Astragaloside IV Attenuates proteinuria in Streptozotocin-Induced Diabetic Nephropathy through inhibition ER Stress

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Aim: To investigate the effect and mechanism of Astragaloside IV (AS-IV) on proteinuria of diabetes rats induced with streptozotocin (STZ).

Methods: Male SD rats were randomly divided into four groups: normal control group (Normal group), diabetic nephropathy group (Model group), diabetic nephropathy plus AS-IV treatment group (AS-IV group) and diabetic nephropathy plus 4-PBA treatment group (PBA group). Endoplasmic reticulum stress in cultured Human podocytes was induced with tunicamycin (TM) and pretreated with or without AS-IV. At the end of 8 weeks, serum creatinine (Scr), blood urea nitrogen (BUN) and 24 hour urinary protein excretion rate (UAER) were detected by HITACHI automatically, morphology of kidney was examined by special staining of histological evaluation (HE). Apoptosis of podocytes cells were measured with flow cytometric analysis. The total and phosphorylation of eIF2α, PERK and JNK were determined by Western blotting, whereas expression of mRNA or protein for GRP78 and ORP150 were determined by real-time PCR or Western blotting separately.

Results: AS-IV treatment significantly reduced the urinary albumin excretion, plasma creatinine, and blood urea nitrogen, prevent the glomerular pathological alteration induced by STZ. AS-IV treatment significantly inhibit the apoptosis of podocytes induced with TM, prevent the phosphorylation of eIF2α, PERK and JNK, inhibit the expression of GRP78 and ORP150 markedly both in vivo and vitro.

Conclusion: AS-IV treatment significantly reduced proteinuria and renal pathogenesis of rats with diabetes through inhibition of endoplasmic reticulum stress.
Diabetic nephropathy (DN) is the major cause of chronic kidney disease (CKD) throughout the world and is the largest cause of end stage of renal disease (ESRD) in the United States\textsuperscript{1, 2}. Currently, the fundamental therapy for DN are control of hyperglycemia and BP, inhibition of the renin-angiotensin-aldosterone system\textsuperscript{3, 4}. These therapeutic can be effective in slowing progression but ineffective in reversing established complications. Therefore, it will be necessary to develop new strategies to slow the progression of the disease.

The endoplasmic reticulum (ER) plays an important role in folding and processing of newly synthesized proteins. Pathophysiological stress conditions will lead to accumulation of misfolded and unfolded proteins, invoking a well-conserved intracellular signaling, known as the unfolded protein response (UPR). There are three canonical arms of UPR: PKR-like eukaryotic initiation factor 2A kinase (PERK), which rapidly attenuates protein translation; the activating transcription factor-6 (ATF6) and the inositol-requiring enzyme-1 (IRE1\textsubscript{α}) cascades transcriptionally upregulate ER chaperone genes that promote proper folding and ER-associated degradation of proteins, allowing the folding machinery of the ER to catch up with the backlog of unfolded proteins. However, excessive and prolong upregulation of UPR may leads to cell injury and death.

ER stress plays a key role in cardiovascular and neurodegenerative diseases, cancer and diabetes\textsuperscript{5-8}. Recent experimental studies showed that ER stress also take part in the development of diabetes complications, such as nephropathy and early neuropathy and therefore being recognized as an emerging target for therapy\textsuperscript{9-11}.

Astragaloside IV (AS-IV) is a small molecular saponin found in Astragalus membranaceus (Fisch) Bge, which is a widely used herbal in China for thousands years. Recent studies have showed the molecule has diverse pharmacological activities such as anti-inflammation, anti-hypertension, anti-diabetes, and myocardial protection both in vitro and vivo experiments\textsuperscript{12-15}. Studies also showed that AS-IV can attenuation the injury of podocyte, ameliorate proteinuria in adults of idiopathic nephrotic syndrome\textsuperscript{16}. Combination with Angelica (Angelica sinesis) and
Ligustrazine not only improved clinical symptoms, increased serum albumin and lowered blood lipid level, but also reduced urinary protein excretion\textsuperscript{17, 18}. However, the mechanism of AS-IV on ameliorate proteinuria in DN have not been well clarified, this study will elucidate the renoprotective effects of AS-IV through reduce ER stress and then provide a novel therapeutic approach for the treatment of DN.
RESEARCH DESIGN AND METHODS

Animals. SD rats weighing 180-200 g were kept under pathogen-free conditions, all experiments were performed in accordance with the Animal Care and Use Committee of Huazhong University of Science and Technology.

STZ-induced diabetes and analysis. Rats were injected intraperitoneally with 40 mg/kg body weight STZ in 100 mmol/L citrate buffer (pH 4.6) for 5 consecutive days (after an overnight fast). Rats with a blood glucose level over 300 mg/dl were considered as diabetic rats\textsuperscript{19}, and treated with AS-IV or PBA after 2 weeks of STZ injection, drugs were administered via oral gavage to the rats for 8 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 24h urine collection at the end 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 measured using an ELISA Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s method. Urine creatinine was measured by an automatic biochemistry analyzer (Hitachi Model 7600, Japan). At the end of 8 weeks of treatment, rats were 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. Animals were killed and half of each kidney was snap-frozen for RNA preparation/protein extraction, and another half was processed for histology and immunostaining.

Human podocyte culture. Human podocytes were cultured and differentiated in RPMI culture medium containing 10% FBS and 1% penicillin/streptomycin as previously described\textsuperscript{20}. In brief, immortalized normal human podocytes were propagated at 33°C and then thermoshifted for differentiation for 14 days at 37°C. Terminally differentiated podocytes were serum and insulin starved in 0.2% FBS for other experiments.

FACScan flow cytometric assay. Apoptosis was assessed by annexin V-FITC and PI
staining followed by the analysis with flow cytometry (Beckman-Coulter, USA). Briefly, after the indicated treatment, the cells were harvested by trypsinization, fixed with 70% (v/v) alcohol at 4°C for 30 min and washed with PBS. After centrifugation, cells were incubated in 0.1 mL of phosphate-citric acid buffer (0.2 mol/L Na₂HPO₄, 0.1 mol/L citric acid, pH 7.8) for 30 min at room temperature. The cells were centrifuged and resuspended with 0.5 mL PI solution containing Triton X-100 (0.1% v/v), RNase (100 µg/mL) and PI (80 µg/mL), then the cell suspension was ready for the analysis by the flow cytometry.

**Renal histopathology.** The kidneys were fixed with 10% buffered formalin and embedded in paraffin for histological evaluation (HE). The sections were then examined by light microscopy. Glomerular injury was evaluated by mesangial expansion in sections stained with HE. In brief, the mesangial area was counted as mesangial expansion, which was determined in 20 consecutive glomeruli from each rat. All slides were observed independently by two blinded investigators. Relative mesangial expansion was described as the fold change from the normal control group.

**Quantitative real-time PCR.** Total RNA extraction from kidney or podocytes using TRIzol regent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. cDNA from total RNA was synthesized with reverse-transcription reaction using a ThermoScript RT-PCR system (Toyobo, Osaka, Japan). The primers of GRP78, ORP150 and actin were synthesized by Sangon Biotechnology Company (Shanghai, China). Real-time PCR analysis was performed in a final volume of 25 µL containing 12.5 µL SYBR Green I fluorescence using a LightCycler instrument (Roche Diagnostic, Mannheim, Germany). The following normal cycling profile for PCR was used: one cycle at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, and at 58 °C for 5 s, with a final extension step at 72 °C for 30 min. Then Real-time PCR products were analyzed by melting curve to confirm the amplification. The housekeeping gene actin was used for confirmation of similar cDNA loading. The following primers were used: R-ORP150-F, GATCACCCTGCCCAGCTTTT; R-ORP150-R, CCTCCTTAGTCTT CACCCTTT; R-GRP78-F, TCGTATGTGGCCTTCACTCC; R-GRP78-R, TTCT
Western blotting. Total protein concentration was measured using BCA method, and 50µg protein per lane was separated by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) on 8% or 10% polyacrylamide gels and transferred to poly vinylidene fluoride (PVDF) membranes. Membranes were incubated in primary antibodies for either 2 h at room temperature or overnight at 4°C. The following antibodies were used (1:1,000 dilution unless otherwise indicated): total eIF2α (CST #5324), phospho-eIF2α (CST #3398), total PERK (PTG 20582-1-AP), phospho-PERK (Santa sc-32577), total JNK (CST #9258), phospho-JNK (CST #4668), GRP78 (EPI 3216-1) and ORP150 (EPI 3905-1). Equal loading of protein between lanes was confirmed by subsequent β-actin immunoblots (β-actin 1:5,000; Sigma). After incubation with horseradish peroxidase–conjugated goat anti-mouse or donkey anti-rabbit (1:5,000; Jackson ImmunoResearch Laboratories, West Grove, PA) antibody for 1 h at room temperature, immunodetection was performed by chemiluminescence.

Statistical analysis. Data are expressed as means ± SEM. One-way ANOVA was used to determine statistically significant differences between groups. P value <0.05 was considered significant.
RESULTS

**Characteristics of experimental mice.** Table 1 showed the characteristics of four groups of rats at the end of the experimental period. Compared to Normal group, the rat injected with STZ to induced diabetes showed significantly higher of right kidney weights, Kidney/body weight ratio, glucose, and more serious impaired of kidney function. After 8 weeks of treatment with AS-IV, rat showed significantly reduced the urinary albumin excretion, plasma creatinine, and blood urea nitrogen, the kidney/body weight ratio decreased either; however, there were no differences in body weight and blood glucose between Normal group and AS-IV treated. PBA, a chemical chaperones has been approved for renoprotection showed same effects to AS-IV, our study demonstrated it can lower blood glucose, that is difference to AS-IV, we therefor presumed that AS-IV ameliorates the functional abnormality of diabetic nephropathy independently to the hypoglycemia effect.

**Table 1.** Effects of AS-IV on body weight, kidney weight, blood glucose and proteinuria in rats after 8 weeks of treatment.

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Model</th>
<th>AS-IV</th>
<th>PBA</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>240.25±14.30</td>
<td>225.62±11.45</td>
<td>226.38±8.42</td>
<td>230.72±12.00</td>
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<tr>
<td>Kidney weight</td>
<td>1.37±0.10</td>
<td>1.58±0.39</td>
<td>1.39±0.14</td>
<td>1.42±0.21</td>
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<tr>
<td>Kidney/body weight ratio(‰)</td>
<td>5.70±0.51</td>
<td>7.06±0.96</td>
<td>6.32±0.40</td>
<td>6.16±0.45</td>
</tr>
<tr>
<td>SCr(µmol/L)</td>
<td>25.53±10.83</td>
<td>51.01±11.45</td>
<td>33.39±8.71</td>
<td>27.61±6.86</td>
</tr>
<tr>
<td>BUN(mmol/L)</td>
<td>15.36±3.93</td>
<td>53.80±11.80</td>
<td>26.09±2.29</td>
<td>25.38±2.53</td>
</tr>
<tr>
<td>Urinary albumin excretion (µg/day)</td>
<td>571.68±72.45</td>
<td>1510±392.68</td>
<td>799.40±121.47</td>
<td>757.88±105.53</td>
</tr>
<tr>
<td>Blood glucose (mmol/L)</td>
<td>8.48±0.86</td>
<td>21.93±5.40</td>
<td>19.82±1.18</td>
<td>12.28±4.93</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD. ΔP<0.05 vs Normal, *P<0.05 vs Model, #P<0.05 vs Model.

**Changes in kidney morphology.** Figure 1 shows the representative photomicrographs of mesangial matrix accumulation in the HE-stained kidneys for the four groups. After 8 weeks of STZ injection, the diabetic rats showed mesangial matrix expansion in the glomeruli compared to normal control rats, and mean mesangial area was significantly increased in diabetic rats as quantification of renal pathology. Whereas AS-IV treated showed significantly ameliorated the mesangial
cells proliferation and mesangial expansion compared with the untreated diabetic rats.

**Fig.1.** AS-IV ameliorates the mesangial matrix expansion in diabetic nephropathy rats.

Representative photomicrographs of HE-stained kidney(A-D). Data are results of independent experiments in each group with four rats per group. Original magnification: ×400. A: Normal, B: Model, C: AS-IV, D: PBA.

**AS-IV reduced TM induced apoptosis in podocytes.** The TM-induced apoptotic of podocytes was confirmed by flow cytometry analysis co-stained with annexin V and propidium iodide. As showed figure.2, compared with untreated control, apoptotic events were significantly induced when podocytes treated with TM. Our data showed that AS-IV has a similar anti-apoptotic effect to PBA, it not only decreased early apoptotic events (located in the lower right quadrant of the flow cytometry diagrams), but also reduce late apoptosis (in the upper right quadrant).
Fig. 2. AS-IV exhibits anti-apoptosis on podocyte induced with TM.

Podocytes were treated for co-stained with propidium iodide (PI) and annexin V-FITC (A-V) followed by flow cytometric analysis. Dead cells refer to percentage of cells encompassing both A-V single positive and A-V/PI double-positive cells.

**AS-IV alleviates ER stress.** Compare to normal rats, phosphorylation of PERK, eIF2α and JNK were upregulated in diabetes rats, whereas total PERK, eIF2α and JNK were unchanged, GRP78 and ORP150 were increased in both of mRNA and protein levels, these results together indicated the ER stress was activated in glomeruli of diabetic rat(Fig. 3 and 5A). PBA is a low-molecular weight fatty acid and has been well proved restoring ER function, our study also showed that URP was blocked after treated with PBA. Interestingly, After treated with 8 weeks of AS-IV, the activation of URP in rat was blunted, the phosphorylated level of PERK, eIF2α and JNK level were reduced compared to untreated rats, AS-IV also reduced the express of GRP78 and ORP150 both in mRNA and protein level, all these properties were resemble to the treatment of PBA.

We also examined the effects of AS-IV on ER stress in podocytes induced with TM(Fig. 4 and 5B). Our results showed that the expression of ORP150 and GRP78 were increased, phosphorylated of PERK, eIF2α and JNK level were upregulated in podocytes treated with TM, that meant podocytes were induced to ER stress with TM. However, the potency of TM on ER stress was blocked when cells were pretreated
with AS-IV or PBA. Our findings demonstrated that ORP150 and GRP78 were inhibited both on mRNA and protein levels when cells were treated with AS-IV, in the same time, the expression of phosphorylated PERK, eIF2α and JNK level were downregulated when cells were treated with AS-IV. We therefor presume that the renoprotection effect of AS-IV was associated with inhibition of the ER stress on podocytes.

**Fig. 3.** AS-IV alleviates ER stress on DN rats induced with STZ. Representative Western blot analyses of total and phosphorylated PERK, total and phosphorylated eIF2α, total and phosphorylated JNK and expression of GRP78, ORP150. Mean ±SEM, N=6 per group. △P<0.05 vs Normal, *P<0.05 vs Model, #P<0.05 vs Model.

Representative Western blot analyses of total and phosphorylated PERK, total and phosphorylated eIF2α, total and phosphorylated JNK and expression of GRP78, ORP150. Mean ±SEM, N=6 per group. △P<0.05 vs Normal, *P<0.05 vs Model, #P<0.05 vs Model.
Fig. 4. Astragaloside IV alleviates ER stress on podocytes treated with TM.

Representative Western blot analyses of total and phosphorylated PERK, total and phosphorylated eIF2α, total and phosphorylated JNK and expression of GRP78, ORP150. Mean ±SEM, N=6 per group. \( ^\triangle P<0.05 \) vs Normal, \( ^* P<0.05 \) vs Model, \( ^\# P<0.05 \) vs Model.
Fig.5. Astragaloside IV decreased the expression of GRP78 and ORP150.

The expression of GRP78 and ORP150 mRNA in rat injected with STZ to induced diabetes (A). The expression of GRP78 and ORP150 mRNA in podocytes treated with TM (B). Mean ±SEM, N=6 per group. △P<0.05 vs Normal, *P<0.05 vs Model, #P<0.05 vs Model.
DISCUSSION

The endoplasmic reticulum (ER) folds and modifies proteins to maintain homeostasis, however, aberrant metabolic conditions such as hyperlipidemia, hyperglycemia, and reactive oxygen species can differentially affect ER and activate the unfolded protein response (UPR), excessive and long-term upregulation of UPR resultant ER stress. A number of researches have documented the activation of ER stress in the kidney both in mammalian model of diabetes and in patients of established diabetes. In streptozotocin (STZ)-treated rats, the expression of GRP78 was increased both in glomerular and tubular cells, ER stress marker CHOP and JNK were upregulated. Study also showed that compared to 9 month old mice and controls, the expression of BiP, CHOP, p-PERK, and p-eIF2α were increased in 22 month old mice induced to diabetic kidneys. Moreover, microarray studies of biopsies obtained from patients of established DN demonstrate higher expression of glucose-regulated protein 78 (GRP78 or BiP), oxygen-regulated protein 150 (ORP150) and XBP-1 compared with mild diabetes. In this study, we authenticated that ER stress were activated in DN rat induced with STZ, whereas AS-IV showed the potential biological effect of ameliorating the renal injury, our research demonstrated that these effects may relate to its potency of alleviating ER stress, such potential benefits could be verified in podocytes induced to ER stress with TM. To the best of our knowledge, this is the first study demonstrating that AS-IV attenuated DN in diabetic rats through improving ER stress.

Persistent proteinuria is a strong prognostic indicator for progression of DN, furthermore, proteinuria and hyperglycemia may generate reactive oxygen species and require a marked increase in the synthesis of membrane proteins in the kidney, which may result in ER stress in kidney. In this study, we found AS-IV not only restored the functional abnormality of DN, but also decreased urinary albumin excretion in diabetic rat. However, AS-IV showed no effect on blood glucose, that is difference to PBA, a chemical chaperones which has been approved for renoprotection and hypoglycemia effect, we therefor presumed that the renoprotection potency of AS-IV
may independent of the hypoglycemia effect.

Diabetic glomerulosclerosis is defined by increase in glomerular extracellular matrix, that is mainly synthesized by mesangial cells and leading to renal dysfunction in diabetes patients. Our research showed that AS-IV significantly ameliorate mesangial expansion and deposit of extracellular matrix in diabetic rat, we therefore proved that AS-IV attenuate ECM accumulation in DN.

Accumulating evidence indicates that ER stress contributes to glomerular diseases with proteinuria, including puromycin aminonucleoside nephrosis, protein overload, and experimental and human DN. Studies also have showed that both the pathway of PERK/ eIF2α and IRE1/JNK were activated in DN. Several studies assessed the presence of ER stress markers in human kidney biopsies, they found that such as MCD, FSGS, membranous nephropathy and DN were associated with increased podocyte ORP150/HYOU1 and GRP78 expression. On the other hand, chemical chaperones, which reduce misfolded proteins and thereby mitigate ER stress, have been shown to ameliorate STZ-induced DN. In the study we found that AS-IV significantly alleviate the ER stress in STZ-treated DN rat, it not only blocked the activation of PERK/eIF2α and IRE1/JNK pathway, but also downregulated GRP78 and ORP150. In view of the important role of ER stress in the pathyology of kidney impair, we therefor presume that the renoprotection effect of AS-IV can ascribe to its biological effect of attenuating ER stress.

The podocyte plays a crucial role in maintenance the permeability properties of the glomerular filtration barrier, damage of the podocyte may result to proteinuria, which is a hallmark of glomerular diseases. Studies have demonstrated that ER stress is induced in podocytes from an advanced DN model mouse, the decline in protein-folding capacity in podocytes triggers cellular dysfunction which in turn lead to derangement of the structure of slit diaphragms and thereby causes proteinuria. In the present study, we first showed that AS-IV significantly prevented podocytes from apoptosis induced with TM, our research demonstrated that the antiapoptosis property of AS-IV related with its biologic activity of alleviated ER stress, we also found that AS-IV can deduced the phosphorylation of PERK and eIF2α, all of them are upstream
of CHOP- a mainly mediator of ER stress induced apoptosis\textsuperscript{8,27}. Recent study showed that sustained JNK activity during prolonged ER stress may inhibit antiapoptotic members of the Bcl-2 family\textsuperscript{28}. JNK also activates proapoptotic BH3-only proteins, such as Bid and Bim\textsuperscript{29}, all these events lead to execution of the intrinsic apoptotic process\textsuperscript{30}. Our research also showed that treated with AS-IV can deduced the phosphorylation of JNK. We therefor presume that AS- IV blocked ER stress and inhibited apoptosis.

In aggregate, these studies support a new paradigm that AS-IV ameliorated the structural and functional abnormalities of STZ-induced diabetic rats through improves the ER stress. This novel finding will provide an alternative therapy for treatment of DN by targeting inhibition of ER stress.


