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Astragaloside IV ameliorates diabetic nephropathy involving protection of podocytes in streptozotocin induced diabetic rats

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ABSTRACT

Podocyte loss and dysfunction play key role during the development of diabetic nephropathy (DN). The aim of this study was to observe the protective effects of astragaloside IV on podocyte in diabetic rats and explore its mechanisms preliminary. Healthy male Sprague-Dawley (SD) rats were randomized into normal control group, diabetic nephropathy group and diabetic nephropathy with AS-IV treatment group. DN was induced by intraperitoneal injection of streptozotocin (STZ). AS-IV treatment started 2 weeks before STZ injection and lasted 14 weeks. 24 h Urinary proteins were measured 4, 8 and 12 weeks after STZ injection. Body weight, blood glucose, blood urea nitrogen (BUN), creatinine (Cr), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured 12 weeks after STZ injection. Renal pathology, podocyte morphological changes, podocyte density, protein and mRNA expression of integrin α3, integrin β1 and integrin-linked kinase (ILK) were detected by histopathology, electron microscopy, immunohistochemistry, western blot and real-time PCR, respectively. Hyperglycemia, proteinuria, mesangial expansion and podocyte loss, increased protein expression of ILK and decreased protein expression of integrin α3 and integrin β1 were detected in diabetic rats. AS-IV treatment ameliorated podocyte loss, renal histopathology and podocyte foot process effacement, decreased proteinuria, partially restored protein expression of integrin α3, integrin β1 and ILK. These findings suggested that AS-IV may protect podocyte and ameliorate diabetic nephropathy by inhibiting the expression of ILK and restoring the expression of integrin α3β1 in diabetic rats.

1. Introduction

Chronic kidney disease (CKD) has been recognized as a major public health problem of the world which develops to end stage renal disease (ESRD) irrespective of underlying causes (Khwaja et al., 2007; Zhang et al., 2008). Diabetic nephropathy is a serious complication of diabetes. The prevalence of diabetic nephropathy increased strikingly in recent decades and currently diabetic nephropathy has become the leading cause of the ESRD (Andersen et al., 1983; Stephens et al., 1990). During the progression of diabetic nephropathy podocytes injuries play the central role in the deterioration of renal function (Marshall, 2007). In normal conditions podocytes anchor at the outer side of the glomerular basement membrane (GBM) by integrin and establish the glomerular filtration barrier together with GBM and glomerular capillary endothelial cells (Drumond and Deen, 1994). During the process of glomerular injury, the foot processes of podocyte retract and broaden and then podocyte may detach from the GBM, because podocytes are highly differentiated cells with almost no capability to undergo cell division (Pagtalunan et al., 1997). The consequence of podocyte detachment is the irreversible reduction of podocyte number and the failure to cover the outer side of the GBM completely by remaining podocytes. Glomerular parietal epithelial cells may adhere to the naked areas of GBM and lead to segmental glomerular sclerosis (Kriz et al., 1995, 1998).

Integrin-linked kinase is an intracellular serine/threonine kinase that interacts with many integrins (Hannigan et al., 1996). ILK has been reported to be involved in the regulation of cellular activities and signaling pathways including cell adhesion (Dedhar et al., 1999; Wu, 1999). Previous studies showed that ILK dysregulation was involved in podocyte injury of DN (Guo et al., 2001). *Astragalus membranaceus* (Fisch) Bge is a widely used herb for the treatment of cardiovascular diseases, kidney diseases and diabetes in Chinese traditional medicine for centuries (Ai et al., 2008; Rios and Waterman, 1997). AS-IV is one of the main active ingredients

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of *Astragalus membranaceus* (Fisch) Bge and has been reported to have many pharmacological activities. 3-0-Beta-D-xylopyranosyl-6-0-beta-D-glucopyranosylcycloastra-genol is the chemical name of AS-IV. AS-IV could synergize with ferulic acid to inhibit renal tubulointerstitial fibrosis in rats with obstructive nephropathy (Meng et al., 2011) and reduce ischemic acute kidney injury in rats (Tan et al., 2013). Our previous study showed that AS-IV could improve high glucose-induced cell adhesion dysfunction in cultured mouse podocytes (Chen et al., 2008). In this study we will observe the effect of AS-IV on expression of α3β1 integrin and ILK and investigate their correlations with diabetic podocyte injury in vivo.

2. Materials and methods

2.1. Drug preparation

Astragaloside IV ($C_{41}H_{68}O_{14}$, molecular weight=784, CAS no. 84687-43-4) purchased from Xi'an Sobeo Pharmaceutical Technology Company, Limited (Xi'an, China) was suspended in 1% carboxymethyl cellulose (CMC) solution and was given to the diabetic rats by oral gavage with 3 different dosages as described before (Gui et al., 2012).

2.2. Animal study and experimental design

Healthy male Sprague-Dawley (SD) rats weighing 180–200 g were purchased from Experimental Animal Center, Zhejiang University, Zhejiang province, China. They were housed in a room with air-conditioned temperature at 23 ± °C and alternating 12 h cycles of light and dark. Animals were fed with standard diet and free to water. The rats were randomly divided into five groups (n=8/each group): (1) normal control rats (NC), (2) diabetic rats (DN), (3) diabetic rats treated with low dose of AS-IV at 2.5 mg/kg/d (DN+AL), (4) diabetic rats treated with moderate dose of AS-IV at 5 mg/kg/d (DN+AM) and (5) diabetic rats treated with high dose of AS-IV at 10 mg/kg/d (DN+AH). Diabetes mellitus was induced by single intraperitoneal injection of streptozotocin (STZ) diluted with 0.1 M citrate buffer (pH 4.5). The STZ dosage was 65 mg/kg. Normal control rats were intraperitoneal injected with equal volume of vehicle. Forty eight hours after STZ injection, tail vein blood glucose was measured. Rats with blood glucose beyond 300 mg/dl were considered as diabetic rats. AS-IV treatment started 2 weeks before STZ injection and lasted 14 weeks. Rats of AS-IV treatment groups were given AS-IV suspended in 1% CMC by oral gavage once daily. Normal control rats were given the same volume of CMC. At the end of 4, 8 and 12 weeks after STZ injection, 24 h urine of each group were collected, centrifuged at 800g for 10 min at 25 °C and stored at −80 °C. Urinary protein was assayed by pyrogallol red colorimetric assay kit according to the instructions of the manufacturer (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). At the end of 12th week after STZ injection rats were weighed and then sacrificed. Blood samples were collected from the abdominal aorta. Blood glucose, blood urea nitrogen (BUN), creatinine (Cr), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by automatic biochemistry analyzer (Hitachi Model 7600, Japan). The kidneys were collected and cut into pieces for histopathology, electron microscopy, immunohistochemistry, western blot and real-time PCR analysis. All the work was performed according to the "Guide for the Care and Use of Laboratory Animals" published by the Zhejiang University and was approved by the Animal Ethics Committee of Zhejiang University, Zhejiang province, China.

2.3. Histology

The kidneys were fixed with 10% neutral buffered formalin and embedded in paraffin, cut into 4 μm sections for hematoxylin and eosin, Periodic Acid-Schiff and Masson staining. Mesangial matrix expansion in the glomeruli was evaluated in PAS-stained sections using Image-Pro Plus 4.5 (So et al., 2013). The mean percent area of PAS-stained glomeruli was calculated for 20 randomly selected fields of each kidney section. The glomerulosclerosis in each glomerulus was scored semi-quantitatively as follows: 0, no sclerosis; 1, sclerosis in <25% of glomerulus; 2, sclerosis in 25–50% of glomerulus; and 3, sclerosis in >50% of glomerulus (Fujihara et al., 2000). To evaluate interstitial fibrosis, 20 fields for each section were assessed on Masson-stained sections. Semi-quantitative analysis in each field was assessed as follows: 0, no fibrosis; 1, fibrosis less than 10% of areas; 2, fibrosis 10% to approximately 25% of areas; 3, fibrosis 25% to approximately 50% of areas; and 4, fibrosis more than 50% of areas. The averages of interstitial fibrosis scores were calculated from the total evaluated interstitial lesions in each section. The pathologic changes were assessed by a renal pathologist who was blinded to this study.

2.4. Immunohistochemistry

Wilm's Tumor 1 protein (WT1) is a characteristic protein of podocyte and is necessary for podocyte maturation. In scientific researches WT1 is commonly used as molecular marker of podocyte (Michaud and Kennedy, 2007; Su et al., 2010). In this study we used WT1 for the analysis of podocytes density (podocyte numbers per glomerulus) by immunohistochemistry under 4 μm paraffin-embedded sections. Rabbit anti-WT1 antibody (Santa Cruz, USA) was used as the first antibody. The color was visualized by diaminobenzidine and counterstained with hematoxylin. The WT1 signal was quantified by light microscope with image analyzer. Podocyte density was expressed as a percentage of WT1 immunostained area occupied by total glomerular area. Twenty consecutive glomerular sections and an average of 20 glomeruli per rat were observed.

2.5. Electron microscopy studies

Electron microscopic sections were performed by routine procedures. Renal cortex was cut into pieces on ice, fixed with 2.5% glutaraldehyde dissolved in 0.1 M sodium cacodylate (pH 7.4) at 4 °C overnight and washed in the same buffer. The tissue fragments were postfixed in 1% cacodylate-buffered OsO_4 for 2 h, dehydrated, and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined by electron microscopy. The number of podocyte foot processes present in each micrograph was divided by the total length of GBM regions in each image to determine the average density of podocyte foot processes. The electron microscope photos were evaluated in a blind fashion.

2.6. Western blotting

Kidney cortex was homogenized in lysis buffer on ice with a homogenizer. The supernatants were collected after centrifuging at 10,000 rpm for 5 min at 4 °C. Protein concentration of the supernatants was measured by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). The whole tissue lysates were mixed with equal amount of 2× SDS loading buffer (125 mmol/l Tris–HCl, 4% SDS, 20% glycerol, 100 mmol/l dithiothreitol, and 0.2% bromphenol blue). Samples were separated by 10% sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis and electro-transferred to a polyvinylidene difluoride (PVDF)