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Dipeptidyl Peptidase (DPP) IV Inhibitor Attenuates Kidney Injury in Streptozotocin Induced Diabetic Rats

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Running title: DPP IV inhibitor in DN

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ABBREVIATIONS

cAMP cyclic adenosine monophosphate

DPP IV dipeptidyl peptidase IV

GBM glomerular basement membrane

GLP-1 glucagone like peptide 1

TGF-β1 transforming growth factor-β1

8-OhdG 8-Hydroxy-Deoxyguanosine

Section: Endocrine and Diabetes

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ABSTRACT

Dipeptidyl peptidase (DPP) IV inhibitors are likely beneficial for preventing diabetic complication and modulating glucagon-like peptide-1 receptor (GLP-1R) expression. The aim of this study was to determine if the DPP IV inhibitor, LAF237, has renoprotective qualities in streptozotocin-induced diabetic rats. Diabetic and non-diabetic rats were treated with an oral dose of 4 or 8mg/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-OhdG excretion, and GLP-1R, cleaved caspase 3 and transforming growth factor (TGF)-β1 expression. LAF237 significantly decreased 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. Notably, LAF237 markedly down-regulated DPP IV activity and increased active GLP-1 levels, which likely 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 incretin action and activating the GLP-1R.

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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 (GIP), which are secreted from L-cells of the gastrointestinal tract in response to food digestion in humans and rodents. Greater 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 5h after oral administration of DPP IV inhibitor, the plasma GLP-1 concentration remains elevated in diabetics receiving 50mg/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 beta-cell failure, stimulate insulin release, improve glycemic and hemoglobin A1c 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 (Golubovic et al., 1996; Mitic et al., 2008). These findings indicate that 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

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diabetic nephropathy has 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 signalling 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 anti-apoptotic effect (Kim et al., 2010; Shimoda et al., 2011). Similarly, exendin-4 exerts renoprotective effects partly via the action against 8-OHdG excretion and caspase 3 expression regulated by GLP-1R in both type 1 and type 2 diabetic rodents (Kodera et al., 2011; Park et al., 2007). And 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 level 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 kidney of streptozotocin-treated type 1 diabetic rat.

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Materials and Methods

Animals. Male Sprague-Dawley rats (5-7 weeks) 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; 60 mg/kg body weight) dissolved in 0.1 mol/L citrate buffer (pH 4.5). One week after the verification of diabetes (designated as week 0), diabetic and non-diabetic rats were stochastically divided into 3 and 2 groups (n=6-9 per group), respectively. In 12 h of nighttime, LAF237 (Vildagliptin; Novartis, Basel, Switzerland) was 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 non-diabetic rats for 24 weeks. Diabetic and non-diabetic 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 to all of five groups. Body weight and tail blood glucose were measured every two weeks after 8 h of fasting (in the daytime) throughout the study period. Hemoglobin A1c (HbA1c) was determined by an aminophenyl-boronate-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 in Chonbuk National University Medical School.

Plasma Parameters. After overnight fasting of the rats in weeks 12 and 24, blood samples were collected at 2h, 4h, 8h postmeal via the tail vein and plasma was prepared. Plasma insulin and active GLP-1 levels were measured using ELISA

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(enzyme-linked immunosorbent assay) kits (Linco Research, St. Charles, Missouri, USA).

Renal Function. In weeks 12 and 24, the urine from each rat was collected with a metabolic cage (Nalgene; Sybron, Bend, OR, USA) every 4 hours at room temperature, and then transferred to 4°C. When the final 24-hour 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, China). Plasma and urine creatinine and urea concentrations were assayed with an automatic biochemistry analyzer (Olympus-2000, Tokyo, Japan). Creatinine and urea clearance were calculated using an index of glomerular filtration rate (GFR).

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 previously described (Kuhad and Chopra, 2009). TGF-β1 was quantified using the Quantikine Rat TGF-β1 immunoassay kit (R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions. DPP IV activity was assayed by measuring the release of p-nitroaniline resulting from the hydrolysis of glycylprolyl-p-nitroanilide tosylate at 380 nm, as described previously (Yang et al., 2007).

24-hour Urinary 8-OhdG and cAMP Excretions. Urinary 8-Hydroxy-Deoxyguanosine (8-OhdG) concentration, a marker of oxidative damage to DNA, was measured in weeks 12 and 24 by ELISA (8-OHdG check; Japan Institute

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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 Brooker, 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 reaction or Masson trichrome. Glomerular injury was evaluated by mesangial expansion and glomerulosclerosis index (GSI) in sections stained with periodic acid-Schiff (PAS) reagent as described previously (Teles et al., 2009). Briefly, 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 were subsequently computed. The fraction of renal cortex occupied by interstitial tissue (%INT) was quantitatively evaluated in Masson-stained sections 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 anti-cleaved caspase 3 (1:150; Chemicon International, Inc., Temecula, CA), anti-GLP-1R (1:150; Abcam, Inc., Cambridge, MA) and anti-rabbit Alexa Fluor (488) (1:500; Invitrogen, Lidingo, Sweden) antibodies were used when stained. Photomicrographs were captured using a Carl Zeiss Axioskop2 plus microscope (Carl Zeiss, Goettingen, Germany) and a digital camera (Axiocam HRC, Carl Zeiss, Goettingen, Germany) with final

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magnifications of 400× for glomeruli and 100× for tubules. From each tissue, 150-200 random glomeruli (10 sections) and 30 tubular fields (5 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 stained as described previously (Wijnhoven et al., 2007). Glomerular basement membrane (GBM) thickness was measured by the orthogonal intercept method in electron photomicrographs under 20,000× magnification using 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 RIPA buffer and protease inhibitors. 50 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℃ with rabbit antibodies raised against cleaved-caspase 3 (1:400; Cell Signaling Technology, Beverly, MA, USA), and β-actin (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, the membranes were probed with secondary anti-rabbit IgG-HRP-linked antibody (1:2000; Enzo Life Science, Farmingdale, NY, USA) for 1 hour at room temperature. The bands were detected using an enhanced chemiluminescence (ECL) solution (Amersham Biosciences,

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Uppsala, Sweden) and followed by exposure to X-ray film. The optical density for quantification was determined using software Bandscan 4.0 (Glyko, USA).

Real-time PCR. Total RNA was extracted from isolated renocortical tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and treated with RNase-free DNase (Invitrogen, Carlsbad, CA, USA). First-strand complementary DNA (cDNA) was generated with random primers by reverse transcriptase (TaKaRa, Otsu, Japan). The PCR reaction was carried out using a SYBR Green master mix kit and the ABI Prism 7900HT Sequence Detection System (Applied BioSystems, Foster City, CA, USA). 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' –CATCCACCTGAACCTGTTTGC-3' and 5'-GGGCAGCGTCTTTGATGAA-3' (GLP-1R), and 5'-CGTGAAAAGATGACCCAGATCA-3' and 5'-TGGTACGACCAGAGGCATACAG-3' (β- actin).

Statistical Analysis. Data are presented as the mean ± standard error of 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 using SPSS 12.0 software.

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Results

Effect on Food Intake, Body Weight, Blood Glucose, HbA1c and Insulin Levels. Food intake, fasting blood glucose, HbA1c and plasma insulin levels were significantly higher, while body weight was markedly lower, in diabetic rats compared with non-diabetic 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, respectively, significantly reduced diabetic albuminuria ($P<0.05$ and $P<0.05$), proteinuria ($P<0.05$ and $P<0.05$), serum creatinine ($P<0.05$ and $P<0.05$), blood urea nitrogen ($P<0.05$ and $P<0.05$) and albumin/creatinine ratio ($P<0.05$ and $P<0.05$) 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 non-diabetic groups. Treatment with LAF237 significantly lowered

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the fractional mesangial area ($P<0.05$ and $P<0.01$) and reduced the GSI ($P<0.05$ and $P<0.01$) 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 nine-fold higher in untreated diabetic rats than non-diabetic 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 hyperglycemic rats displayed significant thickening of the GBM compared to age-matched, non-diabetic 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 to age-matched control animals at week 24 ($P<0.01$, Fig. 3A). Treatment with both 4 and 8mg/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 non-diabetic 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 3 and 4 times higher, respectively, at 2h postmeal, and 2 and 3 times higher at both 4h and 8h postmeal, compared to the untreated diabetic groups at the corresponding time point. Differences between treated

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and untreated non-diabetic 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 non-diabetic 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). Interestingly, the 24-week period of hyperglycemia resulted in a lower intensity of GLP-1R staining in the kidney, but this decrease was prevented by either 4 or 8mg/day LAF237 treatment. 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 to non-diabetic animals. Gene expression was significantly up-regulated following 8mg/kg LAF237 therapy, 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 vs. 79 ± 17 and 850 ± 114 vs. 86 ± 14 ng/24h, $P<0.01$ and $P<0.01$, respectively, Fig. 5B) while cAMP level decreased (47.2± 4.24 vs. 62.2 ± 5.77 and 36.8 ± 3.89 vs. 56.3 ± 5.30 ng/24h, $P=0.06$ and $P<0.05$, respectively, Fig. 5A) in untreated diabetic rats in weeks 12 and 24, compared with non-diabetic rats. Treatment with 4 or 8mg/kg LAF237 significantly suppressed the increase in 8-OHdG excretion and attenuated the reduction in cAMP level. The cAMP content was also elevated in LAF237-treated non-diabetic rats compared to normal control rats.

Assessment of Cleaved Caspase 3 Expression. Very few cleaved caspase

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3-stained cells were found in the glomeruli of non-diabetic rats. Glomerular apoptosis was significantly greater in placebo-treated diabetic rats in week 24 (Fig. 6A-C). The increase was inhibited by 31% ($P<0.05$) or 34% ($P<0.05$) after treatment with 4 or 8mg/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. 6D). This activity was lowered significantly ($P<0.05$) in the diabetic group treated with 8mg/kg LAF237 compared with the untreated diabetic group. Levels were also lower in the diabetic group treated with 4mg/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 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 Sprague-Dawley rats. TGF-β1 expression was significantly inhibited following 4mg/kg LAF237 therapy in diabetic rats (305±45 vs. 438±38 pg/mg protein, $P<0.05$, Fig. 7), but there was no additive effect on TGF-β1 expression when the dose was increased to 8mg/kg. In contrast, there was no such change in non-diabetic rats.

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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 and Fig. 1). LAF234 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 isolated pancreatic islet of 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 likely 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 likely an important sign or booster of the progression of diabetic nephropathy (Golubovic 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

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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 8h postmeal in both non-diabetic and diabetic rats (Fig. 3B), which is consistent with 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. 4), whereas treatment with the DPP IV inhibitor up-regulated GLP-1R expression in both the glomerulus and tubules. Thus, in addition to 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 neuropathy 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 anti-hypertensive 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.

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Accumulating evidence has confirmed that GLP-1R is coupled to the cAMP second messenger pathway, which elicits beta-cell survival, insulin secretion, neuroprotection and anti-hypertensive 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 non-diabetic and diabetic rats (Fig. 5A), indicating that GLP-1R, the expression of which was up-regulated in diabetic rats treated with LAF237 compared to placebo, might directly affect the kidneys through a cascade involving the second messenger. Although the specific mechanism remains unclear, anti-oxidation and anti-apoptosis through GLP-1R activation are likely the main pathways for tissue protection. It has been reported that GLP-1R signaling, directly or via reducing thioredoxin interacting protein (TxNIP) expression level, modulates the endoplasmic reticulum and oxidative stress, leading to promotion of beta cell adaptation and survival (Yu and Jin, 2010; Yusta et al., 2006). An anti-apoptotic effect mediated by GLP-1R in beta and neural cells following hyperglycemia or other insults has also been suggested (Li et al., 2003; Li et al., 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 following LAF237 treatment (Fig. 5B and 6). However, LAF237 therapy did not change renal SOD and MDA levels (data not shown). These findings suggest that the reduction in oxidative DNA damage (rather than lipid peroxidation) and cell apoptosis

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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. 7). This finding likely indicates that overproduced TGF-β1 is one of the factors involved in the pathogenesis of diabetic nephropathy, and that 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 anti-oxidative and anti-apoptotic 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

Participated in research design: W.J. Liu, Xie, Y.N. Liu, Kim and T.S. Park.

Conducted experiments: W.J. Liu, Xie, Shao, and Jin

Performed data analysis: Y.N. Liu, Kim, and S.K. Park.

Wrote or contributed to the writing of the manuscript: W.J. Liu, Xie, and T.S. Park.

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Figure Legends

Fig. 1. Histological studies. (A-E) Representative micrographs of PAS-stained kidney sections show mesangial expansion and glomerulosclerosis; 1.5 μm renal cortex sections were collected from non-diabetic 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. (F-H) Bar graph representation of the percent glomerular area occupied by the mesangium (F), glomerulosclerosis index (G) and fractional interstitial area (INT%) (H) in week 24. Data are expressed as mean±S.E.M. ^^P<0.01 as compared with nonDM; *P<0.05, **P<0.01 as compared with DM. nonDM=non-diabetic control; DM=diabetic control; 4LAF/8LAF=treated with 4 or 8mg/kg/day LAF237. Bar=50 μm.

Fig. 2. Effects of LAF237 on ultrastructural changes in the kidneys of non-diabetic and diabetic rats. Representative electron photomicrographs were obtained from non-diabetic 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. (F) Bar graph shows the changes in glomerular basement membrane (GBM) thickness in week 24. Data are expressed as mean±S.E.M. ^^P<0.01 as compared with nonDM; *P<0.05 as compared with DM. nonDM=non-diabetic control; DM=diabetic control; 4LAF/8LAF=treated with 4 or 8mg/kg/day LAF237. Bar=500 nm

Fig. 3. Effects of LAF237 on renal DPP-IV activity (A) and plasma GLP-1 (active)

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level (B) in non-diabetic and diabetic rats in week 24. Data are expressed as mean±S.E.M. ^^P<0.01 as compared with nonDM; *P<0.05, **P<0.01 as compared with DM. nonDM=non-diabetic control; DM=diabetic control; 4LAF/8LAF=treated with 4 or 8mg/kg/day LAF237.

Fig. 4. Effect of LAF237 on GLP-1 receptor expression in the renal cortex of non-diabetic and diabetic rats in week 24. (A-E) Immunostaining for the GLP-1 receptor in 10 μm-sections. Representative microphotographs of glomeruli (G) and tubules (T) in non-diabetic 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. (F) mRNA expression levels of the GLP-1 receptor were quantified by real-time PCR. Data are expressed as mean±S.E.M. ^P<0.05 as compared with nonDM; *P<0.05 as compared with DM. nonDM=non-diabetic control; DM=diabetic control; 4LAF/8LAF=treated with 4 or 8mg/kg/day LAF237. Bar=50 μm.

Fig. 5. Effects of LAF237 on 24h urinary cAMP (A) and 8-hydroxyl-2'-deoxyguanosine (8-OHdG) (B) excretion in non-diabetic and diabetic rats in week 24. Data are expressed as mean±S.E.M. ^P<0.05 and ^^P<0.01 as compared with nonDM; *P<0.05 as compared with DM. nonDM=non-diabetic control; DM=diabetic control; 4LAF/8LAF=treated with 4 or 8mg/kg/day LAF237.

Fig. 6. Effect of LAF237 on cleaved caspase 3 expression in the renal cortex of

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non-diabetic and diabetic rats in week 24. (A) Double-labeling fluorescence of glomerular cells in non-diabetic and diabetic rats that were treated with or without LAF237. Arrows indicate the nuclei of glomerular cells that were dual-stained by anti-cleaved caspase 3 (green) and DAPI (blue-white). (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 as compared with nonDM; *P<0.05 as compared with DM. nonDM=non-diabetic control; DM=diabetic control; 4LAF/8LAF=treated with 4 or 8mg/kg/day LAF237. Bar=50 μm.

Fig. 7. Effects of LAF237 on TGF-β1 levels in kidney cytoplasmic lysates of non-diabetic and diabetic rats in week 24. Data are expressed as mean±S.E.M. ^^P<0.01 as compared with nonDM; *P<0.05 as compared with DM. nonDM=non-diabetic control; DM=diabetic control; 4LAF/8LAF=treated with 4 or 8mg/kg/day LAF237.

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TABLE 1

Effects of LAF237 on body weight, food intake, blood glucose, HbA1c and plasma insulin levels in non-diabetic and diabetic rats.

Groups	nonDM	nonDM+8LAF	DM	DM+4LAF	DM+8LAF
Body weight (g)	599±41	539±38	237±11[a]	222±11	215±14
Food intake (g/day)	27.2±1.7	24.1±1.6	48.9±3.2[a]	47.2±1.4	46.8±0.9
Blood glucose (mg/dl)	101±4.30	99±4.51	468±2.50[a]	455±9.99	440±7.99
HbA1c (%)	4.78±0.11	4.55±0.09	12.14±0.51[a]	11.02±0.59	10.45±0.76
Insulin (ng/dl)	1.69±0.09	1.74±0.12	0.29±0.03[a]	0.34±0.03	0.36±0.04

Empty body weight, daily food intake (over 24 weeks), fasting blood glucose, HbA1c level and plasma insulin (4h postmeal) in week 24 for each group are expressed as mean±S.E.M. [a]$P<0.01$ as compared with nonDM. nonDM=non-diabetic control; DM=diabetic control; 4LAF/8LAF=treated with 4 or 8mg/kg/day LAF237.

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TABLE 2

Effects of LAF237 on renal function in non-diabetic and diabetic rats.

	Time	nonDM	nonDM+8LAF	DM	DM+4LAF	DM+8LAF
Serum creatinine (mg/dl)	week 12	0.48±0.03	0.44±0.02	0.96±0.07[b]	0.69±0.07[c]	0.67±0.07[c]
	week 24	0.47±0.01	0.51±0.02	1.31±0.11[b]	0.75±0.03[d]	0.62±0.02[d]
blood urea nitrogen (mg/dl)	week 12	25.2±1.47	24.5±1.25	52.7±3.19[b]	43.4±2.34[c]	41.4±3.15[c]
	week 24	30.5±0.59	28.5±0.61	65.5±2.31[b]	46.3±1.03[d]	52.6±0.99[d]
Urinary albumin (ug)	week 12	251±22	244±24	944±79[b]	701±68[c]	677±63[c]
	week 24	257±69	249±77	1410±99[b]	1006±85[c]	801±36[d]
Urinary protein (mg)	week 12	11.1±1.3	12.7±1.5	19.9±1.4[a]	14.2±1.1[c]	14.3±1.4[c]
	week 24	13.1±1.1	14.4±0.9	24.5±1.8[a]	15.1±1.4[d]	17.0±1.3[d]
Albumin (ug)/creatinine (mg)	week 12	14.8±1.1	15.7±1.3	65.7±5.4[b]	44.2±3.4[c]	40.7±3.8[c]
	week 24	16.1±1.2	17.4±1.1	88.1±4.6[b]	64.8±3.1[d]	60.0±2.8[d]
Creatinine clearance (ml/min)	week 12	1.98±0.28	2.04±0.19	0.87±0.09[a]	1.25±0.11[c]	1.28±0.12[c]
	week 24	1.92±0.20	1.86±0.22	0.73±0.07[a]	1.16±0.10[d]	1.30±0.12[d]

Data are expressed as mean±S.E.M. [a]$P<0.05$, [b]$P<0.01$ as compared with nonDM; [c]$P<0.05$, [d]$P<0.01$ as compared with DM. nonDM=non-diabetic control; DM=diabetic control; 4LAF/8LAF=treated with 4 or 8mg/kg/day LAF237.

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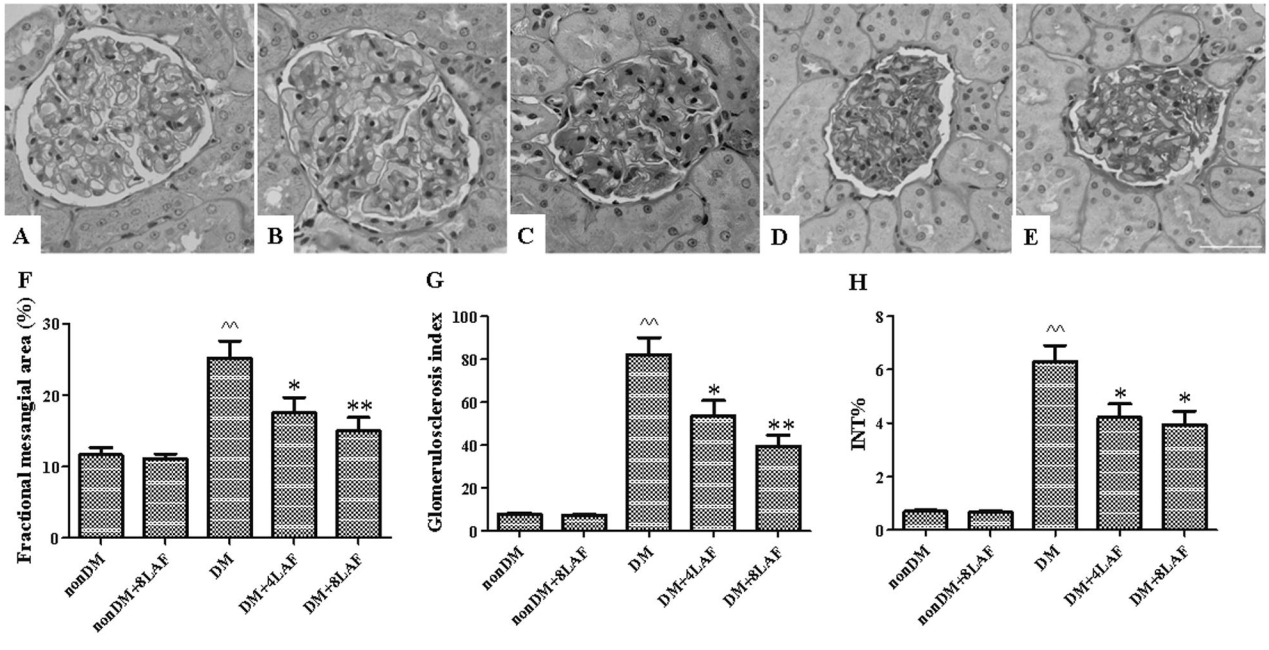
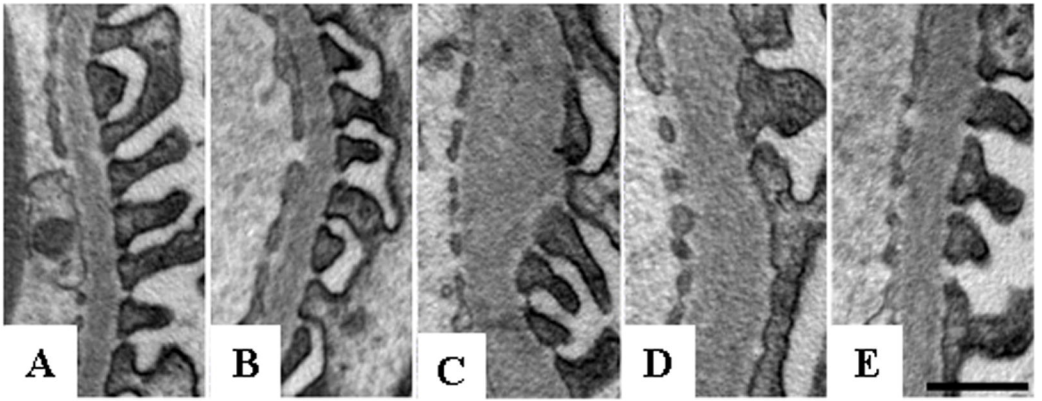

A
B
C
D
E
F
Fractional mesangial area (%)
30
20
10
0
^^
*
**
nonDM
nonDM-8LAF
DM
DM+4LAF
DM-8LAF
G
Glomerulosclerosis index
100
80
60
40
20
0
^^
*
**
nonDM
nonDM-8LAF
DM
DM+4LAF
DM-8LAF
H
INT%
8
6
4
2
0
^^
*
*
nonDM
nonDM-8LAF
DM
DM+4LAF
DM-8LAF

Fig. 1


A
B
C
D
E

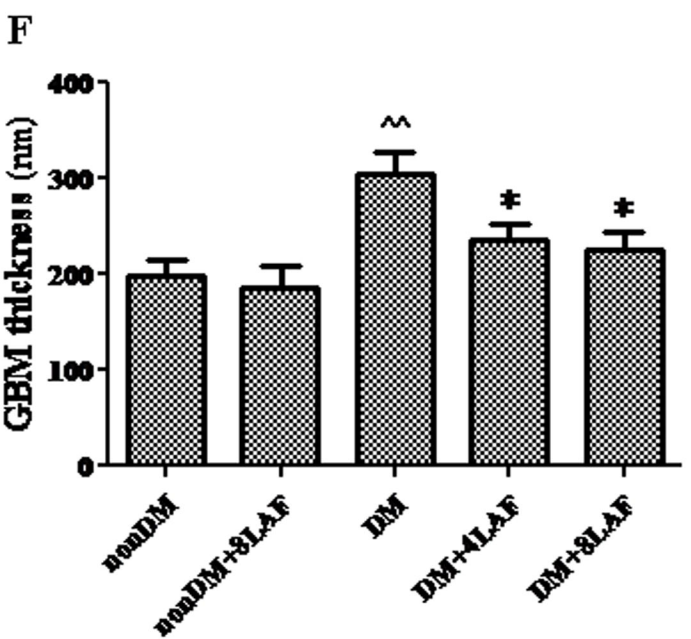

F
GBM thickness (nm)
400
300
200
100
0
^^
*
*
nonDM
nonDM+8LAF
DM
DM+4LAF
DM+8LAF

Fig. 2

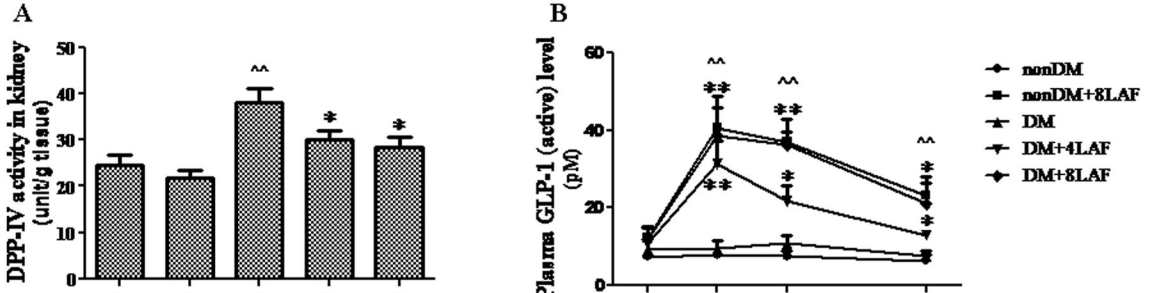

A
DPP-IV activity in kidney (unit/g tissue)
50
40
30
20
10
0
^^
*
*
nonDM
nonDM+8LAF
DM
DM+4LAF
DM+8LAF
B
Plasma GLP-1 (active) level (pM)
60
40
20
0
overnight fasting
postmeal 2h
postmeal 4h
postmeal 8h
nonDM
nonDM+8LAF
DM
DM+4LAF
DM+8LAF

Fig. 3

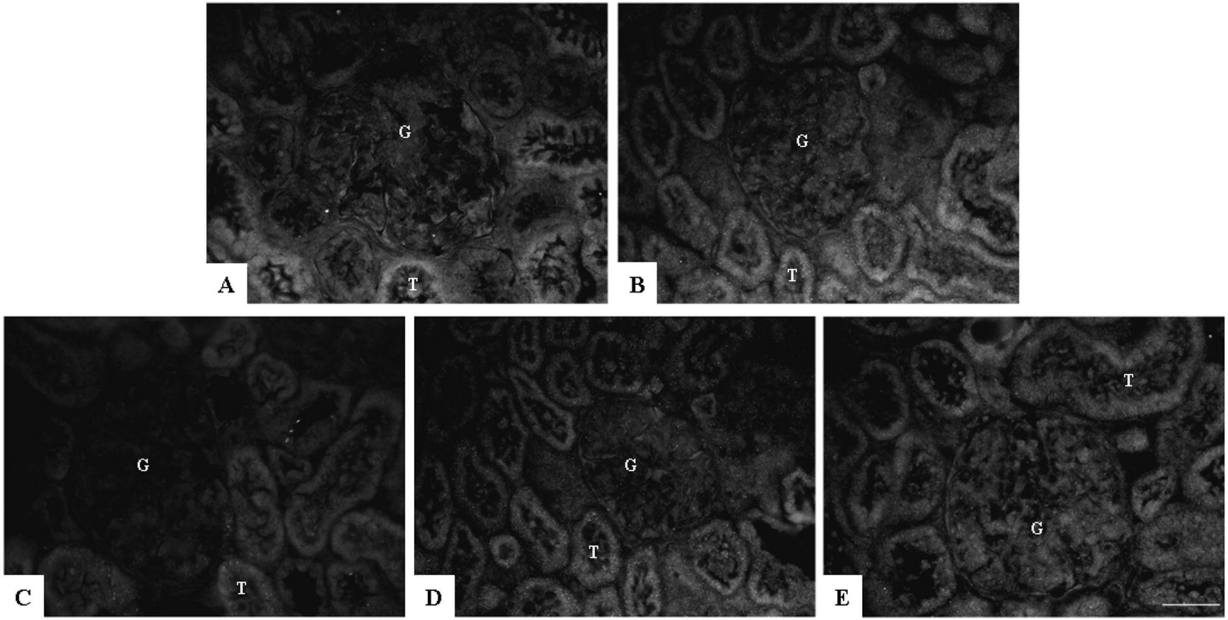

A
G
T
B
G
T
C
G
T
D
G
T
E
T
G
F
mRNA levels of GLP-1 receptor (% of nonDM)
150
100
50
0
^
*
nonDM
nonDM+8LAF
DM
DM+4LAF
DM+8LAF

Fig. 4

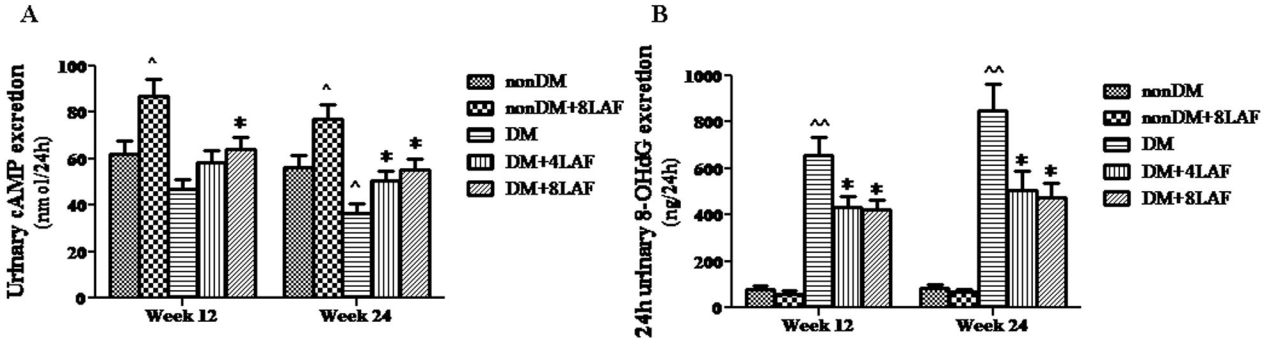

A
Urinary cAMP excretion
(nmol/24h)
100
80
60
40
20
0
Week 12
Week 24
nonDM
nonDM+8LAF
DM
DM+4LAF
DM+8LAF
B
24h urinary 8-OHdG excretion
(ng/24h)
1000
800
600
400
200
0
Week 12
Week 24
nonDM
nonDM+8LAF
DM
DM+4LAF
DM+8LAF

Fig. 5

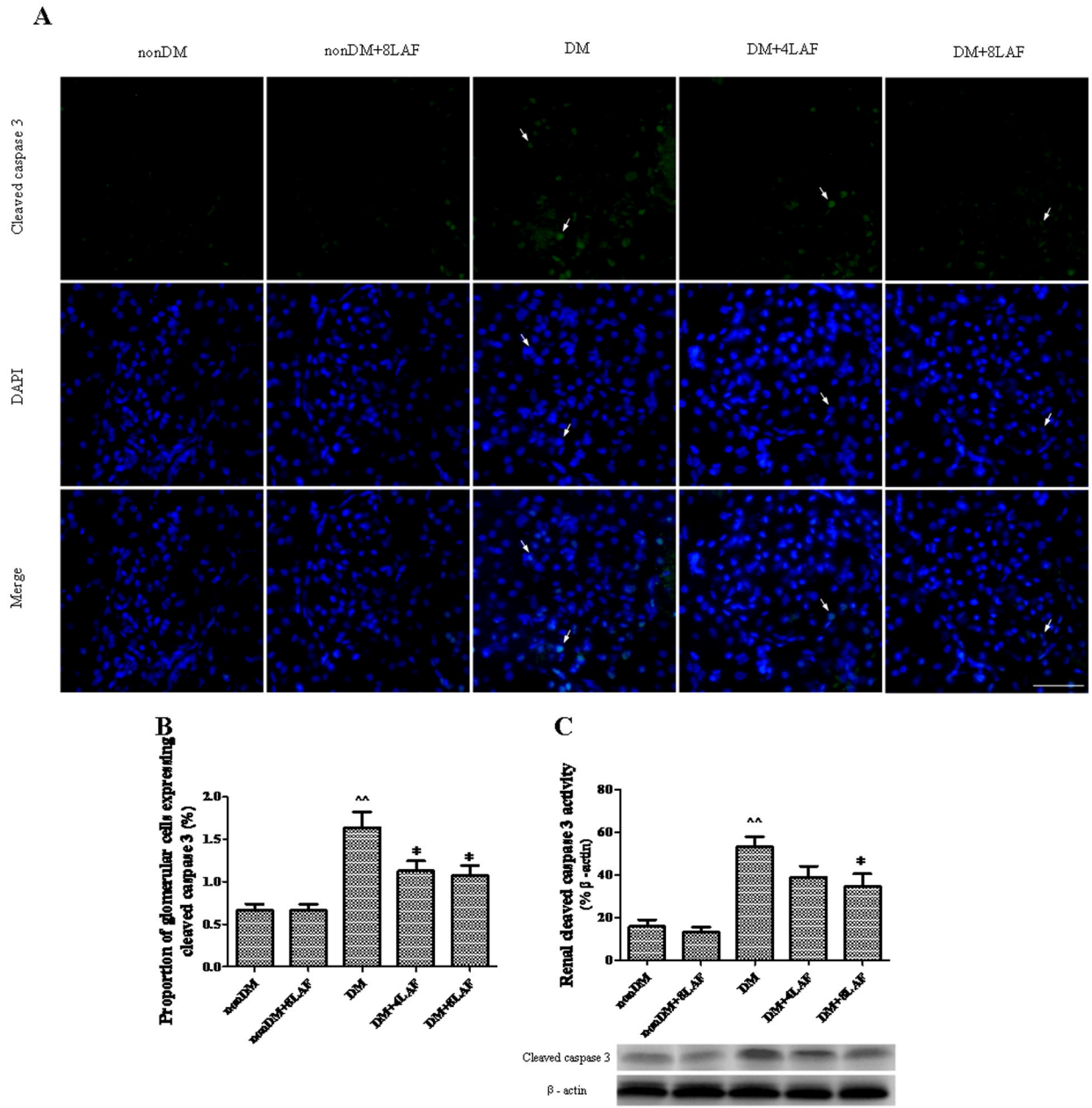

A
nonDM
nonDM+8LAF
DM
DM+4LAF
DM+8LAF
Cleaved caspase 3
DAPI
Merge
B
Proportion of glomerular cells expressing cleaved caspase 3 (%)
2.0
1.5
1.0
0.5
0.0
^^
*
*
nonDM
nonDM+8LAF
DM
DM+4LAF
DM+8LAF
C
Renal cleaved caspase3 activity (% β -actin)
80
60
40
20
0
^^
*
*
nonDM
nonDM+8LAF
DM
DM+4LAF
DM+8LAF
Cleaved caspase 3
β - actin

Fig. 6

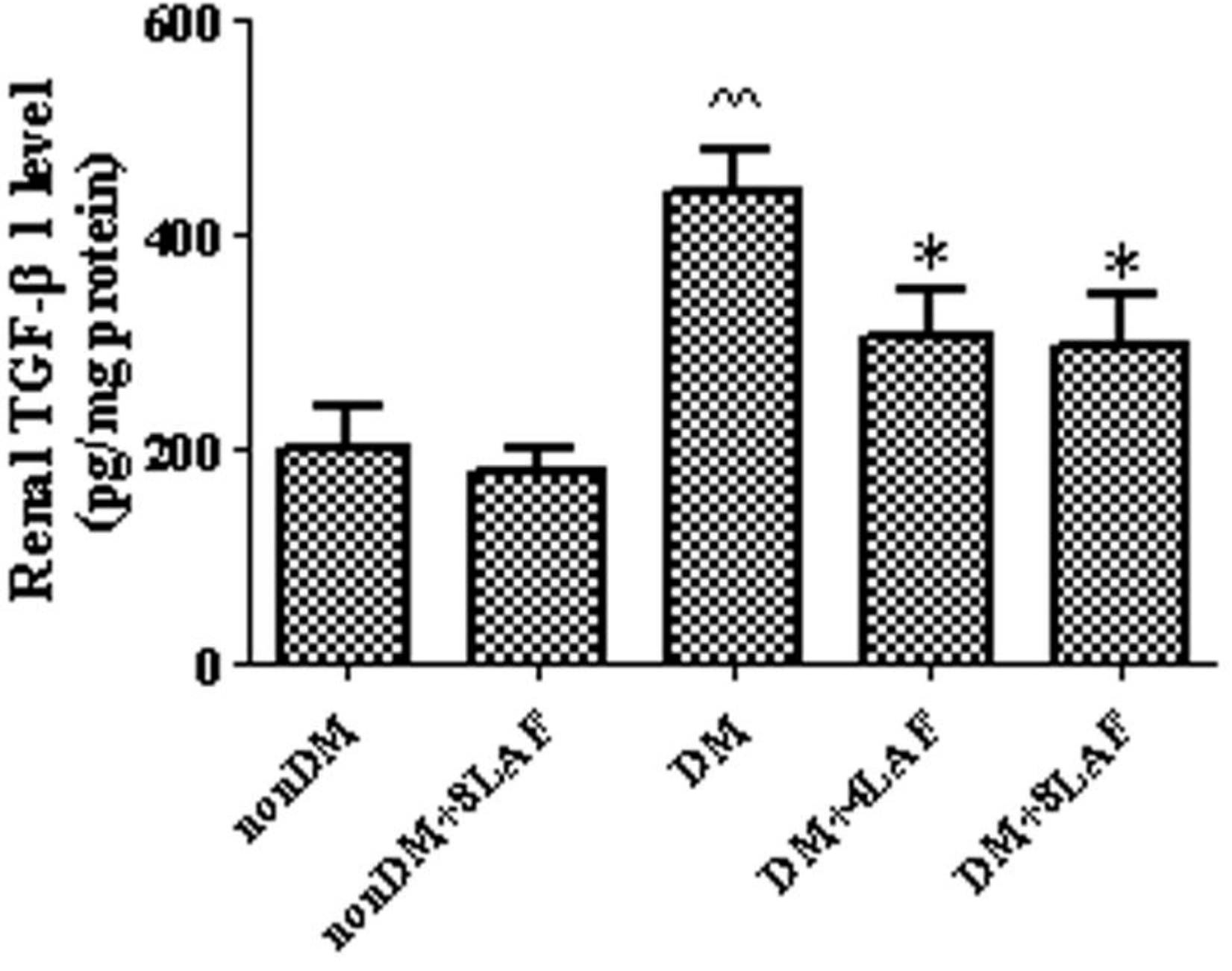

Renal TGF-β 1 level
(pg/mg protein)
600
400
200
0
^^
*
*
nonDM
nonDM+8LAF
DM
DM+4LAF
DM+8LAF

Fig. 7