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Original Paper

Down-Regulation of PERK-ATF4-CHOP Pathway by Astragaloside IV is Associated with the Inhibition of Endoplasmic Reticulum Stress-Induced Podocyte Apoptosis in Diabetic Rats

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Key Words
Astragaloside IV • Endoplasmic reticulum stress • PERK-ATF4-CHOP pathway • Podocyte apoptosis • Diabetic nephropathy

Abstract
Background: Endoplasmic reticulum (ER) stress-induced podocyte apoptosis plays a critical role in the development of diabetic nephropathy (DN). Here, we tested the hypothesis that suppression of PERK-ATF4-CHOP pathway by Astragaloside IV (AS-IV) is associated with inhibition of ER stress-induced podocyte apoptosis in streptozotocin (STZ)-induced diabetic rats. ***Methods:*** Diabetic rats were treated with AS-IV at 5 and 10 $mg \cdot kg^{-1} \cdot d^{-1}$, p.o., for 12 weeks. Albuminuria examination, hematoxylin & eosin staining and TUNEL analysis were performed. Immunohistochemistry, western blot, and real-time PCR were used to detect renal expression of ER chaperone GRP78 and ER-associated apoptosis proteins. ***Results:*** Treatment with AS-IV ameliorated albuminuria and renal histopathology in diabetic rats. Diabetic rats had significant increment in podocyte apoptosis as well as phosphorylated PERK and eIF2α in the kidneys, which were attenuated by AS-IV treatment. Furthermore, diabetic rats were found to have increased protein and mRNA expressions of GRP78 and ER-associated apoptosis proteins, such as ATF4, CHOP and TRB3, which were also attenuated by AS-IV treatment. Increased Bax expression and decreased Bcl-2 expression were detected in diabetic rats, and these changes were partially restored by AS-IV treatment. ***Conclusion:*** The protective effect of AS-IV on ER stress-induced podocyte apoptosis is associated with inhibition of PERK-ATF4-CHOP pathway. Down-regulation of PERK- ATF4-CHOP pathway by AS-IV may be a novel strategy for the treatment of DN.

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Introduction

Diabetic nephropathy (DN) is a common and serious complication of diabetes mellitus, which leads to end-stage renal disease (ESRD). Recently, many studies have demonstrated that podocyte apoptosis plays a critical role in the pathogenesis of proteinuria and glomerulosclerosis in DN [1-3]. However, the mechanisms of podocyte apoptosis are not fully known, and appropriate approaches to minimize these risks are still being explored.

The normal functional role of the endoplasmic reticulum (ER) is to fold, modify, and degrade secretory and transmembrane proteins. ER stress refers to physiological or pathological states that result in accumulation of misfolded proteins in the ER. Growing evidence demonstrates that ER acts as an important stress sensor that through ER stress-triggered signaling pathways or unfolded protein response (UPR) and regulates cell energy metabolism, redox status, inflammation, and cell survival [4-6]. Reactive oxygen species, free fatty acids, and other mediators related to the diabetic environment exacerbate protein-misfolding stress in the ER. ER stress is prominent in a mouse model of type 2 diabetes. Treatment with 4-PBA or TUDCA attenuated ER stress, restored insulin sensitivity, and normalized hyperglycemia in diabetic mice [5]. Treatment with 4-PBA also reduced the induction of ER stress markers, proteinuria, and improved renal histopathology in streptozotocin-induced diabetic rats [7]. Another study demonstrated an analogous effect of 4-PBA in the same diabetic nephropathy model [8]. Moreover, 4-PBA treatment ameliorated BiP expression and the loss of podocytes in mice with podocyte-specific activation of mTORC1, which recapitulated features of DN [9]. There is evidence that ER is involved in the intrinsic pathway of apoptosis [10]. Another study reported that modulation of AT-1R/CHOP-JNK-Caspase12 pathway by olmesartan treatment attenuated ER stress-induced renal apoptosis in streptozotocin-induced diabetic mice [11].

In podocytes, an intact ER is important to the metabolic function of these cells, the integrity of proteins of the slit diaphragm and those mediating cell-matrix interactions. ER perturbation could impair protein maturation, thereby disrupt these important characteristics of the podocytes and cause proteinuria. Alternatively, impairment of the unfolded protein response potentially exacerbates podocyte injury and contributes to podocyte death and depletion [12]. It was reported that apoptosis induced by ER stress occurred in diabetic kidney, which may contribute to the development of DN [13]. A recent study indicated that palmitic acid increased podocyte apoptosis and induced podocyte ER stress [14]. Palmitate could cause podocyte apoptosis via ER stress, indicating that podocyte apoptosis and consequent proteinuria induced by lipotoxic free fatty acid could be alleviated by inhibition of ER stress [15]. These studies indicated that ER stress-induced podocyte apoptosis played a critical role in the development of DN. Thus, targeting inhibition of ER stress could be a novel therapeutic approach for treatment of DN. However, there are no current interventions for DN specifically inhibiting ER stress.

Astragaloside IV(AS-IV) is a novel saponin purified from Astragalus membranaceus (Fisch) Bge, which has been widely used in traditional Chinese medicine to treat renal diseases. We previously reported that AS-IV inhibited the renal oxidative stress and podocyte apoptosis in STZ-induced diabetic rats [16]. This study aims to further test the hypothesis that suppression of PERK-ATF4-CHOP pathway by AS-IV is associated with the inhibition of ER stress-induced podocyte apoptosis in STZ-induced diabetic rats, and then provide a potential therapy for the treatment of DN.

Materials and Methods

Drug preparation

Astragaloside IV(AS-IV) was purchased from Xi'an Sobeo Pharmaceutical Technology Company, Limited (purity above 98%, Xi'an, China). The chemical structure of Astragaloside IV ($C_{41}H_{68}O_{14}$, molecular weight=784) was previously described [17]. AS-IV was suspended in 1% carboxymethyl cellulose (CMC)

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solution as a vehicle for its administration and was given to rats by oral gavage as previously described [16]. AS-IV administration was done once daily. The dosage of AS-IV used in this study was chosen as indicated by our previous study [16].

In vitro study

Conditionally immortalized mouse podocytes were kindly provided by Dr. Peter Mundel (Division of Nephrology, Massachusetts General Hospital, Harvard University) and were conducted as described in our previous study [16]. The podocytes were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco, USA), 100U/ml penicillin and 100μg/ml streptomycin. Cells were grown at 33°C with 10 U/ml mouse recombinant interferon-γ (IFN-γ, Sigma Chemical Corporation, USA). At confluence podocytes were incubated at 37°C on collagen-coated dishes for 14 days deprived of IFN-γ to allow differentiation. Differentiated podocytes were cultured for 24 h in RPMI 1640 medium and 1% FCS before being exposed to various experimental conditions. The cells were then divided into the following groups: (1) normal glucose group (NG) as controls incubated in RPMI 1640 containing 5 mM glucose, (2) high glucose group (HG) incubated in RPMI 1640 containing 30 mM glucose, (3) AS-IV group incubated in HG medium treated with different doses of AS-IV(50, 100, 200 μg/ml) for 24 h. All the glucose used in the present study was D-glucose. All experimental groups were cultured in quadruplicate.

Animal study

All work with rat was approved by the Animal Ethics Committee of Fudan University, Shanghai, China and were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the Chinese National Institutes of Health. Healthy male Sprague-Dawley rats weighing 180 to 200g were purchased from Experimental Animal Center, Fudan University, Shanghai, China. Rats were housed in an air-conditioned room at 23 ± 1°C with alternating 12 h cycles of light and dark. Animals were fed a standard diet and given water ad libitum. Diabetes was induced in rats by a single intraperitoneal injection of streptozotocin (STZ) at 65 mg/kg. Age-matched control rats received an equal volume of vehicle (0.1M citrate buffer, pH 4.5). Forty-eight hours after injection of STZ, the blood glucose level was measured from the tail vein. Rats with a blood glucose level over 300 mg/dl were considered to be diabetic and included in the study. Two weeks after STZ injection, rats were randomly divided into three groups (n=8/each group): (1) STZ-induced diabetic rats (DN); (2) diabetic rats treated with AS-IV at 5 $mg \cdot kg^{-1} \cdot d^{-1}$ (ASL); (3) diabetic rats treated with AS-IV at 10 $mg \cdot kg^{-1} \cdot d^{-1}$ (ASH). The normal rats were chosen as nondiabetic control group (Con, n = 8). AS-IV was started at 2 weeks after STZ injection and was administered via oral gavage to the rats for 12 weeks. The normal control and diabetic control rats received the equal volume of vehicle within the same time. Rats were kept in individual metabolic cages for 24 h urine collection at the end of 3, 6 and 12 weeks of treatment. Urine was centrifuged at 800g for 10 min at 25°C .Whole urine was stored at -70°C and thawed just before use. Urinary albumin concentrations were detected using an ELISA Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's method. At the end of 12 weeks of treatment, blood samples obtained from the tail vein were assayed for blood glucose level. Rats were then anesthetized with pentobarbital sodium and the blood samples were taken through the abdominal aorta for measuring biochemical parameters, including blood urea nitrogen (BUN) and creatinine (Cr) by an automatic biochemistry analyzer (Hitachi Model 7600, Japan). Animals were then killed and the kidneys were harvested immediately. The cortex from transversely bisected left kidneys was snap-frozen in liquid nitrogen and stored at -70°C for protein and total RNA extraction.

Light microscopy

That of the right kidneys was fixed with 10% buffered formalin and embedded in paraffin for histological evaluation. The kidneys were cut into 4 μm sections and stained with hematoxylin and eosin. The sections were then examined by light microscopy in a blind fashion.

Immunohistochemistry

Immunohistochemical analysis was performed to detect the tissue expression of Bax and Bcl-2. Four-micrometer-thick sections were deparaffinized and rehydrated. Immunoperoxidase staining was performed using mouse monoclonal Bax and Bcl-2 antibodies (Santa Cruz Biotechnology, CA, USA). Immunostaining procedures were performed according to the manufacturer's instructions. The sections incubated with PBS, instead of the primary antibody, served as the negative controls.

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Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nickend-labeling

A TUNEL assay was performed to detect apoptotic nuclei in kidney sections as previously described [16]. TUNEL staining using the TUNEL detection kit (Roche Diagnostics, Mannheim, Germany) was conducted according to the manufacturer's instruction. A minimum of five fields per slide and six slides per group were scored for apoptotic nuclei. Positive cells were counted as podocytes when residing on the outer surface of GBM. Cells residing in areas circumscribed by GBM were counted as endocapillary or mesangial cells. Apoptotic cells with nuclei staining dark brown were counted by light microscopy. For quantitative analysis, TUNEL-positive cells were quantified by counting five different fields at 400× magnification with two independent pathologist in a blind fashion. Data are expressed as the fold change from the normal control group.

Western blotting

Tissue samples from the kidneys and cultured podocytes under different experimental conditions were homogenized and the tissue protein was separated by sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membranes were then blocked by incubation in Tris-buffered saline Tween (TBS and 0.1% Tween 20) containing 5% bovine serum albumin and incubated overnight at 4°C with the following primary antibodies: p-IRE1, IRE1 and ATF6 (Abcam), ATF4 (Cell Signaling), CHOP (Proteintech), TRB3 (Calbiochem), Bax, Bcl-2, GRP78, p-PERK, PERK, p-eIF2α and eIF2α (Santa Cruz Biotechnology) antibodies. Negative controls were performed without primary antibody. After washing, the secondary antibody was added and incubated 1h at room temperature. Chemiluminescence detection was performed with the KC™ Detection kit (KC-420, KangChen Biotechnology, Shanghai). GAPDH is used as the internal control. Relative protein expression was described as the fold change from the normal control group.

Real-time quantitative RT-PCR

Total RNA was extracted from tissue samples by the Trizol reagent (Invitrogen, Carlsbad, CA). Then, 2μg of total RNA was reverse transcribed using the SuperScript RT kit from Invitrogen (Invitrogen, Carlsbad, CA). Quantitative RT-PCR was performed using the ABI PRISM7900 Sequence Detection System (Applied Biosystems) with SYBR Green Master Mix. The oligonucleotide primers for target genes were used as follows: GRP78: forward 5'-AACCCAGATGAGGCTGTAGCA-3' and reverse 5'-ACATCAAGCAGAACCAGGTCAC-3'; CHOP: forward 5'-CCAGCAGAGGTCACAAGCAC-3' and reverse 5'-CGCACTGACCACTCTGTTTC-3'; ATF4: forward 5'-GTTGGTCAGTGCCTCAGACA-3' and reverse 5'-CATTCGAAACAGAGCATCGA-3'; TRB3: 5'-TGATGCTGTCTGGATGACAA-3' and reverse 5'-GTGAATGGGGACTTTGGTCT-3'; GAPDH: forward 5'-GGAAAGCTGTGGCGTGAT-3' and reverse 5'-AAGGTGGAAGAATGGGAGTT-3'. In order to confirm amplification specificity, the PCR products from each primer pair were subjected to a melting curve analysis and subsequent agarose gel electrophoresis. A control without cDNA was run in parallel with each assay. Each reaction was amplified in triplicate and ratio results were calculated based on the $2^{-\Delta\Delta CT}$ method as described previously [18]. Relative mRNA levels were normalized to those of GAPDH and described as the fold change from the normal control group.

Statistical analysis

Results were expressed as means ± standard deviation (SD). The significance of differences among experimental groups was assessed by one-way analysis of variance (ANOVA) followed by the Dunnett's multiple range test. Results were considered statistically significant when P value < 0.05. All analyses were performed by SPSS 13.0 software.

Results

Effects of AS-IV on serum and urine levels of biochemical markers in diabetic rats

The levels of albuminuria, blood glucose, blood urea nitrogen (BUN) and creatinine were shown in Figure 1. The STZ-induced diabetic rats showed severe albuminuria (expressed as the mg/d) when compared with the nornmal control rats (P < 0.05). Treatment with AS-IV significantly ameliorated albuminuria in diabetic rats. This protective effect was dose-

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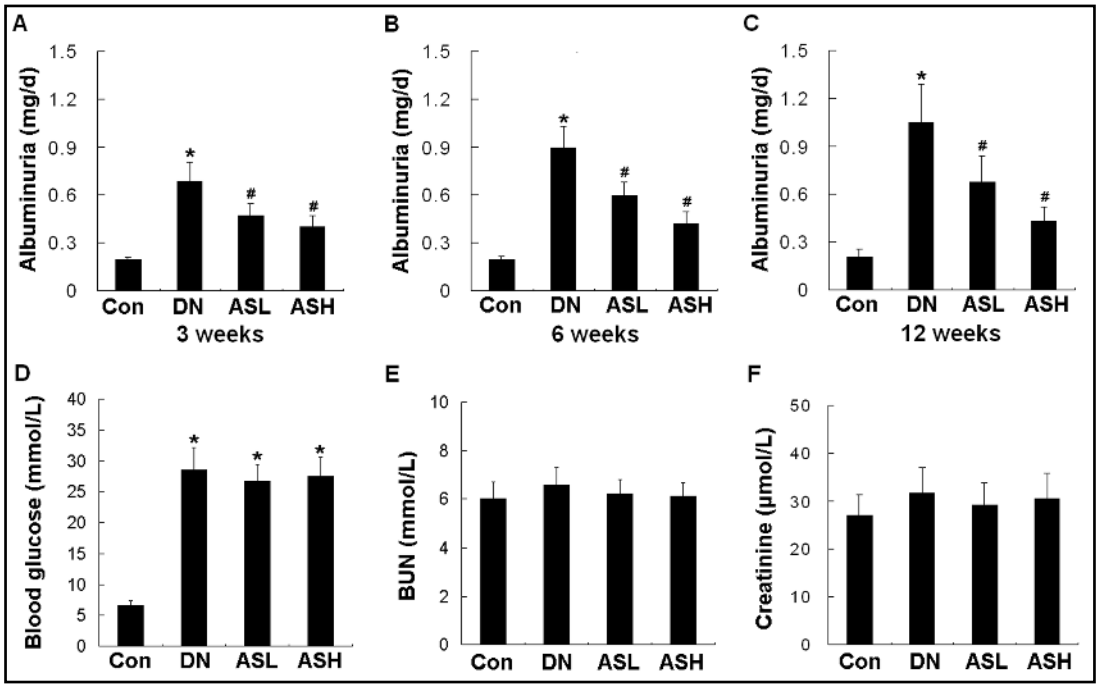

Fig. 1. Effects of AS-IV on serum and urine levels of biochemical markers in diabetic rats. AS-IV treatment was started 2 weeks after STZ injection and lasted 12 weeks. Con, normal control rats; DN, STZ-induced diabetic rats; ASL, DN rats treated with AS-IV (5 mg·kg^{-1}·d^{-1}); ASH, DN rats treated with AS-IV (10 mg·kg^{-1}·d^{-1}). Results are expressed as the means ± SD (n=8/ each group). *P< 0.05 vs Norm. $^{\#}P$< 0.05 vs DN.

dependent, which was evident at a dose as low as 5mg·kg^{-1}·d^{-1}(ASL group) and reached the peak effect at 10mg·kg^{-1}·d^{-1}(ASH group) (Fig. 1). AS-IV also time-dependently reduced albuminuria in diabetic rats, which was evident in as little as 3 weeks after AS-IV treatment (Fig. 1A). Treatment with AS-IV at the dose of 10 mg·kg^{-1}·d^{-1} for 12 weeks apparently decreased the albuminuria, which reached the peak effect (Fig. 1C). Moreover, level of BG (Fig. 1D) was significantly increased in STZ-induced diabetic rats when compared with normal control rats (P < 0.05). However, no differences in levels of BUN (Fig. 1E) and creatinine (Fig. 1F) were observed between AS-IV treated and untreated STZ-induced diabetic rats, which indicated that AS-IV did not cause apparent toxicity to the kidney.

Effects of AS-IV on renal histopathology and podocyte apoptosis in diabetic rats

Mesangial matrix expansion is considered a hallmark of DN. At 14 weeks after STZ injection, the diabetic rats showed focal mesangial matrix expansion compared to normal control rats. However, AS-IV treatment significantly ameliorated mesangial expansion when compared with the untreated STZ-induced diabetic rats (Fig. 2A). To assess podocyte apoptosis in the diabetic kidney, the tissue sections were labeled with an in situ TUNEL assay. At 14 weeks after STZ injection, the diabetic rats showed increased apoptotic podocytes when compared with the normal control rats (P < 0.05). However, there was a significant decrease in apoptotic podocytes in kidney sections from STZ-induced diabetic rats treated with AS-IV. This antiapoptotic effect was dose-dependent, which was as little as 5mg·kg^{-1}·d^{-1}(ASL group), and the maximal inhibition was achieved by 10mg·kg^{-1}·d^{-1}(ASH group) (Fig. 3). The above results indicated that AS-IV significantly alleviated renal histopathology and podocyte apoptosis in diabetic rats.

Effects of AS-IV on Bax and Bcl-2 expression in diabetic rats

ER stress-induced apoptosis was associated with decreased Bcl-2 expression. As shown in Figure 2, immunohistochemistry study showed increased Bax expression and decreased Bcl-2 expression in the renal tissue from STZ-induced diabetic rats when compared with

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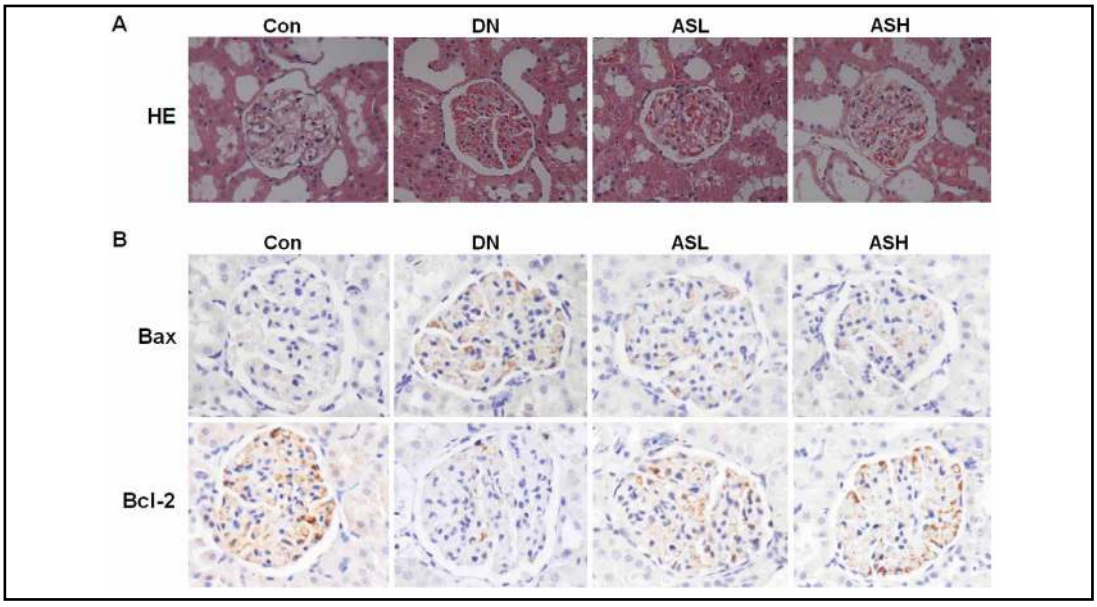

Fig. 2. Effects of AS-IV on renal histopathology, as well as Bax and Bcl-2 expression in diabetic glomeruli detected by immunohistochemical staining. AS-IV treatment was started 2 weeks after STZ injection and lasted 12 weeks. Con, normal control rats; DN, STZ-induced diabetic rats; ASL, DN rats treated with AS-IV (5 mg·kg^{-1}·d^{-1}); ASH, DN rats treated with AS-IV (10 mg·kg^{-1}·d^{-1}) (n=4/ each group).

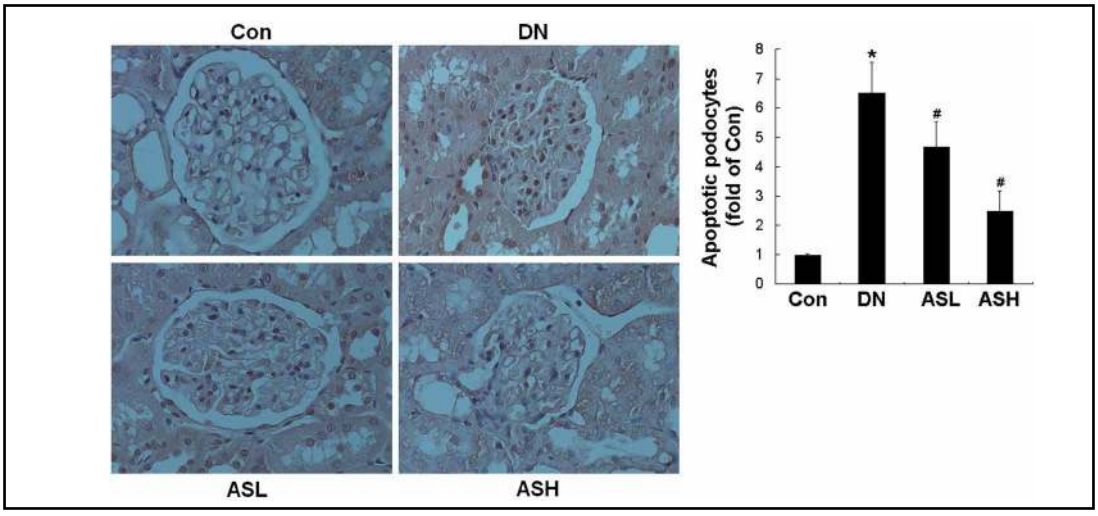

Fig. 3. Effects of AS-IV on podocyte apoptosis in diabetic rats. TUNEL assay was performed to assess podocyte apoptosis in diabetic glomeruli. AS-IV treatment was started 2 weeks after STZ injection and lasted 12 weeks. Con, normal control rats; DN, STZ-induced diabetic rats; ASL, DN rats treated with AS-IV (5 mg·kg^{-1}·d^{-1}); ASH, DN rats treated with AS-IV (10 mg·kg^{-1}·d^{-1}). Results are expressed as the means ± SD (n=4/ each group). *P< 0.05 vs Norm. $^{\#}P$< 0.05 vs DN.

the normal control rats. However, treatment with AS-IV restored the balance of Bax and Bcl-2 expression in STZ-induced diabetic rats in a dose-dependent manner (Fig. 2B). The result of Western blot analysis further confirmed these findings (Fig. 4A). The above results demonstrated that the protective effect of AS-IV on ER stress-induced podocyte apoptosis was associated with the regulation of the balance of Bax and Bcl-2 expression.

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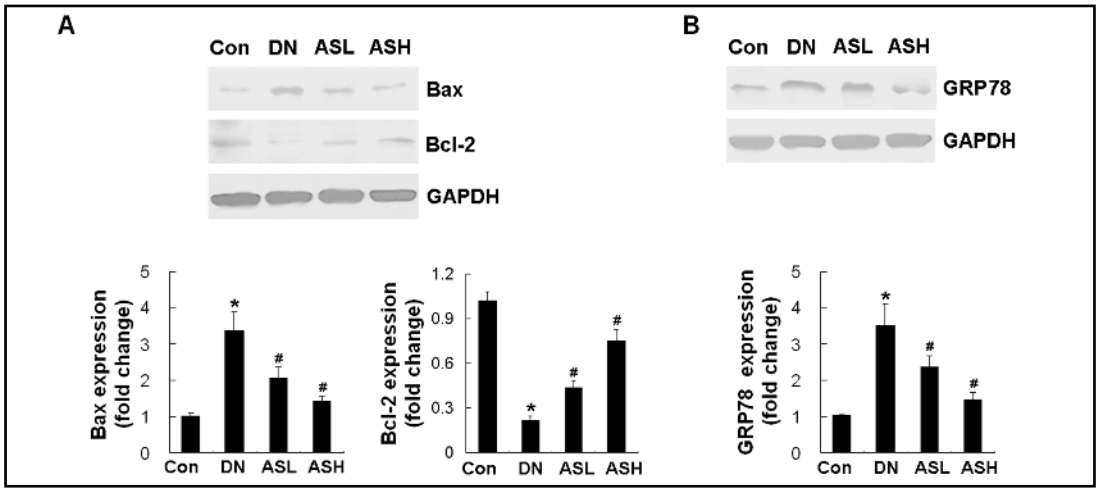

Fig. 4. Effects of AS-IV on Bax, Bcl-2 and GRP78 protein expression in diabetic kidneys examined by Western blotting. AS-IV treatment was started 2 weeks after STZ injection and lasted 12 weeks. Con, normal control rats; DN, STZ-induced diabetic rats; ASL, DN rats treated with AS-IV (5 mg·kg^{-1}·d^{-1}); ASH, DN rats treated with AS-IV (10 mg·kg^{-1}·d^{-1}). Results are expressed as the means ± SD (n=4/ each group). *P< 0.05 vs Norm. $^{\#}P$< 0.05 vs DN.

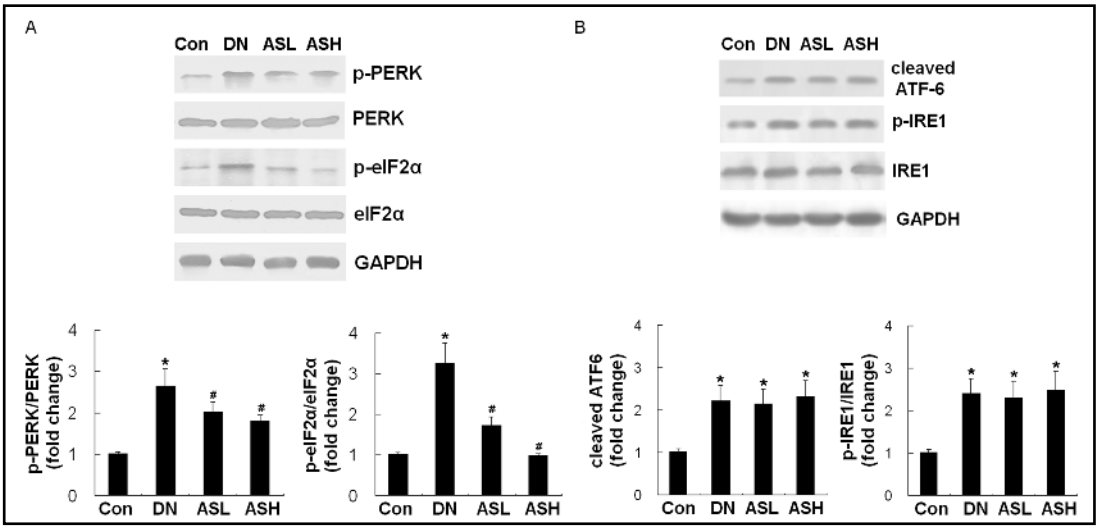

Fig. 5. Effects of AS-IV on phosphorylated PERK and eIF2α protein expression, as well as ATF6 and IRE1 activation in diabetic kidneys examined by Western blotting. AS-IV treatment was started 2 weeks after STZ injection and lasted 12 weeks. Con, normal control rats; DN, STZ-induced diabetic rats; ASL, DN rats treated with AS-IV (5 mg·kg^{-1}·d^{-1}); ASH, DN rats treated with AS-IV (10 mg·kg^{-1}·d^{-1}). Results are expressed as the means ± SD (n=4/ each group). *P< 0.05 vs Norm. $^{\#}P$< 0.05 vs DN.

Effects of AS-IV on renal ER stress in diabetic rats

GRP78, one important molecular chaperone localized in ER, has been extensively considered as an important indicator for the induction of ER stress. The up-regulated expression of GRP78 indicates the activation of ER stress. As can be seen in Fig. 4B, Western blot analysis showed that GRP78 was abundantly expressed in the renal tissue from diabetic rats when compared with the normal control rats (P < 0.05). However, treatment with AS-IV apparently decreased the protein expression of GRP78 in diabetic kidneys. These effects were dose-dependent, which were evident at a dose as low as 5 mg·kg^{-1}·d^{-1} and reached the peak effect at 10 mg·kg^{-1}·d^{-1} of AS-IV (Fig. 4B). Furthermore, real-time PCR analysis showed

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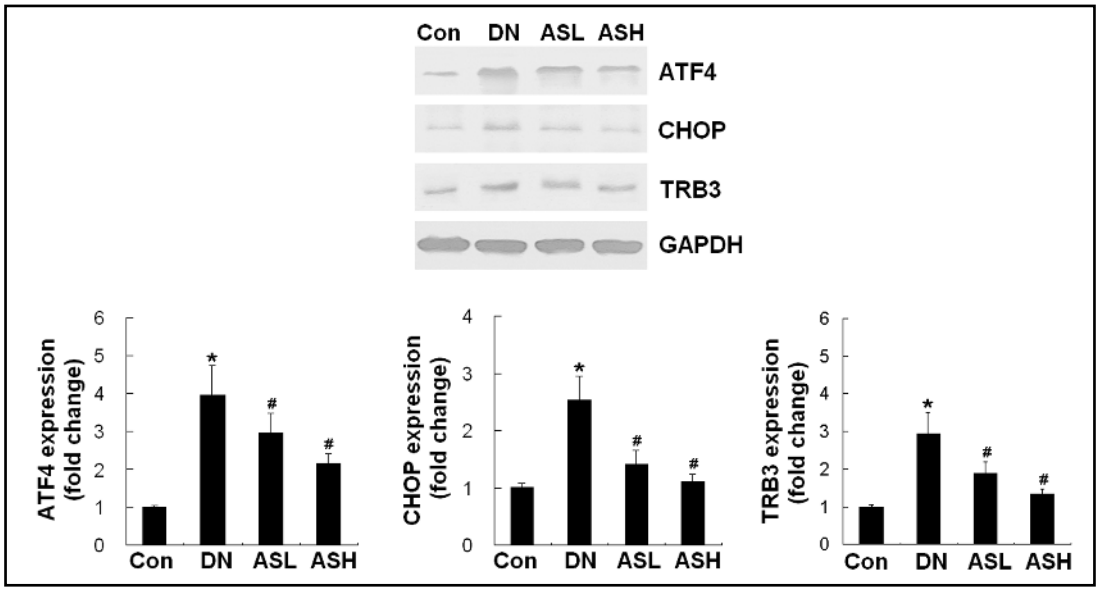

Fig. 6. Effects of AS-IV on ATF4 ,CHOP and TRB3 protein expression in diabetic kidneys examined by Western blotting. AS-IV treatment was started 2 weeks after STZ injection and lasted 12 weeks. Con, normal control rats; DN, STZ-induced diabetic rats; ASL, DN rats treated with AS-IV (5 mg·kg^{-1}·d^{-1}); ASH, DN rats treated with AS-IV (10 mg·kg^{-1}·d^{-1}). Results are expressed as the means ± SD (n=4/ each group). *P< 0.05 vs Norm. $^{\#}P$< 0.05 vs DN.

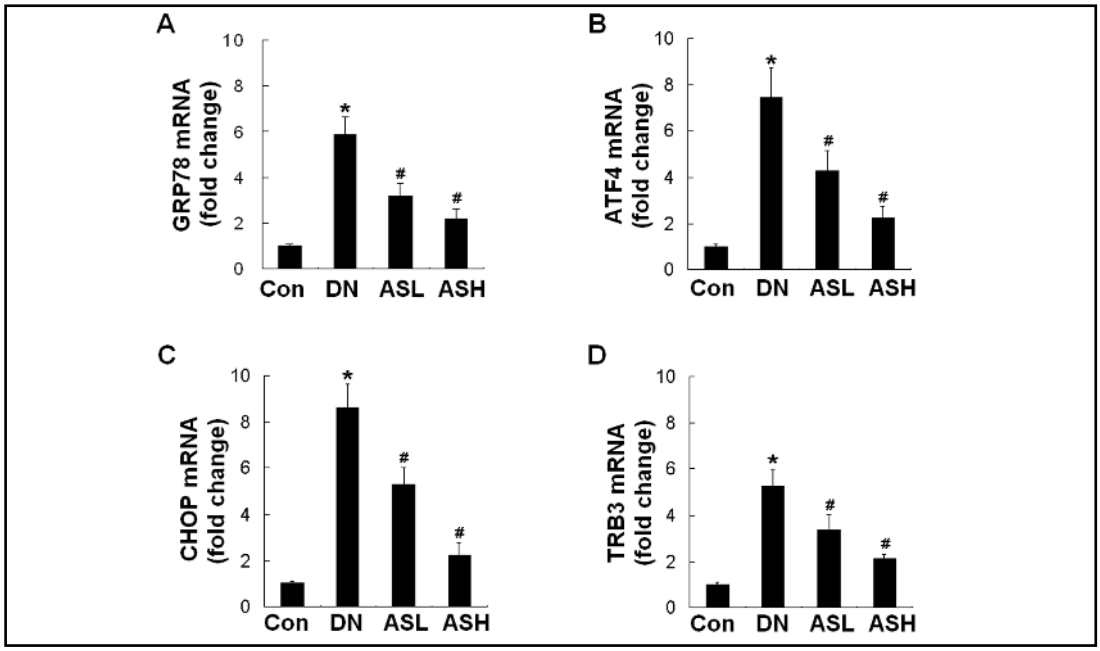

Fig. 7. Effects of AS-IV on GRP78, ATF4, CHOP and TRB3 mRNA expression in diabetic kidneys detected by real-time RT-PCR. AS-IV treatment was started 2 weeks after STZ injection and lasted 12 weeks. Con, normal control rats; DN, STZ-induced diabetic rats; ASL, DN rats treated with AS-IV (5 mg·kg^{-1}·d^{-1}); ASH, DN rats treated with AS-IV (10 mg·kg^{-1}·d^{-1}). Results are expressed as the means ± SD (n=4/ each group). *P< 0.05 vs Norm. $^{\#}P$< 0.05 vs DN.

the same trend of GRP78 at mRNA level (Fig. 7A). All these findings indicated that AS-IV significantly inhibited the activation of ER stress in diabetic kidneys.

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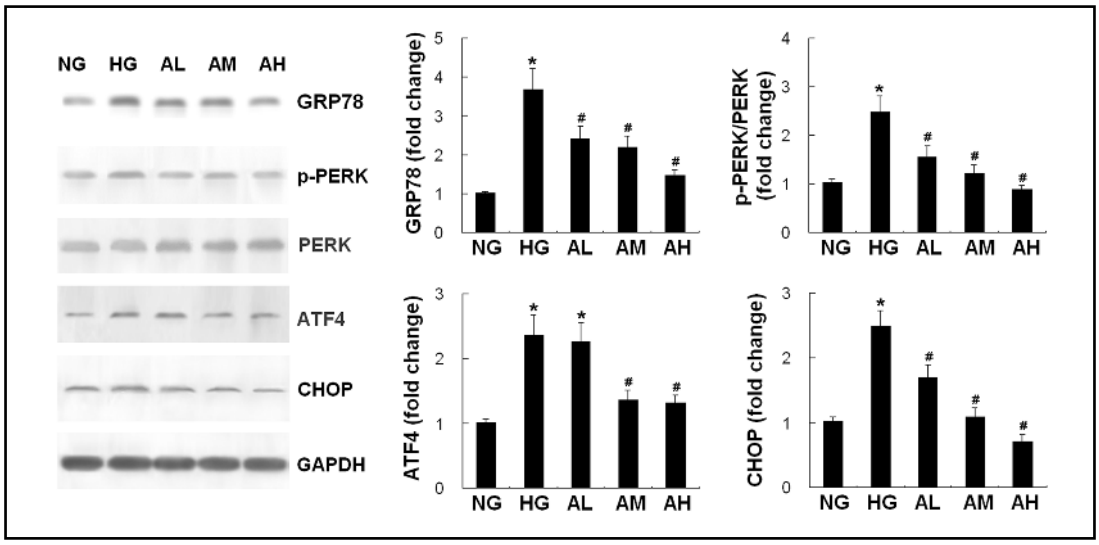

Fig. 8. Effects of AS-IV on ER stress and ER-associated apoptosis pathways in high glucose-stimulated podocytes. The levels of GRP78, ATF4, CHOP and phosphorylated PERK were examined by Western blotting. Podocytes were exposed to NG, HG, HG with 50, 100, 200 μg/ml of AS-IV for 24 h, respectively. NG, normal glucose (5 mM glucose); HG, high glucose (30 mM glucose). Results are expressed as the means ± SD (n=4/each group). **P*< 0.05 vs NG. #*P*< 0.05 vs HG.

Effects of AS-IV on ER-associated apoptosis pathways in diabetic rats

PERK, eIF2α, ATF4 and CHOP are the key molecules in the three branches of ER-associated apoptosis pathways. Firstly, western blots analysis showed that the levels of phosphorylated PERK and eIF2α were dramatically activated in diabetic kidneys. However, the activations were significantly inhibited by AS-IV administration (Fig. 5A).

We also examined the effect of AS-IV on ATF6 and IRE1 activation in diabetic rats. As shown in Figure 5B, the levels of cleaved ATF6 and phosphorylated IRE1 were not affected by AS-IV treatment.

Moreover, the protein expression of ATF4 ,CHOP and TRB3 were also increased in the STZ-induced diabetic kidneys when compared with the normal control rats. AS-IV treatment inhibited signal transduction in PERK signaling pathway via deactivating PERK and down-regulating ATF4, CHOP and TRB3 protein expression (Fig. 6). Changes in RNA expression of ATF4, CHOP and TRB3 were quantified by real-time quantitative RT-PCR. Expressions of ATF4, CHOP and TRB3 were significantly increased in the diabetic kidneys ($P < 0.05$), paralleled with their enhanced protein expression. However, treatment with AS-IV apparently down-regulated the mRNA expression of ATF4 (Fig. 7B), CHOP (Fig. 7C) and TRB3 (Fig. 7D) in diabetic rats. These effects were also dose-dependent, which were evident at a dose as low as 5 $mg \cdot kg^{-1} \cdot d^{-1}$ and reached the peak effect at 10 $mg \cdot kg^{-1} \cdot d^{-1}$ of AS-IV. These results indicated that the protective effect of AS-IV on ER stress-induced podocyte apoptosis was associated with the suppression of PERK-ATF4-CHOP signaling pathway.

Effects of AS-IV on ER stress and ER-associated apoptosis pathways in high glucose-stimulated podocytes

Treatment with AS-IV for 24 h decreased the protein expression of GRP78, ATF4 and CHOP in high glucose-stimulated cultured podocytes (Fig. 8). Treatment with AS-IV for 24 h also inhibited the phosphorylated PERK in high glucose-stimulated cultured podocytes (Fig. 8). Therefore, AS-IV had direct effects on the inhibition of ER stress and ER-associated apoptosis pathways.

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Discussion

The involvement of ER stress in the pathophysiology of DN is a relatively new area of research. Targeting inhibition of ER stress may provide new therapeutic approaches for DN. In this study, the STZ-induced diabetic rats were found to have increased protein and mRNA expressions of GRP78, ATF4, CHOP and TRB3, which were significantly blunted by AS-IV treatment. Furthermore, the diabetic rats had significant increment in podocyte apoptosis as well as phosphorylated PERK and eIF2α in the kidneys, which were also attenuated by AS-IV treatment. Thus, AS-IV inhibited ER stress-induced podocyte apoptosis and ameliorated structural and functional abnormalities in diabetic rats. The protective effect of AS-IV was associated with the inhibition of PERK-ATF4-CHOP pathway. AS-IV might be a novel drug for treatment of DN through suppression of renal ER stress.

There is mounting evidence demonstrating that ER stress plays an important role in the development of DN [19, 20]. Previous studies reported that ER stress was induced during the development of DN and ER-induced apoptosis contributed to the loss of renal cells [11, 13]. Consistent with these studies, the present study also showed that ER stress and renal apoptosis was induced in diabetic kidneys. We previously reported that AS-IV, a novel antioxidant, prevented podocyte apoptosis and reduced albuminuria in DN [16]. However, it is still unknown whether the inhibited effects of AS-IV on podocyte apoptosis in DN might be mediated through inhibition of ER stress. Thus, we further investigated the protective effects of AS-IV on renal ER stress and podocyte apoptosis in STZ-induced diabetic rats. The ER chaperone protein GRP78 is a central regulator of ER homeostasis and is involved in activation of the ER stress response. Under normal conditions, GRP78 binds to the ER luminal domains of the ER stress sensors PERK, IRE-1α, and ATF6 to form an inactive complex [21]. During ER stress, these transmembrane sensors dissociate from GRP78 and are autophosphorylated and subsequently activated [22]. In this study, we detected increased podocyte apoptosis and higher GRP78 levels in diabetic kidneys, which were significantly attenuated by AS-IV treatment. Therefore, the protective effects of AS-IV on podocyte apoptosis was partly attributed to the inhibition of renal ER stress. These findings might strengthen the therapeutic rationale for AS-IV in the treatment of DN and also provide new insights into the development of novel drug through directly targeting renal ER stress.

We next examined the effects of AS-IV on ER stress-triggered signaling pathways in diabetic kidneys. The three major arms of the mammalian UPR include protein kinase RNA (PKR)-like ER kinase (PERK), inositol-requiring protein-1 (IRE1), and activating transcription factor-6 (ATF6) pathways [19]. PERK is a transmembrane protein with an ER luminal stress-sensing domain that binds GRP78 and a cytosolic kinase domain [23]. When ER stress is prolonged, PERK multimerizes and phosphorylates eukaryotic translation initiation factor-α (eIF2α) [24]. Phosphorylation of eIF2α suppresses general protein translation. However, a subset of genes that includes ATF4 [24] is preferentially translated by phosphorylated eIF2α. ATF4 in turn drives the transcription of specific UPR target genes, which include C/EBP homologous protein GADD34 [25] and TRB3 [26]. In this study, the protein and mRNA expression of ATF4 was increased in the STZ-induced diabetic kidneys when compared with the normal control rats. AS-IV treatment decreased ATF4 protein and mRNA expression. This result indicated that the protective effect of AS-IV on ER stress-induced podocyte apoptosis was associated with inhibition of ATF4 expression. CHOP, a transcription factor that can heterodimerize with other members of the C/EBP/ATF family, has been considered to induce apoptosis [27, 28]. Furthermore, previous study reported that when ER stress was induced, it promoted apoptosis by the transcriptional induction of CHOP expression [29]. It has been demonstrated that overexpression of CHOP enhanced apoptosis of mammalian cells [28] and its deficiency protected the cells from an ER stress-induced apoptosis under diabetic conditions [30]. Transcription of the CHOP gene can be mediated by PERK [31]. Consistent with the previous report [11], we also found that the expression of CHOP was up-regulated in the diabetic kidneys in comparison to normal control animals. However, rats treated with AS-IV had shown significant attenuation of CHOP protein and mRNA expression in diabetic

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kidneys. Furthermore, the levels CHOP upstream kinases, such as p-PERK and p-eIF2α were dramatically activated in diabetic kidneys, which were significantly inhibited by AS-IV treatment. We also examined the effect of AS-IV on ATF6 and IRE1 activation in diabetic rats. Western blots analysis showed that the levels of cleaved ATF6 and phosphorylated IRE1 were not affected by AS-IV treatment. It may be reasonable to conclude that the effect of AS-IV was directed at PERK. Possibly, AS-IV might reduce protein misfolding in the ER or improved ER function by some other mechanism. Our previous study demonstrated that AS-IV had an antioxidant effect and could prevent glucose-induced podocyte apoptosis partly through restoring the balance of Bax and Bcl-2 expression and inhibiting caspase-3 activation [16]. Thus, it is possible to show only that there was an association of the protective effect of AS-IV on ER-induced podocyte apoptosis in diabetic rats with the inhibition of PERK-ATF4-CHOP pathway.

CHOP can activate TRB3 [26, 32] and also recduce expression of the prosurvival factor Bcl-2 [33]. We further investigated the effects of AS-IV on the expression of TRB3, Bcl-2 and Bax. This study in STZ-induced diabetic rats has demonstrated the elevated expression of Bax and decreased expression of Bcl-2 in renal glomeruli detected by immunohistochemical staining, which were consistent with the previous studies [2, 34]. However, AS-IV significantly decreased Bax expression while increased Bcl-2 expression in diabetic glomeruli detected by immunohistochemical staining and Western blotting. We further examined the effect of AS-IV on protein and mRNA expression of TRB3 by Western blotting and real-time quantitative RT-PCR. We found that the protein and mRNA levels of TRB3 were dramatically up-regulated in diabetic kidneys, which were significantly suppressed by AS-IV treatment in a dose-dependent manner. Taken together, this study showed that ER stress induced podocyte apoptosis in diabetic rats, accompanied by an increase in TRB3 and Bax expression, as well as a decrease in Bcl-2 expression, which were ameliorated by AS-IV treatment. Thus, AS-IV inhibited ER-induced podocyte apoptosis partly by restoring the balance of TRB3, Bcl-2 and Bax proteins.

Finally, we tested the effects of AS-IV on ER stress and ER-associated apoptosis pathways *in vitro*. AS-IV treatment for 24 h inhibited the protein expression of GRP78, ATF4 and CHOP and the level of phosphorylated PERK in high glucose-stimulated cultured podocytes. The inhibited effects of AS-IV on high glucose-induced podocyte apoptosis have been demonstrated in our previous study [16]. Therefore, AS-IV had direct effects on the inhibition of ER stress and podocyte apoptosis.

In conclusion, this study clearly demonstrated that activation of PERK-ATF4-CHOP signaling pathway was involved in ER stress-induced podocyte apoptosis in diabetic rats and AS-IV inhibited ER stress-induced podocyte apoptosis partly through suppression of PERK-ATF4-CHOP pathway. These novel findings will provide an alternative therapy for treatment of DN through targeting inhibition of renal ER stress.

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Disclosure Statement

The authors have declared that no competing interests exist.

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