Involvement of the NF-κB signaling pathway in the renoprotective effects of isorhamnetin in a type 2 diabetic rat model

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Abstract. The aim of the present study was to investigate the renoprotective effects of isorhamnetin (ISO) in type 2 diabetic rats and its effects on the nuclear factor-κB (NF-κB) signaling pathway, which is associated with diabetic nephropathy. The type 2 diabetic rat model was established by a high-fat diet plus streptozocin injection and the rats were subsequently treated with two dosages of ISO, respectively. The levels of blood glucose were determined. Urinary osteopontin, kidney injury molecule-1 (KIM-1) and albumin were measured to evaluate the renal function of the rats. Renal NF-κB signaling activity was assessed by measuring the levels of NF-κB p65, phospho-NF-κB p65, inhibitor of NF-κB (IκBα) and phospho-IκBα, and the NF-κB p65 DNA-binding activity. Downstream inflammatory mediators [tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, intercellular adhesion molecule-1 (ICAM-1) and transforming growth factor-β1 (TGF-β1)] of the NF-κB signaling pathway were investigated to evaluate the renal inflammatory response. Renal levels of malondialdehyde and total superoxide dismutase were detected to access the oxidative stress. Furthermore, glomerular mesangial cells (GMCs) were treated with lipopolysaccharide and ISO. In the cellular experiment, the NF-κB signaling activity, levels of TNF-α, IL-1β, IL-6, ICAM-1 and TGF-β1, and oxidative stress were also investigated. The results showed that ISO decreased the levels of urinary osteopontin, KIM-1 and albumin. ISO also inhibited the NF-κB signaling activity, decreased the production of inflammatory mediators and attenuated oxidative stress in diabetic rats and GMCs. The present investigations revealed that ISO had ameliorative effects on diabetes-induced renal damage and the activity may be associated with the negative regulation of NF-κB signaling pathway.

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Introduction

As a leading cause of clinical end-stage renal disease, diabetic nephropathy (DN) is the second most prevalent diabetes-associated complication (1). A number of factors are associated with the pathogenesis of DN. However, the exact mechanisms have not been well elucidated. Therefore, no specific therapies have been developed.

Accumulating evidence indicates that inflammation is one of the major factors that has significant roles in the initiation and progression of DN (2-4). The activation of the nuclear factor-κB (NF-κB) signaling pathway has been suggested to be associated with DN (5-7). The roles of the downstream inflammatory mediators of the NF-κB signaling pathway, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, intercellular adhesion molecule-1 (ICAM-1) and transforming growth factor-β1 (TGF-β1), have been well demonstrated (8-10). They can promote the glomerular hypertrophy, expansion of the mesangial matrix and thickening of the glomerular and tubular basement membranes, ultimately resulting in proteinuria, glomerulosclerosis and tubulointerstitial fibrosis.

Excessive production of reactive oxygen species has been found in diabetic animals and patients (11). Recently, studies have demonstrated that oxidative stress is an important pathogenic factor of diabetic complications including nephropathy (12-14). Oxidative stress and inflammation are linked, as each begets and amplifies the other. NF-κB has an important role between the interaction of oxidative stress and inflammation.

Considering the crucial roles of inflammation and oxidative stress in the pathogenesis of DN, antioxidant therapy and anti-inflammation therapy have been tested in DN. Cumulative evidence suggests that these managements can preserve renal function and prevent or slow the progression of DN (15-17). Isorhamnetin (ISO) is a plant flavonoid abundant in herbal medicinal plants, such as *Hippophae rhamnoides* L. In recent

studies, ISO exhibited strong antioxidant and anti-inflammatory properties. Its protective effects on lipopolysaccharide (LPS)-induced acute lung injury in mice (18), apoptosis in TNF-α-induced HUVECs injury (19) and chemically-induced inflammatory bowel disease (20) have been reported. A previous study showed that ISO could negatively regulate the NF-κB signaling cascade in gastric cancer (21). However, its effects on kidney function, the NF-κB signaling pathway, inflammatory response and oxidative stress in diabetic subjects have not been evaluated. Therefore, the present study was designed to test the hypothesis that ISO presents renoprotective effects in type 2 diabetic rats and to detect its possible mechanisms.

Materials and methods

Animals. Sprague-Dawley rats (8 weeks old and 180-220 g) obtained from the SLAC Lab Animal Center (Shanghai, China) were used in this study. Procedures used in the current study were approved by the Ethics Committee of Affiliated Hospital of Weifang Medical College (Weifang, Shandong, China). Animals were housed 5/cage in a controlled environment (22±1˚C, 12-h light/dark cycle).

Induction of diabetes in rats. Type 2 diabetes mellitus (DM) was induced according to the method described in the literature (22). Rats were fed a high-fat diet consisting of a total kcal value of 40 kJ/kg (22% protein, 20% fat and 45% carbohydrate). A single dose of streptozocin (STZ) [freshly diluted with 0.01 M citrate buffer, (pH 4.5), 30 mg/kg] was intraperitoneally injected into the rats after 4 weeks of the high-fat diet. On day 7 post-STZ injection, blood glucose was measured using the OneTouch®Ultra machine (LifeScan, Livingstone, UK). Rats with hyperglycemia (>16.7 mmol/l) were considered to be diabetic and were included for further study. Normal control rats were fed a regular chow diet with a total kcalories value of 20 kJ/kg (20% protein, 5% fat and 52% carbohydrate) without STZ injection.

Experimental groups and ISO treatment. The diabetic rats were randomly divided into 3 groups (n=10 in each group): DM, 50 mg/kg ISO (ISO-50) and 150 mg/kg ISO (ISO-150) groups. Rats in the ISO-50 and ISO-150 groups were orally administered ISO (50 and 150 mg/kg/day, respectively) after grouping for consecutive 12 weeks. Rats in the control group (n=10 in each group) and the DM group were orally administrated with the same volume of saline.

Preparation of renal homogenate and urine. On the last day of ISO treatment, the rats were placed in metabolic cages and 24-h urine was collected. The kidney was removed and rinsed with ice-cold saline. A section of kidney tissue was homogenized (100 mg renal tissue/ml saline) and centrifuged at 1,050 x g for 10 min. The supernatant was collected and further centrifuged at 10,000 x g for 10 min.

Measurements of renal damage biochemical markers and blood glucose. Levels of urinary osteopontin and kidney injury molecule-1 (KIM-1) were measured using ELISA kits according to the instructions provided by the manufacturer (Boster Biological Technology, Ltd., Wuhan, Hubei, China). The levels of urinary albumin were measured using an automatic biochemistry analyzer. Fasting blood glucose (FBG) was measured using OneTouch®Ultra machine.

Cell culture with LPS and ISO. Rat glomerular mesangial cells (GMCs) (Chinese Center for Typical Culture Collection, Wuhan, Hubei, China) were maintained at 5% CO_2 and 37˚C in Dulbecco's modified Eagle's medium (Wisent Bioproducts, St. Bruno, Quebec, Canada) containing fetal bovine serum, 100 μg/ml streptomycin 100 U/ml penicillin. GMCs were randomly divided into the following 4 groups: Normal control (NC), LPS [with the presence of 10 nmol/ml LPS (Sigma-Aldrich, St. Louis, MO, USA) in the medium], ISO-5 (10 nmol/ml LPS and 5 μM ISO in the medium) and ISO-10 (10 nmol/ml LPS and 10 μM ISO in the medium) groups. The cells in each group were cultured for 72 h and the medium was collected. Cells were lysed with lysis buffer and the supernatants of the cell lysates were collected after centrifugation at 18,000 x g for 10 min at 4˚C.

Measurements of NF-κB p65, phospho-NF-κB p65, inhibitor of NF-κB (IκBα) and phospho-IκBα. The levels of NF-κB p65, phospho-NF-κB p65, IκBα and phospho-IκBα in the cell lysate and in the renal homogenate were measured using ELISA kits, according to the manufacturer's protocols. NF-κB p65 ELISA kits were purchased from Cusabio Biotech Co., Ltd. (Wuhan, China), phospho-NF-κB p65 and phospho-IκBα ELISA kits were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA), and the IκBα ELISA kits were purchased from Jining Co. (Shanghai, China).

NF-κB p65 DNA-binding activity. Nuclear protein was extracted from the kidney and rat GMCs. NF-κB DNA-binding activity was measured using the NF-κB p65 transcription factor ELISA assay kits (Cayman Chemical Co., Ann Arbor, MI, USA) according to the manufacturer's protocol.

Measurements of inflammatory mediators. The levels of the NF-κB downstream inflammatory mediators, TNF-α, IL-1β, IL-6, ICAM-1 and TGF-β1, in the cell culture medium and in the renal homogenate were measured using ELISA kits from R&D Systems, Inc. (Minneapolis, MN, USA) according to the manufacturer's protocol.

mRNA expression levels of NF-κB p65, TNF-α, IL-1β, IL-6, ICAM-1 and TGF-β1. Total RNA was isolated from fresh kidney tissue and GMCs using TRIzol reagents (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA). cDNA was synthesized using the cDNA synthesis kit (Takara Bio, Inc., Kyoto, Japan) and amplified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The primer sequences were: TNF-α forward, 5'-TGATCGGTC CCAACAAGGA-3' and reverse primer, 5'-TGCTTGGTG GTTTGCTACGA-3'; IL-1β forward, 5'-ACTATGGCAACT GTCCCTGAAC-3' and reverse primer, 5'-GTGCTTGGG TCCTCATCCTG-3'; IL-6 forward, 5'-AGTTGCCTTCTT GGGACTGA-3' and reverse primer 5'-CAGAATTGCCAT TGCACAAC-3'; NF-κB p65 forward, 5'-TGCAGGCTCCTG TGCGAGTG-3' and reverse primer, 5'-TCCGGTGGCGAT

Table I. Effects of ISO treatment on renal damage parameters and FBG.

Treatment	Osteopontin, pg/ml	KIM-1, pg/ml	Albumin, mg/24 h	FBG, mmol/l
Control	10.69±2.17	8.10±1.34	0.41±0.08	5.36±0.88
DM	48.10±7.07[a]	25.17±3.05[a]	4.12±0.70[a]	11.68±2.01[a]
ISO-50	35.08±5.33[b]	19.44±2.28[b]	3.25±0.51[b]	10.91±2.55
ISO-150	25.05±4.04[b,c]	14.27±2.11[b,c]	1.83±0.38[b,c]	10.04±2.69

Data are expressed as mean ± standard deviation. P<0.05 vs. [a]control; [b]DM; and [c]ISO-50 groups. FBG, fasting blood glucose; KIM-1, kidney injury molecule-1; DM, diabetes mellitus; ISO-50, 50 mg/kg/day isorhamnetin; ISO-150, 150 mg/kg/day isorhamnetin.

Table II. Effects of ISO treatment on renal NF-κB signaling.

Treatment	NF-κB p65, pg/ml	phospho-NF-κB p65, pg/ml	IκBα, pg/ml	phospho-IκBα, pg/ml	NF-κB p65 DNA-binding activity, OD
Control	35.17±6.33	13.50±2.63	30.09±4.36	8.66±1.05	0.25±0.03
DM	98.09±12.51[a]	65.28±8.11[a]	29.82±4.22	21.05±3.17[a]	0.89±0.13[a]
ISO-50	71.20±9.07[b]	48.79±6.90[b]	31.07±5.34	15.10±2.02[b]	0.65±0.11[b]
ISO-150	55.83±6.86[b,c]	30.83±5.04[b,c]	33.10±5.8	11.34±1.61[b,c]	0.48±0.08[b,c]

Data are expressed as mean ± standard deviation. P<0.05 vs. [a]control; [b]DM; and [c]ISO-50 groups. NF-κB, nuclear factor-κB; IκBα, inhibitor of NF-κB; OD, optical density; DM, diabetes mellitus; ISO-50, 50 mg/kg/day isorhamnetin; ISO-150, 150 mg/kg/day isorhamnetin.

CGTCTGTGT-3'; ICAM-1 forward, 5'-CGTGGCGTCCATTTACACCT-3' and reverse primer, 5'-TTAGGGCCTCCTCCTGAGC-3'; TGF-β1 forward, TGGCGTTACCTTGGTAACC and reverse primer, GGTGTTGAGCCCTTTCCAG; and β-actin forward, 5'-AGGCCCCTCTGAACCCTAAG-3' and reverse primer, 5'-CCAGAGGCATACAGGGACAAC-3'. The PCR program involved 95˚C for 30 sec and 40 PCR cycles (95˚C for 5 sec and 60˚C for 30 sec). The PCR reactions were perforemd with an iQ5 Real-Time PCR Detection system (Bio-Rad, Hercules, CA, USA).

Measurements of oxidative stress activity and antioxidant activity. Oxidative stress activity was assessed by measuring the levels of malondialdehyde (MDA) in the cell culture medium and in the renal homogenate using chemichromatometry kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Antioxidant activity was assessed by measuring levels of total superoxide dismutase (T-SOD) using chemichromatometry kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Statistical analysis. Statistical analyses were carried out using SPSS software 14.0 (SPSS, Inc., Chicago, IL, USA). Data are reported as mean ± standard deviation and were analyzed using one-way analysis of variance followed by Student-Newman-Keuls test for multiple comparisons. For all the tests, P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of ISO on renal damage biochemical markers. The DM group had higher levels of urinary osteopontin, KIM-1 and albumin compared to the control group (P<0.05). The ISO-50 and ISO-150 groups had lower levels of these renal damage markers compared to the DM group (P<0.05), and those levels of the ISO-150 group were lower compared to the ISO-50 group (P<0.05), indicating that ISO dose-dependently improved the renal function in the diabetic rats (Table I).

Effects of ISO on blood glucose. No significant differences in the FBG levels were observed among the DM, the ISO-50 and the ISO-150 groups (P>0.05). All three groups had higher levels of FBG compared to the control group (P<0.05) (Table I).

Effects of ISO on NF-κB p65, phospho-NF-κB p65, IκBα and phospho-IκBα. In the *in vivo* study, the high-fat diet plus STZ injection activated the renal NF-κB signaling by increasing levels of NF-κB p65 (protein and mRNA), phospho-NF-κB p65 and phospho-IκBα in the DM group when compared to the control group (P<0.05). The NF-κB activation was inhibited by ISO if compared to the DM group (P<0.05), and the inhibitory effects were dose-dependent in the ISO-50 group and the ISO-150 group (P<0.05). In the *in vitro* study, LPS increased the generation of NF-κB p65 (protein and mRNA), phospho-NF-κB p65 and phospho-IκBα in GMCs when compared to the normal control cells (P<0.05). ISO dose-dependently (5 and 10 μM) inhibited the overproduction of NF-κB p65 (protein and mRNA), phospho-NF-κB p65 and phospho-IκBα if compared to the LPS group (P<0.05). No significant differences in levels IκBα were observed among the groups in the *in vivo* and *in vitro* studies (P>0.05) (Tables II and III).

Effects of ISO on NF-κB p65 DNA-binding activity. In the *in vivo* study, the DM group had a higher NF-κB p65

Table III. Effects of ISO treatment on NF-κB signaling in the glomerular mesangial cells.

Treatment	NF-κB p65, pg/ml	phospho-NF-κB p65, pg/ml	IκBα, pg/ml	phospho-IκBα, pg/ml	NF-κB p65 DNA-binding activity, OD
NC	17.55±3.04	6.11±1.03	15.66±3.05	6.14±1.02	0.22±0.03
LPS	56.07±7.11[a]	40.73±6.35[a]	16.11±1.64	12.28±1.54[a]	0.93±0.15[a]
ISO-5	41.26±4.88[b]	28.05±4.19[b]	16.80±2.55	9.31±1.07[b]	0.62±0.09[b]
ISO-10	30.10±4.14[b,c]	14.66±3.71[b,c]	17.13±3.10	7.03±1.10[b,c]	0.40±0.08[b,c]

Data are expressed as mean ± standard deviation. P<0.05 vs. [a]control; [b]LPS; and [c]ISO-5 groups. NF-κB, nuclear factor-κB; IκBα, inhibitor of NF-κB; OD, optical density; NC, normal control; LPS, lipopolysaccharide; ISO-5, 10 nmol/ml LPS and 5 μM isorhamnetin; ISO-10, 10 nmol/ml LPS and 10 μM isorhamnetin.

Table IV. Effects of ISO treatment on the renal inflammatory mediators.

Treatment	TNF-α, pg/ml	IL-1β, pg/ml	IL-6, pg/ml	ICAM-1, pg/ml	TGF-β1, pg/ml
Control	34.07±5.44	51.16±8.10	25.03±4.15	41.05±6.32	67.71±9.45
DM	90.11±11.20[a]	132.07±16.39[a]	65.34±8.27[a]	96.17±12.85[a]	212.53±30.36[a]
ISO-50	66.19±8.07[b]	100.29±12.24[b]	50.37±6.01[b]	73.54±8.35[b]	152.96±21.88[b]
ISO-150	49.58±6.21[b,c]	76.44±9.89[b,c]	38.03±4.14[b,c]	56.62±6.98[b,c]	117.51±15.30[b,c]

Data are expressed as mean ± standard deviation. P<0.05 vs. [a]control; [b]DM; and [c]ISO-50 groups. TNF-α, tumor necrosis factor-α; IL, interleukin; ICAM-1, intercellular adhesion molecule-1; TGF-β1, transforming growth factor-β1; DM, diabetes mellitus; ISO-50, 50 mg/kg/day isorhamnetin; ISO-150, 150 mg/kg/day isorhamnetin.

Table V. Effects of ISO treatment on the inflammatory mediators in the glomerular mesangial cells.

Treatment	TNF-α, pg/ml	IL-1β, pg/ml	IL-6, pg/ml	ICAM-1, pg/ml	TGF-β1, pg/ml
NC	12.82±2.12	23.03±4.57	14.08±1.99	8.01±1.09	20.31±4.75
LPS	43.58±5.33[a]	71.79±9.04[a]	45.31±6.05[a]	21.22±3.15[a]	51.12±7.08[a]
ISO-5	32.04±4.97[b]	52.21±5.93[b]	33.20±4.52[b]	16.38±2.01[b]	39.63±4.11[b]
ISO-10	20.15±3.06[b,c]	36.48±5.09[b,c]	23.94±3.03[b,c]	12.09±1.71[b,c]	30.34±4.62[b,c]

Data are expressed as mean ± standard deviation. P<0.05 vs. [a]control; [b]LPS; and [c]ISO-5 groups. TNF-α, tumor necrosis factor-α; IL, interleukin; ICAM-1, intercellular adhesion molecule-1; TGF-β1, transforming growth factor-β1; NC, normal control; LPS, lipopolysaccharide; ISO-5, 10 nmol/ml LPS and 5 μM isorhamnetin; ISO-10, 10 nmol/ml LPS and 10 μM isorhamnetin.

DNA-binding activity compared to the control group (P<0.05). ISO (50 and 150 mg) dose-dependently decreased the NF-κB p65 DNA-binding activity when compared to the DM group (P<0.05). In the *in vitro* study, the LPS group showed higher NF-κB p65 DNA-binding activity compared to the normal control cells (P<0.05), and ISO dose-dependently (5 and 10 μM) inhibited the NF-κB p65 DNA-binding activity when compared to the LPS group (P<0.05) (Tables II and III).

Effects of ISO on inflammatory mediators. The changes (protein and mRNA) of TNF-α, IL-1β, IL-6, ICAM-1 and TGF-β1, which are the downstream inflammatory mediators of NF-κB signaling pathway, were investigated. In the *in vivo* study, there were higher levels of these inflammatory mediators in the DM group compared to the control group (P<0.05). ISO (50 and 150 mg) dose-dependently decreased these inflammatory mediators when compared to the DM group (P<0.05). In the *in vitro* study, there were higher levels of these inflammatory mediators in the LPS group compared to the normal control cells (P<0.05). ISO (5 and 10 μM) dose-dependently decreased these inflammatory mediators when compared to the LPS group (P<0.05) (Tables IV-VII).

Effects of ISO on oxidative stress. The levels of MDA and T-SOD were investigated. In the *in vivo* study, the DM group had higher levels of MDA and lower levels of T-SOD compared to the control group (P<0.05). ISO (50 and 150 mg) dose-dependently reversed the changes of MDA and T-SOD when compared to the DM group (P<0.05). In the *in vitro* study, the LPS group showed higher levels of MDA and lower levels of T-SOD compared to the normal control

Table VI. Effects of ISO treatment on the renal mRNA expression levels of the inflammatory mediators.

	mRNA expression level					
Treatment	NF-κB p65	TNF-α	IL-1β	IL-6	ICAM-1	TGF-β1
Control	1.00±0.16	1.00±0.08	1.00±0.11	1.00±0.08	1.00±0.12	1.00±0.11
DM	2.65±0.31[a]	3.03±0.36[a]	2.76±0.29[a]	2.89±0.31[a]	2.93±0.36[a]	3.43±0.47[a]
ISO-50	2.01±0.25[b]	2.34±0.31[b]	2.01±0.25[b]	2.16±0.27[b]	2.20±0.26[b]	2.56±0.40[b]
ISO-150	1.53±0.18[b,c]	1.65±0.27[b,c]	1.44±0.20[b,c]	1.51±0.20[b,c]	1.69±0.21[b,c]	1.85±0.28[b,c]

Data are expressed as mean ± standard deviation. P<0.05 vs. [a]control; [b]DM; and [c]ISO-50 groups. NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α; IL, interleukin; ICAM-1, intercellular adhesion molecule-1; TGF-β1, transforming growth factor-β1; DM, diabetes mellitus; ISO-50, 50 mg/kg/day isorhamnetin; ISO-150, 150 mg/kg/day isorhamnetin.

Table VII. Effects of ISO treatment on mRNA expression of inflammatory mediators in the glomerular mesangial cells.

	mRNA expression level					
Treatment	NF-κB p65	TNF-α	IL-1β	IL-6	ICAM-1	TGF-β1
NC	1.00±0.11	1.00±0.098	1.00±0.13	1.00±0.14	1.00±0.12	1.00±0.11
LPC	3.69±0.52[a]	3.25±0.41[a]	2.91±0.30[a]	3.21±0.44[a]	2.43±0.21[a]	2.68±0.24[a]
ISO-5	2.45±0.41[b]	2.12±0.29[b]	2.17±0.32[b]	2.52±0.35[b]	1.95±0.15[b]	2.05±0.11[b]
ISO-10	1.67±0.25[b,c]	1.51±0.23[b,c]	1.60±0.19[b,c]	1.73±0.21[b,c]	1.53±0.16[b,c]	1.61±0.12[b,c]

Data are expressed as mean ± standard deviation. P<0.05 vs. [a]control; [b]LPS; and [c]ISO-5 groups. NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α; IL, interleukin; ICAM-1, intercellular adhesion molecule-1; TGF-β1, transforming growth factor-β1; NC, normal control; LPS, lipopolysaccharide; ISO-5, 10 nmol/ml LPS and 5 μM isorhamnetin; ISO-10, 10 nmol/ml LPS and 10 μM isorhamnetin.

Table VIII. Effects of ISO treatment on the renal oxidative stress markers.

Treatment	MDA, mmol/g protein	T-SOD, U/mg protein
Control	0.61±0.11	73.53±10.39
DM	1.58±0.20[a]	33.14±5.32[a]
ISO-50	1.19±0.14[b]	45.28±6.27[b]
ISO-150	0.93±0.15[b,c]	59.95±6.09[b,c]

Data are expressed as mean ± standard deviation. P<0.05 vs. [a]control; [b]DM; and [c]ISO-50 groups. MDA, malondialdehyde; T-SOD, total superoxide dismutase; DM, diabetes mellitus; ISO-50, 50 mg/kg/day isorhamnetin; ISO-150, 150 mg/kg/day isorhamnetin.

Table IX. Effects of ISO treatment on the oxidative stress markers in the glomerular mesangial cells.

Treatment	MDA, mmol/g protein	T-SOD, U/mg protein
NC	0.15±0.02	30.12±5.04
LPS	0.62±0.10[a]	14.38±2.37[a]
ISO-5	0.45±0.05[b]	19.85±2.86[b]
ISO-10	0.30±0.05[b,c]	25.41±2.91[b,c]

Data are expressed as mean ± standard deviation. P<0.05 vs. [a]control; [b]LPS; and [c]ISO-5 groups. MDA, malondialdehyde; T-SOD, total superoxide dismutase; NC, normal control; LPS, lipopolysaccharide; ISO-5, 10 nmol/ml LPS and 5 μM isorhamnetin; ISO-10, 10 nmol/ml LPS and 10 μM isorhamnetin.

cells (P<0.05). ISO (5 and 10 μM) dose-dependently reversed the changes of MDA and T-SOD when compared to the LPS group (P<0.05) (Tables VIII and IX).

Discussion

The prevalence of DN is rapidly increasing worldwide due to a rise in the prevalence of type 2 DM (23); however, no specific therapies for DN have been developed. Adequate glycemic control is believed to contribute to the decrease of renal vascular damage risks. The renin-angiotensin system blockade with angiotensin-converting-enzyme inhibitor is currently employed as a first-line treatment for DN (24). Angiotensin II type 1 receptor antagonist is also used to slow the progression of DN (25). However, DN still progresses with these drugs, and therefore, more novel agents are required.

ISO is a plant flavonoid abundant in herbal medicinal plants, such as *Hippophae rhamnoides* L. It has been tested in inflammation-associated diseases (18,20,26) and oxidative stress-associated diseases (27,28). However, to the best of our knowledge, no studies had evaluated its effects on kidney function in DN animals, which is also associated with inflammation

and oxidative stress. In the study, a type 2 diabetes model was established in rats fed a high-fat diet and a small dose of STZ, as described previously (22). Levels of urinary osteopontin and KIM-1, which are specific biomarkers of the early-stage renal damage and urinary albumin, were determined to evaluate the renal function damage of the animals. The DM group showed markedly higher levels of urinary osteopontin, KIM-1 and albumin compared to the control group, indicating that the high-fat diet and STZ injection induced significant renal function damage. However, ISO dose-dependently inhibited the increases of the three parameters when compared to the DM group. Therefore, the results of the present analyses indicated that ISO possesses renoprotective effects in type 2 diabetic rats.

Regulation of blood glucose is believed to contribute to a reduced renal risk. The blood glucose levels of the animals were evaluated and the DM and ISO groups had comparable levels of blood glucose. The results indicated that the recovery of renal damage did not benefit from blood glucose reduction.

Among the complex mechanisms leading to DN, inflammation has a key role (2-4). Increased levels of the inflammatory factors are associated with the initiation and progression of DN (8-10,29). Among the inflammatory factors, activated NF-κB can induce inflammatory cascade reactions, ultimately leading to renal damage. The present study showed that the DM group had increased protein and mRNA levels of NF-κB p65 and its active form phospho-NF-κB p65. The result was consistent with previous studies, which found increased NF-κB activity in diabetic animals (5,7,30). IκBα is an inhibitor of the activation of NF-κB p65, which can suppress the phosphorylation of NF-κB p65. However, significant differences were not found in the levels of IκBα among all the groups. As the phosphorylation of IκBα can induce the NF-κB activation, the levels of phospho-IκBα were measured and the DM group had higher levels of phospho-IκBα compared to the control group. Theoretically, the activated NF-κB p65 can be translocated to the nucleus to promote the transcription of the target genes. Consistently, the DM group had higher NF-κB p65 DNA-binding activity compared to the control groups. Furthermore, the investigations of the downstream inflammatory mediators of NF-κB signaling pathway also showed that the DM group had increased protein and mRNA levels of TNF-α, IL-1β, IL-6, ICAM-1 and TGF-β1. The roles of TNF-α, IL-1β, IL-6, ICAM-1 and TGF-β1 in the initiation and progression of DN have been well demonstrated in previous studies (8-10). Controlling the overexpression of these inflammatory mediators has exhibited beneficial effects in DN subjects (17,31). Notably, the present study found that ISO dose-dependently inhibited the increased production of NF-κB p65, phospho-NF-κB p65, phospho-IκBα and inflammatory cytokines, and decreased the NF-κB p65 DNA-binding activity as well when compared to the DM group. The result indicated that ISO could negatively regulate the NF-κB signaling pathway. This was consistent with the studies reporting that ISO inhibited the NF-κB activity in inflammatory disorders (18,20,32) and tumors (21). On the basis of those prior studies and the present data, it is hypothesized that the inhibitory effects on the NF-κB-mediated inflammatory response may be beneficial for the recovery of renal damage.

In addition to mediating an inflammatory response, NF-κB also triggers the oxidative reactions. NF-κB has an important role between the interaction of oxidative stress and inflammation. Oxidative stress is involved in numerous disorders (33). In the present study, the DM group had higher levels of oxidative marker MDA and lower levels of antioxidative marker T-SOD. This was consistent with the studies that reported increased oxidative activity in diabetic subjects (34,35). There is increasing evidence from pre-clinical and clinical studies, which suggests that antioxidant agents have potential ameliorative effects on diabetic complications, including DN (17,36,37). In the present study, ISO dose-dependently inhibited the overgeneration of MDA and increased the T-SOD activity, which suggested antioxidative activity of ISO in diabetic rats. Similarly, certain studies have also reported its antioxidative activity in other disorders (26-28,38). Therefore, the ameliorative effects on renal damage may also benefit from the suppression of oxidative stress.

In order to confirm the anti-inflammatory and antioxidative activity of ISO, rat GMCs were cultured with the presence of LPS or LPS+ISO. LPS is extensively used to induce an inflammatory state *in vitro* and *in vivo* (39). The present study showed that rat GMCs with LPS stimulation produced higher levels of NF-κB p65, phospho-NF-κB p65, phospho-IκBα, TNF-α, IL-1β, IL-6, ICAM-1 and TGF-β1, and increased the NF-κB p65 DNA-binding activity and oxidative stress, when compared to the normal control cells. Notably, ISO inhibited the changes induced by LPS stimulation. This was consistent with the *in vivo* investigations in the present study.

Collectively, ISO had renoprotective effects in type 2 diabetic rats. The recovery of renal damage may be associated with the inhibition of the NF-κB signaling pathway.

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