Kidney & Blood Pressure Research

Kidney Blood Press Res 2018;43:987-999
DOI: 10.1159/000490688
Published online: 18 June, 2018
Accepted: 7 June, 2018

Original Paper

Anti-Inflammatory Action of Sitagliptin and Linagliptin in Doxorubicin Nephropathy

Chor Ho Jo[a] Sua Kim[a] Joon-Sung Park[b] Gheun-Ho Kim[a,b]

[a]Institute of Biomedical Science, Hanyang University College of Medicine, Seoul, [b]Division of Nephrology, Department of Internal Medicine, Hanyang University College of Medicine, Seoul, Korea

Key Words
Blood pressure • Dipeptidyl peptidase-4 • Doxorubicin • Inflammasome • NADPH oxidase

Abstract
Background/Aims: Dipeptidyl peptidase-4 (DPP4) inhibitors are known to have a protective effect on diabetic kidney disease, possibly via reduction of oxidative stress and inflammation in the kidney. However, whether these potential mechanisms play a role in non-diabetic proteinuric kidney diseases is not clear. ***Methods:*** Two different animal experiments were carried out using sitagliptin and linagliptin for DPP4 inhibition. In each experiment, male Sprague-Dawley rats were uninephrectomized and randomly divided into vehicle-treated and doxorubicin-treated rats, with or without DPP4 inhibition. Administration of a DPP4 inhibitor was performed daily by oral gavage over six weeks. ***Results:*** A single intravenous injection of doxorubicin resulted in hypertension and remarkable proteinuria. Linagliptin, but not sitagliptin, lowered systolic blood pressure in rats with doxorubicin nephropathy. By contrast, sitagliptin ameliorated tubulointerstitial injury, inflammatory cell infiltration, and interstitial fibrosis in rat kidneys with doxorubicin nephropathy. Quantitative polymerase chain reaction analysis revealed that mRNA expression of NLRP3, caspase-1, ASC, and IL-1β was remarkably increased in rat kidneys with doxorubicin nephropathy, and that this upregulation of the major components of the NLRP3 inflammasome was effectively suppressed by treatment with either sitagliptin or linagliptin. Additionally, upregulation of IL-6 was reversed by linagliptin, but not by sitagliptin. On the other hand, sitagliptin, but not linagliptin, reversed the increase in mRNA expression of $gp91^{phox}$, $p47^{phox}$, and $p67^{phox}$ in rat kidneys with doxorubicin nephropathy. ***Conclusion:*** NLRP3 inflammasome activation was shown in our rat model of doxorubicin nephropathy. DPP4 inhibitors can suppress the activity of NLRP3, with or without relieving NADPH oxidase 2-related oxidative stress.

Gheun-Ho Kim MD, PhD

Department of Internal Medicine, Hanyang University College of Medicine,
222-1 Wangsimni-ro, Seongdong-gu, Seoul, 04763 (South Korea)
Tel. +82 2 2290 8318, Fax +82 2 2298 9183, E-Mail kimgh@hanyang.ac.kr

Introduction

Dipeptidyl peptidase-4 (DPP4) inhibitors were originally introduced for the treatment of type 2 diabetes mellitus, and their glucose-lowering effect is exerted by increasing the levels of incretin hormones, such as glucagon-like peptide-1 (GLP-1) [1]. In addition to stimulating insulin secretion from pancreatic β cells, DPP4 inhibitors can exhibit pleiotropic action to protect the cardiovascular system, mainly via the type 1 glucagon-like peptide receptor (GLP-1R) [2]. Furthermore, the renoprotective effects of DPP4 inhibition have been demonstrated in diabetic kidney disease models [3-8] because GLP-1R expression was localized to the vascular smooth muscle cells, glomerular capillaries, and proximal tubule cells in the kidney [9, 10]. Previous *in vitro* studies have shown that GLP-1 can play a renoprotective role via anti-inflammatory action in mesangial cells [11] and inhibition of angiotensin II in the glomerular endothelium [12].

The anti-inflammatory action of DPP4 inhibitors has also been explored in non-diabetic animal models, including renal mass reduction [13], unilateral ureteral obstruction [14], apolipoprotein E knockout mice [15], and obesity-induced renal injury [16]. However, the effect of DPP4 inhibitors in non-diabetic 'proteinuric' kidney diseases is not clear. Recently, Higashijima et al. reported that DPP4 inhibitors reduced macrophage infiltration in a rat Thy-1 nephritis model [17].

In glomerulonephritis, proteinuria determines the progression of renal injury toward interstitial inflammation and fibrosis [18]. It has been recently elucidated that this injury process involves the inflammasome, a complex of cytosolic proteins that typically consists of a sensor (a NOD-like receptor, NLR, or non-NLR), an adapter protein (usually apoptosis-associated speck-like protein containing a caspase activation and recruitment domain, ASC), and caspase-1 [19]. In particular, upregulation of the NLR containing pyrin domain 3 (NLRP3) inflammasome was demonstrated in the setting of proteinuria in cultured proximal tubule cells [20], podocytes [21], and a mouse strain with hereditary nephritic syndrome [22]. Here, we extended these investigations to a representative animal model of nephrotic syndrome pathologically characterized by extensive tubular injury, interstitial inflammation, and renal fibrosis [23]. For this, nephropathy was induced by doxorubicin administration, and we tested whether the NLRP3 inflammasome was activated and whether its activation can be ameliorated by DPP4 inhibition in association with changes in renal histopathology and markers of oxidative stress and inflammation.

Materials and Methods

Animal experiments

Pathogen-free, male Sprague-Dawley rats weighing 200–220 g (Orient Bio Inc., Seongnam, Korea) were uninephrectomized and then used in two separate animal experiments for the different DPP4 inhibitors, sitagliptin and linagliptin. In each experiment, the rats were randomly divided into three groups: vehicle-treated controls (VCs, n=4 or 5), doxorubicin-treated controls (DCs, n=4 or 5), and doxorubicin/sitagliptin-cotreated (DS, n=5) or doxorubicin/linagliptin-cotreated (DL, n=5) rats. Doxorubicin (Ildong Pharmaceutical, Seoul, Korea) was administered via the femoral vein as a single bolus (5 mg/kg), and either sitagliptin (10 mg/kg/d; MSD Korea, Seoul, Korea) or linagliptin (3 mg/kg/d; Boehringer Ingelheim Korea, Seoul, Korea) was given by oral gavage once daily for six weeks in cotreated rats. To test whether the DPP4 inhibitors were delivered to the kidney, DPP4 activity was determined from renal tissue homogenates by using a fluorometric assay kit (#K779-100; BioVision, Milpitas, CA, USA). Systolic blood pressure was measured using the tail-cuff method (P-98A, Softron; Tokyo, Japan). The experimental protocol was approved by the Institutional Animal Care and Use Committee of Hanyang University (No. 2013-0113A).

Renal histopathology

Renal tissue samples were fixed in 4% neutral-buffered formalin and embedded in paraffin. Paraffin sections (4 μm thick) were stained with periodic acid-Schiff reagent (PAS) and Sirius red. Tubulointerstitial injury was scored semiquantitatively on PAS-stained tissues, with a 20x objective. Specifically, tubulointerstitial injury was defined as tubular dilation, tubular atrophy, sloughing of tubular epithelial cells, or thickening of the tubular basement membrane and was scored on a scale of 0 to 4, as follows: 0, no tubulointerstitial injury; 1, <25% of tubulointerstitial injured; 2, 25 to 50% of the tubulointerstitial injured; 3, 51 to 75% of the tubulointerstitial injured; 4, >75% of the tubulointerstitial injured [24]. Areas of tubulointerstitial fibrosis were quantified on Sirius red-stained kidney sections. Ten photographs of randomly selected cortical areas per animal were taken at ×200 magnification using a digital inverted microscope (DMI4000B; Leica Microsystems, Wetzlar, Germany). The photographs were scanned as JPEG files with a resolution of 200 pixels/inch. The total positively-stained area in each image was quantified using Leica Application Suite software (Leica Microsystems).

Immunohistochemistry

Immunohistochemical staining of CD3 and ED1 as markers of T lymphocytes and macrophages, respectively, was performed on formalin-fixed, paraffin-embedded sections using the microwave antigen retrieval method [25]. The primary antibodies were rabbit monoclonal anti-CD3 (Abcam Inc., Cambridge, MA, USA) and mouse monoclonal anti-rat ED1 (Serotec, Oxford, UK). A point-counting technique was used to calculate the number of positively stained interstitial cells in at least 20 consecutive high-power fields [26].

Quantitative polymerase chain reaction (qPCR) analysis

Total RNA was isolated from the rat whole kidney with TRIzol® Reagent (Life Technologies, Carlsbad, CA, USA). RNA was quantified by spectrophotometry, and cDNA synthesis was performed using 3 μg of RNA with SuperScript® III Reverse Transcriptase (Life Technologies). For quantitative polymerase chain reaction (qPCR), 100 ng of cDNA served as a template for PCR amplification using the Brilliant SYBR green QPCR master mix, according to the manufacturer's instructions (FastStart DNA Master SYBR Green I; Roche Molecular Biochemicals, Mannheim, Germany). A serial dilution (1 ng–fg/μL) of cDNA was used as a template to generate a standard curve. Nested primers were used to amplify the standard and kidney cDNA samples (Table 1). The standard and unknown samples were amplified in duplicate in 96-well plates. The thermal profile of the LightCycler® Instrument (Roche Molecular Biochemicals) was optimized with an initial denaturation for 10 minutes at 95°C and 45 amplification cycles, each consisting of 10 seconds at 95°C, 10 seconds at 60°C, and 10 seconds at 72°C. The comparative Ct method was used to determine the relative amounts of target-mRNA levels, expressed for each sample as a percentage of GAPDH mRNA level. Ct ratios were analyzed using LightCycler® software (Version 4.05). Specificity was verified by a post-run melting-curve analysis [27].

ELISA for IL-1β measurement from renal tissue

The concentration of IL-1β from renal tissues was measured using sandwich ELISA [28]. Briefly, monoclonal capture antibody (IL-1β, R&D Systems, Minneapolis, MN, USA) was added to a 96-well plate (Nunc, Roskilde, Denmark) and incubated overnight at 4 °C, following which the plate was washed five times with PBS containing 0.05% Tween-20. Following incubation with blocking solution for 1 hour at room temperature, the test samples and recombinant IL-1β standards were added to the plate. The plate was incubated for 2 hours at room temperature, following which it was washed five times. Biotin-conjugated anti-human IL-1β (eBioscience, San Diego, CA, USA) was added and incubated for 1 hour at room temperature; the plate was then washed. Avidin horseradish peroxidase (eBioscience) was then added, with the reaction allowed to proceed for 30 minutes at room temperature. The plate was then washed five times and 3, 3',5, 5'-tetramethylbenzidine solution was added to induce the color reaction, which was attenuated by the addition of 2N H_2SO_4. The optical density at 450 nm was measured using an automated microplate reader (VersaMax, Molecular Devices, Palo Alto, CA, USA).

Table 1. Primer sequences for qPCR. qPCR, quantitative polymerase chain reaction; NOX, nicotinamide adenine dinucleotide phosphate oxidase; CuZn-SOD, intracellular superoxide dismutase; Mn-SOD, mitochondrial SOD; TNF-α, tumor necrosis factor-α; IkB-α, inhibitor of kB-α; MCP1, monocyte chemotactic protein-1; RANTES, regulated on activation, normal T cell expressed and secreted; NLRP3, NOD-like receptor family, pyrin domain containing 3; CASP1, caspase-1; ASC, apoptosis-associated speck-like protein containing a caspase activation and recruitment domain; IL, interleukin

Gene	Forward (F) and reverse (R) primer sequences	PCR product (bp)	GenBank Accession No.
NOX1	F 5′- GGAGTTGCAGGAGTCCTCATTTT -3′ R 5′- TTCTGCCGGGAGCGATAA -3′	119	NM_053683.1
gp91^{phox}	F 5′- AAAGGAGTGCCCAGTACCAAAGT -3′ R 5′- TACAGGAACATGGGACCCACTAT -3′	79	AF298656
p47^{phox}	F 5′- ACGCTCACCGAGTACTTCAACA -3′ R 5′- TCATCGGGCCGCACTTT -3′	96	AY029167
p67^{phox}	F 5′- GCTTCGGAACATGGTGTCTAAGA -3′ R 5′- AGAGTCAGGCAGTAGTTTTTCACTTG -3′	220	AB002664
p22^{phox}	F 5′- ACCTGACCGCTGTGGTGAA -3′ R 5′- GTGGAGGACAGCCCGGT -3′	69	AJ295951.1
NOX4	F 5′- AGAATGAGGATCCCAGAAAGCTT -3′ R 5′- ATGAGGAACAATACCACCACCAT -3′	89	NM_053524.1
CuZn-SOD	F 5′-TGTGTCCATTGAAGATCGTGTGA-3′ R 5′-TCTTGTTTCTCGTGGACCACC-3′	85	NM_017050
Mn-SOD	F 5′-TTAACGCGCAGATCATGCA-3′ R 5′-CCTCGGTGACGTTCAGATTGT-3′	76	NM_017051
TNF-α	F 5′-GCTCCCTCTCATCAGTTC C-3′ R 5′-CTCCTCTGCTTGGTGGTTTG-3′	110	X66539.1
IκB-α	F 5′-CTGCAGGCCACCAACTACAA-3′ R 5′-GTAGCCATGGATAGAGGCTAAGTG-3′	61	FQ226288.1
MCP1	F 5′- GGTCTCTGTCACGCTTCTG -3′ R 5′- TTCTCCAGCCGACTCATTG -3′	145	NM_031530
RANTES	F 5′- CACCTGCCTCCCCATATG -3′ R 5′- TTCCTTCGAGTGACAAAGACG-3′	146	NM_031116
NLRP3	F 5′- CAGACCTCCAAGACCACGACTG -3′ R 5′- CATCCGCAGCCAATGAACAGAG -3′	128	NM_001191642.1
CASP1	F 5′- TGCCTGGTCTTGTGACTTGGAG -3′ R 5′- ATGTCCTGGGAAGAGGTAGAAACG -3′	134	NM_012762.2
ASC	F 5′- TTATGGAAGAGTCTGGAGCTGTGG -3′ R 5′- AATGAGTGCTTGCCTGTGTTGG -3′	102	NM_172322.1
IL-1β	F 5′- GAGGCTGACAGACCCCAAAAGAT -3′ R 5′- GCACGAGGCATTTTTGTTGTTCA -3′	339	NM_031512
IL-6	F 5′- GAAATACAAAGAAATGATGGATGCT -3′ R 5′- TTCAAGATGAGTTGGATGGTCT -3′	312	NM_012589

Statistics

Values are presented as means ± SD. Differences among the groups were analyzed using the Kruskal-Wallis test. The Mann-Whitney U-test was used for post-hoc comparisons between groups. P-values ≤ 0.05 were considered statistically significant. All statistical analyses were performed using Statview software (Abacus Concepts, Berkeley, CA, USA).

Results

Overt nephropathy was induced by a single intravenous injection of doxorubicin in rats with unilateral right nephrectomy, as evidenced by significant elevation of systolic blood pressure (SBP), urinary protein excretion, and elevated plasma creatinine level. Fig. 1 shows that hypertension in doxorubicin nephropathy was significantly controlled by linagliptin but not by sitagliptin treatment. Proteinuria was unaffected by either DPP4 inhibitor (Tables 2 and 3). An improving tendency in plasma creatinine and creatinine clearance was observed, but did not reach statistical significance. Table 3 shows that, in response to linagliptin treatment, proteinuria and plasma creatinine level had a decreasing tendency, but did not reach statistical significance. Plasma glucose level was not affected by DPP4 inhibition in either animal experiment. However, DPP4 activity determined from renal tissues was significantly suppressed by daily oral administration of DPP4 inhibitors; When it was compared with doxorubicin-treated controls, both sitagliptin (69 ± 19 vs. 100 ± 10%, P <0.05) and linagliptin (38 ± 2 vs. 100 ± 32%, P <0.05) were effective.

Fig. 2 shows the results of renal histopathology from the sitagliptin experiment. A semiquantitative assessment of tubulointerstitial damage in PAS-stained cortical sections revealed that the DS group had a significantly lower tubulointerstitial injury score than the DC group (2.00 ± 0.47 vs. 3.48 ± 0.47 points, P<0.05). The extent of interstitial fibrosis was also lesser in

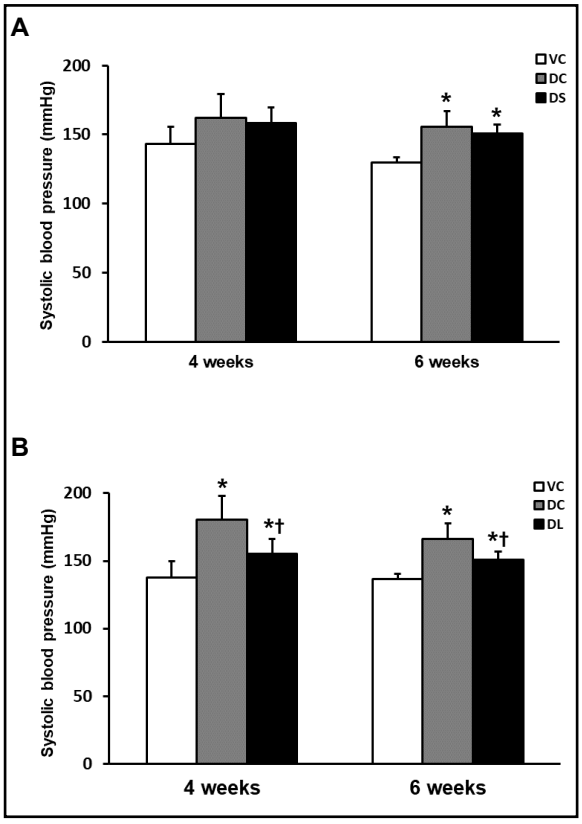

Fig. 1. Systolic blood pressure changes from the sitagliptin (A) and linagliptin (B) experiments**.** Systolic blood pressure was measured from rat tails at four weeks and six weeks after initial administration of doxorubicin and either DPP4 inhibitor. VC, vehicle-treated controls; DC, doxorubicin-treated controls; DS, doxorubicin/sitagliptin-cotreated rats; DL, doxorubicin/linagliptin-cotreated rats. The numbers of animals are shown in Tables 2 and 3. *P<0.05 vs. VC; †P<0.05 vs. DC by Mann-Whitney U-test.

Table 2. Functional parameters from the 6-week sitagliptin experiment. Values are given as mean ± SD. VC, vehicle-treated controls; DC, doxorubicin-treated controls; DS, doxorubicin/sitagliptin-cotreated rats; BUN, blood urea nitrogen; Cr, creatinine. *Kruskal-Wallis test. †P<0.05 vs. VC by Mann-Whitney U-test

Parameters	VC (n = 5)	DC (n = 5)	DS (n = 5)	P-value*
Urine volume (mL/day/100 g BW)	3.8 ± 1.2	9.4 ± 3.3†	9.4 ± 3.3†	0.012
Proteinuria (mg/day/100 g BW)	2 ± 1	218 ± 53†	244 ± 27†	<0.001
Plasma total protein (g/dL)	5.8 ± 0.1	5.1 ± 0.1†	5.0 ± 0.3†	0.008
Plasma albumin (g/dL)	3.5 ± 0.2	2.7 ± 0.2†	2.6 ± 0.2†	0.009
Plasma glucose (mg/dL)	209 ± 34	183 ± 44	190 ± 15	0.523
BUN (mg/dL)	21 ± 2	34 ± 13†	32 ± 7†	0.019
Cr (mg/dL)	0.39 ± 0.30	0.77 ± 0.31†	0.67 ± 0.18†	0.009
Cr clearance (μL/min/100 g BW)	523 ± 118	299 ± 115†	365 ± 136	0.039

Table 3. Functional parameters from the 6-week linagliptin experiment. Values are given as mean ± SD. VC, vehicle-treated controls; DC, doxorubicin-treated controls; DL, doxorubicin/linagliptin-cotreated rats; BUN, blood urea nitrogen; Cr, creatinine. *Kruskal-Wallis test. †P<0.05 vs. VC by Mann-Whitney U-test

Parameters	VC (n = 4)	DC (n = 4)	DL (n = 5)	P-value*
Urine volume (mL/day/100 g BW)	4.5 ± 1.3	8.4 ± 2.4	7.3 ± 2.5	0.116
Proteinuria (mg/day/100 g BW)	3 ± 0	206 ± 52†	150 ± 63†	0.015
Plasma total protein (g/dL)	5.7 ± 0.1	5.0 ± 0.3†	5.2 ± 0.3†	0.022
Plasma albumin (g/dL)	3.5 ± 0.1	2.7 ± 0.2†	2.8 ± 0.1†	0.016
Plasma glucose (mg/dL)	248 ± 27	204 ± 40	223 ± 27	0.391
BUN (mg/dL)	19 ± 2	32 ± 11	26 ± 6	0.124
Cr (mg/dL)	0.44 ± 0.05	0.63 ± 0.14	0.53 ± 0.16	0.183
Cr clearance (μL/min/100 g BW)	564 ± 46	404 ± 11†	496 ± 174	0.174

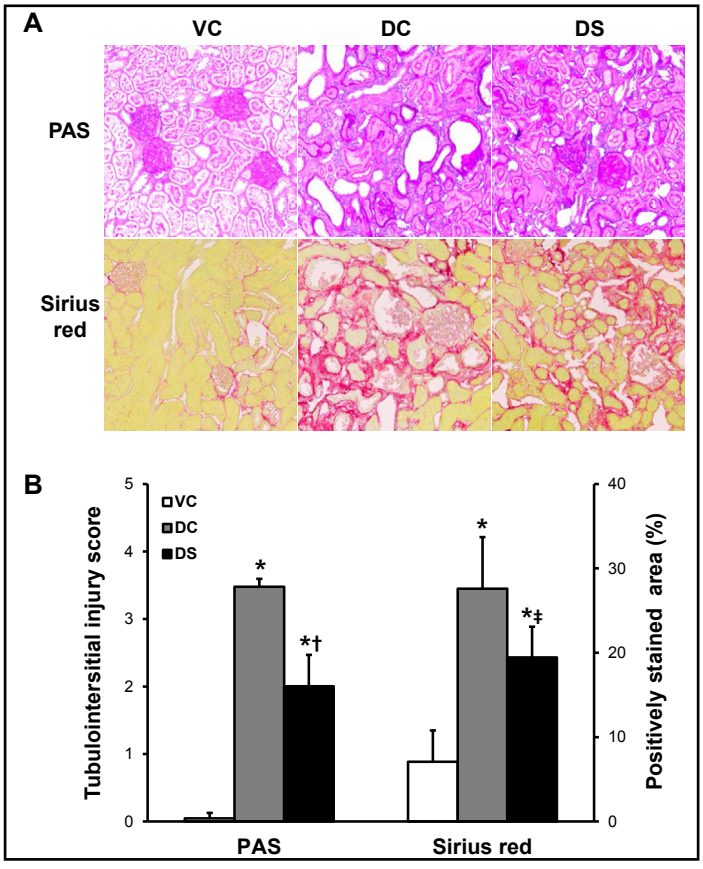

Fig. 2. Periodic acid-Schiff (PAS) and Sirius red staining of the renal cortex from the sitagliptin experiment. Images of representative tissue sections (A) and bar graphs of the results of a semiquantitative assessment (B) are shown. VC, vehicle-treated controls (n=5); DC, doxorubicin-treated controls (n=5); DS, doxorubicin/sitagliptin-cotreated rats (n=5). *P<0.05 vs. VC; †P<0.05 vs. DC; ‡P=0.08 vs. DC by Mann-Whitney U-test.

the DS group than in the DC group when estimated by Sirius red staining (19.5 ± 3.6 vs. 27.6 ± 6.1%, *P*<0.05). Linagliptin treatment produced similar responses in tubulointerstitial injury and interstitial fibrosis (Fig. 3).

Fig. 4 shows the results of immunohistochemical staining of cortical sections for CD3 and ED1. Compared with the DC group, the DS group had significantly fewer interstitial CD3-positive T lymphocytes (45 ± 21 vs. 157 ± 70 cells/HFP, P<0.05) and ED1-positive macrophages (20 ± 6 vs. 31 ± 8 cells/HFP, P<0.05).

Next, we performed a qPCR analysis to investigate the effects of DPP4 inhibition on oxidative stress-related and NLRP3 inflammasome-associated gene expression. As shown in Fig. 5, mRNA expression of the major components of the NLRP3 inflammasome (NLRP3, caspase-1, and IL-1β) and certain inflammatory cytokines were remarkably upregulated in rat kidneys with doxorubicin nephropathy. Figures 5A and 5B summarize the effects of DDP4 inhibition via the two separate inhibitors. The DS group had significantly lower mRNA levels

Kidney & Blood Pressure Research

Kidney Blood Press Res 2018;43:987-999
DOI: 10.1159/000490688
Published online: 18 June, 2018

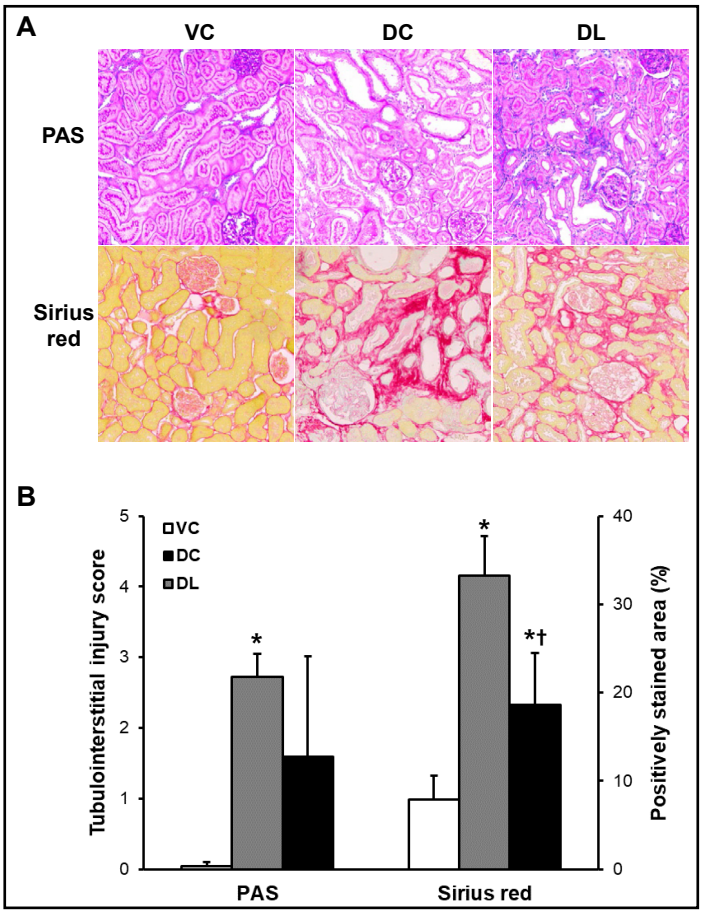

Fig. 3. Periodic acid-Schiff (PAS) and Sirius red staining of the renal cortex from the linagliptin experiment. Images of representative tissue sections (A) and bar graphs of the results of a semiquantitative assessment (B) are shown. VC, vehicle-treated controls (n=4); DC, doxorubicin-treated controls (n=4); DS, doxorubicin/linagliptin-cotreated rats (n=5). *P<0.05 vs. VC; †P<0.05 vs. DC by Mann-Whitney U-test.

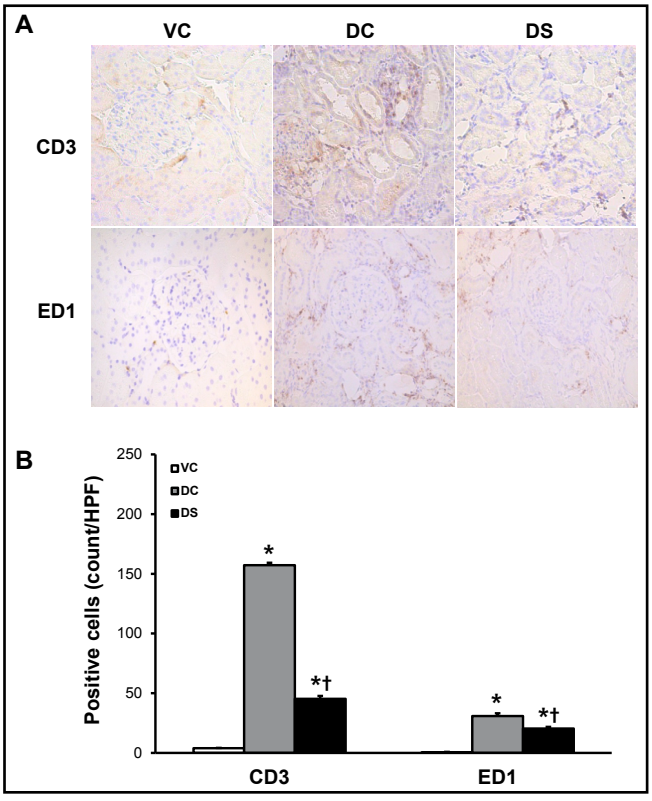

Fig. 4. Immunohistochemical staining for CD3 and ED1 in the renal cortex from the sitagliptin experiment. Images of representative tissue sections (A) and bar graphs of the number of positively stained cells in the interstitium (B) are shown. VC, vehicle-treated controls (n=5); DC, doxorubicin-treated controls (n=5); DS, doxorubicin/sitagliptin-cotreated rats (n=5). *P<0.05 vs. VC; †P<0.05 vs. DC by Mann-Whitney U-test.

of MCP-1 (3.7 ± 0.3 vs. 5.8 ± 0.8, P<0.05), RANTES (1.0 ± 0.1 vs. 1.7 ± 0.1, P<0.05), NLRP3 (3.0 ± 0.4 vs. 5.4 ± 1.0, P<0.05), caspase-1 (2.7 ± 0.5 vs. 5.0 ± 0.7, P<0.05), ASC (2.3 ± 0.3 vs. 5.7 ± 0.4, P<0.05), and IL-1β (2.5 ± 0.1 vs. 4.6 ± 0.5, P<0.05) than in the DC group. Similarly, the DL group had significantly lower mRNA levels of NLRP3 (1.3 ± 0.2 vs. 3.0 ± 0.4, P<0.05), caspase-1 (1.4 ± 0.2 vs. 2.3 ± 0.3, P<0.05), ASC (2.2 ± 0.2 vs. 3.5 ± 0.5, P=0.05), IL-1β (1.8 ± 0.3 vs. 3.5 ± 0.4, P<0.05), and IL-6 (4.0 ± 1.0 vs. 9.7 ± 1.7, P<0.05) than in the DC group. We confirmed that compared with VC (15 ± 3 pg/mg protein), the IL-1β protein concentration determined by ELISA was increased in DC (21 ± 1 pg/mg protein, P<0.05) but was reversed in DL (16 ± 3 pg/mg protein, P<0.05).

Fig. 6 shows that doxorubicin-induced nephropathy is accompanied by the upregulation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzymes (NOX1 and NOX2 components) and downregulation of reactive oxygen species neutralizing enzymes, such as superoxide dismutases (CuZn-SOD and Mn-SOD). Compared with the DC group, the DS group had significantly lower mRNA levels of $gp91^{phox}$ (4.6 ± 0.4 vs. 8.1 ± 0.4, P<0.05), $p47^{phox}$ (4.2 ± 0.6 vs. 5.6 ± 0.3, P<0.05), and $p67^{phox}$ (5.3±0.7 vs. 8.1 ± 1.0, P<0.05). However, the decreased mRNA levels of CuZn-SOD and Mn-SOD in doxorubicin nephropathy were not restored by sitagliptin treatment (Fig. 6A). Interestingly, linagliptin treatment was not successful in blocking the upregulation of oxidative stress-related gene expression in doxorubicin nephropathy (Fig. 6B).

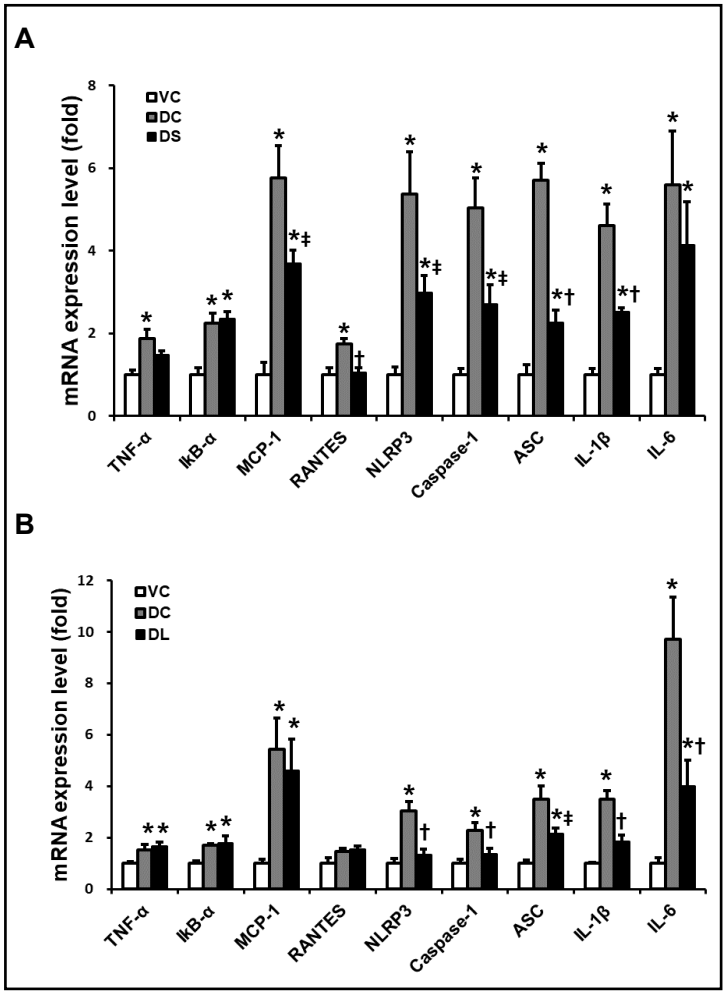

Fig. 5. Quantitative polymerase chain reaction (qPCR) data for mRNA levels of inflammatory mediators. qPCR results from the sitagliptin (A) and linagliptin (B) experiments are shown. TNF-α, tumor necrosis factor-α; IkB-α, inhibitor of kB-α; MCP1, monocyte chemotactic protein-1; RANTES, regulated on activation, normal T cell expressed and secreted; NLRP3, NOD-like receptor family, pyrin domain containing 3; ASC, apoptosis-associated speck-like protein containing a caspase activation and recruitment domain; IL, interleukin. VC, vehicle-treated controls (n=5 for each experiment); DC, doxorubicin-treated controls (n=4 for each experiment); DS, doxorubicin/sitagliptin rats (n=5), DL, doxorubicin/linagliptin-cotreated rats (n=5). *P<0.05 vs. VC; †P<0.05 vs. DC; ‡P=0.05 vs. DC by Mann-Whitney U-test.

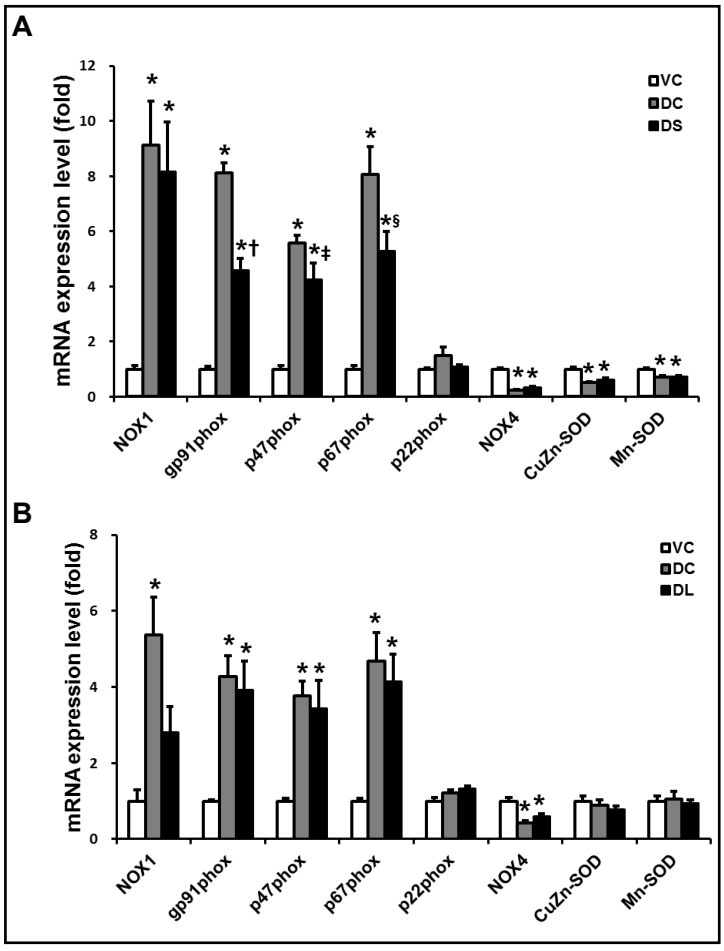

Fig. 6. Quantitative polymerase chain reaction (qPCR) data for mRNA levels of oxidative stress-related biomarkers. qPCR results from the sitagliptin (A) and linagliptin (B) experiments are shown. CuZn-SOD, intracellular superoxide dismutase (SOD); Mn-SOD, mitochondrial SOD; NOX, nicotinamide adenine dinucleotide phosphate oxidase. VC, vehicle-treated controls (n=5); DC, doxorubicin-treated controls (n=4); DS, doxorubicin and sitagliptin-cotreated rats (n=5). *P<0.05 vs. VC; †P<0.05 vs. DC; ‡P=0.05 vs. DC; §P=0.09 vs. DC by Mann-Whitney U-test.

Discussion

In this study, we demonstrate that DPP4 inhibitors can suppress NLRP3 inflammasome activation in doxorubicin nephropathy and lead to amelioration of tubulointerstitial injury and interstitial fibrosis. These effects were independent of blood glucose level, and the observed antioxidant effects were inconsistent between sitagliptin and linagliptin.

DPP4 inhibition has been a plausible treatment strategy for management of diabetic nephropathy [29]. However, only a few previous studies have shown that DPP4 inhibitors play a role in preventing the progression of non-diabetic kidney disease. Lee et al. recently reported that DA-1229, a new DPP4 inhibitor, improved proteinuria, renal fibrosis, and inflammation in doxorubicin-treated mice [30]. They suggested a role of DA-1229 in preventing podocyte injury. In non-diabetic rats with 5/6 nephrectomy, linagliptin reduced albuminuria in association with upregulation of downstream targets of atrial natriuretic peptide [31]. Although we failed in reversing heavy proteinuria by using sitagliptin or linagliptin in our rat model of doxorubicin nephropathy, both DPP4 inhibitors were successful in ameliorating tubulointerstitial injury and interstitial fibrosis, most likely via anti-inflammatory action. Previous studies have shown that DPP4 inhibition can improve renal histopathology in animal models of diabetic nephropathy [29, 32]. Among a variety of potential mechanisms by which DPP4 inhibitors exert this renoprotective effect on damaged kidneys, we focused on the regulation of NLRP3 inflammasome activity. Similarly to previous studies [29, 33], we found no improvement in serum creatinine levels by DPP4 inhibition.

Proteinuria contributes to tubulointerstitial inflammation, leading to renal fibrosis. Renal production of inflammatory cytokines is stimulated in rats with heavy proteinuria [34]. Consistent with this, we found that renal mRNA expression of MCP-1 and RANTES was increased in doxorubicin nephropathy. Morphologically, we showed that peritubular infiltration of CD3- and ED1-positive cells was associated with tubulointerstitial injury and renal fibrosis. Finally, we showed that these inflammatory processes were effectively blocked by sitagliptin treatment. Previous studies have shown that sitagliptin treatment reduces renal inflammation and oxidative stress in animal models of type 2 diabetes [29, 35].

To the best of our knowledge, our results represent the first report demonstrating the activation of the NLRP3 inflammasome in doxorubicin nephropathy. Previous studies have shown that treatment of mice with doxorubicin induced IL-1β release from bone marrow-derived macrophages via activation of the NLRP3 inflammasome [36, 37]. Recently, Kobayashi et al. suggested that NLRP3 plays a role in doxorubicin-induced cardiotoxicity [38]. Instead of IL-1β expression, however, it was connected to IL-10 production in macrophages. We found that, in doxorubicin nephropathy, renal mRNA expression of NLRP3, ASC, caspase-1, and IL-1β was remarkably increased. Furthermore, we demonstrated that these effects of NLRP3 inflammasome activation were suppressed by both sitagliptin and linagliptin.

How do DPP4 inhibitors reduce the activity of NLRP3? The role of reactive oxygen species in mediation of NLRP3 activation is the most plausible [39, 40]. Consistent with this, we showed that renal mRNA expression of NOX1 and NOX2 ($gp91^{phox}$, $p47^{phox}$, and $p67^{phox}$) was increased in doxorubicin nephropathy, and that this upregulation of $gp91^{phox}$, $p47^{phox}$, and $p67^{phox}$ mRNA was effectively blocked by sitagliptin treatment. In contrast, the downregulation of NOX4, CuZn-SOD, and Mn-SOD was not altered by DPP4 inhibition. We believe that there exist mechanisms other than antioxidant action by which DPP4 inhibitors suppress the activity of NLRP3, because the effects of linagliptin treatment on the expression of the NLRP3 inflammasome and NOX2 were not parallel.

It is not surprising that the two DPP4 inhibitors, sitagliptin and linagliptin, have different effects on doxorubicin nephropathy. Each of the DPP4 inhibitors might have unique drug-specific effects, because sitagliptin and linagliptin show different activities in their diverse effects on endothelial cell biology [41]. Besides, characteristic pharmacologic actions might be derived from the different binding modes of DPP4 inhibitors in the active site [42]. In this study, linagliptin, but not sitagliptin, had an antihypertensive effect on doxorubicin nephropathy. This discrepancy may be explained by different anti-inflammatory actions between sitagliptin and linagliptin. In our rat kidneys with doxorubicin nephropathy, the upregulation of IL-6 was effectively reversed by linagliptin, but not by sitagliptin. Previous studies using animal models of hypertension have shown that IL-6 may have a role in pathogenesis of hypertension [43, 44]. Furthermore, inhibition of IL-6 was connected to attenuation of associated renal damage [45-47]. Thus, we believe that antihypertensive effect of linagliptin may be derived from reversing IL-6 upregulation in doxorubicin nephropathy. Besides, the antihypertensive action of linagliptin can be explained by its reported ability to upregulate endothelial nitric oxide synthase (eNOS) and restore endothelium-dependent vasodilation [48, 49].

Conclusion

We showed that inflammatory processes accompanying NLRP3 inflammasome activation are prominent in doxorubicin nephropathy, and that DPP4 inhibitors can suppress the activity of NLRP3 with or without relieving NOX2-related oxidative stress. Thus, DPP4 inhibitors might be a novel agent to offer anti-inflammatory action in proteinuric kidney disease.

Acknowledgements

Gheun-Ho Kim designed the experiments. Chor Ho Jo, Joon-Sung Park, and Sua Kim performed the experiments. Chor Ho Jo, Joon-Sung Park, and Gheun-Ho Kim wrote the manuscript.

We are grateful to Dr. Il Hwan Oh for his technical assistance.

Disclosure Statement

The authors declare no conflicts of interest regarding the publication of this article.

References

1 Makino Y, Fujita Y, Haneda M: Dipeptidyl peptidase-4 inhibitors in progressive kidney disease. Curr Opin Nephrol Hypertens 2015;24:67-73.

2 Hocher B, Reichetzeder C, Alter ML: Renal and cardiac effects of DPP4 inhibitors from preclinical development to clinical research. Kidney Blood Press Res 2012;36:65-84.

3 Mega C, de Lemos ET, Vala H, Fernandes R, Oliveira J, Mascarenhas-Melo F, Teixeira F, Reis F: Diabetic nephropathy amelioration by a low-dose sitagliptin in an animal model of type 2 diabetes (Zucker diabetic fatty rat). Exp Diabetes Res 2011;2011:162092.

4 Alter ML, Ott IM, von Websky K, Tsuprykov O, Sharkovska Y, Krause-Relle K, Raila J, Henze A, Klein T, Hocher B: DPP-4 inhibition on top of angiotensin receptor blockade offers a new therapeutic approach for diabetic nephropathy. Kidney Blood Press Res 2012;36:119-130.

5 Liu WJ, Xie SH, Liu YN, Kim W, Jin HY, Park SK, Shao YM, Park TS: Dipeptidyl peptidase IV inhibitor attenuates kidney injury in streptozotocin-induced diabetic rats. J Pharmacol Exp Ther 2012;340:248-255.

6 Marques C, Mega C, Gonçalves A, Rodrigues-Santos P, Teixeira-Lemos E, Teixeira F, Fontes-Ribeiro C, Reis F, Fernandes R: Sitagliptin prevents inflammation and apoptotic cell death in the kidney of type 2 diabetic animals. Mediators Inflamm 2014;2014:538737.

7 Kanasaki K, Shi S, Kanasaki M, He J, Nagai T, Nakamura Y, Ishigaki Y, Kitada M, Srivastava SP, Koya D: Linagliptin-mediated DPP-4 inhibition ameliorates kidney fibrosis in streptozotocin-induced diabetic mice by inhibiting endothelial-to-mesenchymal transition in a therapeutic regimen. Diabetes 2014;63:2120-2131.

8 Kodera R, Shikata K, Takatsuka T, Oda K, Miyamoto S, Kajitani N, Hirota D, Ono T, Usui HK, Makino H: Dipeptidyl peptidase-4 inhibitor ameliorates early renal injury through its anti-inflammatory action in a rat model of type 1 diabetes. Biochem Biophys Res Commun 2014;443:828-833.

9 Yu HC, Burrell LM, Black MJ, Wu LL, Dilley RJ, Cooper ME, Johnston CI: Salt induces myocardial and renal fibrosis in normotensive and hypertensive rats. Circulation 1998;98:2621-2628.

10 Tanaka T, Higashijima Y, Wada T, Nangaku M: The potential for renoprotection with incretin-based drugs. Kidney Int 2014;86:701-711.

11 Ishibashi Y, Nishino Y, Matsui T, Takeuchi M, Yamagishi S: Glucagon-like peptide-1 suppresses advanced glycation end product-induced monocyte chemoattractant protein-1 expression in mesangial cells by reducing advanced glycation end product receptor level. Metabolism. 2011;60:1271-1277.

12 Mima A, Hiraoka-Yamomoto J, Li Q, Kitada M, Li C, Geraldes P, Matsumoto M, Mizutani K, Park K, Cahill C, Nishikawa S, Rask-Madsen C, King GL: Protective effects of GLP-1 on glomerular endothelium and its inhibition by PKC beta activation in diabetes. Diabetes 2012;61:2967-2979.

13 Joo KW, Kim S, Ahn SY, Chin HJ, Chae DW, Lee J, Han JS, Na KY: Dipeptidyl peptidase IV inhibitor attenuates kidney injury in rat remnant kidney. BMC Nephrol 2013;14:98.

14 Min HS, Kim JE, Lee MH, Song HK, Kang YS, Lee MJ, Lee JE, Kim HW, Cha JJ, Chung YY, Hyun YY, Han JY, Cha DR: Dipeptidyl peptidase IV inhibitor protects against renal interstitial fibrosis in a mouse model of ureteral obstruction. Lab Invest 2014;94:598-607.

15 Li J, Guan M, Li C, Lyv F, Zeng Y, Zheng Z, Wang C, Xue Y: The dipeptidyl peptidase-4 inhibitor sitagliptin protects against dyslipidemia-related kidney injury in apolipoprotein E knockout mice. Int J Mol Sci 2014;15:11416-11434.
16 Nistala R, Habibi J, Lastra G, Manrique C, Aroor AR, Hayden MR, Garro M, Meuth A, Johnson M, Whaley-Connell A, Sowers JR: Prevention of obesity-induced renal injury in male mice by DPP4 inhibition. Endocrinology 2014;155:2266-2276.
17 Higashijima Y, Tanaka T, Yamaguchi J, Tanaka S, Nangaku M: Anti-inflammatory role of DPP-4 inhibitors in a non-diabetic model of glomerular injury. Am J Physiol Renal Physiol 2015;308:878-887.
18 Zoja C, Abbate M, Remuzzi G: Progression of renal injury toward interstitial inflammation and glomerular sclerosis is dependent on abnormal protein filtration. Nephrol Dial Transplant 2015;30:706-712.
19 Chang A, Ko K, Clark MR: The emerging role of the inflammasome in kidney diseases. Curr Opin Nephrol Hypertens 2014;23:204-210.
20 Fang L, Xie D, Wu X, Cao H, Su W, Yang J.: Involvement of endoplasmic reticulum stress in albuminuria induced inflammasome activation in renal proximal tubular cells. PLoS One 2013;8:e72344.
21 Okamura K, Dummer P, Kopp J, Qiu L, Levi M, Faubel S, Blaine J: Endocytosis of albumin by podocytes elicits an inflammatory response and induces apoptotic cell death. PLoS One 2013;8:e54817.
22 Nishi Y, Satoh M, Nagasu H, Kadoya H, Ihoriya C, Kidokoro K, Sasaki T, Kashihara N: Selective estrogen receptor modulation attenuates proteinuria-induced renal tubular damage by modulating mitochondrial oxidative status. Kidney Int 2013;83:662-673.
23 Park JS, Kim S, Jo CH, Oh IH, Kim GH: Effects of dietary salt restriction on renal progression and interstitial fibrosis in adriamycin nephrosis. Kidney Blood Press Res 2014;39:86-96.
24 Nangaku M, Pippin J, Couser WG: Complement membrane attack complex (C5b-9) mediates interstitial disease in experimental nephritic syndrome. J Am Soc Nephrol 1999;10:2323-2331.
25 Park JS, Jo CH, Kim S, Kim GH: Acute and chronic effects of dietary sodium restriction on renal tubulointerstitial fibrosis in cisplatin-treated rats. Nephrol Dial Transplant 2013;28:592-602.
26 Yu XQ, Wu LL, Huang XR, Yang N, Gilbert RE, Cooper ME, Johnson RJ, Lai KN, Lan HY: Osteopontin expression in progressive renal injury in remnant kidney: role of angiotensin II. Kidney Int 2000;58:1469-1480.
27 Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Methods 2001;25:402-408.
28 Jang J, Yoon Y, Oh DJ: A calpain inhibitor protects against fractalkine production in lipopolysaccharide-treated endothelial cells. Kidney Res Clin Pract 2017;36:224–231.
29 Mega C, Teixeira-de-Lemos E, Fernandes R, Flávio F: Renoprotective effects of the dipeptidyl peptidase-4 inhibitor sitagliptin: A review in type 2 diabetes. J Diabetes Res 2017;2017: 5164292.
30 Lee JE, Kim JE, Lee MH, Song HK, Ghee JY, Kang YS, Min HS, Kim HW, Cha JJ, Han JY, Han SY, Cha DR: DA-1229, a dipeptidyl peptidase IV inhibitor, protects against renal injury by preventing podocyte damage in an animal model of progressive renal injury. Lab Invest 2016;96:547-560.
31 Tsuprykov O, Ando R, Reichetzeder C, von Websky K, Antonenko V, Sharkovska Y, Chaykovska L, Rahnenführer J, Hasan AA, Tammen H, Alter M, Klein T, Ueda S, Yamagishi SI, Okuda S, Hocher B: The dipeptidyl peptidase inhibitor linagliptin and the angiotensin II receptor blocker telmisartan show renal benefit by different pathways in rats with 5/6 nephrectomy. Kidney Int 2016;89:1049-1061.
32 Vavrinec P, Henning RH, Landheer SW, Wang Y, Deelman LE, Dokkum RP, Buikema H: Vildagliptin restores renal myogenic function and attenuates renal sclerosis independently of effects on blood glucose or proteinuria in zucker diabetic fatty rat. Curr Vasc Pharmacol 2014;12:836-844.
33 Kröller-Schön S, Knorr M, Hausding M, Oelze M, Schuff A, Schell R, Sudowe S, Scholz A, Daub S, Karbach S, Kossmann S, Gori T, Wenzel P, Schulz E, Grabbe S, Klein T, Münzel T, Daiber A: Glucose-independent improvement of vascular dysfunction in experimental sepsis by dipeptidyl-peptidase 4 inhibition. Cardiovasc Res 2012;96:140-149.
34 Eddy AA, Giachelli CM: Renal expression of genes that promote interstitial inflammation and fibrosis in rats with protein-overload proteinuria. Kidney Int 1995;47:1546-1557.

35 Ferreira L, Teixeira-de-Lemos E, Pinto F, Parada B, Mega C, Vala H, Pinto R, Garrido P, Sereno J, Fernandes R, Santos P, Velada I, Melo A, Nunes S, Teixeira F, Reis F: Effects of sitagliptin treatment on dysmetabolism, inflammation, and oxidative stress in an animal model of type 2 diabetes (ZDF rat). Mediators Inflamm 2010;2010:592760.

36 Sauter KA, Wood LJ, Wong J, Iordanov M, Magun BE: Doxorubicin and daunorubicin induce processing and release of interleukin-1β through activation of the NLRP3 inflammasome. Cancer Biol Ther 2011;11:1008-1016.

37 Wong J, Tran LT, Magun EA, Magun BE, Wood LJ: Production of IL-1β by bone marrow-derived macrophages in response to chemotherapeutic drugs: synergistic effects of doxorubicin and vincristine. Cancer Biol Ther 2014;15:1395-1403.

38 Kobayashi M, Usui F, Karasawa T, Kawashima A, Kimura H, Mizushina Y, Shirasuna K, Mizukami H, Kasahara T, Hasebe N, Takahashi M: NLRP3 deficiency reduces macrophage interleukin-10 production and enhances the susceptibility to doxorubicin-induced cardiotoxicity. Sci Rep 2016;6:26489.

39 Liu D, Xu M, Ding LH, Lv LL, Liu H, Ma KL, Zhang AH, Crowley SD, Liu BC: Activation of the Nlrp3 inflammasome by mitochondrial reactive oxygen species: a novel mechanism of albumin-induced tubulointerstitial inflammation. Int J Biochem Cell Biol 2014;57:7-19.

40 Joshi S, Wang W, Peck AB, Khan SR: Activation of the NLRP3 inflammasome in association with calcium oxalate crystal induced reactive oxygen species in kidneys. J Urol 2015;193:1684-1691.

41 Shi S, Kanasaki K, Koya D: Linagliptin but not Sitagliptin inhibited transforming growth factor-β2-induced endothelial DPP-4 activity and the endothelial-mesenchymal transition. Biochem Biophys Res Commun 2016;471:184-190.

42 Nabeno M, Akahoshi F, Kishida H, Miyaguchi I, Tanaka Y, Ishii S, Kadowaki T: A comparative study of the binding modes of recently launched dipeptidyl peptidase IV inhibitors in the active site. Biochem Biophys Res Commun 2013;434:191-196.

43 Lee DL, Sturgis LC, Labazi H, Osborne JB Jr, Fleming C, Pollock JS, Manhiani M, Imig JD, Brands MW: Angiotensin II hypertension is attenuated in interleukin-6 knockout mice. Am J Physiol Heart Circ Physiol 2006;290:H935-H940.

44 Crosswhite P, Sun Z: Ribonucleic acid interference knockdown of interleukin 6 attenuates cold-induced hypertension. Hypertension 2010:55:1484-1491.

45 Hashmat S, Rudemiller N, Lund H, Abais-Battad JM, Van Why S, Mattson DL: Interleukin-6 inhibition attenuates hypertension and associated renal damage in Dahl salt-sensitive rats. Am J Physiol Renal Physiol 2016;311:F555-F561.

46 Zhang W, Wang W, Yu H, Zhang Y, Dai Y, Ning C, Tao L, Sun H, Kellems RE, Blackburn MR, Xia Y: Interleukin 6 underlies angiotensin II-induced hypertension and chronic renal damage. Hypertension 2012;59:136-144.

47 Cha DR: Interleukin-6 signaling in podocyte hypertrophy. Kidney Res Clin Pract 2016;35:195-196.

48 Aroor AR, Sowers JR, Bender SB, Nistala R, Garro M, Mugerfeld I, Hayden MR, Johnson MS, Salam M, Whaley-Connell A, Demarco VG: Dipeptidylpeptidase inhibition is associated with improvement in blood pressure and diastolic function in insulin-resistant male Zucker obese rats. Endocrinology 2013;154:2501-2513.

49 Aroor AR, Sowers JR, Jia G, DeMarco VG: Pleiotropic Effects of the dipeptidylpeptidase-4 inhibitors on the cardiovascular system. Am J Physiol Heart Circ Physiol 2014;307:H477-H492.