Dipeptidyl Peptidase IV Inhibitor Attenuates Kidney Injury in Streptozotocin-Induced Diabetic Rats

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ABSTRACT

Dipeptidyl peptidase (DPP) IV inhibitors are probably beneficial for preventing diabetic complication and modulating glucagon-like peptide-1 receptor (GLP-1R) expression. The aim of this study was to determine whether the DPP IV inhibitor LAF237 (vildagliptin) has renoprotective qualities in streptozotocin-induced diabetic rats. Diabetic and non-diabetic rats were treated with an oral dose of 4 or 8 mg/kg/day LAF237 or placebo for 24 weeks, and renal injury was observed by light and electron microscopy. We also assessed DPP IV activity, active GLP-1 level, cAMP and 8-hydroxy-deoxyguanosine excretion, and proteinuria, albuminuria, and urinary albumin/creatinine ratio, improved creatinine clearance, and dose-dependently inhibited interstitial expansion, glomerulosclerosis, and the thickening of the glomerular basement membrane in diabetic rats. It is noteworthy that LAF237 markedly down-regulated DPP IV activity and increased active GLP-1 levels, which probably prevented oxidative DNA damage and renal cell apoptosis by activating the GLP-1R and modulating cAMP. Renoprotection was also associated with a reduction in TGF-β1 overexpression. Our study suggests that DPP IV inhibitors may ameliorate diabetic nephropathy as well as reduce the overproduction of TGF-β1. The observed renoprotection is probably attributable to inhibition of DPP IV activity, mimicking of incretin action, and activation of the GLP-1R.

Introduction

LAF237 (vildagliptin), a highly selective dipeptidyl peptidase (DPP) IV inhibitor, may successfully prevent the degradation of two major incretins, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide, which are secreted from L-cells of the gastrointestinal tract in response to food digestion in humans and rodents. More than 50% of either incretin is inactivated within several minutes by the enzyme DPP IV, whereas a DPP IV inhibitor suppresses this enzyme and extends the activities of both incretins (Freeman, 2007). Within 5 h after oral administration of DPP IV inhibitor, plasma GLP-1 concentration remains elevated in diabetics receiving 50 mg/day LAF237 compared with placebo, although there are no differences under fasting conditions (Vella et al., 2007).

Considerable research has confirmed that DPP IV inhibitors can prevent β-cell failure, stimulate insulin release, improve glycemic and hemoglobin A1c (HbA1c) control, and decrease triglyceride and free fatty acid levels in diabetic patients (Azuma et al., 2008; Campbell and White, 2008). DPP IV activity increases in the urine of patients with diabetes, especially those with microalbuminuria (Golubović et al., 1996; Mitic et al., 2008). These findings indicate that the change of DPP IV in urine is probably associated with diabetic nephropathy. DPP IV inhibitors or incretin-based agents reportedly ameliorate diabetic neuropathy and retinopathy, at least in part, through delaying the degradation of GLP-1 and its binding affinity to the GLP-1R (Jin et al., 2009; Zhang et al., 2009). These observations suggest that DPP IV inhibitors, partly by increasing GLP-1 level, can exert renoprotective effects in the hyperglycemic state, because dia-

ABBR EVIATI ONS: DPP, dipeptidyl peptidase; GBM, glomerular basement membrane; GLP-1, glucagon-like peptide 1; GLP-1R, GLP-1 receptor; TGF-β1, transforming growth factor-β1; 8-OHdG, 8-hydroxy-deoxyguanosine; STZ, streptozotocin; HbA1c, hemoglobin A1c; PAS, periodic acid-Schiff; DM, diabetic control; nonDM, nondiabetic control; 4LAF/8LAF, treatment with 4 or 8 mg/kg/day LAF237; PCR, polymerase chain reaction; DAPI, 4,6-diamidino-2-phenylindole; GSI, glomerulosclerosis index; %INT, fractional interstitial area.

W.J.L. and S.H.X. contributed equally to this work.

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betic nephropathy has a close relationship with other diabetic microvascular complications (Girach and Vignati, 2006).

GLP-1R is also expressed in the proximal tubules and glomerulus of the kidney. GLP-1R expression decreases in the glomerulus, and treatment with a GLP-1 agonist up-regulates receptor expression and ameliorates renal lesions in db/db mice (Park et al., 2007; Schlatter et al., 2007). There has been much evidence to suggest that GLP-1 signaling through GLP-1R enhances cAMP as a second messenger (Doyle and Egan, 2007). It is well known that incretin-related agents preserve pancreatic β-cell mass by suppressing oxidative stress and antiapoptotic effect (Kim et al., 2010; Shimoda et al., 2011). Likewise, exendin-4 exerts renoprotective effects partly via the action against 8-hydroxy-deoxyguanosine (8-OHdG) excretion and caspase 3 expression regulated by GLP-1R in both type 1 and type 2 diabetic rodents (Park et al., 2007; Kodera et al., 2011). TGF-β1 expression is also down-regulated after incretin treatment in these studies. Thus, we tested the hypothesis that inhibition of DPP IV enhances active GLP-1 levels and modulates renal GLP-1R expression, which, in turn, will suppress TGF-β1 expression and prevent oxidative stress and apoptosis. The net effect of these changes would be retardation of diabetic nephropathy. Accordingly, we examined the effects of a DPP IV inhibitor, LAF237, on the kidneys of streptozotocin-treated type 1 diabetic rats.

Materials and Methods

Animals. Male Sprague-Dawley rats (5–7 weeks; Damool Science, Daejeon, Korea), weighing 180 to 200 g each, were housed in a 12-h light/dark altered room at a constant temperature of 24°C, with food and water available ad libitum. Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ; Sigma, St. Louis, MO; 60 mg/kg body weight) dissolved in 0.1 M citrate buffer, pH 4.5. One week after the verification of diabetes (designated as week 0), diabetic and nondiabetic rats were stochastically divided into three and two groups (n = 6–9 per group), respectively. During 12 h of nighttime LAF237 (vildagliptin; Novartis, Basel, Switzerland) was administered orally at 4 or 8 mg/kg/day to diabetic rats and 8 mg/kg/day to nondiabetic rats. Enough water was offered in the daytime, and food was available ad libitum during the nighttime LAF237 (vildagliptin; Novartis, Basel, Switzerland) was administered orally at 4 or 8 mg/kg/day to diabetic rats and 8 mg/kg/day to nondiabetic rats, dissolved in an appropriate volume of water and administered orally at 4 or 8 mg/kg/day to diabetic rats and 8 mg/kg/day to nondiabetic rats for 24 weeks. Diabetic and nondiabetic control rats received the equal volume of vehicle within the same time. Enough water was offered in the daytime, and food was available ad libitum during the entire experimental period for all five groups. Body weight and tail blood glucose were measured every 2 weeks after 8 h of fasting (in the daytime) throughout the study period. HbA1c was determined by an aminoethyl-beranate-agarose affinity chromatographic method (Glyc-Affin GHb; Seikagaku Kogyo, Tokyo, Japan) at week 24. All animal procedures adhered to the Institutional Animal Care and Use Guidelines at Chonbuk National University Medical School.

Plasma Parameters. After overnight fasting of the rats in weeks 12 and 24, blood samples were collected at 2, 4, and 8 h postmeal via the tail vein, and plasma was prepared. Plasma insulin and active GLP-1 levels were measured by using enzyme-linked immunosorbent assay kits (Linco Research, St. Charles, MO).

Renal Function. In weeks 12 and 24, the urine from each rat was collected in a metabolic cage (Nalgene; Sybron, Bend, OR) every 4 h at room temperature and then transferred to 4°C. When the final 24-h urine was obtained, the parameter values were determined immediately as follows. Urine albumin concentrations were measured by time-resolved fluorometric immunoassay (Feng Hua Bioengineering Corporation, Guangzhou, China). Plasma and urine creatinine and urea concentrations were assayed with an automatic biochemistry analyzer (Olympus-2000; Olympus, Tokyo, Japan). Creatinine and urea clearance were calculated by using an index of glomerular filtration rate.

Kidney Cytoplasmic Lysate and Homogenate Analysis. In week 24, the animals were killed, renal cortices were rinsed and weighed, and the cytoplasmic fractions were prepared as described previously (Kuhad and Chopra, 2009). TGF-β1 was quantified by using the Quantikine Rat TGF-β1 immunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. DPP IV activity was assayed by measuring the release of p-nitroaniline resulting from the hydrolysis of glycyrlprolyl-p-nitroanilide tosylate at 380 nm as described previously (Yang et al., 2007).

24-h Urinary 8-OHdG and cAMP Excretions. Urinary 8-OHdG concentration, a marker of oxidative damage to DNA, was measured in weeks 12 and 24 by enzyme-linked immunosorbent assay (8-OHdG check; Japan Institute for the Control of Aging, Shizuoka, Japan) according to the manufacturer’s instructions. Urinary cAMP excretion was measured by equilibrated radioimmunoassay as described previously (Harper and Brocker, 1975).

Histological and Immunohistochemical Analysis. For histological study, kidneys were fixed with 4% paraformaldehyde and embedded with JB-4. Sections 1.5 μm thick were stained by periodic acid-Schiff (PAS) reaction or Masson trichrome. Glomerular injury was evaluated by mesangial expansion and glomerulosclerosis index (GSI) in sections stained with PAS reagent as described previously (Teles et al., 2009). In brief, the mesangial area with periodic acid-Schiff positive material was counted as mesangial expansion, which was determined in 50 consecutive glomeruli from each rat by a point-counting technique. For sclerosis calculation of a GSI, the degree of sclerosis in each glomerulus was first graded on a scale of 0 to 10, and a weighted average of these scores was subsequently computed. The fraction of renal cortex occupied by interstitial tissue (%INT) was quantitatively evaluated in Masson-stained sections by using a point-counting technique under a 176-point grid (Jepsen and Mortensen, 1979). Immunohistochemical analysis in renal cortex was as described previously (Liu et al., 2011). The anticleaved caspase 3 (1:150; Millipore Bioscience Research Reagents, Temecula, CA), anti-GLP-1R (1:150; Abbcam, Inc., Cambridge, MA), and anti-rabbit Alexa Fluor (488) (1:500; Invitrogen, Lidingo, Sweden) antibodies were used when stained. Photomicrographs were captured with a Carl Zeiss Axioskop2 plus microscope (Carl Zeiss GmbH, Jena, Germany) and a digital camera (Axiocam HRC; Carl Zeiss GmbH) with final magnifications of 400× for glomeruli and 100× for tubules. From each tissue, 150 to 200 random glomeruli (10 sections) and 30 tubular fields (five sections) were counted in a blinded fashion by two independent investigators.

Electron Microscopic Evaluation. To study the renal ultrastructure, the renal cortex was fixed, embedded, and then stained with lead citrate and uranyl acetate. Glomerular basement membrane (GBM) thickness was measured by the orthogonal intercept method in electron photomicrographs under 20,000× magnification with a JEM 1200 EXII electron microscope (JEOL, Tokyo, Japan). Thirty measurements were performed on each glomerulus, and 20 glomeruli were measured for every animal.

Western Blot Assay. Kidney tissues were homogenized with radioimmunoprecipitation assay buffer and protease inhibitors. Fifty micrograms of total protein were loaded in a stacking polyacrylamide gel and resolved on an 8%–15% polyacrylamide gel with biotinylated molecular weight standard markers. The samples were then transferred to a 0.2-micron nitrocellulose membrane. After blocking for 1 h, the blots were incubated overnight at 4°C with rabbit antibodies raised against cleaved-caspase 3 (1:400; Cell Signaling Technology, Danvers, MA), and β-actin (1:1000 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After washing, the membranes were probed with secondary anti-rabbit IgG-hors eradish peroxidase-linked antibody (1:2000; Enzo Life Sciences, Inc., Farmingdale, NY) for 1 h at room temperature. The bands were detected by using an enhanced chemiluminescence solution (GE Healthcare, Chalfont St.
Giles, Buckinghamshire, UK) and followed by exposure to X-ray film. The optical density for quantification was determined by using Glyko Bandscan 4.0 software (Prozyme, Hayward, CA).

**Real-Time PCR.** Total RNA was extracted from isolated renal cortical tissues by using TRIzol reagent (Invitrogen, Carlsbad, CA) and treated with RNase-free DNase (Invitrogen). First-strand cDNA was generated with random primers by reverse transcriptase (Takara, Otsu, Japan). The PCR was carried out by using a SYBR Green Master mix and the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). All reactions were conducted in triplicate as described previously (Xu et al., 2007). The obtained value was adjusted with a control gene (β-actin) and expressed as a percentage of the value in normal control extracts. The sequences of the primers were (forward and reverse, respectively): 5’-CATCCACCTGAACTGTGTC-3’ and 5’-GGGCAGCGTGCTTTGATGAA-3’ (GLP-1R) and 5’-CGTGAAGATGACCCGATCGA-3’ and 5’-TGTTACGACCAGGCCATACAG-3’ (β-actin).

**Statistical Analysis.** Data are presented as the mean ± S.E.M., and a one-way analysis of variance with Duncan’s post hoc test was used. Data were considered statistically significant if P < 0.05. Statistical analysis was performed by using SPSS version 12.0 software (SPSS Inc., Chicago, IL).

**Results**

**Effect on Food Intake, Body Weight, Blood Glucose, HbA1c, and Insulin Levels.** Food intake, fasting blood glucose, and HbA1c levels were significantly higher, whereas body weight and plasma insulin levels were markedly lower, in diabetic rats compared with nondiabetic animals. LAF237 (4 or 8 mg/day) treatment did not significantly attenuate the changes. Plasma insulin content remained at rather low levels in STZ-induced diabetic rats in our study, indicating that single DPP IV inhibitor therapy had less of a hypoglycemic effect in STZ-induced type 1 diabetes than typical type 2 diabetes (Jin et al., 2009) (Table 1).

**Effect on Renal Function.** Diabetic rats exhibited marked polyuria, increased urinary albumin and protein excretion, high serum creatinine and blood urea nitrogen levels, enhanced albumin/creatinine ratio, and decreased creatinine clearance at weeks 12 and 24. Repeated treatments with LAF237 at doses of 4 and 8 mg/kg/day significantly reduced diabetic albuminuria (P < 0.05), proteinuria (P < 0.05), serum creatinine (P < 0.05), blood urea nitrogen (P < 0.05), and albumin/creatinine ratio (P < 0.05) in diabetic rats in week 12. Creatinine clearance was also significantly improved in both LAF237-treated diabetic groups (P < 0.05) compared with the diabetic control group. Data obtained at week 24 were consistent with those of week 12 (Table 2).

**Renal Histological Examination.** The fractional mesangial area and extent of segmental glomerulosclerosis were significantly higher in the untreated diabetic group compared with the nondiabetic groups. Treatment with LAF237 significantly lowered the fractional mesangial area (DM + 4LAF vs. DM, P < 0.05 and DM + 8LAF vs. DM, P < 0.01) and reduced the GSI (DM + 4LAF vs. DM, P < 0.05 and DM + 8LAF vs. DM, P < 0.01) in diabetic rats in a dose-dependent manner (Fig. 1A-G). Another component of renal injury in diabetes, interstitial expansion (characterized by INT% in our study), was 9-fold higher in untreated diabetic rats than nondiabetic rats. Treatment with 4 and 8 mg/kg LAF237 reduced the INT% of diabetic rats by 33% (Fig. 1H; P < 0.05) and 38% (P < 0.05), respectively.

**Effects on GBM Thickness.** The GBM between endothelial and epithelial cells was observed by electron microscopy. The 24-week-old hyperglycemic rats displayed significant thickening of the GBM compared with age-matched, nondiabetic animals. There was, however, a significant decrease in the thickness of the GBM in both LAF237-treated diabetic groups in comparison with the untreated diabetic controls (Fig. 2; P < 0.05).

**Renal DPP IV Activity and Plasma GLP-1 (Active) Level.** Renal DPP IV activity was significantly up-regulated in STZ-induced diabetic rats compared with age-matched control animals at week 24 (P < 0.01; Fig. 3A). Treatment with both 4 and 8 mg/kg LAF237 normalized enzyme activity in diabetic rats. Active GLP-1 level increased after food intake and then decreased gradually with the passage of time in LAF237-treated diabetic or nondiabetic rats; this pattern was not observed in untreated rats. As expected, GLP-1 (active) levels in the 4 and 8 mg/kg LAF237-treated diabetic groups were almost three and four times higher, respectively, at 2 h postmeal, and two and three times higher at both 4 and 8 h postmeal, compared with the untreated diabetic groups at the corresponding time points. Differences between treated and untreated nondiabetic rats were similar to those seen in diabetic animals, and similar GLP-1 (active) levels were observed in week 12 (data not shown).

**Effect on GLP-1R Expression.** GLP-1R expression was clearly visualized by immunohistochemical analysis in both the glomeruli and tubules of nondiabetic rats. It was reported previously that strong expression of GLP-1R was detected in some of the tubules, probably in the proximal rather than distal tubules (Schlatter et al., 2007). It is noteworthy that the 24-week period of hyperglycemia resulted in a lower intensity of GLP-1R staining in the kidney, but this decrease was prevented by treatment with either 4 or 8 mg/day LAF237. A similar result was derived when quantifying receptor mRNA expression using real-time PCR. mRNA expression levels were dramatically lower in untreated diabetic rats compared with nondiabetic animals. Gene expression

**TABLE 1**

<table>
<thead>
<tr>
<th>Table 1: Effects of LAF237 on body weight, food intake, blood glucose, HbA1c, and plasma insulin levels in nondiabetic and diabetic rats</th>
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<tbody>
<tr>
<td><strong>Group</strong></td>
</tr>
<tr>
<td>Body weight, g</td>
</tr>
<tr>
<td>Food intake, g/day</td>
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<tr>
<td>Blood glucose, mg/dl</td>
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<tr>
<td>HbA1c, %</td>
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<td>Insulin, ng/dl</td>
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*a P < 0.01 compared with nonDM.*
TABLE 2
Effects of LAF237 on renal function in nondiabetic and diabetic rats
Data are expressed as mean ± S.E.M.

<table>
<thead>
<tr>
<th>Group</th>
<th>Week</th>
<th>nonDM</th>
<th>nonDM + 8LAF</th>
<th>DM</th>
<th>DM + 4LAF</th>
<th>DM + 8LAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum creatinine, mg/dl</td>
<td>12</td>
<td>0.48 ± 0.03</td>
<td>0.44 ± 0.02</td>
<td>0.96 ± 0.07</td>
<td>0.69 ± 0.07</td>
<td>0.67 ± 0.07</td>
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<td></td>
<td>24</td>
<td>0.47 ± 0.01</td>
<td>0.51 ± 0.02</td>
<td>1.31 ± 0.11c</td>
<td>0.75 ± 0.03c</td>
<td>0.62 ± 0.02c</td>
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<tr>
<td>Blood urea nitrogen, mg/dl</td>
<td>12</td>
<td>25.2 ± 4.87</td>
<td>24.5 ± 1.25</td>
<td>52.7 ± 3.19d</td>
<td>43.4 ± 2.34b</td>
<td>41.4 ± 3.15b</td>
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<tr>
<td></td>
<td>24</td>
<td>30.5 ± 0.59</td>
<td>28.5 ± 0.61</td>
<td>65.5 ± 2.31d</td>
<td>46.3 ± 1.03d</td>
<td>52.6 ± 0.99d</td>
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<td>Urinary albumin, µg</td>
<td>12</td>
<td>251 ± 22</td>
<td>244 ± 24</td>
<td>944 ± 79c</td>
<td>701 ± 68c</td>
<td>677 ± 63b</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>257 ± 69</td>
<td>249 ± 77</td>
<td>1410 ± 99c</td>
<td>1006 ± 85c</td>
<td>801 ± 36c</td>
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<tr>
<td>Urinary protein, mg</td>
<td>12</td>
<td>11.1 ± 1.3</td>
<td>12.7 ± 1.5</td>
<td>19.9 ± 1.4c</td>
<td>14.2 ± 1.1c</td>
<td>14.3 ± 1.4b</td>
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<td>24</td>
<td>13.1 ± 1.1</td>
<td>14.4 ± 0.9</td>
<td>24.5 ± 1.8d</td>
<td>15.1 ± 1.4c</td>
<td>17.0 ± 1.3c</td>
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<tr>
<td>Albumin, µg/creatinine, mg</td>
<td>12</td>
<td>14.8 ± 1.1</td>
<td>15.7 ± 1.3</td>
<td>65.7 ± 5.4d</td>
<td>44.2 ± 3.4b</td>
<td>40.7 ± 3.8b</td>
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<td>24</td>
<td>16.1 ± 1.2</td>
<td>17.4 ± 1.1</td>
<td>88.1 ± 4.6c</td>
<td>64.8 ± 3.1c</td>
<td>60.0 ± 2.8c</td>
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<td>Creatinine clearance, ml/min</td>
<td>12</td>
<td>1.98 ± 0.28</td>
<td>2.04 ± 0.19</td>
<td>0.87 ± 0.09d</td>
<td>1.25 ± 0.11b</td>
<td>1.28 ± 0.12b</td>
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<td>24</td>
<td>1.92 ± 0.20</td>
<td>1.86 ± 0.22</td>
<td>0.73 ± 0.07d</td>
<td>1.16 ± 0.10b</td>
<td>1.30 ± 0.12b</td>
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*p < 0.01 compared with nonDM.

**p < 0.05 compared with DM.

†p < 0.01 compared with DM.

&p < 0.05 compared with nondiabetic control (nonDM).

A to E, representative micrographs of PAS-stained kidney sections show mesangial expansion and glomerulosclerosis; 1.5-µm renal cortex sections were collected from nondiabetic rats without (A) and with 8 mg/kg (B) LAF237 treatment, and diabetic rats without (C) and with 4 or 8 mg/kg (D or E) LAF237 treatment. Bar, 50 µm. F to H, bar graph representation of the percentage of glomerular area occupied by the mesangium (F), glomerulosclerosis index (G), and INT% (H) in week 24. Data are expressed as mean ± S.E.M. &P < 0.01 compared with nondiabetic control (nonDM). *P < 0.05 and **P < 0.01 compared with diabetic control (DM). 4LAF/8LAF, treated with 4 or 8 mg/kg/day LAF237.

Fig. 1. Histological studies. A to E, representative micrographs of PAS-stained kidney sections show mesangial expansion and glomerulosclerosis; 1.5-µm renal cortex sections were collected from nondiabetic rats without (A) and with 8 mg/kg (B) LAF237 treatment, and diabetic rats without (C) and with 4 or 8 mg/kg (D or E) LAF237 treatment. Bar, 50 µm. F to H, bar graph representation of the percentage of glomerular area occupied by the mesangium (F), glomerulosclerosis index (G), and INT% (H) in week 24. Data are expressed as mean ± S.E.M. *P < 0.01 compared with nondiabetic control (nonDM). *P < 0.05 and **P < 0.01 compared with diabetic control (DM). 4LAF/8LAF, treated with 4 or 8 mg/kg/day LAF237.

was significantly up-regulated after therapy with 8 mg/kg LAF237, although no marked changes were observed after treatment with 4 mg/kg LAF237.

**Urinary cAMP and 8-OHdG Excretion.** Urinary 8-OHdG excretion significantly increased (655 ± 78 versus 79 ± 17 and 850 ± 114 versus 86 ± 14 ng/24 h; P < 0.01 and P < 0.01, respectively; Fig. 4B), whereas cAMP level decreased (47.2 ± 4.24 versus 62.2 ± 5.77 and 36.8 ± 3.89 versus 56.3 ± 5.30 ng/24 h; P = 0.06 and P < 0.05, respectively; Fig. 4A) in untreated diabetic rats in weeks 12 and 24 compared with nondiabetic rats. Treatment with 4 or 8 mg/kg LAF237 significantly suppressed the increase in 8-OHdG excretion and attenuated the reduction in cAMP levels. The cAMP content was also elevated in LAF237-treated nondiabetic rats compared with normal control rats.

**Assessment of Cleaved Caspase 3 Expression.** Very few cleaved caspase 3-stained cells were found in the glomeruli of nondiabetic rats. Glomerular apoptosis was significantly greater in placebo-treated diabetic rats in week 24 (Fig. 5, A and B). The increase was inhibited by 31% (P < 0.05) or 34% (P < 0.05) after treatment with 4 or 8 mg/kg LAF237, respectively. Accordingly, markedly elevated cleaved caspase 3 activity, determined by Western blot analysis, was noted in the renocortical tissue of the untreated diabetic group (Fig. 5C). This activity was lowered significantly (P < 0.05) in the diabetic group treated with 8 mg/kg LAF237 compared with the untreated diabetic group. Levels were also lower in the diabetic group treated with 4 mg/kg LAF237, although the difference did not reach statistical significance.
Effect on TGF-β1 Expression. TGF-β1 promotes renal cell hypertrophy and induces fibrosis through the stimulation of the extracellular matrix in kidney injury caused by hyperglycemia (Hoffman et al., 1998), so we examined TGF-β1 expression to evaluate the effect of LAF237 on the progression of nephropathy. Hyperglycemia induced an increase in TGF-β1 level in the cortex of 24-week-old Sprague-Dawley rats. TGF-β1 expression was significantly inhibited after therapy with 4 mg/kg LAF237 in diabetic rats (305/11006 45 versus 438/11006 38 pg/mg protein; P<0.05; Fig. 6), but there was no additive effect on TGF-β1 expression when the dose was increased to 8 mg/kg. In contrast, there was no such change in nondiabetic rats.

Discussion

Our data provide evidence that DPP IV inhibitors can decrease proteinuria, albuminuria, urinary albumin/creatinine ratio, and serum creatinine, improve creatinine clearance, and delay glomerular and tubulointerstitial fibrosis in diabetic rats (Table 2; Fig. 1). LAF237 therapy at both experimental doses also prevented the GBM from thickening, while failing to affect blood glucose, insulin level, and food intake throughout 24 weeks. Circulating insulin concentration remained very low in our diabetic rats, suggesting that STZ might have almost induced destruction of the whole pancreas, which was further proved by the fact that insulin content was nearly undetected in the isolated pancreatic islet of the diabetic rat (data not shown). It limited the potential of LAF237 as an antidiabetic agent. On the other hand, our findings indicate that the renoprotective action of DPP IV inhibitors is probably not attributable to a hypoglycemic effect.

Increased DPP IV activity in the kidney or urine is a well recognized hallmark of diabetic patients and animals with renal dysfunction, which is probably an important sign or booster of the progression of diabetic nephropathy (Golubović et al., 1996; Yang et al., 2007; Mitic et al., 2008). In addition, patients with diabetes mellitus show dramatic loss of GLP-1 potency (Hojbjerg et al., 2007; Holst et al., 2009). These findings suggest that impaired GLP-1 action also exists in the kidney in the hyperglycemic condition, which might be the result of increased DPP IV activity. However, LAF237 therapy significantly decreased DPP IV activity in the kidneys of diabetic rats (Fig. 3A). Therefore, it is not surprising that the DPP IV inhibitor ameliorated mesangial expansion and protected the GBM against thickening probably, in part, by inhibiting renal DPP IV activity and improving GLP-1 function.

Moreover, administration of LAF237 dose-dependently augmented plasma active GLP-1 levels throughout at least 8 h postmeal in both nondiabetic and diabetic rats (Fig. 3B), which is consistent with the results of previous studies (Deacon et al., 1998; Vella et al., 2007). It is important to note that the down-regulation of GLP-1R expression is also observed in the pancreas and kidneys of diabetic animals (Park et al., 2007; Xu et al., 2007). We observed a similar result (Fig. 7), whereas treatment with the DPP IV inhibitor up-regulated GLP-1R expression in both the glomerulus and tubules. Thus, in addition to the inhibition of DPP IV activity, enhancement of the circulating level of active GLP-1 by binding GLP-1R to mediate several pathological processes represents another possible mechanism underlying the observed renoprotection. Therapeutic intervention based on GLP-1 pathways, such as GLP-1 level elevation and receptor activation, has been shown to have a beneficial effect on diabetic neu-
ropathy and retinopathy (Jin et al., 2009; Zhang et al., 2009). In insulin-resistant obese men, intravenous infusions of GLP-1 reduce glomerular hyperfiltration and enhance sodium excretion, suggesting an action mediated by GLP-1R in the kidneys (Gutzwiller et al., 2004). Moreover, GLP-1R agonists offer antihypertensive and renoprotective properties, at least partly, through their binding affinity for GLP-1R in db/db mice (Park et al., 2007; Hirata et al., 2009). Therefore, mimicking the action of GLP-1 correlated with GLP-1R activation has therapeutic possibilities for both diabetic complications and nephropathy.

Accumulating evidence has confirmed that GLP-1R is coupled to the cAMP second-messenger pathway, which elicits β-cell survival, insulin secretion, neuroprotection, and antihypertensive effects (Perry et al., 2002; Park et al., 2006; Hirata et al., 2009). Repeated DPP IV inhibitor therapy resulted in a significant increase in urinary cAMP excretion in both nondiabetic and diabetic rats (Fig. 4A), indicating that GLP-1R, the expression of which was up-regulated in diabetic rats treated with LAF237 compared with placebo, might directly affect the kidneys through a cascade involving the second messenger. Although the specific mechanism remains unclear, antioxidant and antiapoptosis through GLP-1R activation are probably the main pathways for tissue

**Fig. 4.** Effects of LAF237 on 24-h urinary cAMP (A) and 8-OHdG (B) excretion in nondiabetic and diabetic rats in week 24. Data are expressed as mean ± S.E.M., †, P < 0.05 and ‡, P < 0.01 compared with nonDM. *, P < 0.05 compared with DM.

**Fig. 5.** Effect of LAF237 on cleaved caspase 3 expression in the renal cortex of nondiabetic and diabetic rats in week 24. A, double-labeling fluorescence of glomerular cells in nondiabetic and diabetic rats that were treated with or without LAF237. Arrows indicate the nuclei of glomerular cells that were dual-stained by anticleaved caspase 3 (green) and DAPI (blue-white). Bar, 50 μm. B, bar graphs show cleaved caspase 3-stained glomerular cells presented as a percentage of the total number of DAPI-stained cells. C, cleaved caspase 3 activity in kidney lysates detected by Western blotting. Data are expressed as mean ± S.E.M. †, P < 0.01 compared with nonDM. *, P < 0.05 compared with DM.
protection. It has been reported that GLP-1R signaling, directly or via reducing thioredoxin interacting protein expression level, modulates the endoplasmic reticulum and oxidative stress, leading to promotion of cell adaptation and survival (Yusta et al., 2006; Yu and Jin, 2010). An antiapoptotic effect mediated by GLP-1R in neural cells after hyperglycemia or other insults has also been suggested (Li et al., 2003, 2009). Moreover, activation of GLP-1R is associated with protecting renal and retinal cells via the amelioration of oxidative DNA damage and apoptosis in diabetic microvascular complications (Park et al., 2007; Zhang et al., 2009). In the present study, renal cleaved caspase 3 expression and urinary 8-OHdG excretion were higher with hyperglycemia, and then were attenuated after LAF237 treatment (Figs. 4B and 5). However, LAF237 therapy did not change renal superoxide dismutase and malondialdehyde levels (data not shown). These findings suggest that the reduction in oxidative DNA damage (rather than lipid peroxidation) and cell apoptosis might represent a potential target for renoprotection mediated by GLP-1R and a second messenger.

Elevated TGF-β1 levels have been observed in the urine, serum, glomeruli, and tubulointerstitium in diabetic patients, especially those with nephropathy (Yamamoto et al., 1993; Tsakas and Goumenos, 2006). Treatment with antisense TGF-β1 oligodeoxynucleotides, and an inhibitor of TGF-β type I and II receptor kinases significantly decreases renal hypertrophy and fibrosis (Han et al., 2000; Petersen et al., 2008). In our study, we found that a DPP IV inhibitor could also ameliorate the increase in renal TGF-β1 expression induced by hyperglycemia (Fig. 6). This finding probably indicates that overproduced TGF-β1 is one of the factors involved in the pathogenesis of diabetic nephropathy, and DPP IV inhibition participates in conferring renoprotection through down-regulation of the TGF-β1 system, although the relevant mechanism remains to be elucidated.

In conclusion, the DPP IV inhibitor LAF237 prevented the progression of diabetic nephropathy by decreasing proteinuria and albuminuria, improving creatinine clearance, and retarding the development of interstitial expansion, glomerulosclerosis, and GBM thickening. This renoprotection was probably the result of the inhibition of DPP IV activity and the enhancement of active GLP-1 level, which activated GLP-1R, resulting in antioxidative and antiapoptotic effects. The processes were also associated with down-regulation of TGF-β1 expression. Thus, DPP IV inhibitors provide a promising, novel approach for treating diabetic nephropathy.
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Authorship Contributions

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References


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