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Original Paper

Chronic Administration of Visfatin Ameliorated Diabetic Nephropathy in Type 2 Diabetic Mice

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Key Words
Visfatin • Diabetes mellitus • Diabetic nephropathy

Abstract
Background/Aims: Visfatin is a known adipokine which may improve insulin resistance in obesity and have an anti-diabetic effect via the insulin receptor. We studied the effects of visfatin on diabetic nephropathy in type 2 diabetic mice. ***Methods:*** Diabetic male *db/db* mice were treated with intraperitoneal injections of visfatin. Basal parameters were measured in all mice and glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed in diabetic mice. The histopathological and molecular changes were evaluated in diabetic nephropathy. ***Results:*** Visfatin treatment had no effect on body weight, water and food intake, urinary volume, blood glucose, and HbA1c level. However, visfatin improved HOMA-IR, GTT, ITT and decreased plasma insulin and visfatin level, but not adiponectin level. Plasma cholesterol and triglyceride level were also improved by visfatin treatment. Significantly, visfatin decreased albuminuria in diabetic mice. Glomerulosclerotic change and mesangial expansion in the kidneys were significantly reduced. In addition, visfatin inhibited the expression of proinflammatory and profibrotic cytokines such as MCP-1, TGFβ1, type IV collagen, and PAI-1. The enzymes related to lipid metabolism in the kidney, HMG-CoAR was suppressed by visfatin treatment, whereas FXR and ABCA1 were significantly elevated by treatment. ***Conclusion:*** Visfatin might have a protective effect in diabetic nephropathy without the hypoglycemic effect.

Introduction

The kidney has been a main target organ of complications in the metabolic syndrome of obesity and diabetes mellitus [1-3]. Although the kidney can be damaged by hyperglycemia

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in diabetes mellitus, its role has not been detailed in glucose metabolism and insulin resistance. However, it has been suggested that the kidney plays an important role in glucose metabolism, and there are frequently abnormalities in glucose metabolism in patients with chronic kidney disease (CKD) [4]. There is also considerable evidence from animal studies for renal glucose production and utilization [5-8]. However, the role of the kidney in glucose metabolism and energy homeostasis is still unclear.

Recent works have revealed that adipose tissue is a highly metabolic organ with pluripotent functions far beyond the mere storage of energy. Adipose tissue is now known to be an endocrine organ that secretes a large number of adipokines, bioactive proteins which have essential roles in energy homeostasis, glucose and lipid metabolism, insulin resistance, inflammation, immunity, and atherosclerosis [9-12]. Therefore, the cross-talk between adipose tissue and the kidney may be possible in metabolic syndrome, particularly in CKD related to insulin resistance [11]. They possess several active molecules released by adipocytes like as leptin, resistin, adiponectin, and visfatin, as well as cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, macrophage/monocyte chemoattractant protein (MCP)-1, and interleukin (IL)-1. Interestingly, the circulating levels of these adipokines are disordered in experimental animals and patients with CKD, which might be an independent risk factor for cardiovascular mortality. Moreover, their production, secretion, and regulatory actions are not limited to adipose tissue, but are determined on other organ tissues. For example, the kidney itself is able to alter the clearance of adipokines and produce them.

Visfatin was originally characterized from human peripheral blood lymphocytes and is preferentially produced by visceral adipose tissue [13]. Its enzymatic basis and structure has been established as a ubiquitous intracellular protein that is also called nicotinamide phosphoribosyltransferase (NAMPT) / pre-B cell colony-enhancing factor (PBEF)-1 [14]. NAMPT is a component of a nuclear nicotinamide adenine dinucleotide (NAD^+) salvage/ recycling pathway that regulates the functions of NAD^+-dependent enzymes, such as the protein deacetylase sirtuin (SIRT)-1 [15]. Visfatin may be related to the aging-dependent circadian cycle, which lead to the decline of pancreatic cell function in type 2 diabetes and could be an effective target in type 2 diabetes. Angiotensin Ⅱ play a role in the prolongation of life span in mice [16]. Mice lacking angiotensin developed a longevity phenotype and upregulated visfatin expression in the kidney.

We have previously observed that visfatin synthesis is increased by high glucose in mesangial cells, podocytes, and proximal tubular cells [17, 18]. Visfatin treatment induced rapid uptake of glucose into renal cells through glucose transporter (GLUT)-1 translocation. Interestingly, visfatin upregulated profibrotic and proinflammatory molecules increased by high glucose stimulation in renal cells *in vitro* and visfatin synthesis was increased in the renal glomeruli of type 2 diabetic animals [17, 18]. These results suggest that this adipokine is produced in the renal cells and has an important role in the pathogenesis of diabetic nephropathy.

However, controversy remains concerning the role of visfatin in various organ injuries. Thus, our objective is to evaluate the effect of visfatin administration on diabetic nephropathy. We also investigated its effect on insulin resistance and lipid metabolism in type 2 diabetic animals.

Materials and Methods

Animal experiments

We purchased male *db/db* mice from Otsuka Pharmaceutical Co. (Tokyo, Japan) as type 2 diabetic models. Age-matched male *db/m* mice served as the genetic control for *db/db* mice. Mice were fed a standard chow (Cargill Agri Purina Korea Inc, Seoul, Korea). All 8 week-old mice were divided into 4 groups (each group: n = 8) as follows: Control and diabetic groups with or without visfatin treatment (Merck & Co. Inc, Rahway, NJ, USA). 100pmol of visfatin treatment was injected intraperitoneally daily for 3 months. Mice had free access to food and tap water and were caged individually under controlled temperature (23±2°C) and humidity (55±5%) with an artificial light cycle. Daily water intake was checked at regular intervals to confirm the dose of the administered drug. At the end of the study period, systolic blood pressure was mea-

sured using tail-cuff plethysmography (LE 5001-Pressure Meter, Letica SA, Barcelona, Spain). Plasma glucose levels were measured by a glucose oxidase-based method and creatinine levels were determined by a modified Jaffe method. Plasma insulin levels and plasma adiponectin levels were measured using an enzyme-linked immunosorbent assay kit (Linco Research, St Charles, MO, USA). Plasma visfatin levels were measured using an enzyme-linked immunosorbent assay kit (E-EL-M1237, Elabscience, Beijing, China). The homeostasis model assessment index (HOMA-IR) was calculated using fasting glucose (mmol/l) × fasting insulin (mU/l)/22.5. Plasma triglyceride and cholesterol analyses were performed using a GPO-Trinder kit (Sigma, St Louis, MO, USA). Insulin tolerance test (ITT) was performed after 8 hours of fasting, and blood samples were collected through the tail vein. Mice received 0.75 U/kg of regular insulin by intraperitoneal injection, and blood glucose was subsequently measured at 0, 30, 60, 90, and 120 minutes. Glucose tolerance test (GTT) was performed after an 8-hr fasting period, and then blood samples were collected via the tail vein. All mice received 2 g dextrose per kg body weight by intraperitoneal injection for the GTT, and blood glucose levels were measured at 0, 30, 60, 90, and 120 min after glucose loading. To determine urinary albumin excretion, individual mice were caged in a metabolic cage and a 24-h urine sample was collected at the end of the study. Urinary albumin concentration was determined by a competitive enzyme-linked immunosorbent assay (Shibayagi, Shibukawa, Japan) and corrected by urinary creatinine concentration. All mice were killed under anesthesia by an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Experiments were conducted in accordance with the Korea University Guide for Laboratory Animals.

Table 1. Primer sequences for real-time quantitative PCR

Target Gene	Primer sequence('5-'3)
MCP-1, forward	CTGGATCGGAACCAAATGAG
MCP-1, reverse	CGGGTCAACTTCACATTCAA
PAI-1, forward	TCCTCATCCTGCCTAAGTTCTC
PAI-1, reverse	GTGCCGCTCTCGTTTACCTC
TGFβ1, forward	AGCCCGAAGCGGACTACTAT
TGFβ1, reverse	CTGTGTGAGATGTCTTTGGTTTTC
Col-IV, forward	GCTCTGGCTGTGGAAAATGT
Col-IV, reverse	CTTGCATCCCGGGAAATC
HMG-CoA, forward	AGCCGAAGCAGCACATGAT
HMG-CoA, reverse	CTTGTGGAATGCCTTGTGATTG
ABCA-1, forward	CGTTTCCGGGAAGTGTCCTA
ABCA-1, reverse	GCTAGAGATGACAAGGAGGATGGA
FXR, forward	CCAACCTGGGTTTCTACCC
FXR, reverse	CACACAGCTCATCCCCTTT
β -actin, forward	GGACTCCTATGTGGGTGACG
β -actin, reverse	CTTCTCCATGTCGTCCCAGT

ABCA-1, ATP-binding cassette transporter-1; Col-IV, type IV collagen; FXR, farnesoid X receptor; HMG-CoA, cholesterol 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase; MCP-1, monocyte chemoattractant peptide-1; PAI-1, plasminogen activator inhibitor-1; PPARγ, peroxisome proliferator-activated receptor-γ; SREBP-1c, sterol regulatory element-binding protein-1c; TGF-β1, transforming growth factor-β1; In this experiment, each sample was run in triplicate, and the corresponding non-reverse transcribed mRNA samples were used as negative controls. The mRNA level of each sample was normalized to that of β-actin mRNA.

Analysis of gene expression by real-time quantitative PCR

Total RNA was extracted from epididymal fat tissues, renal cortical tissues, liver, and heart using Trizol reagent and further purified using an RNeasy Mini kit (Qiagen, Valencia, CA, USA). Primers were designed from the respective gene sequences using Primer 3 software, and the secondary structures of templates were examined and excluded using the *m*fold software program. The nucleotide sequences of all primers used in this study are shown in Table 1. Quantitative gene expression was performed on a LightCycler 1.5 system (Roche Diagnostics Corporation, Indianapolis, IN, USA) using SYBR Green technology. Real-time reverse transcription-PCR was performed for 10 min at 50 °C and 5 min at 95 °C. Subsequently, 45 cycles were applied, consisting of denaturation for 10 s at 95 °C and annealing with extension for 30 s at 60 °C. At the end of the PCR cycle, samples were heated to 95 °C to check that a single PCR product was obtained. The ratio of each gene to the β-actin level (relative gene expression number) was calculated by subtracting the threshold cycle number (C_t) of the target gene from that of β-actin and raising 2 to the power of this difference.

Immunohistochemistry in tissues

Tissue samples were fixed in 10% buffered formalin and embedded in paraffin. Kidney tissue was cut into 4-μm-thick slices and stained with periodic acid Schiff (PAS), To perform immunohistochemical staining for type IV collagen, transforming growth factor β1 (TGFβ1), plasminogen activator inhibitor-1

(PAI-1), and CD68, kidney sections were transferred to a 10 mmol/l citrate buffer solution adjusted to a pH of 6.0. Slides were the heated at 80 °C for 30 minutes for TGFβ1 and CD68 staining. Alternatively, sections were transferred to Biogenex Retrievit (pH 8.0) (InnoGenex, San Ramon, CA, USA) and microwaved for 10-20 min for plasminogen activator inhibitor (PAI)-1 staining or treated with trypsin (Sigma, St Louis, MO, USA) for 20 min at 37 °C to retrieve antigens for type IV collagen. To block endogenous peroxidase activity, 3.0% H_2O_2 in methanol was applied for 20 min, followed by incubation at room temperature for 60 min with 3% BSA/3% normal goat serum (type IV collagen and CD68), 90 minutes with 5% normal goat serum, and either 15 minutes with 10% power block (PAI-1) or 30 minutes with 20% normal sheep serum (TGFβ1). Slides were incubated overnight at 4 °C with rabbit polyclonal anti-TGFβ1 antibody (1:100; Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA), rat monoclonal anti-CD68 antibody (1:100; AbD Serotec, Raleigh, NC, USA), rabbit polyclonal anti-type IV collagen antibody (1:150; BioDesign International, Sarco, ME, USA), or rabbit polyclonal anti-PAI-1 antibody (1:50; American Diagnostica, Stanford, CT, USA). For coloration, slides were incubated at room temperature with a mixture of 0.05% 3,3′-diaminobenzidine containing 0.01% H_2O_2 and then counterstained with Mayer's hematoxylin. Negative control sections were stained under identical conditions with a buffer solution that was substituted for the primary antibody. Glomerular sclerosis and mesangial expansion were determined on PAS staining result as we described previously [19]. In brief, severity of sclerosis for each glomerulus was graded from 0 to 4+ as follows: 0, no lesion; 1+, sclerosis of <25% of the glomerulus; 2+, 3+, and 4+, sclerosis of 25 to 50%, >50 to 75%, and >75% of the glomerulus, respectively. In addition, glomerular mesangial expansion was scored semi-quantitatively, whereby the percentage of mesangial matrix occupying each glomerulus was rated on a scale from 0 to 4 as follows: 0, 0%; 1, <25%; 2, 25% - 50%; 3, 50% - 75%; and 4, >75 All histologic examinations for glomerulosclerosis and mesangial expansion were carried out by a renal pathologist in a blinded manner, and more than 80 glomeruli were analyzed in kidney sections from each mice. For evaluation of immunohistochemical staining for type IV collagen, TGFβ1, PAI-1 and CD68 results, glomerular fields were graded semi-quantitatively as we described previously [20]. Briefly, For type IV collagen, TGFβ1, and PAI-1, four scores were scaled by extent of positive glomerular field; 0, absent or less than 25% of the area positive; 1, 25%–50% of the area positive; 2, 50%–75% of the area positive; 3, more than 75% of the area positive. To evaluate immunohistochemical staining were counted under a high power field (×400) containing 50 to 60 glomeruli and an average score was calculated and expressed as positive cells per glomerulus. A renal pathologist carried out the histologic examinations in a blinded manner.

Statistical analysis

Results are means ± SE. The ANOVA test was used to compare all four groups with SPSS for Windows 10.0 (SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

Basal characteristic parameters in experimental animals

To examine the role of visfatin in type 2 diabetic mellitus, we first compared the basal biochemical parameters of the experimental animals among four groups (Table 2). Fasting plasma glucose levels were significantly higher in *db/db* mice (556±46 mg/dl) than in *db/m* mice (147±19 mg/dl; $p< 0.05$). *Db/db* mice (45.4±4.37g) had markedly increased body weight compared to *db/m* mice (33.8±0.71g; $p<0.001$). HbA1c levels were also higher in *db/db* mice (8.4±0.4 % vs. 4.5±0.2 %; $p< 0.05$). However, visfatin treatment of both diabetic and non-diabetic mice had no effect on these parameters. *Db/db* mice had significantly greater food intake, water intake, and urinary volume than *db/m* mice over three months. However, visfatin treatment also had no effect on these parameters. Plasma creatinine levels were greater in *db/db* mice than in *db/m* mice, although they were not influenced by visfatin treatment. *Db/db* mice had lower plasma adiponectin levels than *db/m* mice, and visfatin had no effect on plasma adiponectin levels. Plasma visfatin levels were measured in all mice of each group. *Db/db* mice showed higher visfatin level than that in *db/m* mice. However, chronic visfatin treatment for 3 months significantly lowered plasma visfatin level in *db/db* mice, not in *db/m* mice. Systolic blood pressures measured at the end of the 12-week study were similar among the four groups. Table 3 shows that each organ weight was measured and corrected by body weight at the time of sacrifice . *Db/db* mice showed significantly higher weight gain

Table 2. Physical and biochemical parameters in experimental animals

Treatment duration	Group (n = 8 / each group)	Body weight (g)	Urine volume (mL/day)	Food intake (g/day)	Water intake (mL/day)	Plasma creatinine (mg/dL)	Fasting plasma glucose (mg/dL)	Hb_{A1C} (%)	Plasma adiponectin (μg/mL)	Plasma visfatin (ng/mL)	Systolic blood pressure (mmHg)
1month	db/m	31.7±0.49	0.82±0.18	6.71±0.70	8.65±1.66	N.A.	147±11	4.6±0.6	N.A.	N.A.	N.A.
	db/m+visfatin	29.1±0.91	0.65±0.14	4.37±0.52	7.06±0.46	N.A.	139±8.9	5.1±0.4	N.A.	N.A.	N.A.
	db/db	56.4±2.82[a]	1.71±0.22[c]	6.93±0.24	14.6±0.13[a]	N.A.	453±54[a]	8.5±0.7[a]	N.A.	N.A.	N.A.
	db/db+visfatin	45.5±3.45[a,b]	2.19±0.26[c]	9.16±1.09[d]	16.3±1.48[a]	N.A.	565±36[a]	9.7±0.7[a]	N.A.	N.A.	N.A.
2months	db/m	34.3±1.09	0.81±0.06	4.43±0.16	5.18±0.99	N.A.	170±6.9	4.1±0.3	N.A.	N.A.	N.A.
	db/m+visfatin	32.0±1.03	0.87±0.14	4.12±0.43	4.62±0.14	N.A.	156±10	4.5±0.1	N.A.	N.A.	N.A.
	db/db	52.3±3.53[a]	3.32±0.21[a]	6.37±0.21[a]	16.8±0.36[a]	N.A.	589±59[a]	8.5±0.6[a]	N.A.	N.A.	N.A.
	db/db+visfatin	42.6±5.31[a,b]	2.62±0.32[a]	8.48±0.35[a,e]	17.38±0.41[a]	N.A.	528±20[a]	9.3±0.8[a]	N.A.	N.A.	N.A.
3months	db/m	33.8±0.71	0.81±0.14	4.18±0.10	6.12±0.50	0.33±0.01	147±19	4.5±0.2	8.78±1.53	72.53±8.84	102.9±1.09
	db/m+visfatin	32.2±0.99	0.95±0.21	4.87±0.39	6.50±0.34	0.35±0.01	134±25	4.4±0.3	10.3±1.34	148.9±4.07[d]	114.6±1.28
	db/db	45.4±4.37[d]	8.34±0.50[a]	6.93±0.16[a]	17.0±0.34[a]	0.47±0.02[d]	556±46[a]	8.4±0.4[a]	4.79±1.13	124.7±2.90[d]	101.3±1.32
	db/db+visfatin	46.8±6.11[d]	6.46±1.31[a,b]	8.83±0.26[a,e]	15.6±2.18[a]	0.42±0.03[d]	542±37[a]	8.9±0.6[a]	4.17±0.57	116.0±1.46[e]	103.1±1.36

Values are expressed as mean±SEM. [a] $P < 0.05$ *vs db/m* control, [b] $P < 0.05$ *vs db/db* vehicle, [c] $P < 0.01$ *vs db/m* control, [d] $P < 0.001$ *vs db/m* control, [e] $P < 0.001$ *vs db/db* + vehicle.

in the kidney, liver, and adipose tissue compared with *db/m* mice. Visfatin treatment had no effect on the weight of kidney and liver in *db/db* mice. However, visfatin treatment decreased the weight of adipose tissue in *db/db mice*.

Effects of visfatin treatment on metabolic parameters and insulin resistance

We next compared plasma lipid levels among four groups. Diabetic *db/db* mice presented with higher plasma cholesterol and triglyceride levels than non-diabetic *db/m* mice. Visfatin treatment had no effect on cholesterol and triglyceride levels in non-diabetic mice, but its treatment lowered plasma cholesterol and triglyceride levels in diabetic mice (Figure 1, A and B). Visfatin improved HOMA-IR and decreased plasma insulin levels significantly in diabetic mice (Figure 1, C, D). Both ITT and GTT were improved significantly by visfatin treatment in diabetic mice (Figure 1, E, D), which suggests that visfatin may improve the metabolic syndrome in diabetic animals.

Effects of visfatin treatment on diabetic kidney disease in db/db mice

To evaluate the effects of visfatin treatment on renal injury in diabetic mice compared with non-diabetic controls, we measured urinary albumin excretion and investigated histologic changes in both non-diabetic and diabetic kidneys. Urinary albumin excretion was greater in

diabetic *db/db* mice than in non-diabetic *db/m* mice. Visfatin treatment did not influence urinary albumin excretion in non-diabetic *db/m* mice, but it significantly decreased urinary albumin excretion in diabetic *db/db* mice, which was very significant after three months (Figure 2A).

Figure 2, B-E shows representative renal pathologic chan-

Table 3. Summary of organ mass changes in experimental animals

Group (n=8/ each group)	Kidney/ Body weight	Liver/ Body weight	Epididymal fat/ Body weight
db/m	1.06±0.09	4.50±0.39	2.33±0.35
db/m+visfatin	0.99±0.05	4.24±0.12	2.07±0.34
db/db	1.55±0.33[a]	5.86±0.64[a]	5.59±0.97[b]
db/db+visfatin	1.33±0.37	6.58±0.28[a]	4.54±0.56[c]

Values are expressed as mean±SEM. [a] $P < 0.05$ *vs db/m* control, [b] $P < 0.01$ *vs db/m* control, [c] $P < 0.05$ *vs db/db* + vehicle.

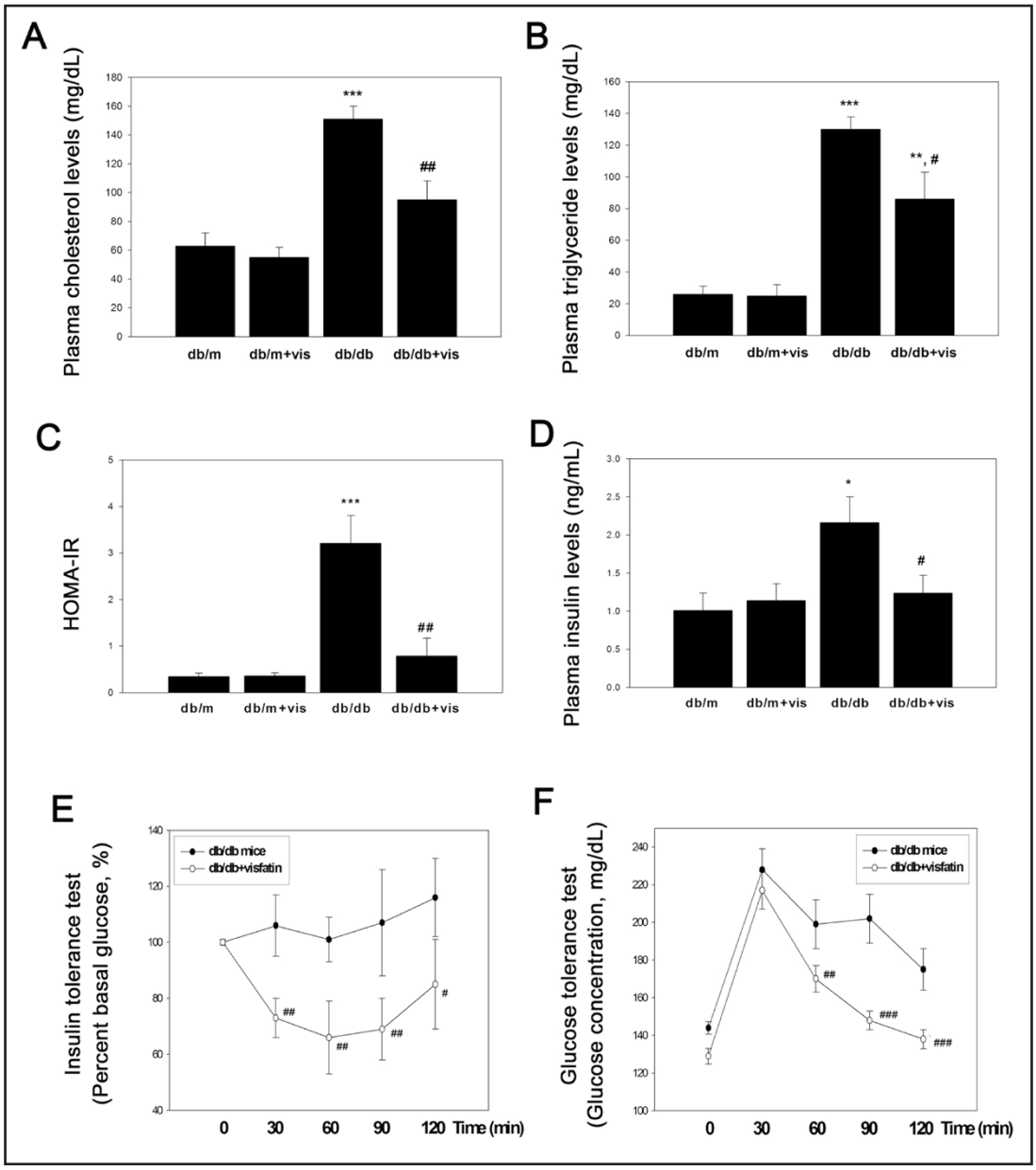

Fig. 1. Effect of visfatin on plasma lipid profile, plasma insulin level, HOMA-IR, ITT and GTT in experimental animals. Plasma cholesterol (A), plasma triglyceride (B), HOMA-IR (C), plasma insulin levels (D), ITT (E),

and GTT (F) are shown. Data are expressed as the means±SEM. *, $P < 0.05$ *db/m vs db/db*, **, $P< 0.01$ *db/m vs db/db*, ***, $P< 0.001$ *db/m vs db/db*, $^{\#,}$ $P< 0.05$ vehicle *vs* visfatin, $^{\#\#,}$ $P< 0.01$ vehicle *vs* visfatin, $^{\#\#\#,}$ $P< 0.001$ vehicle *vs* visfatin. Vis, visfatin; HOMA-IR, The homeostasis model assessment index; ITT, insulin tolerance test; GTT, glucose tolerance test.

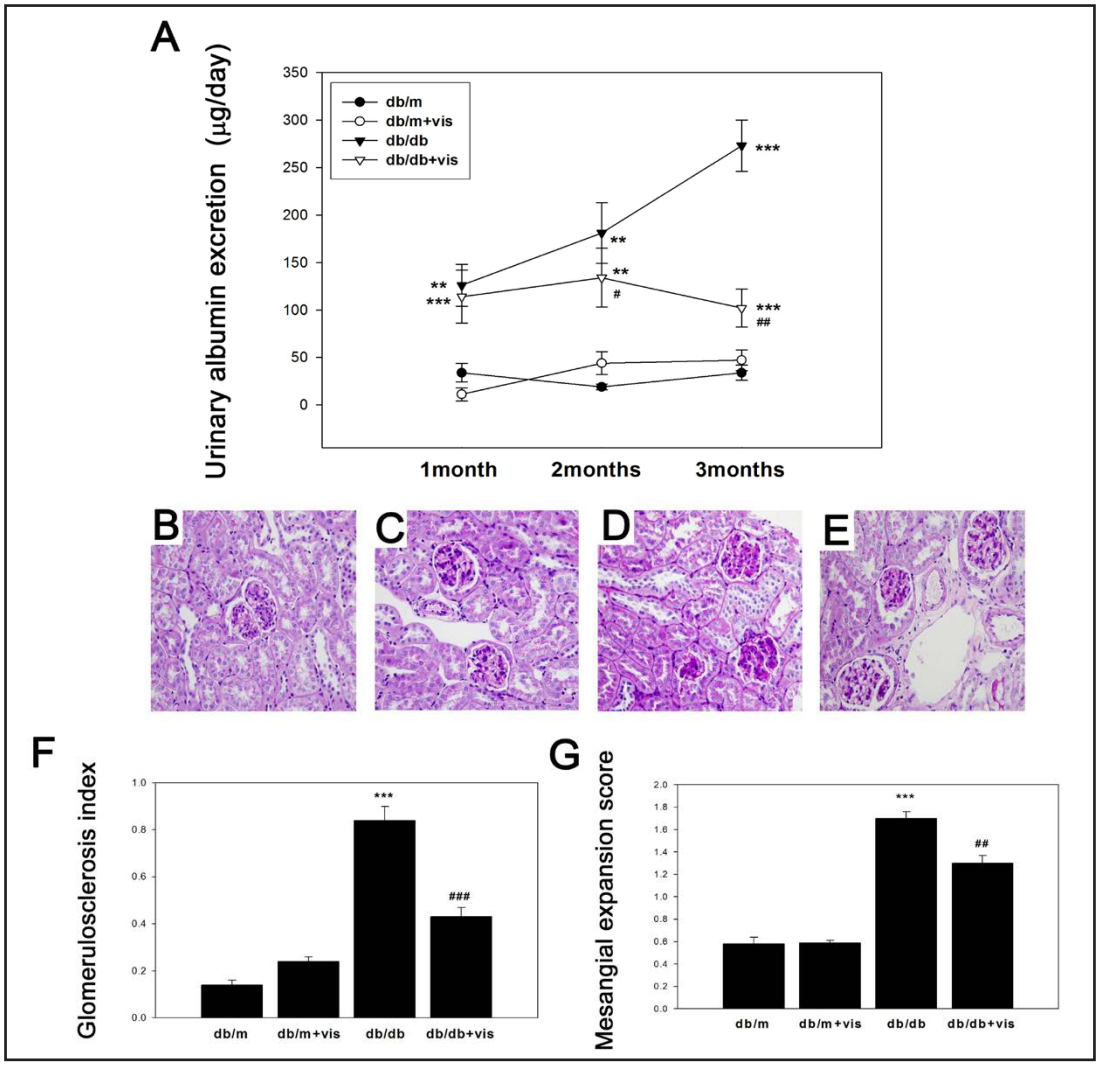

Fig. 2. Effect of visfatin on renal injury in experimental animals. (A) Urinary albumin excretion. (B-E) Renal histologic changes, PAS stain. (B: *db/m*, C: *db/m*+visfatin, D: *db/db*, E: *db/db*+visfatin). (F) Glomerulosclerosis index. (G) Mesangial expansion score. Data are expressed as means±SEM. **, $P< 0.01$ *db/m vs db/db*, ***, $P< 0.001$ *db/m vs db/db*, $^{\#}$, $P< 0.05$ vehicle *vs* visfatin. $^{\#\#}$, $P< 0.01$ vehicle *vs* visfatin, $^{\#\#\#,}$ $P< 0.001$ vehicle *vs* visfatin. Original magnification x 400.

ges in the experimental groups at the end of the study period. Diabetic *db/db* mouse kidneys (Figure 2D) showed more glomerulosclerotic changes and more mesangial expansion than non-diabetic *db/m* mouse kidneys (Figure 2B). Visfatin treatment had no effect on non-diabetic *db/m* mouse kidneys (Figure 2, B and C), whereas it significantly decreased glomerulosclerotic changes and mesangial expansion in diabetic *db/db* mouse kidneys (Figure 2, D and E).

By immunohistochemistry of TGFβ1, type IV collagen, and PAI-1 there were no differences between non-diabetic *db/m* mice with visfatin or vehicle treatment (Figure 3, vehicle: A *vs* visfatin: B). However, visfatin inhibited their expression in diabetic *db/*

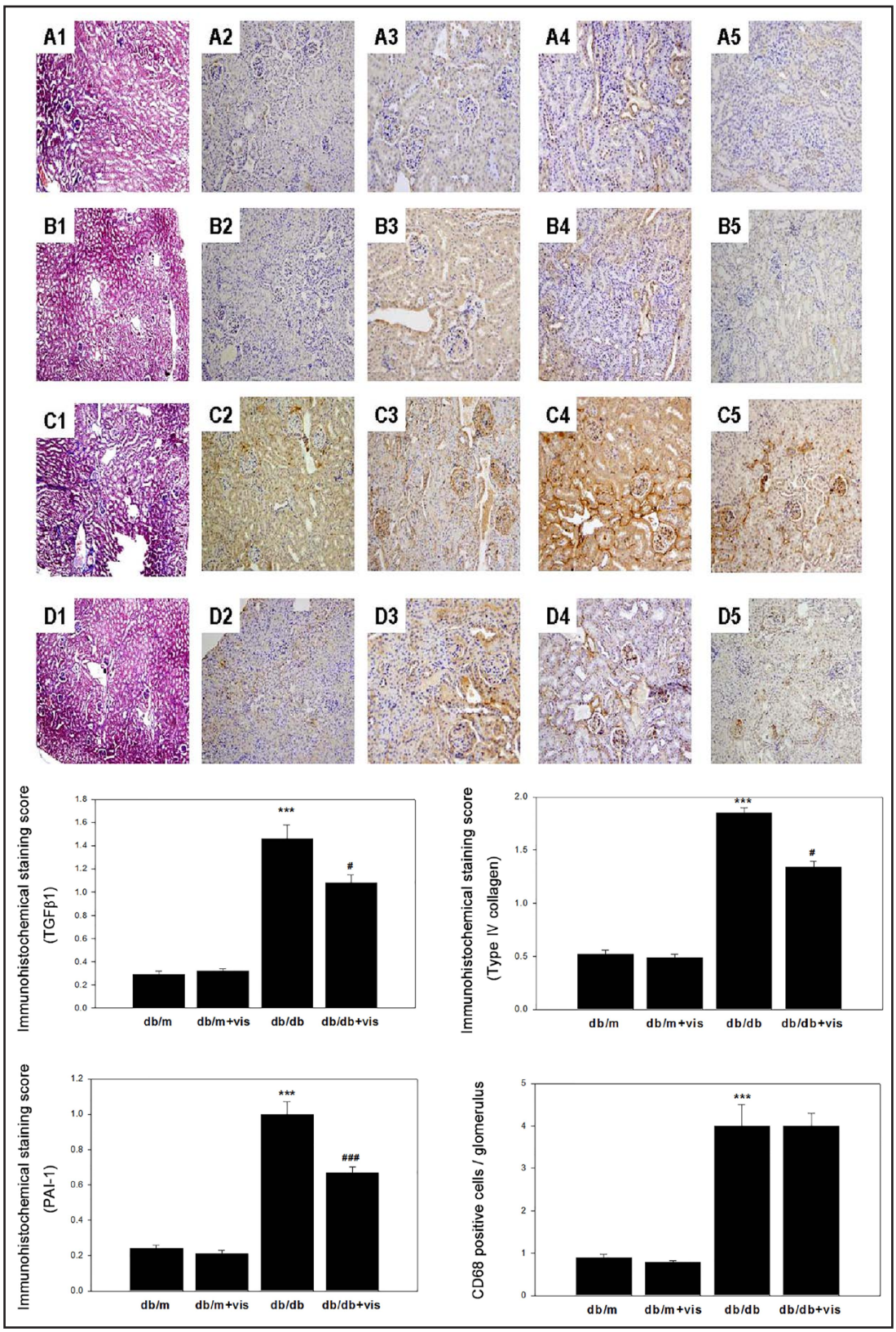

Fig. 3. The immunohistochemistry results and staining scores of profibrotic markers in the kidneys of experimental animals. (A1-D1) MT stain, (A2-D2) CD68, (A3-D3) TGFβ1,(A4-D4) Type IV collagen, and (A5-D5)

PAI-1 are shown. (A: *db/m* control, B: *db/m*+visfatin, C: *db/db*+vehicle, D: *db/db*+visfatin). ***, *P*< 0.001 *db/m vs db/db*, #, *P*< 0.05 vehicle *vs* visfatin, ###, *P*< 0.001 vehicle *vs* visfatin. Original magnification x 400. TGFβ1, transforming growth factor β1; PAI-1, plasminogen activator inhibitor-1; HMG-Co AR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase mRNA, FXR; farnesoid X receptor; ABCA1, adenosine triphosphate-binding cassette transporter A1,

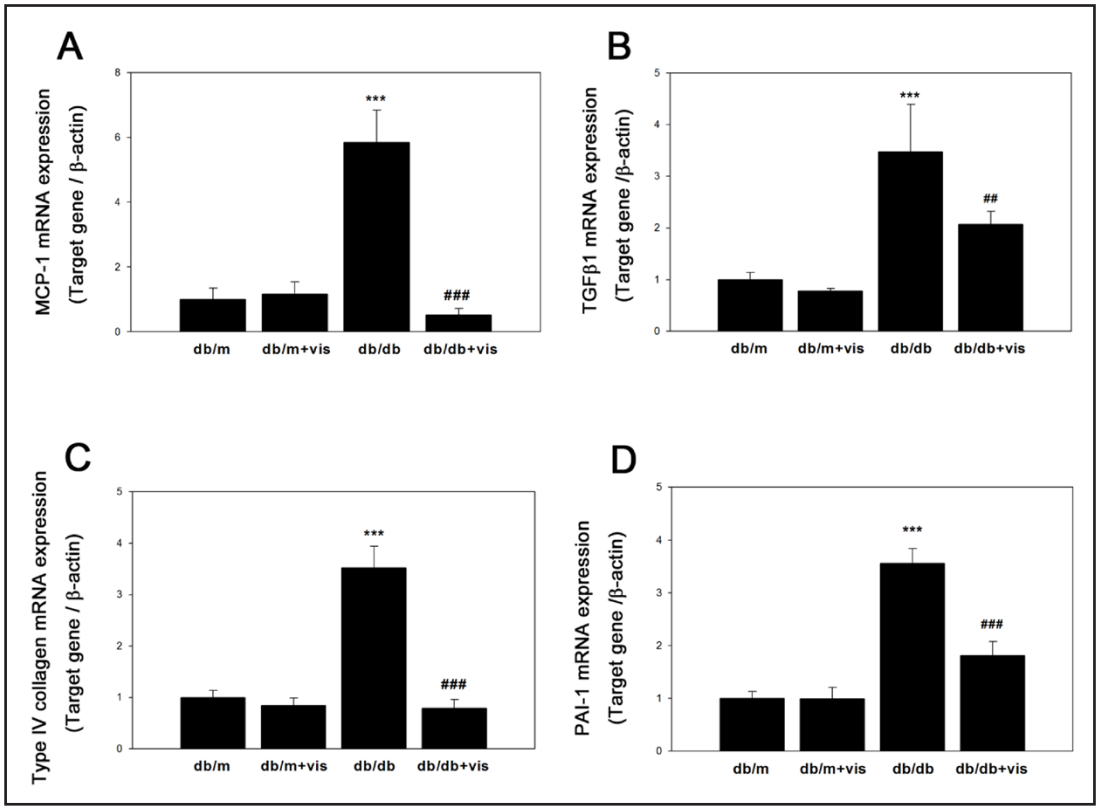

Fig. 4. Effects of visfatin on mRNA expression of the profibrotic markers in the kidneys of the experimental animals (A: MCP-1, B: TGFβ1, C: Type IV collagen, D: PAI-1). *, *P*< 0.05 *db/m vs db/db*, **, *P*< 0.01 *db/m vs db/db*, ***, *P*< 0.001 *db/m vs db/db*, #, *P*< 0.05 vehicle *vs* visfatin, ##, *P*< .01 vehicle *vs* visfatin, ###, *P*< 0.001 vehicle *vs* visfatin. MCP-1, macrophage/monocyte chemoattractant protein-1; TGFβ1, transforming growth factor β1; PAI-1, plasminogen activator inhibitor-1

db mice (Figure 3, vehicle: C *vs* visfatin: D). The scoring indices for immunohistochemistry of TGFβ1, type IV collagen, and PAI-1 are shown. Real-time PCR results for the profibrotic markers of MCP-1, TGFβ1, type IV collagen, and PAI-1, showed an increase in diabetic *db/db* mouse kidneys compared to non-diabetic *db/m* mouse kidneys (Figure 4, A-D). Visfatin did not change these expressions in non-diabetic *db/m* mouse kidneys, but decreased them markedly in diabetic *db/db* mouse kidneys. We next evaluated the changes in the mRNA gene expressions of the enzymes related to lipid metabolism in the kidneys of diabetic *db/db* mice compared to non-diabetic *db/m* mice (Figure 5, A-C). Visfatin treatment inhibited the increased expression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase mRNA (HMG-Co AR; induces cholesterol synthesis). Visfatin also stimulated the expression of farnesoid X receptor (FXR; inhibits fatty acid and triglyceride synthesis) and increased the expression of adenosine triphosphate-binding cassette transporter A1 (ABCA1; induces cholesterol efflux) mRNA. However, visfatin treatment had no effect on their expressions in non-diabetic *db/m* mice kidney. These results suggest that visfatin may have its protective effect on renal injury related to lipid metabolism in diabetic kidney.

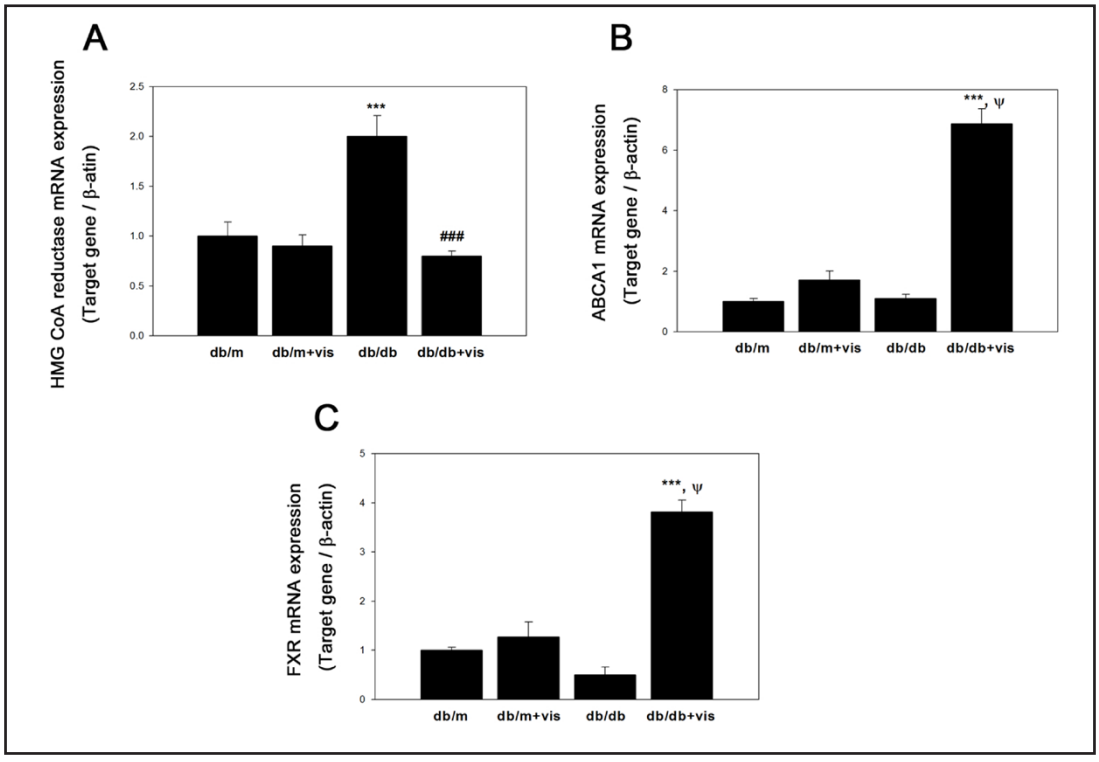

Fig. 5. Effects of visfatin on mRNA expression of the enzymes related lipid metabolism in the kidneys of the experimental animals (A: HMG-CoA reductase, B: ABCA1, C: FXR). *, $P< 0.05$ *db/m vs db/db*, **, $P< 0.01$ *db/m vs db/db*, ***, $P< 0.001$ *db/m vs db/db*, $^{\#}$, $P< 0.05$ vehicle *vs* visfatin, $^{\#\#}$, $P< 0.01$ vehicle *vs* visfatin, $^{\#\#\#}$, $P< 0.001$ vehicle *vs* visfatin. HMG-Co AR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase ; ABCA1, adenosine triphosphate-binding cassette transporter A1 ; FXR, farnesoid X receptor.

Discussion

We showed a protective role of visfatin in diabetic nephropathy. Visfatin improved urinary albumin excretion and pathologic and molecular changes in renal injury without lowering plasma glucose level in diabetic mice. Visfatin dramatically improved plasma lipid levels and insulin resistance in experimental animals and had a beneficial effect on the balance of lipid metabolism in the diabetic kidney.

According to our previous studies, plasma visfatin levels were elevated in diabetic rats and patients [17, 21]. Besides, visfatin was synthesized in renal cultured cells of mesangial cells, proximal tubular cells, and podocytes [17, 18]. We also observed that plasma visfatin levels were higher in *db/db* mice than that in *db/m* mice in the present study. Visfatin was increased in other inflammatory conditions such as acute lung injury, sepsis, and rheumatoid arthritis [22-24] and is considered to be a proinflammatory adipokine or a surrogate marker of systemic inflammation. However, it is unclear whether this elevated visfatin concentration is either a compensatory response or an epiphenomenon. Interestingly, visfatin was first reported to be expressed almost exclusively in visceral adipose tissue [25]. Adipose tissue is now believed to be a special endocrine organ, and the visfatin gene in humans is expressed predominantly in visceral adipose tissue as opposed to subcutaneous adipose tissue [26]. Adipokines, which are secreted by this adipocyte tissue, have been studied in association with insulin resistance and the metabolic syndrome, including obesity, glucose intolerance, and hyperlipidemia.

Initial studies on visfatin in the human population have obtained conflicting results. Elevated plasma visfatin levels have been reported in patients with type 2 diabetes mellitus [27-30]. On the other hand, other investigative results showed decreased plasma visfatin

levels in patients with type 1 diabetes, liver cirrhosis, exercise in type 2 diabetes, and in the 3rd trimester of gestational diabetes [31-35]. Elevated visfatin levels were found in hemodialysis patients[36] and a positive correlation of visfatin levels with all stages of chronic kidney disease was observed [37]. Yilmaz et al. [38] published that visfatin levels were positively associated not only with insulin resistance but also with the degree of albuminuria in type 2 diabetic patients. They suggested that the endothelial dysfunction in early diabetic nephropathy is associated with altered circulating levels of visfatin and low circulating adiponectin. But our study shows that visfatin administration did not lead any change of plasma adiponectin level in both diabetic and non-diabetic mice, even though plasma adiponectin level was lower in diabetic mice than non-diabetic mice. Moreover, in another experimental study, visfatin activated endothelial nitric oxide synthase (eNOS) via Akt, mitogen-activated protein (MAP) kinase, and MCP-1 to improve endothelial cell function, angiogenesis, and atherosclerosis [39, 40]. Our study also shows that visfatin treatment decreased MCP-1 and CD68 expressions in the kidney of diabetic mice. This suggests that the protective effect of visfatin in diabetic kidney injury may occur through the anti-inflammatory mechanism despite we are not sure whether visfatin does affect directly the target organ injury or indirectly through the insulin resistance improvement. However, it is interesting to observe that visfatin had its renal protective effect despite it had no effect on glucose level, HbA1c, blood pressure, and plasma adiponectin level. In addition, we observed that chronic visfatin administration lowered plasma visfatin level in diabetic mice and improved the lipid parameters in this study. Fukuhara and colleagues found that visfatin levels in serum increased in parallel with visceral but not subcutaneous fat in both mice and humans [41]. Our study showed that adipose tissue decreased and lipid levels were improved with decreased visfatin levels in plasma of diabetic mice by chronic visfatin treatment. Therefore, these results suggest that chronic visfatin treatment might have the renoprotective effect via the improvement of lipid metabolism. The different results from our previous studies we investigated could be explained by differences obtained *in vitro vs in vivo* experiment and/or by acute *vs* long-term treatment. Our previous *in vitro* studies had showed that visfatin treatment aggravated high-glucose induced renal injury in cultured renal cells through uptake of glucose into the renal cells [17, 18].

Visfatin has been reported to have an insulin-mimic effect over insulin resistance [25]. Several studies failed to present an association between circulating visfatin and insulin sensitivity [29, 42-44], and it is unclear whether the relationship between visfatin and insulin is synergistic. However, it is interesting that visfatin binds directly to the insulin receptor at a site distinct from insulin and has hypoglycemic effects by reducing glucose release from hepatocytes and stimulating glucose utilization in peripheral tissues [45]. Insulin receptor expression has been ascertained in renal cells such as proximal tubular cells, mesangial cells, and podocytes. In fact, insulin binds either to insulin receptor with a high affinity and to the insulin-like growth factor receptor and the insulin-receptor like receptor in the kidney with a low affinity. In the kidney of an insulin-resistant rat animal model, insulin receptor expression was reduced despite a high plasma insulin level [46]. In our previous study, high glucose stimulation upregulated visfatin synthesis, and then visfatin stimulated glucose uptake via the glucose transporter (GLUT)-1 in renal mesangial cells [18]. Visfatin stimulation in renal mesangial cells upregulated the insulin signaling pathway and induced synthesis of downstream profibrotic molecules [18]. However, in the present study long-term stimulation of visfatin for three months in diabetic mice inhibited the activation of proinflammatory and profibrotic molecules in diabetic nephropathy. This result can be obtained from chronic effect of visfatin stimulation in high glucose-induced injury. We did not investigate the effect of visfatin on insulin receptor expression in kidney. It is possible that there is a difference between *in vitro* and *in vivo* experiment. Therefore, visfatin might have either the direct action on the kidneys or the indirect action via regulating lipid metabolism of the kidneys.

Furthermore, another study suggested that visfatin does not have only the insulin-mimetic action, but also has a regulatory role in glucose-stimulated insulin secretion in pancreatic β-cells *in vitro* and *in vivo* [47]. The authors demonstrated that mice lacking visfatin synthesis develop impaired glucose tolerance and defective insulin secretion, which

are restored by visfatin. This result suggests that visfatin may act differently as a regulatory adipokine, depending on its exposure in a time-dependent manner. Therefore, visfatin may affect plasma adiponectin level during acute phase of visfatin stimulation, but chronic stimulation of visfatin did not affect plasma adiponectin level in the present study.

Visfatin administration in diabetic mice of the present *in vivo* study could not lower plasma glucose. Plasma visfatin levels do not change after feeding and visfatin levels are usually lower, compared to those of insulin, although visfatin and insulin seem to have similar affinities for the insulin receptor [25, 41, 48]. Visfatin does not compete with insulin and binds to different sites of insulin receptor [41]. If high glucose concentration stimulates intrarenal cellular visfatin synthesis, and then the glucose taken up by renal cells activates an intracellular signaling pathway to cause diabetic nephropathy, visfatin may be a compensatory and protective molecule secreted to prevent high-glucose-induced cellular injury. In fact, visfatin also stimulates glucose uptake by cultured adipocytes and muscle cells and inhibit glucose release by cultured hepatocytes [41].

We here could not determine the regulatory and physiologic actions of high glucose, insulin, and visfatin in these experiments. The hypoglycemic effect of visfatin was not of physiological importance. However, long-term visfatin administration might protect the kidney form intrarenal insulin resistance and injury. Visfatin may compensate via autocrine and paracrine pathways to improve insulin resistance and lipid metabolism. In our study, we used intraperitoneal injection, which is a more physiologic route than vascular infusion. This allows visfatin to accumulate directly into the liver and visceral adipose tissue, as main organs regulating lipid and glucose metabolism, before high enough concentrations reach the plasma, heart, and kidney through the systemic circulation. This could cause the indirect effect on the kidneys through regulating lipid metabolism.

Conclusion

Taken together from our results in this study, visfatin had a protective effect in diabetic nephropathy, insulin resistance, and lipid metabolism in type 2 diabetic mice. Visfatin might play a crucial role in the pathogenesis of insulin resistance and diabetic complications despite it is uncertain whether it occurs from direct effect on the kidneys or indirect effect through the insulin resistance improvement. These findings could suggest the new anti-diabetic drug that improves insulin resistance and lipid metabolism. Further studies should be undertaken to understand the exact role of visfatin, and the regulation and physiologic role of visfatin in diabetes needs to be considered in the future.

Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interests and nothing to disclose.

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