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Original Article

The involvement of lectin-like oxidized low density lipoprotein receptor-1 and p38MAPK in early diabetic nephropathy

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Abstract: Backgrounds: The present study aimed to investigate the effects of Lectin-like Oxidized Low Density lipoprotein receptor-1 (LOX-1) as well as the interaction of LOX-1 and p38MAPK pathway in early diabetic nephropathy (DN). Methods: 30 male rats were divided into 3 groups: normal control (NC), diabetes mellitus (DM) and SB203580 (p38MAPK inhibitor) treatment (SB203580) groups. Diabetic rats were induced by Streptozotocin-injection. After the onset of diabetes, rats in SB203580 group were administrated by SB203580. Six weeks afterwards, blood glucose (BG) and serum oxLDL was evaluated. Renal function markers such as serum creatinine (sCr), blood urea nitrogen (BUN), creatinine clearance rate (cCr) and urinary albumin excretion rate (UAER) were measured. Meantime, renal glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) were examined as parameters of oxidative stress. The levels of phosphorylated-p38 MAPK (p-p38MAPK) in renal cortex were evaluated by western blot and immunohistochemistry, the renal expression levels of LOX-1 protein and mRNA also were detected by western blot or real-time PCR. Results: Compared to NC group, the levels of BG, oxLDL, BUN, sCr, cCr and UAER were increased in DM group, while the activities of renal GSH-Px and SOD were significantly decreased. Meanwhile, LOX-1 expression- and p-p38MAPK levels were also upregulated in diabetic rats. However, the inactivation of p38 MAPK alleviated the upregulation of LOX-1 expression induced by diabetes. Meantime, all the parameters to evaluate renal injury were significantly attenuated by the administration of SB203580. Conclusions: The upregulation of LOX-1 by p38MAPK are important to the pathogenesis of DN.

Keywords: Diabetic nephropathy, p38MAPK, lectin-like oxidized low density lipoprotein, oxidative stress, SB203580

Introduction

Microvascular complications, including diabetic nephropathy (DN) are the major cause of fatalities in diabetes. In diabetic kidney, many pathways such as glycolysis, specific defects in the polyol pathway, uncoupling of nitric oxide synthase, xanthine oxidase, NAD(P)H oxidase, and advanced glycation can generate reactive oxygen species (ROS) [1]. The production of oxidants or ROS exceeding local antioxidant capacity results in oxidative stress, which plays a key role in the development of DN [2].

Lectin-like oxidized low density lipoprotein receptor (LOX-1) was first discovered in endothelial cell, and its expression could be upregulated by oxLDL, AGEs and high glucose [3]. Binding of LOX-1 and oxLDL induces the generation of superoxide anion via increasing the activity of NADPH oxidase, results in the quick increase of intracellular ROS [4], while ROS generation can upregulate LOX-1 expression, and a feedback cycle exists between LOX-1 and ROS [5]. Furthermore, series of approaches could attenuate oxidative stress by inhibiting the expression of LOX-1 [6, 7]. In diabetes, LOX-1 has been implicated to play a critical role in development of diabetic nephropathy [8], neuropathy [9] and cardiovascular complications [10].

Motigen-activated protein kinases (MAPKs) are major intracellular signal transduction factors, in which, p38MAPK is proved to be activated by the hexosamine pathway, increased products of glycation reactions, and oxidative stress in-

duced by hyperglycemia in diabetic kidney [11, 12]. Moreover, p38MAPK could activate nuclear factor-κB (NF-κB), which regulates the gene expression of various cytokines and adhesion molecule, subsequently induce the kidney injury [13].

Therefore, we assumed that in diabetic kidney, the overexpression of LOX-1 could induce the early DN caused by oxidative stress via activating p38MAPK pathway. In present study, we investigated the expression of LOX-1 and p38MAPK activity in renal cortex of STZ-induced diabetic rats in early stage with the blockage of a p38MAPK inhibitor, SB203580, and further elucidated the involvement of LOX-1 and p38MAPK pathway in early DN.

Materials and methods

Animal model

Six-week-old male Wistar rats (160-180 g) were obtained from the Experimental Animal Center of Shandong University. Diabetes was induced by a single intraperitoneal injection of STZ (60 mg/kg, Sigma, USA). The normal control rats (NC group, n = 10) were injected with an equal volume of vehicle. After 72 hours following injection, animals with blood glucose higher than 16.7 mmol/L were considered as diabetic rats. Then, randomly divided diabetic rats into two groups: diabetes mellitus rats (DM group, n = 10) and SB203580-treatment diabetic rats (SB203580 group, n = 10, 5 mg/kg/day i.p.). All rats were received humane care in compliance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH publication 86-23, revised 1986). They were maintained on a 12 h light/dark cycle and kept for six weeks with free access to food and water. All protocols were in accordance with the institutional guidelines for animal research.

Metabolic parameters and tissue collection

At the end of six weeks, all rats were kept individually in metabolic cages to collect 24-hour urine for the measurement of urine creatinine (Ucr) and urinary albumin excretion rate (UAER). Then, after fasting overnight, all rats were anesthetized with 3% butylenes (50 mg/kg, i.p.) and sacrificed. Blood samples were collected from the inferior vena cava to detect blood glucose (BG), serum creatinine (sCr), and blood urea nitrogen (BUN). Both kidneys were quickly removed and rinsed with cold isotonic saline and then weighed. An index of renal hypertrophy was estimated by comparing the wet weight of the left kidney to the body weight. Thereafter, the fresh kidney cortices were dissected. Some of them were placed into liquid nitrogen and stored at -70°C for assay of tissue oxidative stress parameters, western blot and real-time PCR. The others were fixed in 10% neutralized formalin and embedded in paraffin for morphological and immunohistochemical analysis.

Measurements of blood glucose and renal function

The levels of sCr and BUN were measured by 7170-A Olympus Biochemistry Test (Olympus Co., Japan). The levels of BG were evaluated by blood glucose meter One-Touch II (Johnson & Johnson, USA). Ccr was used as an index of glomerular filtration rate. UAER was determined by the method of radio-immunity (kit from beifang immunoreagent institute, China). Ccr was calculated with Scr, Ucr and 24-hour urine volume, and subsequently adjusted with body weight.

Assay of oxidative stress parameters

Oxidative stress was evaluated by examining levels of renal superoxide dismutase (SOD) and activities of antioxidant enzyme glutathione peroxidase (GSH-Px). SOD and GSH-PX were measured by commercially available kits according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The data on activities of renal SOD and GSH-Px were both expressed as U per milligram protein (U/mg prot). The protein content was estimated by the dye binding assay of Bradford assay (Bio-Rad, Hercules, California, USA), with BSA used as a standard.

Western blot analysis of LOX-1 and p-p38MAPK protein expression in renal cortices

In a 1 mL solution containing 10 mmol/L HEPES (pH 7.6), 10 mmol/L KCl, 1.5 mmol/L $Mgcl_2$, 0.5% NP-40, 1 mmol/L DTT, and 0.5 mmol/L PMSF, 50 mg freshly frozen kidney samples were homogenized and lysed for 10 minutes on ice in a 1 mL solution containing 10 mmol/L hepes (PH 7.6), 10 mmol/L KCl, 1.5 mmol/L $Mgcl_2$, 0.5% NP-40, 1 mmol/L DTT, and 0.5 mmol/L PMSF. Then, 50 ug of protein were resolved under reducing conditions on 10%

Table 1. Primers used in real-time PCR

Primers	Size (bp)	Sense/antisense
LOX-1	489	5'-AAAAAGTCGGGAGAATTGCCTATC-3'/5'-CCGGGTTTTTGCTTCTGGTCTT-3'
ß-actin	218	5'-CCTCTATGCCAACACAGTGC-3'/5'-GTCCTCCTGCTTGCTGATCC-3'

Table 2. Metabolic parameters and renal function in three groups (mean ± s, n = 10)

Group	BG (mmolg/L)	BW (g)	Left KW/BW (‰)	sCr (μmol/l)	BUN (mmol/L)	cCr (ml/min • kg)	UAER (mg/24 h)
NC	6.00±0.85	325±28	3.42±0.43	32.05±4.32	6.49±1.39	3.78 ±0.69	0.28±0.13
DM	24.14±3.86**	228±17**	6.58±0.85**	44.74±5.28**	17.05±2.38**	5.86±1.23**	2.45±0.92**
SB203580	21.38±2.04**	236±22**	5.01±0.48**,#	39.56±3.86*,#	12.43±1.08**,#	4.22±0.79*,#	1.42±0.18**,##

Note: *P < 0.05, **P < 0.01 vs. NC group; #P < 0.05, ##P < 0.01 vs. DM group.

Table 3. The paramaters of oxidative stress in three groups (mean ± s, n = 10)

Group	SOD (U/mg pr)	GSH-Px (U/mg pr)	OxLDL (μg/dl)
NC	14.06±2.36	26.89±2.36	2.65±0.42
DM	6.56±0.98**	13.65±1.68**	4.86±1.02**
SB203580	11.72±1.47*,##	19.63±1.62**,##	4.19±0.45**

Note: *P < 0.05, **P < 0.01 vs. NC group; ##P < 0.01 vs. DM group.

separation gels with a 6% stacking SDS-polyacrylamide gel. After transferring onto nitrocellulose membrane, the membranes were hybridized with polyclonal anti-LOX-1/anti-p38MAPK/anti-phospho-p38MAPK antibody (Santa Cruz, USA; diluted 1:500), subsequently incubated with horseradish peroxidase-conjugated secondary antibody (Zhongshan Biotechnology Co, Ltd., China; diluted 1:1000), and developed by chemiluminescence. The membranes were then exposed to x-ray film and subsequently developed immunoreactive bands. Housekeeping protein ß-actin was used as a loading control. Densitometry was performed with gel imaging system (Alphaimager 2200, Pharmacia Biotech Co., USA), and densitometric units were measured as the ratio to ß-actin protein.

Immunohistochemical analysis of p-p38MAPK protein expression in renal cortices

4 μm-thick paraffin slices were roasted, deparaffinized and antigen repaired, and then incubated in 0.3% H_2O_2 to remove endogenous peroxidase activity and 5% BSA in PBS to blocked non-specific staining. Subsequently, the slices were incubated with p-p38MAPK antibody to rats (Santa Cruz, USA; diluted 1:200) overnight at 4°C and biotinylation IgG for 1 h at 37°C, and then incubated with peroxidase-conjugated streptavidin for 30 min at 37°C and developed with 3.3-diaminobenzidine to produce a brown color. Sections were then counterstained with hematoxylin and examined under a light microscope. Four serial sections were chosen from each experimental rat and observed randomly four views under a 400-fold microscope. The average of the integral optical density (IOD) of each view was detected by an Image-Pro plus 6.0 analytical systems.

Real-time PCR detection of LOX-1 mRNA in renal cortices

Total RNA of renal cortices was isolated using Trizol (Invitrogen, USA) and reversed transcribed to cDNA using a reverse transcriptase kit (Takara, Dalian, China). Quantitative PCR was performed using ABI 7500 system (Applied Biosystems, USA) by a SYBR green kit (Takara, Dalian, China). The primers are in **Table 1**.

Statistical analysis

All data are expressed as mean ± SEM and analyzed using statistical package SPSS 11.0 for Windows. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test was used to determine statistically significant differences among the three groups. P < 0.05 was considered statistically significant.

Results

The changes of renal function and hypertrophy index in diabetic rats were alleviated by SB203580

In DM group, body weight (BW) was significantly lower than those in NC group, and blood glucose (BG) were markedly higher than that of normal rats (P < 0.01). Compared with NC group, renal hypertrophy index (the left KW/BW) and renal function parameters (Scr, cCr,

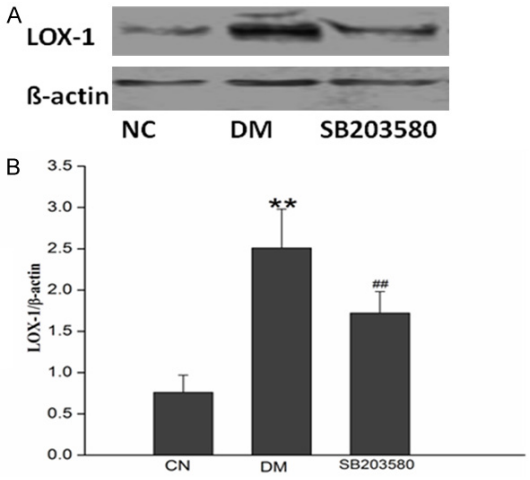

Figure 1. Expression of LOX-1 protein detected by western blot analysis in three groups. A. Representative images of western blotting showing expression of LOX-1 in each group. B. Quantitative analysis of LOX-1. $**P < 0.01$ vs. CN; $##P < 0.01$ vs. DM.

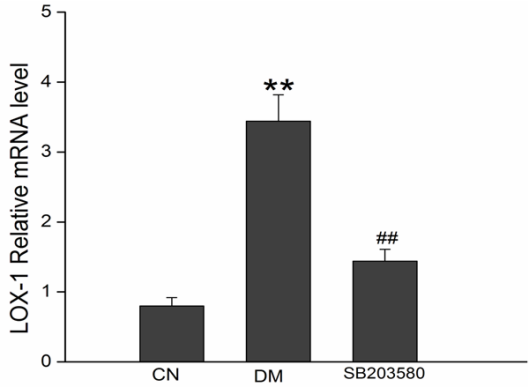

Figure 2. The mRNA levels of LOX-1 in renal cortex of each group were measured by Real-time PCR. β-actin was used as an internal control. $**P < 0.01$ vs. control. $##P < 0.01$ vs. DM.

BUN and UAER) were significantly increased in DM group ($P < 0.01$). Whereas, all these renal parameters were significantly decreased by SB203580 treatment ($P < 0.05$ vs. DM). But there were no significant difference in BW and BG between SB203580 and DM group (**Table 2**).

Oxidative stress in diabetic rats was was mitigated by SB203580

The renal SOD and GSH-Px activities were significantly reduced in diabetic rats, compared to NC group ($P < 0.01$); in SB203580 group, the activities of SOD and GSH-Px were restored largely. The level of serum oxLDL was significantly higher in all diabetic rats compared with normal rats, while in SB203580 group, the level of oxLDL was similar to that in DM group (**Table 3**).

LOX-1 expressions in renal cortices were downregulated by SB203580

Western blot analysis and RT-PCR were employed to assess the expression levels of LOX-1 protein or mRNA in renal cortices. Markedly upregualtion of LOX-1 immunoreactive peptide was present in DM group, the levels of LOX-1 protein were all markedly upregulated in diabetic rats ($P < 0.01$ vs. NC), while this overexpression of LOX-1 protein were significantly decreased by SB203580 treatment ($P < 0.05$ vs. DM) (**Figure 1**). The renal mRNA levels of LOX-1 were presented in **Figure 2**. The mRNA level of LOX-1 in DM group was 4.3 times higher than that of in control group. While after SB203580 treatment for 6 weeks, the mRNA expression of LOX-1 was significantly downregulated to about 42% of that in DM group, all these differences have statistical significance ($P < 0.01$).

Positive expression of p-p38MAPK immunoreactivity in renal cortices was inhibited by SB203580

The immunostaining showed that p-p38MAPK was predominantly localized in mesangial area, endothelial cells and the overlying epithelial cells as well as the proximal and distal renal tubular epithelial cells with staining nucleus. In NC group, only a few positive expression of p-p38MAPK immunoreactivity could be seen, while the p-p38MAPK immunoreactivity was significantly increased in diabetic rats ($P < 0.01$ vs. NC). While the p-p38MAPK positive cells were markedly decreased by SB203580 treatment in diabetic rats ($P < 0.01$ vs. DM) (**Figure 3**).

Renal expression level of p-p38MAPK protein was decreased by SB203580

Densitometric analysis of p-p38MAPK and p38MAPK was detected by western blot. The phosphorylation of p38MAPK means the activation of p38MAPK signal. The result showed significant upregualtion of p-p38MAPK immunoreactive peptide was existed in DM group,

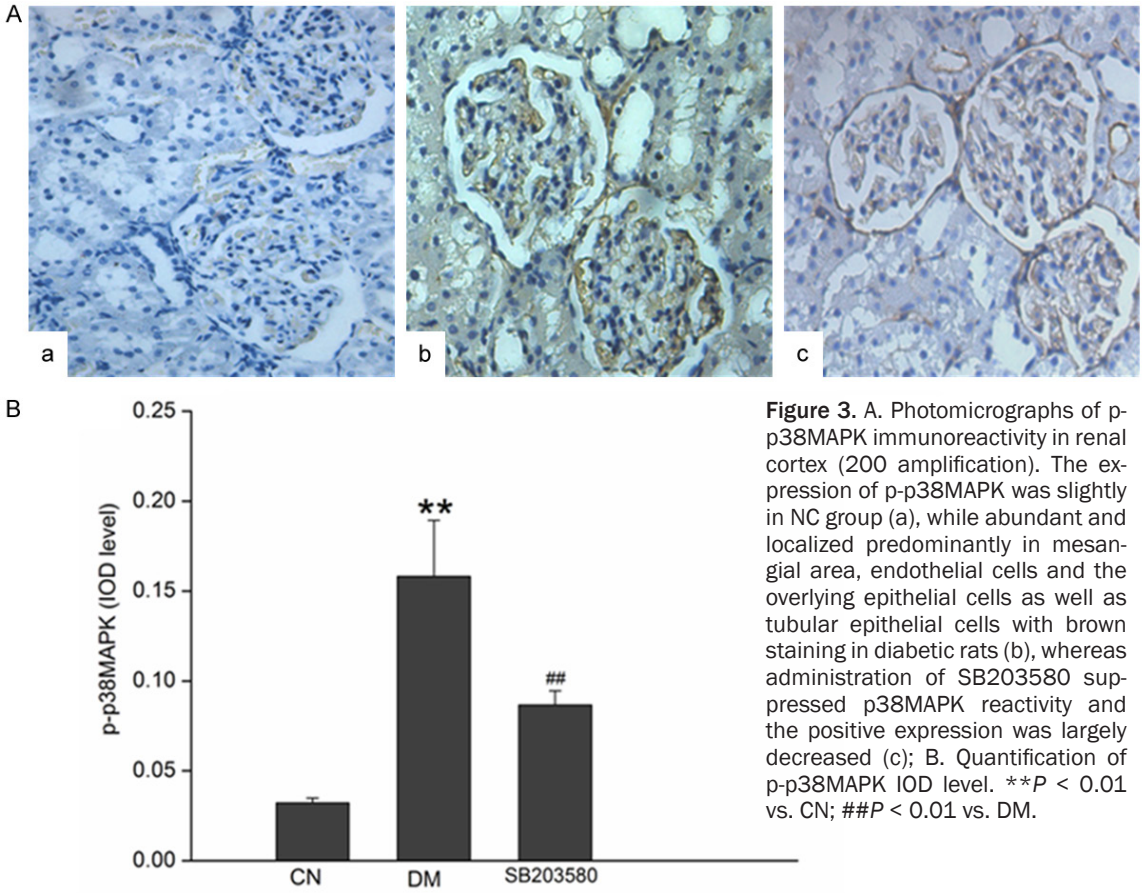

Figure 3. A. Photomicrographs of p-p38MAPK immunoreactivity in renal cortex (200 amplification). The expression of p-p38MAPK was slightly in NC group (a), while abundant and localized predominantly in mesangial area, endothelial cells and the overlying epithelial cells as well as tubular epithelial cells with brown staining in diabetic rats (b), whereas administration of SB203580 suppressed p38MAPK reactivity and the positive expression was largely decreased (c); B. Quantification of p-p38MAPK IOD level. $**P < 0.01$ vs. CN; $##P < 0.01$ vs. DM.

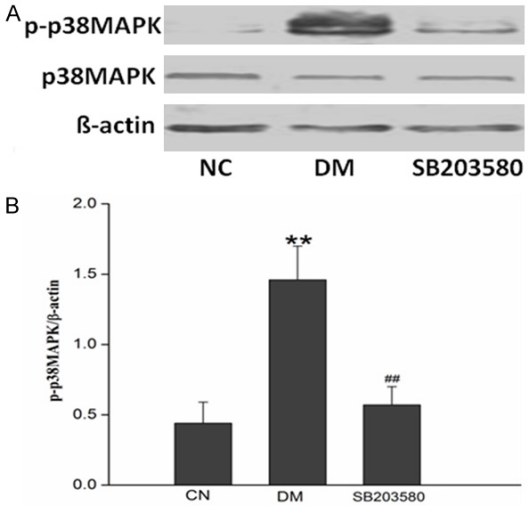

Figure 4. Expression of p-p38MAPK protein detected by western blot in three groups. A. Representative images of western blotting showing expression of LOX-1 and p38MAPK activation in every group. B. Quantitative analysis of LOX-1 and p-p38MAPK. $**P < 0.01$ vs. CN; $##P < 0.01$ vs. DM.

while not p38MAPK. The p-p38MAPK protein level was in DM group was 3.3 times higher than that of in CN group, while the protein expression of p-p38MAPK was largely down-regulated to 39% of that in DM group (**Figure 4**).

Discussion

In the present study, with STZ-induced diabetic rats and the *in vivo* administration of p38MAPK specific inhibitor SB203580 we investigated the importance of LOX-1 and p38MAPK pathway in the early renal injury induced by type 1 diabetes. We found that LOX-1 expression and p38MAPK activity were increased, coinciding with the early renal injury in diabetes which was marked by the increased levels of BUN, Scr, cCr, UAER and enhanced index of renal hypertrophy. The excessive oxidative stress also occurred in diabetic rats, which was indicated by decreased activities of SOD and GSH-Px in renal cortex as well as the increased levels of serum oxLDL. With the *in vivo* administration of p38MAPK specific inhibitor SB203580, we observed that accompanied with inhibition of p38MAPK activity, the renal injury and the excessive oxidative stress in diabetes was significantly alleviated as well as the expression of

LOX-1 was partially inhibited. These results indicate that LOX-1 and p38MAPK plays an essential role in early renal injury induced by type 1 diabetes, p38MAPK signal may be involved in the early kidney injury induced by oxidative stress of diabetes.

Oxidative stress induced by hyperglycemia, participated in the development and progression of diabetic nephropathy [14]. Oxidative stress could mediate p38MAPK activation in mesangial cells [15], and the activation of p38MAPK patyway is a feature of human and experimental diabetic nephropathy [16]. To adapt to the increased oxidative stress, cell has an antioxidative enzyme defense system, including a variety of enzymes such as SOD, GSH-PX, catalase, thioredoxin and glutaredoxin. These enzymes could accompany with non-enzymatic antioxidant defense systems to protect cells from the damages caused by oxidative stress. The susceptibility of LDLs to oxidation is increased in type 1 and type 2 diabetes mellitus [17], and increased plasma oxLDL levels were correlated with the incidence of diabetic nephropathy [18].

In the present study, elevated expression of oxLDL and decreased levels of SOD and GSH-Px in renal cortex were found at six weeks after onset of diabetes. These indicated the imbalance between the production of free radicals and the antioxidant defense system induced by hyperglycemia in early diabetes, which were in agreement with previous reports [19, 20]. As previous studies reported [16, 21], we also found that the activities of p38MAPK increased in renal cortex of diabetic rats, coincided with the enhanced oxidative stress. P38 is a serine-threonine kinase which is activated by phosphorylation and mediates many cellular responses under a variety of chemical and physical insults [22]. It could transmit signals from cell membrane receptors to the nucleus, resulting in activation of many signals such as nuclear factor-kappaB (NF-κB), which can further upregulate the expression of E- and P-selectin, as well as the intracellular and vascular cell adhesion molecules (ICAM-1 and VCAM-1), respectively [23]. While the activation of these selectin and adhesion molecules was an expected event in diabetic rats with severe capillary vasculopathy [24]. P38MAPK plays an important role in the development of diabetic nephropathy also via its downstream signaling events. It may mediate angiotensinogen gene expression via reactive oxygen species generated by high glucose in kidney proximal tubular cells [25]. It is also involved in the apoptosis of mesangial cell and podocyte [26]. cAMP response element-binding protein (CREB), a transcription factor, could be activated by p38MAPK and induced extracellular matrix (ECM) production in vivo [21], while the accumulation of ECM is an ultrastructural hallmark of diabetic nephropathy. Increased urinary albumin excretion is the first manifestation of diabetic nephropathy in human. In our study, we found the increased levels of serum BUN, Scr, cCr and UAER in diabetic rats. Meanwhile, significantly elevated activity of p38MAPK and oxidative stress were also assessed. These results suggested that p38MAPK plays an important role in the development of oxidative stress induced diabetic nephropathy.

OxLDL can mediate endothelial dysfunction, which plays a critical role in the pathogenesis of diabetic vasculopathies. The serum levels of oxLDL were higher in patients with diabetic nephropathy than in patients without diabetic nephropathy [27], indicated that oxLDL plays an important role in the development of diabetic nephropathy. In these patients, the high susceptibility of LDL to oxidative modification is related to the excessive oxidative stress commonly presenting in diabetes. As a major receptor of oxLDL, the up-regulated expression of LOX-1 has been found in renal damages induced by hypertension, ischemia-reperfusion, and hypercholesterolemia [28, 29], which are all intimately associated with increased oxidative stress. Recently, Yamamoto N reported the LOX-1 expression in the tubulointerstitial area, suggested that LOX-1 likely plays an important role in human diabetic nephropathy [30]. LOX-1 has also been shown to activate the NADPH oxidase and subsequent redox signals involving MAPKs and NF-κB in human endothelial cells [31].

In the present study, we found the elevated level of serum oxLDL in diabetic rats. Meanwhile, we observed the up-regulated expression of LOX-1 protein in renal cortex, and simultaneously p38MAPK activity in renal cortex was increased. Inhibition of p38MAPK could down-regulate the expression of LOX-1 protein and gene. Moreover, the renal damage of diabetic

rats was alleviated. Lectin-like oxidized LDL receptor-1 could activate many signaling pathways such as protein kinase C (PKC), and resulted in the induction of several downstream pathways including p38MAPK, p42/44MAPK, NF-κB and activating protein-1 (AP-1) [32, 33]. In return, these induced inflammatory signal mediators up-regulate LOX-1 expression and promote its activity [34]. These could suggest the existence of a feedback between LOX-1 and p38MAPK, which is a possible explanation that expression of LOX-1 was also significantly downregulated with the inhibition of p38MAPK by SB203580 in our study.

In conclusion, the present study demonstrated that renal cortical p38MAPK activity and LOX-1 expression were increased at early stage of diabetic nephropathy. Ox-LDL/LOX-1 system and activation of p38MAPK might be involved in the early DN induced by oxidative stress. Therefore, oxLDL/LOX-1 system might play an important role in the progenesis of DN via activating p38MAPK signaling pathway. Inhibition of p38MAPK activity may be a potential treatment against early DN.

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Disclosure of conflict of interest

None.

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