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Attenuation of diabetic nephropathy in diabetes rats induced by streptozotocin by regulating the endoplasmic reticulum stress inflammatory response

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Abstract

The endoplasmic reticulum (ER) is capable of sensing metabolic and stress parameters and integrating intra- and extracellular signals to support a coordinated cell response. In the present study, we verified the hypothesis that 4-phenylbutyric acid (4-PBA), a chemical chaperone, prevented the progression of diabetic nephropathy (DN). Male Sprague-Dawley rats were randomly divided into 3 groups: a normal control group, a DN group, and a DN model plus 4-PBA treatment group (PBA). The DN model was induced by injection of streptozotocin with uninephrectomy. The dosage of 4-PBA treatment was gavaged at a dose of 1 g/kg body weight each day for 12 weeks. The expression of the ER stress indicators significantly increased in the kidney of DN rats within the indicated period. Moreover, the expression of phosphorylated c-JUN NH_2-terminal kinase, the monocyte chemoattractant protein–1, and the final fibrotic effector all elevated markedly in the kidney of DN rats. Urinary protein excretion rate and the concentration of urinary monocyte chemoattractant protein–1 were higher than those in the normal control group. Treatment with 4-PBA can suppress the expression of the glucose-regulated protein 78 and the phosphorylation of the PKR-like ER kinase, both of which are ER stress indicators; renoinflammatory signal; and the expression of inflammatory cytokines and fibrosis factors. It also can inhibit the increase in urinary protein excretion rate and urinary monocyte chemoattractant protein–1. In conclusion, 4-PBA exerts a marked renoprotective effect possibly due to modulating ER stress and related inflammatory cascade.

1. Introduction

Diabetic nephropathy (DN) has become the principal cause of end-stage renal disease in the Western world and a major cause of morbidity and mortality from diabetic complications [1,2]. Previous studies have suggested the critical importance of metabolic and hemodynamic factors in the progression of renal lesions in patients with DN. In the last few years, much research has focused on the role of low-grade inflammatory processes in DN [3–5]. Thus, exploring the activation mechanism of aseptic inflammation in the kidney of patients with diabetes is very important for developing new strategies to suppress the development of DN.

Recently, it has become clear that the endoplasmic reticulum (ER) is a key player in cellular stress responses, including the cell's response to oxidative stress, metabolic stress, etc [5,6]. On the one hand, cells may restore its intracellular homeostasis by enhancing the defense mechanism such as unfold protein response through ER stress; on the other hand, if the byproducts of stress exceeds the processing capacity of the cells, then the cells will initiate some other pathologic reaction such as apoptosis or inflammation. Therefore, ER stress could be a double-edged sword. Kubota et al [7], Qi et al [8], and Ozcan et al [9] first proved that ER stress plays an important role in the pathogenesis of diabetes and that hyperglycemia will be alleviated by treatment with 4-phenylbutyrate acid (4-PBA), a chemical chaperone, through inhibition of inflammatory signal. We hypothesize that ER stress also plays a pivotal role in the mechanism of DN.

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The aim of the present study was to test the hypothesis that 4-PBA prevents renal damage by restoring ER homeostasis and inhibition of the inflammatory process. The results demonstrated that 4-PBA can suppress the activation of ER stress and expression of inflammatory cytokine, and exerts a marked renoprotective effect.

2. Methods

2.1. Experimental animal

Male Sprague-Dawley rats (initial weight, 200-220 g; average age, 8 weeks; Third Military Medical University Animal Experiment Center, Chongqing, People's Republic of China) were randomly divided into 2 groups: normal control rats (NC, n = 18) and DN rats (n = 54). Diabetes was induced by injection intraperitoneally with streptozotocin (STZ, 40 mg/ kg body weight) dissolved in citrate buffer (pH 4.5, 0.1 mol/L). Blood glucose (Glu) levels were determined at 3 and 7 days after STZ injection, and only 36 rats with blood Glu concentrations of at least 16.7 mmol/L were used. After 4 weeks, diabetic rats were subjected to right nephrectomy to hasten the development of the kidney disease [10,11]. Two weeks later, the microalbumin was examined by urinary automatic analyzer; and the DN model was established. Thirty-six DN rats were once again randomly divided into 2 subgroups: the DN with isotonic sodium chloride solution treatment group (DN, n = 18) and the DN with 4-PBA treatment group (PBA, 1 g/kg body weight each day, by gavage beginning on the day the DN model was established, n = 18) [9]. The experiments were started (week 0). Rats of the NC group (n = 18) received normal chow, whereas rats from the DN and 4-PBA group received high-sugar, high-fat chow [12–14]. All rats had free access to standard chow and tap water. All experimental procedures were carried out in accordance with the recommendations of the Care and Use Committee of the Third Military Medical University. Rats were killed at weeks 4, 8, and 12; and the left kidneys were harvested and weighed. The cortices were divided into 3 pieces, with one piece fixed in 10% formalin for periodic acid-Schiff (PAS) staining. The remaining cortices were frozen in liquid nitrogen and stored at −80°C.

2.2. Biochemical analysis

Blood samples were drawn from the heart and were centrifuged at 3500 rpm for 5 minutes. After proper dilution, the supernatant was used for the determination of concentrations of blood Glu, triglyceride (TG), total cholesterol (TC), and plasma creatinine (Cr) using an automatic biochemistry analyzer (NanJingJianCheng, Nanjing, China; Hitachi 7150, Tokyo, Japan).

2.3. Urinary metabolites

The 24-hour urine samples were collected in metabolic cages at the end of weeks 4, 8, and 12. After the total urine volume (UV) was determined, urine samples were stored at −20°C. The urinary protein was measured using the Coomassie blue method. urinary protein excretion rate (UPER) was calculated by the following formula: 24-hour total volume of urine (in liters) × urinary protein levels (in milligrams per liter). Urine monocyte chemoattractant protein–1 (MCP-1) levels were measured by quantitative sandwich enzyme-linked immunosorbent assay (ELISA) using a commercial kit according to the manufacturer's instructions (Biosource, Camarillo, CA). The assay was performed in duplicate, and the intensity of the color was measured in an ELISA reader at 450 nm.

2.4. Renal morphologic analysis

Cortical tissue for light microscopy was fixed in 10% formalin and embedded in paraffin. Sections were 4 μm thick and were processed for PAS.

In each rat, quantitative analysis of glomerular area (GA) and fractional mesangial area (FMA) in glomeruli stained with PAS reagent was performed as described previously [15]. In each slice, 30 glomeruli were randomly selected. The *GA*, defined as the cross-sectional area containing the vascular pole and the urine pole, and the mean areas were measured and calculated. The FMA was expressed as the percentage of the GA. Morphologic analyses were performed by an experienced pathologist who was blinded to the source of the tissues.

2.5. Immunohistochemistry

Immunohistochemistry for the determination of GRP78 and MCP-1 was performed using the streptavidin-biotin-peroxidase complex method [16]. Briefly, paraffin-embedded renal tissues were deparaffinized in xylene and rehydrated in graded ethanols. Endogenous peroxidase activity in the tissues sections was inactivated with 0.1% hydrogen peroxide. After the sections were blocked with bovine serum for 30 minutes, the primary antibodies that were used included rabbit anti-GRP78 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) and goat anti–MCP-1 (1:200; Santa Cruz Biotechnology). After washing in phosphate-buffered saline, sections were incubated with biotinylated second antibodies for 1 hour followed by an avidin-biotin-peroxidase complex (1:200; Vector, Burlingame, CA) and developed with 3,3-diaminobenzidine. For control, normal immunoglobulin G was used. Finally, the sections were counterstained with hematoxylin for 30 seconds. Microscopic observations were performed under ×200 magnification.

2.6. Quantitative real-time polymerase chain reaction analysis

Total RNA was isolated from the renal cortex using TRIzol extraction (Invitrogen Life Technologies, Shanghai, China) and reverse-transcribed to complementary DNA using ReverTra AceTM (TOYOBO, Osaka, Japan). Quantitative real-time polymerase chain reaction (PCR) was performed with primer

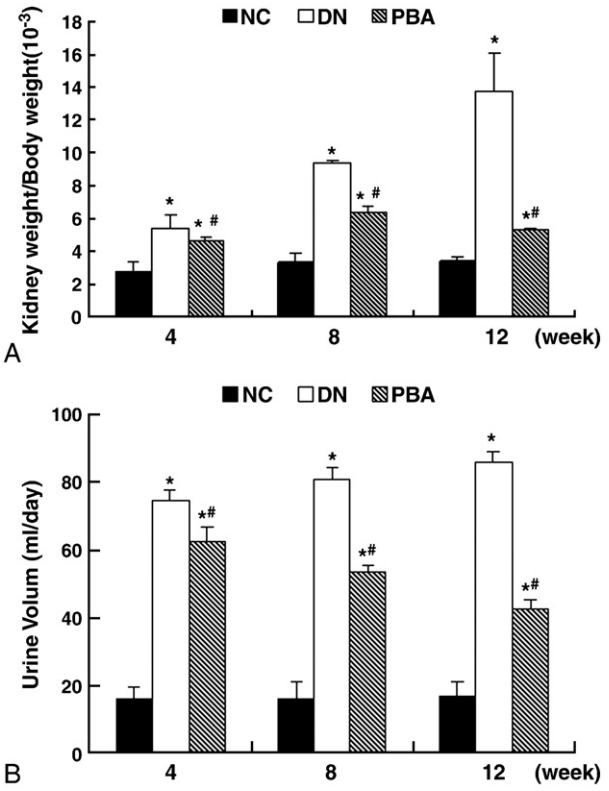

Fig. 1. Effects of 4-PBA on KI and UV in DN rats. A, Kidney weight and body weight were measured and calculated in all rats at the fourth, eighth, and 12th week of the study. B, Urine collections were obtained for measurement of UV in all rats by metabolic cages during the fourth, eighth, and 12th weeks of the study. Data are presented at means ± SE. *$P < .05$ compared with NC group; #$P < .05$ compared with DN group.

pairs and probes on a Rotor-gene 6000 (Corbett Life Science, Sydney, Australia). All samples were analyzed in triplicate, and ddH_2O served as a no-template control. The relative amount of messenger RNA (mRNA) was calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method [17]. The primer and probe sequences were as follows: (1) GRP78 (193 base pairs [bp]), 5′-AACGACCCCTGACAAAAGACAA-3′ and 5′-AGGCGG-TTTTGGTCATTGG-3′; (2) transforming growth factor (TGF)–β1 (236 bp), 5′-GGCTGCTGACCCCCACTGATAC-3′ and 5′-GGTGGCCATGAGGAGCAGGAA-3′; (3) MCP-1 (125 bp), 5′- CGCTTCTGGGCCTGTTGT- TCC-3′ and 5′-GCCGACTCATTGGGATCATC -3′; (4) type I collagen (379 bp), 5′-ACCT- TCCTGCGCCTGATGTC-3′ and 5′-CGGGAGGTCTTGGTGGTTTT-3′; and (5) GAPDH (159 bp), 5′-ACCCATCACCATCTTCCAGGAG-3′ and 5′-GAAGG -GGCGGAGATGATGAC-3′.

2.7. Western blot analysis

Tissue samples from the renal cortex were placed in a buffer containing 20 mmol/L Tris-HCL, pH 6.8, 1 mmol/L EDTA, 1% sodium dodecyl sulfate, 1 mmol/L phenylmethylsulfonyl fluoride, and 1× protease inhibitor cocktail. The protein was separated on 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes. The membranes were incubated with one of the following antibodies: rabbit anti–p-pancreatic ER kinase (PERK) (1:1000; Cell Signaling Technology, Boston, MA), mouse anti–p-JNK (1:200; Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti–p-insulin receptor substrate 1 (IRS-1) (1:200; Santa Cruz Biotechnology), goat anti–MCP-1 (1:200; Santa Cruz Biotechnology), and goat anti–type I collagen 1 (1: 200; Santa Cruz Biotechnology) as the primary antibodies. Horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G was used as the second antibody (1:1000; Sigma, St Louis, MO). All membranes were incubated with a monoclonal anti–β-actin antibody (1:2000; Novus, St. Louis, MO). Immunoreactive bands were visualized with the luminescence method (Western Blot Chemiluminescence Reagent Plus, NEN Life Science Products, Boston, MA) Western Blot Chemiluminescence Reagent Plus. The band density of each gene was normalized to the corresponding density of β-actin at 42 kd.

2.8. Statistical analysis

Data were compared among groups using 1-way analysis of variance, followed by the least significant difference tests. All statistical analyses were performed by the SPSS Statistical Software version 13. 0 (SPSS, Chicago, IL). All values are presented as means ± SE, and a value of $P < .05$ was considered statistically significant. Statistical methods are included in the tables and figures.

3. Results

3.1. Effect of 4-PBA administration on renal growth; 24-hour UV; UPER; and metabolism of Glu, Cr, TG, and TC

Compared with NC rats, the parameters of the kidney weight/body weight index (KI), Glu, TG, TC, UV, and Cr

Table 1
Metabolic parameters in the different groups

Index	Group	4 wk	8 wk	12 wk
Glu	NC (n = 6)	10.35 ± 0.73	10.17 ± 1.18	11.96 ± 1.07
(mmol/L)	DN (n = 6)	33.60 ± 1.68*	29.90 ± 1.78*	32.92 ± 1.59*
	4-PBA (n = 6)	30.64 ± 5.48*	23.79 ± 2.97†	17.42 ± 4.07†
TG	NC (n = 6)	0.63 ± 0.07	0.64 ± 0.10	0.65 ± 0.08
(mmol/L)	DN (n = 6)	2.02 ± 0.31*	2.54 ± 0.34*	2.50 ± 0.69*
	4-PBA (n = 6)	2.40 ± 0.24*	2.65 ± 0.34*	2.48 ± 0.56*
TC	NC (n = 6)	1.23 ± 0.18	1.24 ± 0.13	1.26 ± 0.08
(mmol/L)	DN (n = 6)	2.96 ± 0.57*	3.01 ± 1.05*	3.11 ± 0.60*
	4-PBA (n = 6)	2.95 ± 0.33*	3.07 ± 0.60*	3.12 ± 0.21*
Cr	NC (n = 6)	41.31 ± 3.62	42.05 ± 2.48	39.50 ± 2.66
(μmol/L)	DN (n = 6)	48.26 ± 5.96*	57.54 ± 4.13*	69.75 ± 7.49*
	4-PBA (n = 6)	47.81 ± 6.47*	58.89 ± 5.16*	66.98 ± 7.83*

Values are presented as means ± SE.
* $P < .05$ compared with NC group.
† $P < .05$ compared with DN group.

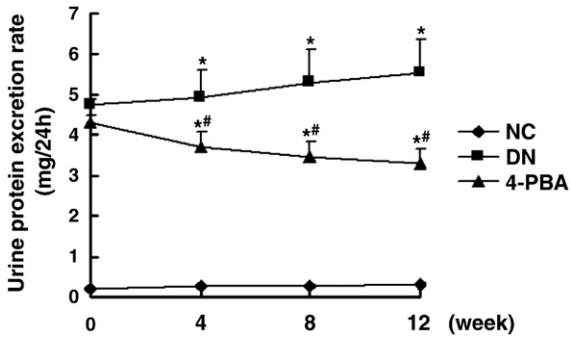

Fig. 2. Time course of changes in UPER. UPER increased progressively in the untreated DN group during the 12-week observation period following induction of diabetes. UPER was significantly reduced by treatment with 4-PBA during weeks 4, 8, and 12 when compared with the DN group. Data are presented as means ± SE. *$P < .05$ compared with NC group; $^{\#}P < .05$ compared with DN group.

were elevated significantly in DN rats (all Ps < .05) (Fig. 1A-B). The 4-PBA treatment suppressed the increase in KI and blood Glu but had no effect on the metabolism of TG, TC, and Cr ($P > .05$) (Table 1). In NC rats, the UPER was kept at a low level during the experimental period. However, the UPER increased progressively with time in DN rats and peaked at the 12th week of the observation period (Fig. 2), suggesting that 4-PBA could prevent urinary protein excretion. These results indicate that 4-PBA could markedly prevent the progression of DN.

3.2. Effect of 4-PBA administration on renal histopathologic changes

We found that typical glomerular damages appeared in the kidneys of DN rats, including mesangial cell proliferation, mesangial matrix accumulation and expansion, as well as the focal thickening of the glomerular basement membrane (Fig. 3B arrow). These changes are not seen in the glomeruli of DN rats treated with 4-PBA (Fig. 3C). Quantitative analysis for the percentage of PAS-positive area in the glomeruli and FMA using a computer image analysis system is summarized in Fig. 3 (D, E). The mean GA and FMA of DN rats were significantly higher than those of NC rats ($P < .05$). The GA and FMA were significantly lower in the 4-PBA group than in the DN group (Fig. 3D-E). These morphologic changes suggest that 4-PBA markedly attenuated the glomerular constructive damage.

3.3. Effects of 4-PBA administration on ER stress in kidneys of DN rats

GRP78, also known as *BIP*, has been used extensively as a key indicator for the induction of ER stress [18,19]. As described in Fig. 4A, the mRNA level of GRP78 was significantly elevated in the kidneys of DN rats, as compared with NC rats ($P < .05$). The phosphorylated PERK serves as a convenient marker for its activation status [20,21]. We examined the phosphorylation status of PERK to further confirm the presence of ER stress, which is a transmembrane

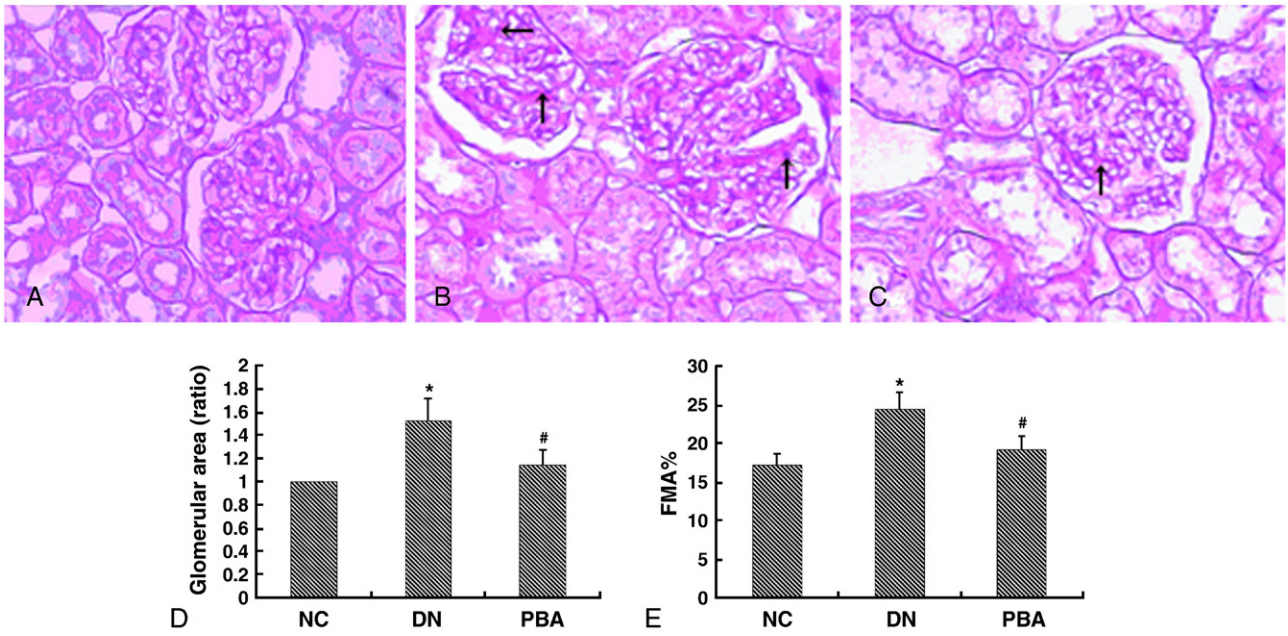

Fig. 3. Periodic acid-Schiff staining of kidney sections for pathologic changes. Original magnification ×200. Quantitative analysis in the kidney was performed using PAS staining. A, Normal control at the end of the study (12 weeks). B, Diabetic nephropathy rats at the end of the study (12 weeks). C, 4-Phenylbutyric acid–treated DN rats at the end of the study (12 weeks). D, Glomerular area (ratio NC group) in all rats. Glomerular area increased in the kidneys of DN rats in comparison to that of the NC group rats (*$P < .05$). Glomerular area decreased in the kidneys of 4-PBA rats in comparison to that of the DN group rats ($^{\#}P < .05$). E, Fractional mesangial area in all rats. Fractional mesangial area increased in the kidneys of DN rats compared with NC rats (*$P < .05$). The improvement in FMA in 4-PBA–treated DN rats was significant compared with that of DN rats ($^{\#}P < .05$). Values are means ± SE. *$P < .05$ compared with NC group; $^{\#}P < .05$ compared with DN group.

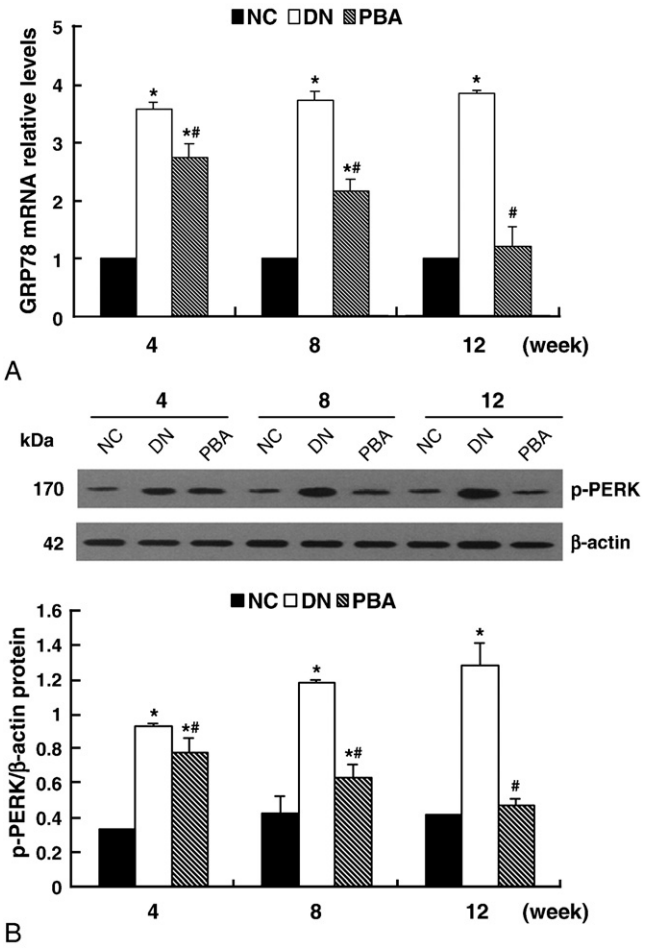

Fig. 4. Effect of 4-PBA on mRNA and protein expression as an indicator of ER stress in all rats. A, Representative GRP78 mRNA levels were measured by real-time PCR and corrected for GADPH mRNA from the 3 groups at 4, 8, and 12 weeks. B, Representative Western blot of p-PERK protein expression in all rats at 4, 8, and 12 weeks. Diabetic-induced ER stress was significantly reduced by treatment with 4-PBA. Values are means ± SE. *$P <$ .05 compared with NC group; #$P <$ .05 compared with DN group.

ER stress signal transducer, by Western blot analysis. Fig. 4B shows that p-PERK expression also increased significantly, compared with that of NC rats. The increases in GRP78 mRNA and p-PERK protein expression were significantly reversed by the 4-PBA treatment for the indicated time ($P <$ .05). When we evaluated the expression of GRP78 using immunohistochemistry, it was overexpressed in the nuclear region of glomerular and tubular cells in the DN group. On the other hand, weak expression of GRP78 was seen in the glomeruli of the 4-PBA group (Fig. 5A-C). These results suggest that 4-PBA can effectively alleviate ER stress in the kidneys of DN rats.

3.4. Effect of 4-PBA administration on JNK phosphorylation in kidneys of DN rats

JNK is a member of the mitogen-activated protein kinase family of protein kinases, which also includes ERK and p38 [22]. Because JNK phosphorylation level, not JNK, reflects the activation of the inflammatory signal in the liver and adipose tissue of patients with diabetes and is also associated with inflammatory injury and ER stress [23–25], we performed Western blot analysis to detect the expression of it in renal tissue in the experimental DN rats. The result in this study shows that p-JNK was significantly increased in the kidney of DN rats as compared with NC rats during the experimental period. The increased expression of p-JNK was prevented by 4-PBA treatment at the indicated time (Fig. 6), therefore indicating that regulation of ER stress with a molecular chaperone is an effective way to prevent JNK pathway-related inflammatory injury in the renal tissue of DN rats.

3.5. Effect of 4-PBA administration on inflammatory cytokines in kidneys of DN rats

Monocyte chemoattractant protein–1 plays an important role in the propagation of focal inflammation and macrophage infiltration [26]. We found that the mRNA and protein levels of MCP-1 were increased significantly in DN rats as compared with NC rats. Moreover, urinary MCP-1 concentration was also significantly higher in DN rats during the experimental period ($P <$ .05) (Fig. 7C). Furthermore, MCP-1 was overexpressed in the nuclear region of glomerular and tubular cells in the DN group. Diffuse low-level expression of MCP-1 was seen in the cytoplasmic region in the mesangial cells of the 4-PBA group (Fig. 5D-F). Therefore, these results effectively suggest that the degree of ER stress is closely associated with inflammation in kidneys of DN rats. These results reflect the increased renal inflammatory response in the kidney of DN rats. Meanwhile, we found in this study that 4-PBA treatment could almost reverse the increase in MCP-1 expression and urine secretion, suggesting that 4-PBA may reduce the high Glu-induced inflammatory activation of mesangial cells ($P <$ .05) (Fig. 7A-B).

3.6. Effect of 4-PBA administration on profibrotic index in kidneys of DN rats

There was an increase in mRNA expression of TGF-β1 and type I collagen in the diabetic kidney, compared with NC rats, as assessed by reverse transcriptase PCR (Fig. 8A-B). The increase in protein expression of type I collagen by Western blot analysis was similar to the increase in mRNA expression (Fig. 8C). Attenuated TGF-β1 and type I collagen mRNA was found in the kidneys of DN rats treated with 4-PBA. Administration of 4-PBA diminished the protein and mRNA expression of TGF-β1 and type I collagen over time ($P <$ .05) (Fig. 8A-C). Together with the morphologic improvement, these results provide direct evidence that regulation of ER stress by 4-PBA could ameliorate renal fibrosis in DN through suppression of profibrotic cytokine expression.

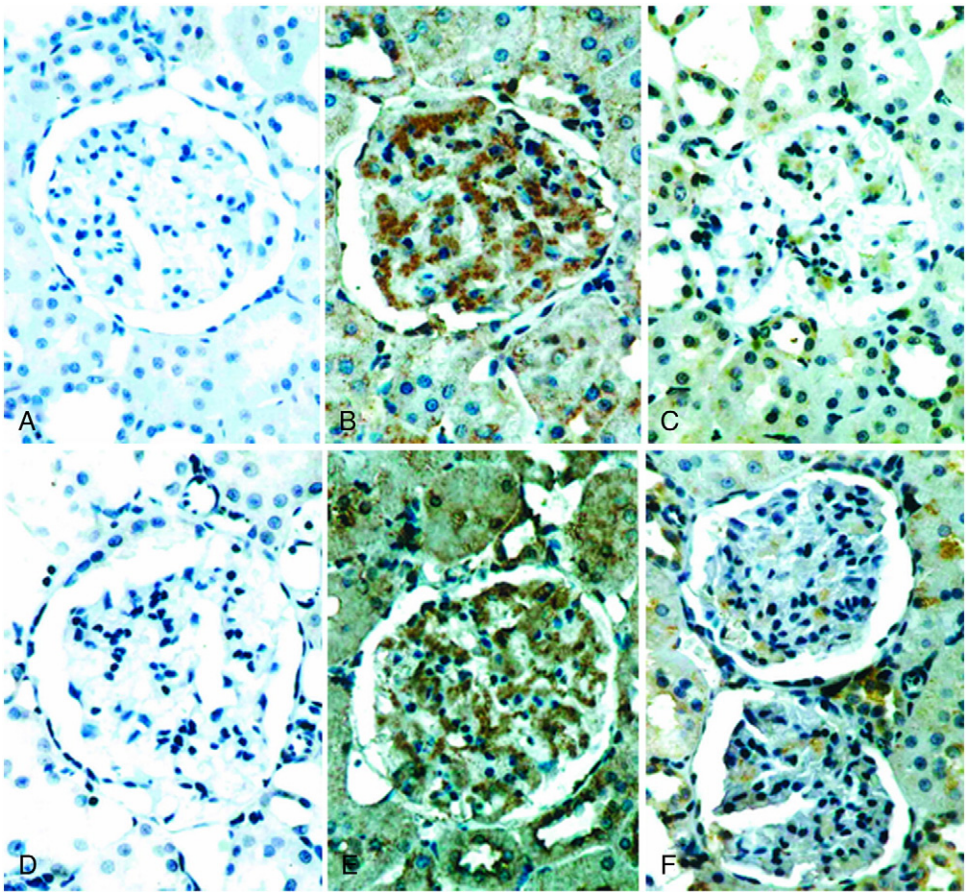

Fig. 5. Immunohistochemical expression of GRP78 and MCP-1 in kidneys of NC, DN, and DN treatment 4-PBA rats at 12 weeks . Expression of GRP78 and MCP-1 in kidneys was evaluated using immunohistochemical staining. GRP78-positive cells had stained nuclei, and more GRP78-positive cells were seen in the DN group compared with 4-PBA group. More MCP-1–positive cells expressed in mesangial cells were seen in the DN group compared with the 4-PBA group. Original magnification ×200. A, The expression of GRP78 in NC rats. B, The expression of GRP78 in DN rats. C, The expression of GRP78 in 4-PBA rats. D, The expression of MCP-1 in NC rats. E, The expression of MCP-1 in DN rats. F, The expression of MCP-1 in 4-PBA rats.

3.7. Effect of 4-PBA administration on insulin resistance in kidneys of DN rats

Insulin receptor substrate 1 is a substrate for the insulin receptor tyrosine kinase, and serine phosphorylation of IRS-1 can reduce insulin receptor signaling. In DN rats, p-IRS-1 was markedly increased for the indicated time, with respect to NC rats. In the DN+ 4-PBA group ($P < .05$), p-IRS-1 was decreased significantly in renal tissue ($P < .05$), although the p-IRS-1 was higher than that in the NC rats. These results indicate that there is down-regulation of ER stress-influenced insulin receptor signaling (Fig. 9).

4. Discussion

The present study demonstrated that 4-PBA treatment could suppress the renal damage, as evidenced by the improvement of glomerular mesangial matrix clustering as well as the decreased KI, UV, and UPER and the marked decrease of the expression of proinflammatory and profibrosis factors, through mitigation of cellular ER stress. These results provide evidence that manipulation of the severity and persistence of ER stress may be a new approach for preventing the progression of DN.

Over the past decades, it has been accepted that the pathologic progression of DN is characterized by mesangial cells proliferation, mesangial matrix accumulation, and sclerosis. However, the molecular mechanism underlying these individual disorders remains unknown. Recently, people have accepted that ER stress may be the headquarters of a variety of stress signals including oxidative stress and inflammatory pathways [5,6]. The most important finding in the present study is that ER stress was induced by hyperglycemia in kidney, especially in glomeruli of kidney. There are several lines of evidence to support this notion. First, the expression of GRP78 (78-kd Glu-regulated/binding immunoglobulin protein, also known as *BIP*) was markedly increased in the kidneys of DN rats and had a tendency to increase with observation time. As is well known, GRP78 is an ER chaperone and an important molecular indicator of ER stress whose expression is increased upon ER stress and is released from the ER membrane to enhance protein folding.

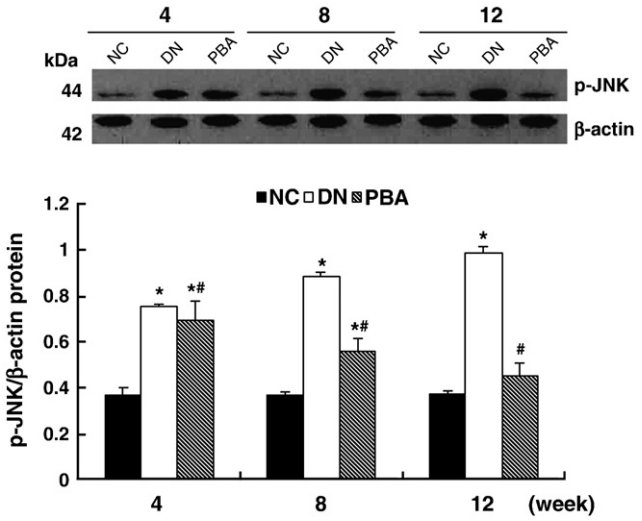

Fig. 6. Effect of 4-PBA on p-JNK protein expression in all rats. Representative Western blot of p-JNK protein expression in the kidneys of NC, DN, and 4-PBA rats at weeks 4, 8, and 12. The level of insulin resistance was significantly decreased by 4-PBA. Data are presented as means ± SE. *$P < .05$ compared with NC group; #$P < .05$ compared with DN group.

Second, PERK (PKR-like kinase) whose phosphorylation status is a key indicator of the presence of ER stress was also induced in the kidneys after DN induction. Third, in our study, enhancing the folding capacity of the ER via long-term administration of 4-PBA resulted in attenuated ER stress (reduced GRP78 expression) and nephropathy progression in rats with diabetes, evidenced by decreased UPER and improved histologic changes. Taken together, it can be concluded that ER stress is involved in the renal deterioration and the development of DN. In other kidney diseases, accumulating studies demonstrate that ER stress was activated; for example, the increased expression of GRP94 and GRP78 was demonstrated in the kidney of rats exposed to nephrotoxic doses of cisplatin and gentamicin, a kind of chemical stress [27]. The heterozygous mutant BIP mice showed significant tubular-interstitial lesions with aging. These results suggest that ER stress plays a significant role in the pathophysiology of chronic renal tubular-interstitial injury in vivo [28]. Furthermore, the induction of UPR by indomethacin was observed in diverse cell types, including podocytes, mesangial cells, and tubular epithelial cells [29]. Recently, Lindenmeyer et al analyzed the microarray data from biopsies of patients with established DN and in control kidneys or biopsies of patients with mild DN, and suggested an association between the degree of DN and UPR gene expression [30]. In conclusion, ER stress may be a common signal transduction pathway in multiple kidney diseases.

Another new finding of the study is that ER stress was the upstream signal of inflammation in the kidney of DN rats. In this study, we found that there was a significant increase in phosphorylation of JNK in the kidneys of DN rats compared with controls. Furthermore, the results also showed that the mRNA and protein levels of MCP-1, which is a major

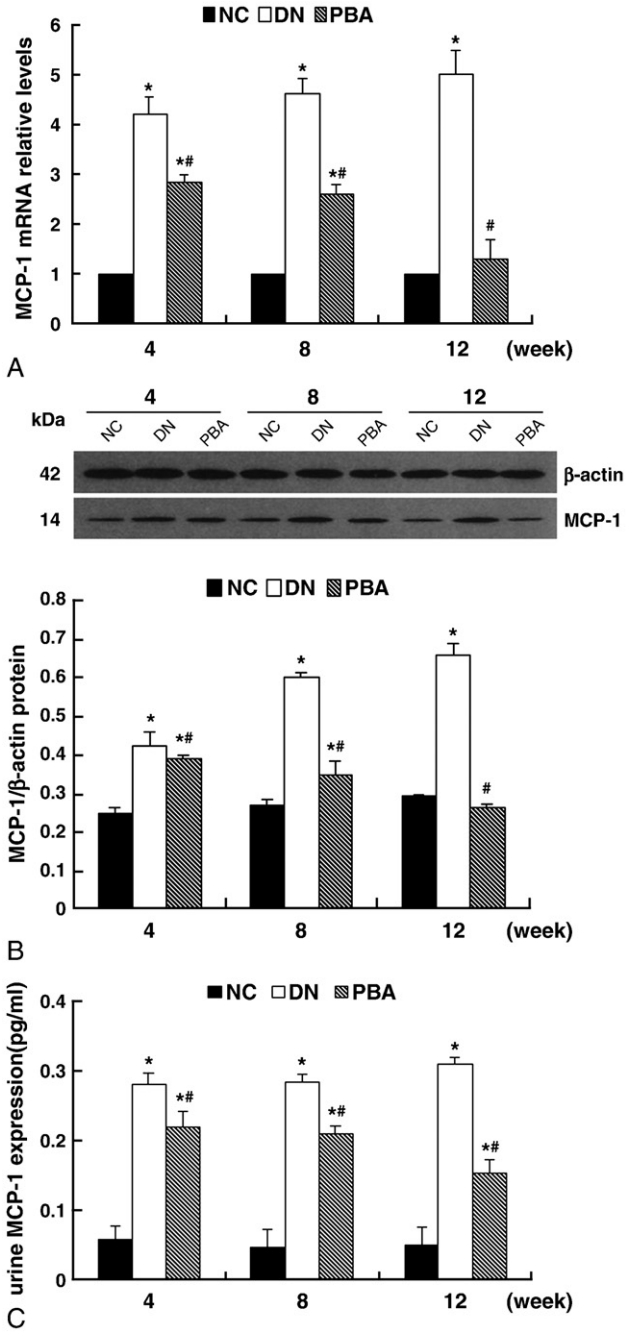

Fig. 7. Effects of MCP-1 expression in the kidney and levels secreted in urine for all rats. A, The MCP-1 mRNA levels were measured by real-time PCR and corrected for by GADPH mRNA in NC, DN, and 4-PBA rats at weeks 4, 8, and 12. B, Representative Western blot of MCP-1 protein expression in the kidneys of all rats at weeks 4, 8, and 12 of the study. C, Urine collections were obtained for MCP-1 measurement in rats by ELISA. The MCP-1 expression in the kidney and levels secreted in urine were markedly down-regulated by treatment with 4-PBA. Values are means ± SE. *$P < .05$ compared with NC group; #$P < .05$ compared with DN group.

proinflammatory cytokine, were significantly elevated in the kidneys of DN rats. More importantly, we found that 4-PBA treatment not only reduced phosphorylation of JNK, but also reduced the expression of MCP-1 in the kidneys of DN rats. Phosphorylation of JNK by endogenous signals initiated in

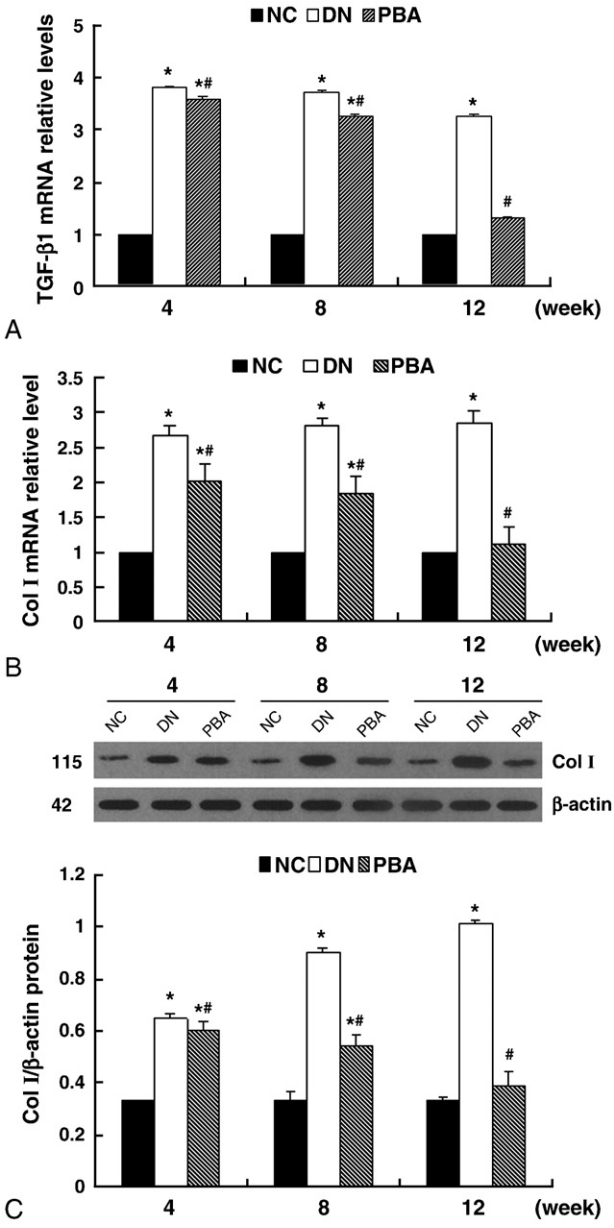

Fig. 8. Effects of 4-PBA on TGF-β1 and type I collagen mRNA and protein expression in rats. A, The TGF-β1 mRNA levels were measured by real-time PCR and were corrected for by GADPH mRNA in NC, DN, and 4-PBA rats at weeks 4, 8, and 12. B, Type I collagen mRNA levels were measured by real-time PCR and corrected for by GADPH mRNA in NC, DN, and 4-PBA rats at weeks 4, 8, and 12. C, Representative Western blot of type I collagen in kidneys of all rats at weeks 4, 8, and 12 of the study. Values are means ± SE. *$P < .05$ compared with NC group; $^{\#}P < .05$ compared with DN group.

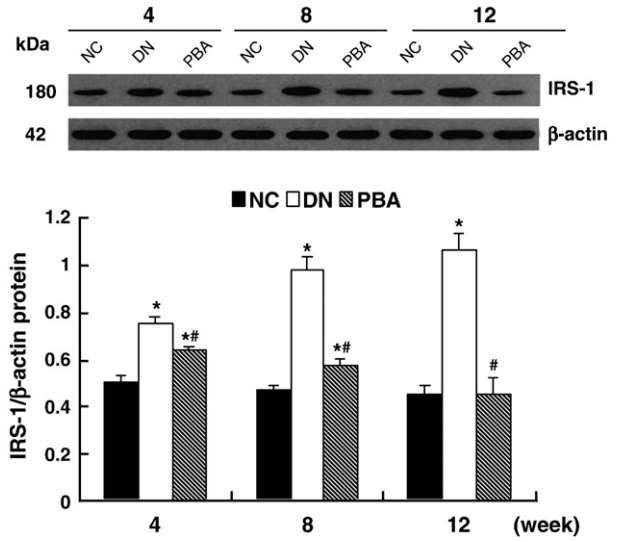

Fig. 9. Effects of 4-PBA on p-IRS-1 protein expression in rats. Western blots were performed on renal tissue in NC, DN, and 4-PBA rats at weeks 4, 8, and 12 of the study. Representative protein expression of p-IRS-1 was normalized with β-actin. Data represent means ± SE. *$P < .05$ compared with NC group; $^{\#}P < .05$ compared with DN group.

the ER proceeds by a pathway similar to that initiated by cell surface receptors in response to extracellular signals. JNK blockade markedly blunted the tumor necrosis factor–α response in cultured macrophages [31]. Macrophage infiltration is directly correlated with the severity of DN. Increases in MCP-1 expression and interstitial macrophage infiltration coincide with the development of hyperglycemia and precede a rise in albuminuria in type 1 DN in mice [32]. In a model of STZ-induced type 1 DN, mice genetically deficient in MCP-1 were found to have reduced renal injury, as compared with wild-type mice with equivalent hyperglycemia. This study also indicated that the absence of MCP-1 resulted in the prevention of albuminuria and elevated plasma Cr at week 18 of diabetes, which coincided with a marked reduction (≥50%) in glomerular and interstitial CD68+ macrophages and histologic damage [33]. Therefore, MCP-1 plays a critical role in inflammation of the kidney and promotes type 2 diabetic renal injury [34]. Taken together, these results suggest that regulating ER stress contributed to the deactivation of the inflammatory signal and proinflammatory cytokine expression.

The third finding is that glomerular fibrosis could be inhibited with 4-PBA treatment by regulation of ER stress in the kidney of DN rats. Glomerular fibrosis is the final manifestation of end-stage renal disease. Transforming growth factor–β1, a well-documented fibrogenic growth factor, is involved in the pathogenesis of DN, including renal inflammation and fibrosis [35,36]. Furthermore, a number of studies have shown that the expression of type I collagen, the major component of ECM, is mediated by TGF-β1 [37,38]. In the current study, we found that TGF-β1 and type I collagen expression was significantly elevated in the kidney of DN rats, which can be attributed to activation of JNK mediated by ER stress. A significant inhibition of TGF-β1 and type I collagen level by 4-PBA observed in our study is indicative of the fact that 4-PBA contributes to the beneficial effects seen in DN. Therefore, it seems reasonable to assume that administration of 4-PBA may constitute a new molecular basis for inhibition of intrarenal fibrosis, which plays a potential role in the progression of nephropathy.

As mentioned above, ER stress is a central feature of type 2 diabetes mellitus and kidney disease at the molecular,

cellular, and organismal levels [39,40]. Therefore, pharmacologic manipulation of this pathway by supplementing with a molecular chaperone, such as 4-PBA, may play a role in restoring ER stress and its related downstream signal disorders, for example, JNK activation and insulin resistance [9,41]. In our study, the expression of p-IRS-1 in the renal tissue of DN rats is increased, which is an indicator of insulin resistance, and is accompanied by activation of the JNK pathway. Multiple lines of evidence indicate the effectiveness of 4-PBA in diverse diseases. For one thing, a previous study suggested that the blood Glu–lowering effect of PBA is due to increased systemic insulin sensitivity [9]. In agreement with this view, Choi et al [42] showed that this might be related to the role of 4-PBA treatment in repairing islet cell function and increasing insulin secretion. Another potential role of 4-PBA is evidenced by dissimilar parts or organs. Recent work reported that pre- or posttreatment with 4-PBA at therapeutic doses attenuated infarction volume, hemispheric swelling, and apoptosis, and also improved neurologic status in a mouse model of brain ischemia, possibly because of a decrease in the protein load retained by the ER [8]. This is also supported by Liu et al [43], whose study indicated that mutated nephrin is rescued by 4-PBA, suggesting the beneficial effect of ER stress modulators in nephrosis induced by nephrin mutation. Taken together, 4-PBA treatment not only decreased the phosphorylation of JNK and p-IRS-1 expression, but also contributed to blood Glu regulation. As mentioned above, there is ample evidence to support that the beneficial role of ER stress is manipulated by 4-PBA treatment.

In summary, there was an increase in GRP78 and p-PERK expression, as well as an increase in the progression of inflammatory changes in the kidney. Increased levels of the chemical chaperone 4-PBA inhibited the increase in GRP78 and p-PERK expression and inflammatory changes in renal tissue in the setting of diabetes. These results suggest that the ER stress-related inflammatory cascade is activated. In terms of therapeutics, our findings suggest that the regulation of the ER stress response offers new opportunities for preventing and treating DN.

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References

[1] Yamamoto N, Toyoda M, Abe M, et al. Lectin-like oxidized LDL receptor–1 (LOX-1) expression in the tubulointerstitial area likely plays an important role in human diabetic nephropathy. Intern Med 2009;48:189-94.
[2] Mora C, Navarro JF. Inflammation and pathogenesis of diabetic nephropathy. Metabolism 2004;53:265-6.
[3] Navarro JF, Mora C. Role of inflammation in diabetic complications. Nephrol Dial Trans 2005;20:2601-4.
[4] Boden G. Endoplasmic reticulum stress: another link between obesity and insulin resistance/inflammation? Diabetes 2009;58:518-9.
[5] Fornoni A, Ijaz A, Tejada T, Lenz O. Role of inflammation in diabetic nephropathy. Curr Diabetes Rev 2008;4:10-7.
[6] Zhang K, Kaufman RJ. From endoplasmic-reticulum stress to the inflammatory response. Nature 2008;454:455-62.
[7] Kubota K, Niinuma Y, Kaneko M, et al. Suppressive effects of 4-phenylbutyrate on the aggregation of Pael receptors and endoplasmic reticulum stress. J Neurochem 2006;97:1259-68.
[8] Qi X, Hosoi T, Okuma Y, Kaneko M, Nomura Y. Sodium 4-phenylbutyrate protects against cerebral ischemic injury. Mol Pharmacol 2004;66:899-908.
[9] Ozcan U, Yilmaz E, Ozcan L, et al. Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. Science 2006;25:1137-40.
[10] Cheng H, Tian XF, Dong HR, Ding SS, Qiu CB, Chen YP. The preliminary studies on the cross action between endothelin-1 and angiotensin II in kidney tissue of rats with diabetic nephropathy. Zhonghua Yi Xue Za Zhi 2004;84:248-52.
[11] Sugano M, Yamato H, Hayashi T, et al. High-fat diet in low-dose-streptozotocin–treated heminephrectomized rats induces all features of human type 2 diabetic nephropathy: a new rat model of diabetic nephropathy. Nutr Metab Cardiovasc Dis 2006;16:477-84.
[12] Zhang F, Ye C, Li G, et al. The rat model of type 2 diabetic mellitus and its glycometabolism characters. Exp Anim 2003;52:401-7.
[13] Reed MJ, Meszaros K, Entes LJ, et al. A new rat model of type 2 diabetes: the fat-fed, streptozotocin-treated rat. Metabolism 2000;49:1390-4.
[14] Danda RS, Habiba NM, Rincon-Choles H, et al. Kidney involvement in a nongenetic rat model of type 2 diabetes. Kidney Int 2005;68:2562-71.
[15] Lee EA, Seo JY, Jiang Z, et al. Reactive oxygen species mediate high glucose-induced plasminogen activator inhibitor–1 up-regulation in mesangial cells and in diabetic kidney. Kidney Int 2005;67:1762-71.
[16] Forbes JM, Thallas V, Thomas MC, et al. The breakdown of preexisting advanced glycation end products is associated with reduced renal fibrosis in experimental diabetes. Faseb J 2003;17:1762-4.
[17] Yuan JS, Reed A, Chen F, Stewart Jr CN. Statistical analysis of real-time PCR data. BMC Bioinformatics 2006;7:85.
[18] Kaufman RJ. Orchestrating the unfolded protein response in health and disease. J Clin Invest 2002;110:1389-98.
[19] Lee AS. The ER chaperone and signaling regulator GRP78/BiP as a monitor of endoplasmic reticulum stress. Methods 2005;35:373-81.
[20] Bertolotti A, Zhang Y, Hendershot LM, Harding HP, Ron D. Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. Nat Cell Biol 2000;2:326-32.
[21] Harding HP, Zhang Y, Ron D. Protein translation and folding are coupled by an endoplasmic reticulum resident kinase. Nature 1999;397:271-4.
[22] Weston CR, Lambright DG, Davis RJ, et al. Signal transduction MAP kinase signaling specificity. Science 2002 2002;296:2345-7.
[23] Wang Y, Ji HX, Xing SH, Pei DS, Guan QH. SP600125, a selective JNK inhibitor, protects ischemic renal injury via suppressing the extrinsic pathways of apoptosis. Life Sci 2007;80:2067-75.
[24] Hotamisligil GS. Inflammation and endoplasmic reticulum stress in obesity and diabetes. Int J Obes 2008;32(Suppl 7):S52-4.
[25] Kaneto H, Matsuoka TA, Nakatani Y, et al. Oxidative stress, ER stress, and the JNK pathway in type 2 diabetes. J Mol Med 2005;83:429-39.
[26] Tesch GH. MCP-1/CCL2: a new diagnostic marker and therapeutic target for progressive renal injury in diabetic nephropathy. Am J Physiol Renal Physiol 2008;294:F697-701.

[27] Peyrou M, Hanna PE, Cribb AE. Cisplatin, gentamicin, and p-aminophenol induce markers of endoplasmic reticulum stress in the rat kidneys. Toxicol Sci 2007;99:346-53.
[28] Kimura K, Jin H, Ogawa M, Aoe T. Dysfunction of the ER chaperone BiP accelerates the renal tubular injury. Biochem Biophys Res Commun 2008;22:1048-53.
[29] Inagi R, Kumagai T, Nishi H, et al. Preconditioning with endoplasmic reticulum stress ameliorates mesangioproliferative glomerulonephritis. J Am Soc Nephrol 2008;19:915-22.
[30] Lindenmeyer MT, Rastaldi MP, Ikehata M, et al. Proteinuria and hyperglycemia induce endoplasmic reticulum stress. J Am Soc Nephrol 2008;19:2225-36.
[31] Ikezumi Y, Hurst L, Atkins RC, Nikolic-Paterson DJ. Macrophage-mediated renal injury is dependent on signaling via the JNK pathway. J Am Soc Nephrol 2004;15:1775-84.
[32] Banba N, Nakamura T, Matsumura M, Kuroda H, Hattori Y, Kasai K. Possible relationship of monocyte chemoattractant protein–1 with diabetic nephropathy. Kidney Int 2000;58:684-90.
[33] Chow FY, Nikolic-Paterson DJ, Ozols E, Atkins RC, Rollin BJ, Tesch GH. Monocyte chemoattractant protein–1 promotes diabetic renal injury in streptozotocin-treated mice. Kidney Int 2006;69:73-80.
[34] Chow FY, Nikolic-Paterson DJ, Ma FY, Ozols E, Rollins BJ, Tesch GH. Monocyte chemoattractant protein–1 induced tissue inflammation is critical for the development of renal injury but not type 2 diabetes in obese db/db mice. Diabetologia 2007;50:471-80.
[35] Okuda S, Languino LR, Ruoslahti E, Border WA. Elevated expression of transforming growth factor-beta and proteoglycan production in experimental glomerulonephritis. Possible role in expansion of the mesangial extracellular matrix. J Clin Invest 1990;86:453-62.
[36] Navarro-Gonzalez JF, Mora-Fernandez C. The role of inflammatory cytokines in diabetic nephropathy. J Am Soc Nephrol 2008; 19:433-42.
[37] Sharma K, Ziyadeh FN, Alzahabi B, et al. Increased renal production of transforming growth factor-β1 in patients with type II diabetes. Diabetes 1997;46:854-9.
[38] Zheng F, Fornoni A, Elliot SJ, et al. Upregulation of type I collagen by TGF-beta in mesangial cells is blocked by PPARgamma activation. Am J Physiol Renal Physiol 2002;282:F639-48.
[39] Ozcan U, Cao Q, Yilmaz E, et al. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. Science 2004;306: 457-67.
[40] Cybulsky AV. Endoplasmic reticulum stress in proteinuric kidney disease. Kidney Int 2010;77:187-93.
[41] Aguirre V, Davis R, White MF, et al. The c-Jun NH2-terminal kinase promotes insulin resistance during association with insulin receptor substrate–1 and phosphorylation of Ser307. J Biol Chem 2000;275: 9047-54.
[42] Choi SE, Lee YJ, Jang HJ, et al. A chemical chaperone 4-PBA ameliorates palmitate-induced inhibition of glucose-stimulated insulin secretion (GSIS). Arch Biochem Biophys 2008;475:109-14.
[43] Liu XL, Doné SC, Yan K, Kilpeläinen P, Pikkarainen T, Tryggvason K. Defective trafficking of nephrin missense mutants rescued by a chemical chaperone. J Am Soc Nephrol 2004;15: 1731-8.