACE inhibitors ameliorate glomerular hypertension by reducing the resistance of efferent arterioles. However, the effect of ACE inhibitors might depend not only on the suppression of the renin-angiotensin system but also on other biochemical effects. N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) is a tetrapeptide normally presenting in human plasma and is exclusively hydrolyzed by ACE. The plasma levels of Ac-SDKP were shown to be increased fivefold after ACE inhibitor treatment in patients (18). We recently reported that Ac-SDKP has an antifibrotic property after demonstrating that Ac-SDKP inhibits TGF-β–induced expressions of plasminogen activator inhibitor-1 and α2 (I) collagen in human mesangial cells. We also found that Ac-SDKP inhibits TGF-β signaling pathway through the suppression of Smad2 and Smad3 activations via nuclear export of Smad7 (19),...
demonstrating that a novel mechanism is involved in the renoprotective effect of ACE inhibitors.

In this study, we therefore investigated the efficacy of the administration of Ac-SDKP in preventing glomerulosclerosis and renal insufficiency in db/db mice, a rodent model of type 2 diabetes. An 8-week course of Ac-SDKP treatment prevented renal insufficiency, excess mesangial expansion, and expression of extracellular matrix proteins. In addition, we ascertained that Ac-SDKP inhibits nuclear translocation of Smad3 in diabetic db/db mice in vivo. This study provides the first evidence that Ac-SDKP is a natural peptide that inhibits TGF-β signaling in vivo and is useful as a novel therapeutic strategy for diabetic nephropathy.

RESEARCH DESIGN AND METHODS

Male diabetic db/db mice and age-matched nondiabetic db/m mice were purchased from CLEA Japan (Tokyo, Japan). At 10 weeks of age, osmotic minipumps (Alzet, Cupertino, CA) were surgically implanted for subcutaneous delivery of 1,000 μg · kg⁻¹ · day⁻¹ Ac-SDKP donated by Daiichi Suntory Biomedical Research (Tokyo, Japan) or balanced salt solution (BSS; saline plus 0.01 N acetic acid). We also tested control tetrapeptide (H-Ser-Leu-Leu-Lys-OH) in parallel. The osmotic pumps were replaced when the mice were 14 weeks of age since each pump lasted only 4 weeks. Body weight, blood glucose, erythrocyte count, and hematocrit were measured almost every 3 weeks for the control mice but did not affect body weights, glucose levels, and mean blood pressures. The kidney weights of db/db mice increased plasma Ac-SDKP levels by approximately threefold compared with BSS-treated mice and is useful as a novel therapeutic strategy for diabetic nephropathy.

**TABLE 1** Characteristics of experimental groups of mice

<table>
<thead>
<tr>
<th></th>
<th>db/m mice</th>
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<th>db/db mice</th>
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<tbody>
<tr>
<td></td>
<td>BSS</td>
<td>Ac-SDKP</td>
<td>BSS</td>
<td>Ac-SDKP</td>
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<tr>
<td>n</td>
<td>13</td>
<td>12</td>
<td>13</td>
<td>13</td>
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<tr>
<td>Body weight (g)</td>
<td>30.6 ± 0.6</td>
<td>30.9 ± 0.4</td>
<td>59.1 ± 1.8*</td>
<td>59.1 ± 0.8*</td>
</tr>
<tr>
<td>Blood glucose (mmol/l)</td>
<td>7.8 ± 0.3</td>
<td>7.5 ± 0.2</td>
<td>23.1 ± 1.4*</td>
<td>22.0 ± 1.3*</td>
</tr>
<tr>
<td>Left kidney weight (mg)</td>
<td>171.1 ± 6.2</td>
<td>174.2 ± 5.6</td>
<td>2085 ± 5.7*</td>
<td>2085 ± 6.2*</td>
</tr>
<tr>
<td>Mean blood pressure (mmHg)</td>
<td>84.5 ± 2.2</td>
<td>85.5 ± 2.4</td>
<td>91.8 ± 2.7*</td>
<td>95.9 ± 2.3*</td>
</tr>
<tr>
<td>Plasma Ac-SDKP concentration (nmol/l)</td>
<td>0.85 ± 0.04</td>
<td>3.37 ± 1.22†</td>
<td>1.39 ± 0.13</td>
<td>4.79 ± 1.03‡</td>
</tr>
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</table>

Data are means ± SE. *P < 0.01 vs. db/m mice; †P < 0.05 vs. db/m mice given BSS; ‡P < 0.05 vs. db/db mice given BSS.

RESULTS

Characteristics of experimental mice. The characteristics of the four groups of mice at the end of the experimental period are presented in Table 1. The body weights of the db/db mice were significantly heavier than those of the db/m mice, and their blood glucose levels were significantly higher. The treatment with Ac-SDKP in both db/db and db/m mice increased plasma Ac-SDKP levels by approximately threefold compared with BSS-treated mice but did not affect body weights, glucose levels, and mean blood pressures. The kidney weights of db/db mice were significantly greater than those of db/m mice, and there...
was no significant difference between BSS- and Ac-SDKP–treated mice. Since Ac-SDKP was shown to inhibit hematopoiesis (22,23), we evaluated whether the treatment with Ac-SDKP affects peripheral erythrocyte number and the percentage of hematocrit. Erythrocyte counts and hematocrits were not different in \( \text{db/m} \) mice treated with BSS (12.0 ± 0.7 × 10⁶/mm³, 51.4 ± 2.3%), \( \text{db/m} \) mice with Ac-SDKP (11.8 ± 0.9 × 10⁶/mm³, 52.7 ± 1.5%), \( \text{db/db} \) mice with BSS (11.8 ± 0.9 × 10⁶/mm³, 52.0 ± 0.3%), and \( \text{db/db} \) mice with Ac-SDKP (12.2 ± 0.3 × 10⁶/mm³, 51.6 ± 0.5%).

**Glomerular histology.** We examined whether treatment with Ac-SDKP attenuated the glomerular hypertrophy and mesangial matrix expansion of diabetic \( \text{db/db} \) mice. The area of PAS-positive matrix in the glomeruli of \( \text{db/db} \) mice was increased compared with that of \( \text{db/m} \) mice. The \( \text{db/db} \) mice treated with Ac-SDKP showed minimal mesangial matrix expansion as compared with the \( \text{db/db} \) mice treated with BSS (Fig. 1). The glomerular surface area was greater in \( \text{db/db} \) than \( \text{db/m} \) mice, and Ac-SDKP treatment significantly attenuated the glomerular hypertrophy in \( \text{db/db} \) mice (Fig. 2A). The mesangial matrix expansion was

**FIG. 1.** Ac-SDKP treatment ameliorates mesangial matrix expansion in diabetic \( \text{db/db} \) mice. Representative photomicrographs of PAS-stained kidney sections from \( \text{db/m} \) with BSS (A), \( \text{db/m} \) with Ac-SDKP (B), \( \text{db/db} \) with BSS (C), and \( \text{db/db} \) with Ac-SDKP (D).

**FIG. 2.** Ac-SDKP treatment ameliorates glomerular hypertrophy and mesangial matrix expansion in diabetic \( \text{db/db} \) mice. Quantitative measurements of glomerular surface area (A) and mesangial matrix area (B). C: Plasma Ac-SDKP level significantly correlates with mesangial matrix expansion (\( r = 0.48, P < 0.05 \)). The graph shows logarithmic expression of Ac-SDKP concentration for the horizontal axis and mesangial matrix expansion for the vertical axis in \( \text{db/db} \) mice treated with Ac-SDKP. Data are means ± SE, \( n = 12 \) for \( \text{db/m} \) group treated with Ac-SDKP and \( n = 13 \) for each other group. \(*P < 0.01\) vs. \( \text{db/m} \) groups; \( †P < 0.01\) vs. \( \text{db/db} \) group treated with BSS.
increased more than twofold in db/db compared with db/m mice. Ac-SDKP treatment significantly attenuated the increase in db/db mice (Fig. 2B). Since the glomerular surface area and mesangial matrix expansion in control tetrapeptide-treated groups were similar to those in BSS-treated groups regardless of their strains (data not shown), we used the BSS treatment as a control in all experiments. Simple regression analysis performed to investigate the effect of Ac-SDKP on the mesangial matrix expansion in db/db mice revealed that the treatment with Ac-SDKP decreased the mesangial matrix area in a dose-dependent manner. There was a significant correlation between the mesangial matrix area and the plasma Ac-SDKP level (Fig. 2C).

Expression of fibronectin and type IV collagen. The long-term Ac-SDKP treatment successfully prevented the mesangial matrix expansion in db/db mice. To evaluate the extracellular matrix components of the mesangial matrix, we performed immunohistochemical studies of fibronectin and type IV collagen. Consistent with the results for mesangial matrix expansion, the diabetic db/db mice demonstrated overexpression of fibronectin and type IV collagen as compared with db/m mice (Fig. 3 and Fig. 4). Administration of Ac-SDKP also reduced the overexpression of fibronectin and type IV collagen in the glomeruli of db/db mice. As shown in Fig. 3E and Fig. 4E, semiquantitative scores for fibronectin expression increased from 1.38 ± 0.05 and 1.04 ± 0.06 in db/m mice to 3.10 ± 0.04 and 2.90 ± 0.04 in db/db mice, respectively. The treatment with Ac-SDKP reduced scores for fibronectin and type IV collagen expression to 2.21 ± 0.08 and 2.16 ± 0.11, respectively.

FIG. 3. The treatment with Ac-SDKP prevents the increase in fibronectin expression in db/db mice. Representative photomicrographs from db/m with BSS (A), db/m with Ac-SDKP (B), db/db with BSS (C), and db/db with Ac-SDKP (D). E: Semiquantitative scores for fibronectin expression. Data are means ± SE, n = 6 for each group. *P < 0.01 vs. db/m groups; †P < 0.01 vs. db/db group treated with BSS.

FIG. 4. The treatment with Ac-SDKP prevents the increase in type IV collagen expression in db/db mice. Representative photomicrographs from db/m with BSS (A), db/m with Ac-SDKP (B), db/db with BSS (C), and db/db with Ac-SDKP (D). E: Semiquantitative scores for type IV collagen expression. Data are means ± SE, n = 6 for each group. *P < 0.01 vs. db/m groups; †P < 0.01 vs. db/db group treated with BSS.
Plasma creatinine and urinary albumin excretion. To evaluate the effect of Ac-SDKP on functional abnormalities in db/db mice, the plasma creatinine concentrations and urinary albumin excretions were examined. The plasma creatinine levels were higher in db/db mice than in db/m mice, but the treatment with Ac-SDKP normalized the elevation (Fig. 5A). The urinary albumin excretion of the BSS-treated db/db mice was ~10-fold that of the db/m mice. The urinary albumin excretion in the Ac-SDKP–treated db/db mice was somewhat decreased compared with that in the BSS-treated db/db mice, but the difference was not statistically significant (Fig. 5B).

The effect of Ac-SDKP on TGF-β signaling. We previously reported that Ac-SDKP inhibits TGF-β signaling via Smad pathway in mesangial cells (19). To ascertain whether this inhibitory effect is operative in vivo, we performed an immunohistochemical study and Western blot analysis for Smad3 (Fig. 6). Since activated Smad3 was shown to translocate into the nucleus, the activation of Smad3 was evaluated by counting cells with nuclear staining using a computer-assisted color image analyzer. The nuclear translocation of Smad3 in glomeruli of the outer cortex was increased in db/db mice (21.7 ± 1.4%) compared with db/m mice (9.3 ± 0.4%). The treatment with Ac-SDKP attenuated the increase in the db/db mice (12.4 ± 0.5%). Furthermore, the similar result was obtained by Western blot analysis (Fig. 6F). These observations demonstrated that Ac-SDKP inhibited TGF-β/Smad signaling in vivo.

Expression of TGF-β1 and TGF-β type II receptor. It is reported that renal expression of TGF-β1 and TGF-β type II receptor were increased in db/db mice (24). To evaluate the effect of Ac-SDKP on renal expression of TGF-β1 and its type II receptor, we performed TGF-β1 enzyme-linked immunosorbent assay and the immunohistochemistry of TGF-β type II receptor. The renal expression of TGF-β1 was increased in db/db mice compared with db/m mice (95.1 ± 4.3 vs. 79.2 ± 1.7 ng/g protein, *P < 0.05). The treatment with Ac-SDKP did not change the expression of TGF-β1 in db/db mice (91.5 ± 4.5 ng/g protein).

Interestingly, the expression of TGF-β type II receptor was upregulated in db/db mice, and the treatment with Ac-SDKP inhibited the expression in db/db mice (Fig. 7).

DISCUSSION
Growing evidence clearly indicates that TGF-β plays a central role in the pathogenesis of diabetic nephropathy, and the inhibition of TGF-β activity is a powerful strategy in treating diabetic kidney diseases (10,12,25). From this view, we focus on Ac-SDKP, which is hydrolyzed by ACE and shows increased plasma levels in individuals taking ACE inhibitors (18). Our previous study (19) clearly demonstrated that Ac-SDKP can inhibit the TGF-β1–induced plasminogen activator inhibitor-1 and α2(I) collagen mRNA expression via inhibition of the Smad activation. Therefore, we hypothesized that in vivo Ac-SDKP treatment could have a favorable effect on the progression of diabetic nephropathy. In this study, the 8-week treatment with Ac-SDKP of db/db mice prevented extracellular matrix overproduction, mesangial matrix expansion, glomerular hypertrophy, and elevation of plasma creatinine values in parallel with significant increases in plasma Ac-SDKP levels despite persistent hyperglycemia. Although we measured plasma creatinine by using enzymatic assay, we should note that plasma creatinine measured by colorimetric method overestimates true creatinine values in mice (26). To evaluate plasma creatinine more accurately, a high-performance liquid chromatography method would be reliable. Furthermore, plasma Ac-SDKP concentration had a significant correlation with mesangial area (see Fig. 2C). This suggests that increasing the concentration of Ac-SDKP can prevent mesangial matrix expansion more completely. On the other hand, albuminuria in db/db mice was not significantly affected by the treatment with Ac-SDKP, although it was slightly diminished. In contrast, in a reported study (12) neutralizing anti–TGF-β antibodies failed to diminish albuminuria at all. The discrepancy suggests that Ac-SDKP may possess some unknown function in addition to the inhibition of TGF-β signaling.
although further studies are needed to elucidate the role of TGF-β in the development of albuminuria.

We next demonstrated that the nuclear translocations of Smad3 were greater in \textit{db/db} than in \textit{db/m} mice by using immunohistochemical studies and Western blot analysis. These results are consistent with a previous report (24) showing increased nuclear accumulation of Smad3 and nuclear binding activity of Smad-binding element in renal glomeruli and tubules of \textit{db/db} mice. The important role of Smad signaling in diabetic kidney disease is evident from the fact that Smad3-null diabetic mice do not exhibit glomerular basement membrane thickening and overexpression of extracellular matrix (27). The treatment with Ac-SDKP significantly attenuated the increase of Smad3 nuclear translocation in \textit{db/db} mice, suggesting that Ac-SDKP inhibited glomerulosclerosis and renal insufficiency through the inhibition of Smad activation in diabetic mice.

We think that Ac-SDKP would be a safe and useful therapeutic agent for several reasons. Firstly, Ac-SDKP is already normally present in the living body, and its level in plasma is increased by ACE inhibitor administration. No harmful effect such as anemia was noted at the dose used in this study. Secondly, ours and other groups (28–30) have reported that several compounds and drugs, such as aminoguanidine, glycosaminoglycan, and thiazolidinediones, prevented diabetic nephropathy via the inhibition of TGF-β expression in diabetic animal models, but Ac-SDKP inhibits TGF-β/Smad signaling specifically. Findings recently reported by Benigni et al. (31) support this notion, since the addition of anti–TGF-β antibody to ACE inhibitors completely arrests proteinuria and renal injury in diabetic rats. Furthermore, in our preliminary study, the combination treatment with Ac-SDKP and captopril increased the plasma Ac-SDKP levels by approximately eightfold and revealed stronger effect on reducing mesangial expansion in \textit{db/db} mice than Ac-SDKP alone (data not shown).

Diabetic nephropathy is a leading cause of end-stage renal disease, accounting for ~40% of all new admissions for renal replacement therapy in many countries. The morbidity and mortality associated with diabetic nephropathy are extremely high, but the recommended therapies...
with strict glycemic and blood pressure control decrease them significantly. Since these therapies nevertheless fail to completely prevent the progression of diabetic nephropathy, therapeutic strategies for diabetic kidney disease are urgently needed. Treatment with Ac-SDKP, which demonstrates specific and secure efficacy of inhibition of TGF-β/Smad signaling, may represent a useful therapy for patients with diabetic nephropathy.

ACKNOWLEDGMENTS

This study was supported by a research grant from Takeda Science Foundation, Suzukien Memorial Foundation and the Japanese Ministry of Education, Culture, Sports, Science and Technology (to D.K.).

REFERENCES


