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Original Article

Astragaloside IV alleviates puromycin aminonucleoside-induced podocyte cytoskeleton injury through the Wnt/PCP pathway

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Abstract: Podocyte injury is a common cause of massive proteinuria. Astragaloside IV (AS-IV) has been reported to protect podocytes in diabetic models. However, the effects and potential mechanism of AS-IV on puromycin aminonucleoside (PAN)-induced podocyte injury remains unclear. The aim of the present study was to investigate the protective effect of AS-IV on PAN-induced podocyte injury both *in vivo* and *in vitro*. *In vivo*, we induced a podocytic-injury model in rats via a single tail vein injection of PAN. The rats in the treatment group received AS-IV intragastrically (i.g.) at a dose of 40 mg/kg/day for 10 days. At the end of the experiment, 24 h urine, serum and kidney samples were collected for examination. *In vitro*, we injured podocytes with 30 μg/ml PAN and treated them with AS-IV at concentrations of 5, 25 and 50 μg/ml. Next, we analyzed podocyte protein expression and the Wnt/planar-cell polarity (PCP) pathway using western blot and immunofluorescence (IF). Our results showed that AS-IV decreased proteinuria in PAN-injured rats, and restored specific protein expression in podocytes. In PAN-induced injuries to human podocytes, AS-IV restored the expression and distribution of F-actin and synaptopodin, and repaired the morphology of the actin-based cytoskeleton. Notably, AS-IV could activate the Wnt/PCP pathway by promoting expression of Wnt5a, protein tyrosine kinase 7 (PTK7), Rho-associated coiled-coil-containing protein kinase 1 (ROCK1), Ras-related C3 botulinum toxin substrate 1 (Rac1) and phospho-SAPK/JNK (Thr183/Tyr185) (p-JNK) *in vivo* and *in vitro*. In conclusion, we demonstrated that AS-IV alleviated PAN-induced injury to the podocyte cytoskeleton, partially by activating the Wnt/PCP pathway.

Keywords: Astragaloside IV, podocyte, cytoskeleton, planar-cell polarity pathway, puromycin aminonucleoside

Introduction

Podocytes are highly differentiated epithelial cells anchored to the outer surface of the glomerular basement membrane (GBM). They are essential for maintaining the integrity of the glomerular filtration barrier [1]. Podocytes are targets of injury in various renal diseases, particularly proteinuric glomerular diseases such as minimal-change disease (MCD) and focal segmental glomerulosclerosis (FSGS) [2]. Podocytopathy, first defined by Martin R Pollak [3], is the process induced by physiological stress and pathological states; it varies from disruptions of the actin cytoskeleton [4, 5], to loss of slit-diaphragm (SD) proteins and structural integrity, to subsequent foot process effacement and detachment of podocytes from the GBM or apoptosis [6, 7]. Therefore, maintaining and repairing the structure and function of podocytes is crucial in treating proteinuric glomerular diseases.

The planar cell polarity (PCP) pathway is regarded as a noncanonical Wnt signaling pathway because it is activated by Wnt5a or Wnt11, but does not depend on beta-catenin [8]. The pathway was discovered in *Drosophila* wings [9], in which several core PCP genes, including *Frizzled*, *Van Gogh*, *Dishevelled* and *Fat*, were revealed by subsequent studies [10-15]. In vertebrates, PCP controls directional cell polarity

in the plane of a tissue, where polarity information is transmitted between neighboring cells [16]. In mammals, PCP regulates formation of actin-based filopodia in neuroepithelial cells, promoting the cells to move directionally during neural-tube closure [17]. In a variety of cell systems, the PCP pathway regulates cytoskeleton arrangement, in which asymmetric localization of mutually antagonistic core proteins results in redistribution of actin-based cytoskeletal filaments and orientation of cellular protrusions [18]. Sima Babayeva et al. have reported that the PCP pathway can regulate actin rearrangement, cell morphology, motility, and nephrin distribution in podocytes [19, 20]. Therefore, we wished to explore the role of this pathway in the repair of injured podocytes.

Astragaloside IV (AS-IV) is a bioactive saponin extracted from the medicinal plant *Astragalus membranaceus*, which has been widely used to treat renal diseases in traditional Chinese medicine. Previous studies have proven that AS-IV exerts anti-oxidative-stress and anti-apoptosis effects on both podocytes and renal tubular epithelial cells (RTECs), and also prevents mesangial-cell proliferation in diabetic nephropathy experimental models [21-25]. However, the protective effects of AS-IV in a puromycin aminonucleoside (PAN)-induced podocytic-injury model and the underlying mechanisms thereof remain unknown. The aim of this study was to test AS-IV's protective effects on injured podocytes and evaluate its potential mechanism.

Materials and methods

Reagents and antibodies

We purchased AS-IV (purity > 98%) from Chengdu Conbon Biotech Co., Ltd. (Chengdu, China) and PAN from MedChemExpress (Monmouth Junction, New Jersey, USA). We purchased the following primary antibodies: rabbit anti-nephrin antibody (nephrin, ab58968) and rabbit anti-nephrosis 2 (NPHS2) antibody (podocin, ab50339; Abcam, Cambridge, UK); rabbit anti-synaptopodin and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Proteintech, Wuhan, China); and rabbit anti-Wnt5a antibody (Wnt5a #2530), rabbit anti-rho-associated, coiled-coil-containing protein kinase 1 (ROCK1) antibody (ROCK1 #4035), rabbit anti-protein tyrosine kinase 7 (PTK7)/cholecystokinin tetrapeptide (CCK4) antibody (PTK7 #25618), rabbit anti-Ras-related C3 botulinum toxin substrates 1, 2 and 3 (Rac1/2/3) antibody (Rac1 #2465), and rabbit anti-phospho-SAPK/JNK (Thr183/Tyr185) antibody (p-JNK, #9251; Cell Signaling Technology) (CST; Danvers, Massachusetts, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies against mouse or rabbit immunoglobulin G (IgG) were purchased from Life Technologies (Carlsbad, California, USA).

Cell culture

Professor Moin A Saleem of Southmead Hospital, University of Bristol (Bristol, UK), kindly provided us with conditionally immortalized AB8/13 human podocytes. We proliferated them in Roswell Park Memorial Institute (RPMI)-1640 medium (GIBCO, Thermo Fisher Scientific, Waltham, Massachusetts, USA) containing 10% fetal bovine serum (FBS), 1% insulin-transferrin-selenium (ITS) supplement (Life Technologies) and penicillin/streptomycin (100 U/ml and 100 mg/ml, respectively; GIBCO) at 33°C in a 5% CO_2 atmosphere. When cell density reached 60%, we switched the podocytes to a 37°C incubator for 12-14 days until they differentiated.

Treatment with PAN and AS-IV

PAN and AS-IV powder were dissolved in dimethyl sulfoxide (DMSO); the concentration of DMSO was < 0.1% in cell culture medium. We then diluted the PAN solution to a concentration of 30 μg/ml with cell culture medium and used it to stimulate the podocytes for 48 h in accordance with methods from previous studies [26]. AS-IV was added to the culture medium at concentrations of 5, 25 and 50 μg/ml. The differentiated podocytes were divided into five groups to detect a potential protective role of AS-IV on PAN-injured podocytes. These were a control group, a PAN-only group, and three groups treated with 5, 25 or 50 μg/ml AS-IV, which was administered simultaneously with PAN for 48 h. Additionally, 25 μg/ml AS-IV was used to investigate underlying mechanism that AS-IV protects PAN-injured podocytes.

Animal model and experimental design

All work with rats was approved by the Animal Ethics Committee of Guangzhou University of

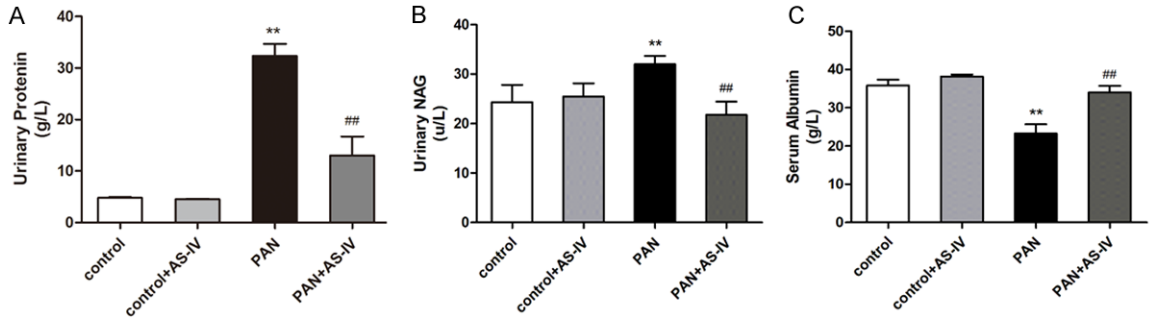

Figure 1. AS-IV ameliorated proteinuria, urinary NAG and ALB abnormalities in PAN-injured rats. AS-IV reduced levels of urinary protein (A), decreased those of urinary NAG (B) and restored ALB levels (C) compared with PAN-treated rats. Control, normal rats; control+AS-IV, normal rats administered AS-IV at 40 mg/kg/day i.g.; PAN, PAN-treated rats; PAN+AS-IV, AS-IV administered i.g. starting at the same time as PAN injection. Data are presented as means ± SEM (n = 6 per group; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* PAN group).

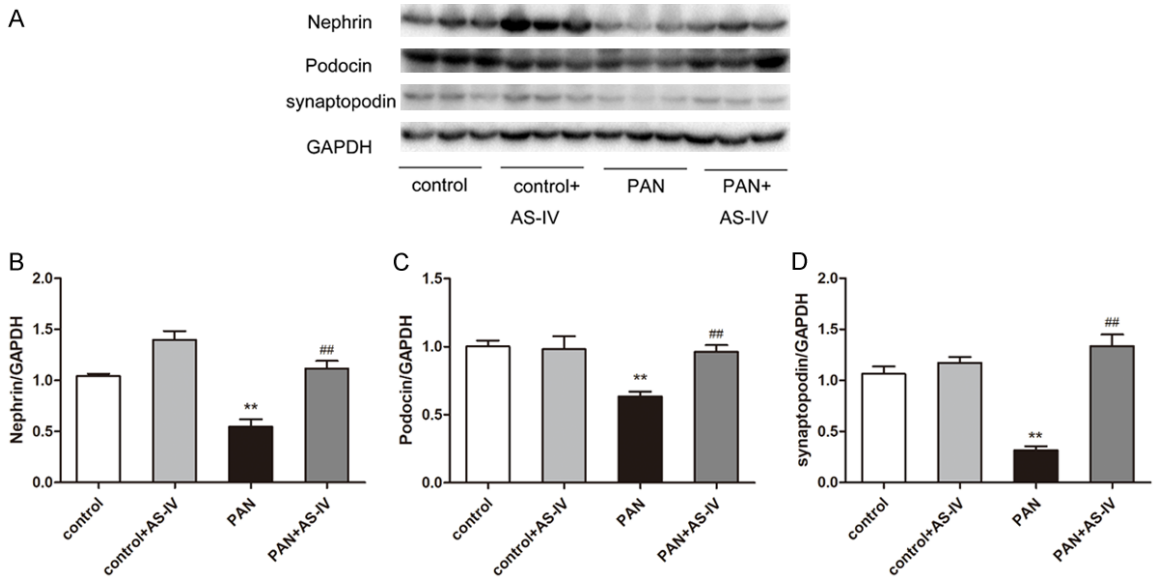

Figure 2. Expression of nephrin, podocin and synaptopodin in podocytes of rats subjected to various treatments. A. Representative western blot images indicate that specific SD and cytoskeletal protein expression was significantly decreased in the PAN group, but restored by AS-IV treatment. B. Densitometric analysis of nephrin expression normalized to GAPDH content. C. Densitometric analysis of podocin expression normalized to GAPDH content. D. Densitometric analysis of synaptopodin expression normalized to GAPDH content. Data are presented as means ± SEM (n = 5 per group; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* PAN group).

Chinese Medicine (Guangzhou, China) and was performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH; Bethesda, Maryland, USA). We purchased 32 healthy male Wistar rats weighing 150-180 g from the Experimental Animal Center of Southern Medical University (Guangzhou, China). They were housed in a specific pathogen-free (SPF) animal facility with alternating 12-h light-dark cycles at the Center for Disease Control and Prevention, Shenzhen, China, and fed a standard diet plus water ad libitum. We induced our rat model via a single tail vein injection of PAN (5 mg/100 g body weight [BW]). Then rats were randomly divided into four groups: (1) normal rats (n = 8), (2) normal rats+AS-IV treatment (n = 8), (3) PAN treatment (n = 8), (4) PAN+AS-IV treatment (n = 8). In the treatment groups, we started AS-IV (40 mg/kg/day) by intragastric administration (i.g.) the same day as PAN injection. At day 11, 24-h

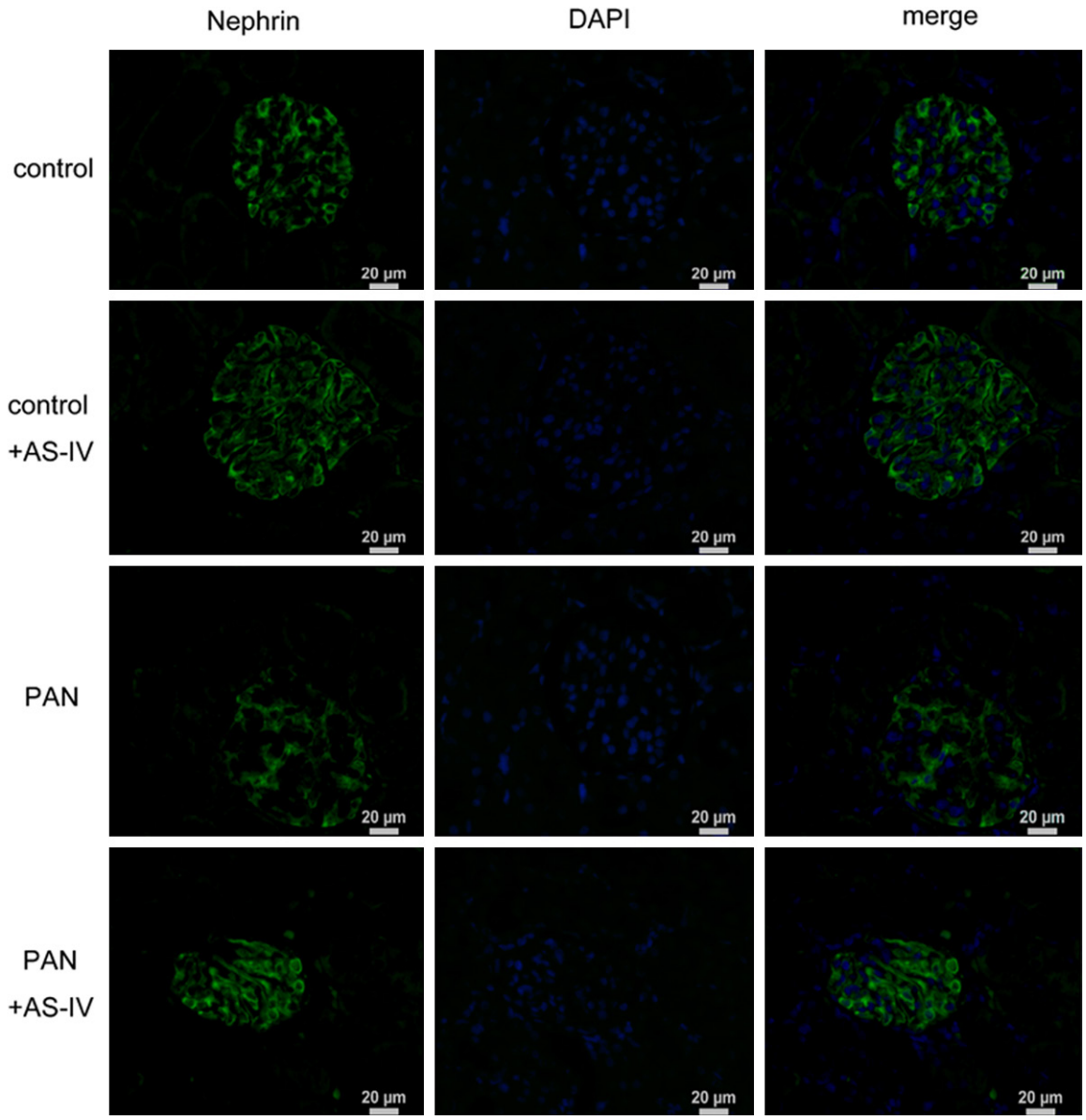

Figure 3. Representative IF staining images of nephrin in podocytes of rats subjected to various treatments. AS-IV restored nephrin expression in PAN-injured glomeruli. All images are shown at ×400 magnification; scale bar = 20 μm.

urine samples were collected using metabolic cages. We then anesthetized the rats with pentobarbital sodium (50 mg/kg BW via intraperitoneal [i.p.] injection), took serum for biochemical analysis, and removed and preserved kidneys for western blotting and immunofluorescence (IF) analyses.

Biochemical analysis

We examined levels of serum creatinine (SCr), blood urea nitrogen (BUN), serum albumin (ALB), urinary total protein (UTP), and urinary N-acetyl-β-D-glucosaminidase (NAG) using a BS-180 Automatic Biochemistry Analyzer (Mindray Bio-Medical Electronics Co., Ltd., Shenzhen, China) per the manufacturer's instructions.

IF analysis

Renal tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned. We then deparaffinized and dehydrated the sections, retrieved their antigens, and blocked the sections with 5% bovine serum albumin (BSA)

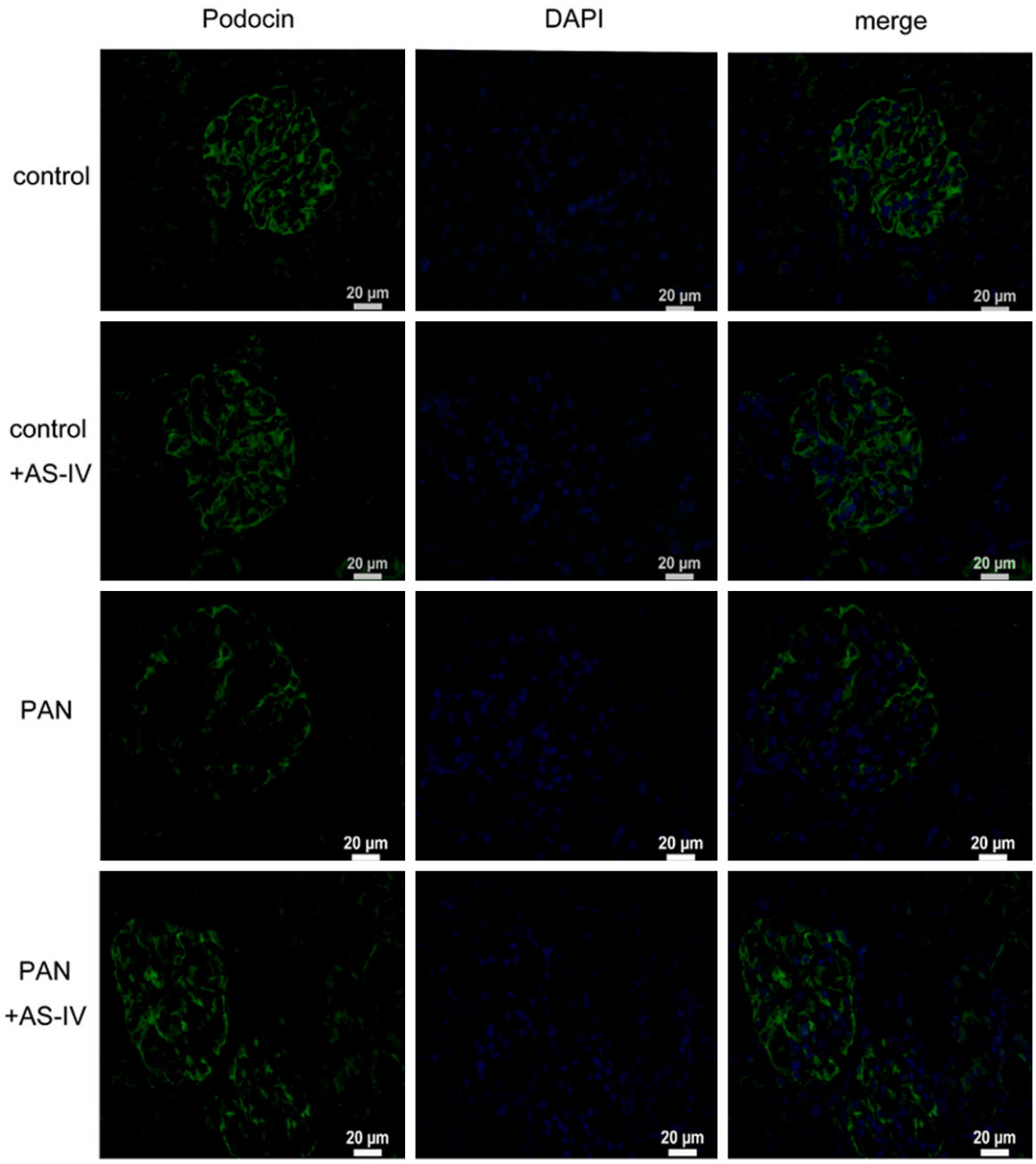

Figure 4. Representative IF staining images of podocin in podocytes of rats subjected to various treatments. AS-IV restored podocin expression in PAN-injured glomeruli. All images are shown at ×400 magnification; scale bar = 20 μm.

plus 10% normal goat serum at room temperature (RT) for 1 h. The cultured podocytes received different treatment *in vitro*: they were fixed in 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) for 5 min, and then blocked. Primary antibodies were incubated overnight at 4°C, followed by appropriate secondary antibodies. We used 4',6-diamidino-2-phenylindole (DAPI) to stain nuclei. F-actin was directly stained with Alexa Fluor 594 phalloidin (Thermo Fisher), blocked with 1% BSA-PBS for 30 min, and then stained again with phalloidin for 20 min. In all cases, we used antibody-negative controls to ensure true-positive staining. The results were imaged with a fluorescence microscope (Nikon Corp., Tokyo, Japan).

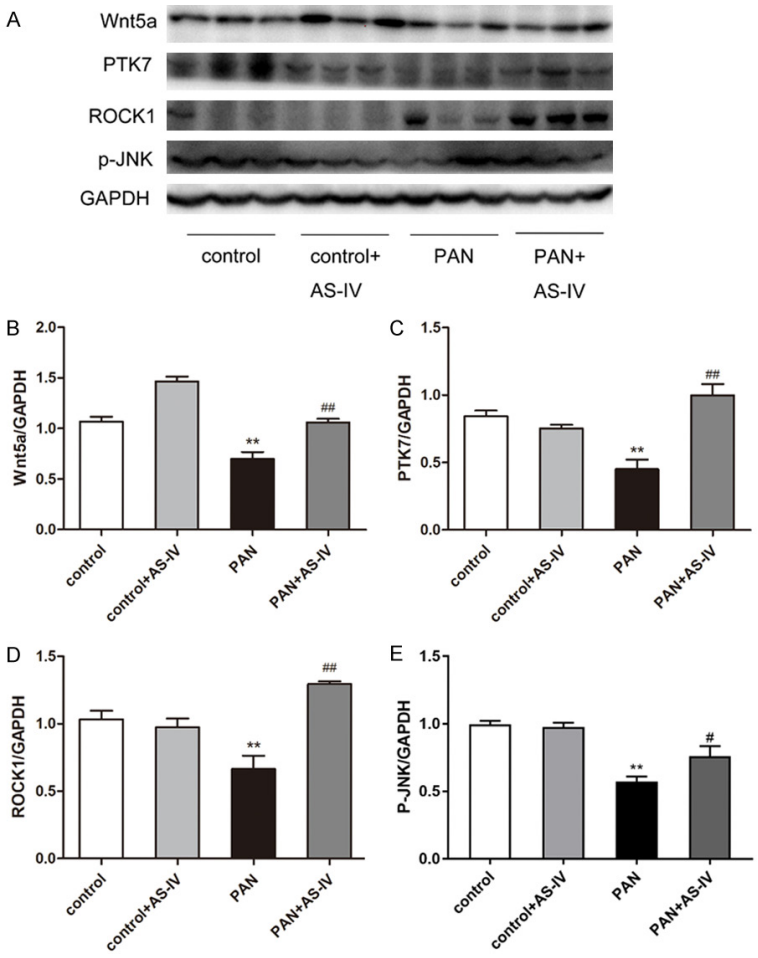

Figure 5. Expression of Wnt5a, PTK7, ROCK1 and p-JNK in renal cortex of rats subjected to various treatments. A. Representative western blot images indicate that expression of Wnt5a, PTK7, ROCK1 and p-JNK was significantly elevated by AS-IV treatment after PAN stimuli. B. Densitometric analysis of Wnt5a expression normalized to GAPDH content. C. Densitometric analysis of PTK7 expression normalized to GAPDH content. D. Densitometric analysis of ROCK1 expression normalized to GAPDH content. E. Densitometric analysis of p-JNK expression normalized to GAPDH content. Data are presented as means ± SEM (n = 5 per group; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* PAN group).

Western blotting

We separated the extracted protein samples from cultured podocytes or rat renal tissues using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred them to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked for 1 h in 5% nonfat dry milk (Bio-Rad Laboratories, Hercules, California, USA) which was dissolved in tris-buffered saline plus Tween 20 (TBS-T) and incubated overnight at 4°C with primary antibodies. We then incubated them in HRP-conjugated secondary antibodies at RT for 1 h. HRP activity was viewed using Clarity Western Electrochemiluminescence (ECL) Substrate (Millipore) and a ChemiDoc MP Imaging System (Bio-Rad). We used Image Lab software version 5.1 for densitometric analysis (Bio-Rad).

Statistical analysis

All data are reported as the mean ± standard error of the mean (SEM). We compared continuous variables between > two groups using one-way analysis of variance (ANOVA), and evaluated differences between groups using the least-significant-difference (LSD) test or the Games-Howell test. $P < 0.05$ was considered statistically significant. We used SPSS software version 25.0 (IBM Corp., Armonk, New York, USA) for all statistical analyses.

Results

AS-IV reversed levels of proteinuria, urinary NAG and ALB in PAN-injured rats

Metabolic parameters related to renal function after ten days of drug treatment in the rats are shown in **Figure 1**. Rats in the PAN group had higher urinary-protein levels (control *vs.* PAN *vs.* PAN+AS-IV, respectively 4.79 ± 1.33 *vs.* 32.34 ± 2.34 *vs.* 13.03 ± 3.70 g/L; *vs.* the PAN group, $P < 0.01$), higher NAG levels (control *vs.* PAN *vs.* PAN+AS-IV, respectively 24.35 ± 3.47 *vs.* 32.02 ± 1.66 *vs.* 21.78 ± 2.66 u/L; *vs.* the PAN group, $P < 0.01$), and lower ALB levels (control *vs.* PAN *vs.* PAN+AS-IV, respectively 35.87 ± 1.50 *vs.* 23.28 ± 2.41 *vs.* 34.02 ± 1.70 g/L; *vs.* the PAN group, $P < 0.01$), which could be reversed by AS-IV treatment. These data suggested that AS-IV was beneficial in reducing urinary-protein excretion and increasing ALB in PAN-injured rats.

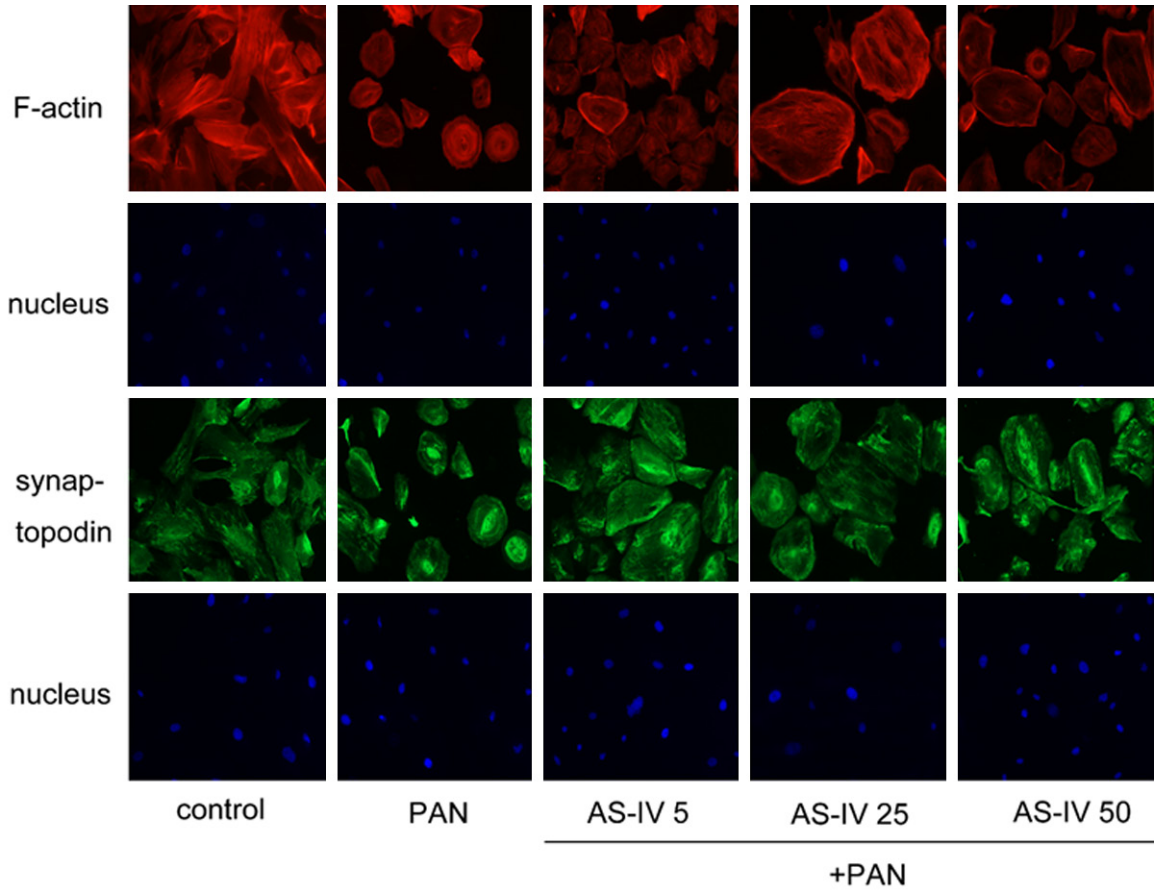

Figure 6. Representative IF images of F-actin and synaptopodin in cultured human podocytes subjected to various treatments. Compared with the PAN-injured podocytes, AS-IV (5, 25 or 50 μg/ml) ameliorated the expression and distribution of F-actin and synaptopodin, increased the number of stress fibers, and repaired the morphology of the actin-based cytoskeleton. All images are shown at ×100 magnification.

AS-IV upregulated SD and cytoskeleton specific protein expression in podocytes of PAN-injured rats

Both nephrin and podocin are known as SD-specific proteins, and synaptopodin is an actin-associated protein in podocyte foot processes [27]. In our study, expression of podocin, nephrin and synaptopodin was inhibited after PAN-induced injury, but was upregulated after treatment with AS-IV (**Figures 2-4**). These results indicated that AS-IV could improve PAN-injured podocytes.

AS-IV activated the PCP signaling pathway in PAN-injured rats

The PCP pathway controls directional cell polarity by regulating asymmetric redistribution of core PCP proteins, which in turn leads to redistribution of actin-based cytoskeletal filaments. Wnt5a is known as a ligand that binds to a *Frizzled* receptor or PTK7 co-receptor to initiate the PCP pathway. Our results demonstrated that PAN downregulated Wnt5a expression compared with the control group. However, AS-IV notably enhanced Wnt5a expression compared with the PAN group. Meanwhile, expression of PTK7 co-receptor as well as the downstream molecule ROCK1 and p-JNK in the PCP pathway were also significantly increased in the AS-IV treatment group (**Figure 5**). These findings implied that AS-IV might activate the PCP signaling pathway to redistribute disruptions of the podocyte actin cytoskeleton after PAN injury.

AS-IV restored cytoskeleton-associated protein expression and distribution in PAN-injured human podocytes

As a disrupted actin cytoskeleton is characteristic of podocyte injury, we assessed cytoskeleton-dependent morphological changes to hu-

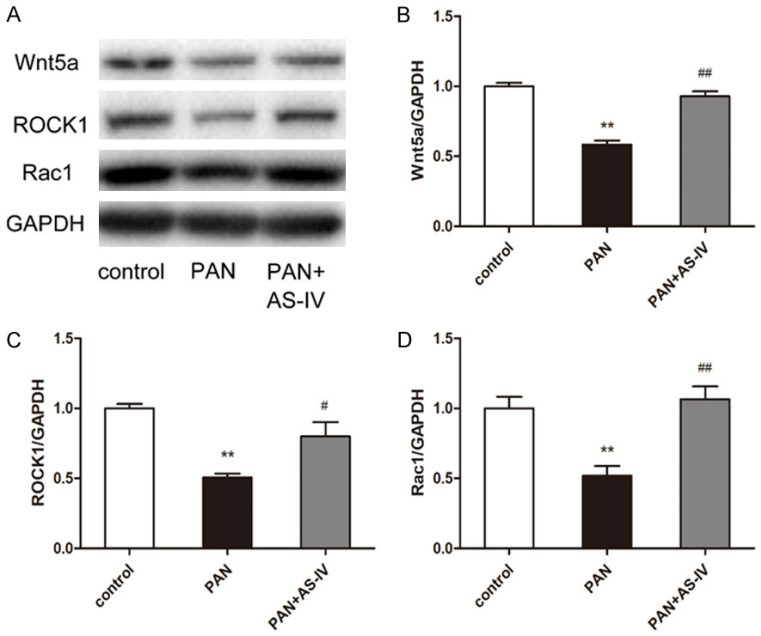

Figure 7. Expression of Wnt/PCP pathway protein in cultured human podocytes subjected to various treatments. A. Representative western blot images indicate that expression of Wnt5a, ROCK1, and Rac1 in the Wnt/PCP pathway was decreased in the PAN group, but significantly improved by AS-IV treatment with 25 μg/ml. B. Densitometric analysis of Wnt5a expression normalized to GAPDH content. C. Densitometric analysis of ROCK1 expression normalized to GAPDH content. D. Densitometric analysis of Rac1 expression normalized to GAPDH content. Data are presented as means ± SEM from 3 independent experiments ($^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* PAN group).

man podocytes after PAN stimulation. Our results showed that PAN caused abnormalities in F-actin and synaptopodin expression and distribution (**Figure 6**). However, treating podocytes with AS-IV improved the expression and distribution of F-actin and synaptopodin, increased the number of stress fibers, and repaired actin-based cytoskeleton morphology.

AS-IV enhanced protein expression of the PCP signaling pathway in PAN-injured human podocytes

To investigate whether the aforementioned changes were consistent *in vivo* and *in vitro*, we also assessed the protein expression of the PCP signaling pathway in human podocytes treated with PAN and AS-IV (25 μg/ml). The results showed that levels of Wnt5a ligand were reduced in the PAN group, but significantly enhanced after AS-IV treatment (**Figure 7**). Meanwhile, expression of downstream proteins of the PCP pathway, such as ROCK1 and Rac1, was also decreased in the PAN group and significantly enhanced by AS-IV treatment. These data indicated that AS-IV might repair PAN-damaged podocyte cytoskeleton by activating the PCP signaling pathway.

Discussion

Injury to podocytes, which are critical components of the glomerular-filtration barrier, can directly trigger proteinuria [1]. Astragali Radix is frequently used to treat many renal diseases in traditional Chinese medicine, and AS-IV is its most bioactive component. Therefore, in this study, we explored whether AS-IV could repair PAN-induced podocyte injury and the hypothetical mechanism of such repair.

We confirmed that AS-IV was effective in a PAN-injured rat model, reducing proteinuria while increasing ALB. These effects might be due to AS-IV's protection of the podocyte cytoskeleton. *In vivo*, it was able to restore expression of nephrin, podocin and synaptopodin, while *in vitro*, it improved distribution of the actin cytoskeleton and increased both the number of stress fibers and the expression of synaptopodin. Additionally, we confirmed that Wnt5a ligand and downstream molecules in the PCP signaling pathway were activated in the AS-IV treatment group both *in vivo* and *in vitro*.

Wnt/PCP is a developmentally conserved polarity pathway that is essential to maintaining polarized epithelial-tissue organization, convergent-extension movements, and to promoting cell migration [28]. Wnt5a is known as a typical Wnt ligand in the PCP pathway. Wnt5a-*Frizzled* binding can initiate the PCP signaling events that mediate cytoskeletal rearrangements, which in turn promote cell directional movements. PTK7 is a transmembrane protein and a known regulator of PCP signaling; it plays a key role as a Wnt co-receptor by interacting with *Frizzled7* and recruiting *Disheveled* (*Dvl*) to the plasma membrane [29, 30]. The precise

molecular cascade downstream is as follows: *Dvl* is recruited to the plasma membrane, followed by activation of the small guanosine triphosphate hydrolases (GTPases) Rho and Rac which leads to c-Jun N-terminal kinase (JNK) phosphorylation [31]. Recent studies suggest PTK7 is required for non-canonical Wnt signaling that controls convergent-extension cell movements as well as PCP in mice, zebrafish and *Xenopus* frogs [32-34]. ROCK1 is a downstream mediator of the PCP signaling pathway and is stimulated by activated Rho, which mediates cytoskeletal reorganization [35].

Podocytes are known as highly polarized glomerular epithelial cells. Babayeva et al. [19, 20] have demonstrated that the PCP pathway regulates nephrin endocytosis for remodeling of cytoskeleton and SD protein complexes during podocyte development. The core PCP protein, Van Gogh-like protein 2 (VANGL2), interacts directly with the SD protein membrane-associated guanylate kinase, WW and PDZ domain-containing protein 2 (MAGI2). Loss of VANGL2 in podocytes affects glomerular development and increases susceptibility to injury in aging glomeruli [36]. In addition, Wnt5a can directly induce actin cytoskeletal reorganization and promote migration of cultured podocytes. In our study, we found that AS-IV significantly elevated the expression of Wnt5a, PTK7, ROCK1, p-JNK and Rac1 after PAN-induced injury. These results indicated AS-IV might initiate the Wnt/PCP pathway to promote cytoskeletal rearrangements in order to resist PAN-induced cytoskeletal allostery.

Conclusions

In conclusion, the present study demonstrated that AS-IV could exert a preventive effect against PAN-induced podocyte skeleton injury. Furthermore, the underlying molecular mechanism might be associated with activation of the Wnt/PCP pathway.

Acknowledgements

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Disclosure of conflict of interest

None.

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