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Astragaloside IV improves high glucose-induced podocyte adhesion dysfunction via $\alpha_3\beta_1$ integrin upregulation and integrin-linked kinase inhibition

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ABSTRACT

Impaired podocyte adhesion to glomerular basement membrane (GBM) may contribute to podocyte detachment from GBM, which represents a novel early mechanism leading to diabetic nephropathy (DN). Here, we examined the effects of Astragaloside IV (AS-IV), a saponin purified from *Astragalus membranaceus* (Fisch) Bge, on high glucose-induced cell adhesion dysfunction in cultured mouse podocytes. Cells were seeded into 96-well plates coated with basement membrane protein complex (BMC). The cells were incubated for 12 h in media containing 30 mM glucose (HG) with 10, 50 and 100 μg/ml of AS-IV. The cells were also exposed to HG media with 100 μg/ml of AS-IV for 3, 6, 12 and 24 h. Cell adhesion assays were performed by fluorescence and centrifugation methods, respectively. Levels of mRNA were determined by quantitative reverse transcriptase real-time PCR and protein expression was analyzed by immunoblotting. HG strongly inhibited adhesion of podocytes to BMC, accompanied by reduction in $\alpha_3\beta_1$ integrin mRNA and protein expression, as well as increase in integrin-linked kinase (ILK) activity and expression. When podocytes under HG stimulation were treated with AS-IV, a dose- and time-dependent increase in cell–matrix adhesion was observed, which was significant from 10 μg/ml of AS-IV and from 6 h of incubation of AS-IV with 100 μg/ml. This was accompanied by significant increases in $\alpha_3\beta_1$ integrin mRNA and protein expression, as well as inhibition of ILK activation and overexpression. These results suggest that AS-IV improve HG-induced podocyte adhesion dysfunction, which is partly attributed to $\alpha_3\beta_1$ integrin upregulation and ILK inhibition.

1. Introduction

Diabetic nephropathy (DN) is a serious and common complication of type 1 and type 2 diabetes leading to end-stage renal disease (ESRD) in up to 30% of individuals with diabetes [1]. Recently, much work has underlined the important role of the podocyte in the development of DN [2]. In human and experimental diabetes mellitus, podocyte damage occurs at a relatively early stage, and the cells may ultimately become detached from the glomerular basement membrane (GBM) [3,4]. Moreover, the decreased number of podocytes per glomerulus was the strongest predictor of progression of DN, where fewer cells predicted more rapid progression [5]. Recent study confirmed, by direct visualization and quantitation of podocyte and GBM denudation that podocyte detachment correlated with classical DN lesions and renal function in patients with type 1 diabetes mellitus [6]. Thus, the concept of podocyte loss or detachment as a major player in the

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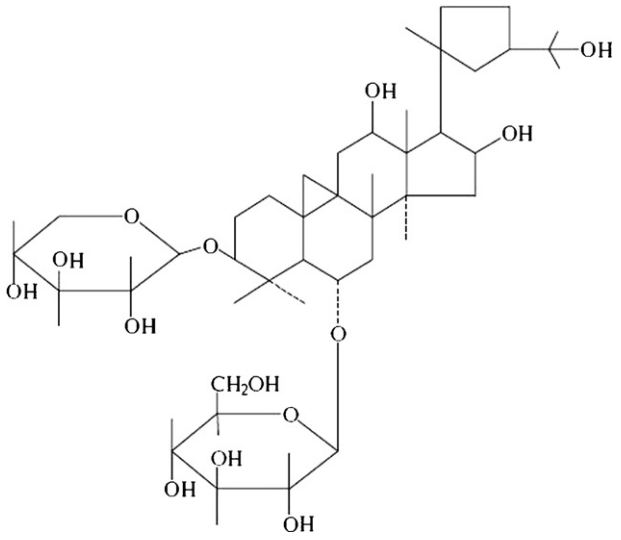

Fig. 1 – Chemical structure of Astragaloside IV ($C_{41}H_{68}O_{14}$, molecular weight = 784).

glomerulosclerotic process in diabetes is well documented. Alterations in adhesive interaction between podocytes and GBM are most likely to be critical in podocyte detachment [7]. The inhibitory effects of hyperglycemia on podocyte adhesion to GBM have been determined in both cell culture and in vivo models of diabetes mellitus [8]. One recent report demonstrated that increased glucose concentrations reduced matrix-related cell adhesion in cultured human podocytes, leading to decreased binding to the GBM components [9]. Thus, impaired adhesion to GBM may contribute to the podocyte detachment from GBM, which represents a novel early mechanism in the pathogenesis of DN [8]. The promise of this new insight is the development of novel and effective strategies for the prevention and treatment of DN.

The saponin Astragaloside IV (AS-IV; Fig. 1), a 3-*O*-β-D-xylopyranosyl-6-*O*-β-D-glucopyranosylcycloastragenol, was purified from *Astragalus membranaceus* (Fisch) Bge, which has long been widely used in traditional Chinese medicine to treat renal diseases [10]. AS-IV has been shown to reduce the blood glucose level, intensity of oxidative stress and AGEs level in DN rats [11]. AS-IV also has antioxidative effects and may prevent the development of early DN in rats [12]. However, the protective effects of AS-IV on podocyte adhesion have not been investigated yet.

This study aimed to examine the effects of AS-IV on cell adhesion in cultured differentiated mouse podocytes under high glucose stimulation and to explore its possible mechanisms.

2. Materials and methods

2.1. Cell line and culture

The immortalized mouse podocyte cell line was a generous gift from Prof. Luigi Gnudi (King's College London School of Medicine, London, UK). Cells were grown on type 1 collagen-coated dishes (BD Bioscience, Bedford, MA, USA) at 33 °C in the presence of 10 U/ml mouse recombinant interferon-γ (IFN-γ, Sigma Chemical Corporation, St. Louis, MO, USA) in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco, USA), 100 U/ml penicillin and 100 mg/ml streptomycin. To induce differentiation, podocytes were maintained at 37 °C under 5% CO_2 and 95% air without IFN-γ to allow differentiation for at least 7 days. Differentiated cells were identified by their large arborized shape and by the cell expression of synaptopodin, a known differentiation marker [13]. Differentiated podocytes were cultured for 24 h in RPMI 1640 medium containing 5 mM D-glucose and 1% FCS before being exposed to various experimental conditions.

2.2. AS-IV treatment

AS-IV was purchased from Sigma (St. Louis, MO, USA; purity above 99.8%, HPLC). The cells were divided into four groups: (1) normal glucose group (NG) as control incubated in RPMI 1640 containing 5 mM glucose; (2) high glucose group (HG) incubated in RPMI 1640 containing 30 mM glucose; (3) mannitol group (MA) incubated in NG medium supplemented with 25 mM D-mannitol (Sigma, St. Louis, MO, USA) as an osmotic control; (4) AS-IV group incubated in HG medium treated with increasing concentrations of AS-IV (10, 50 and 100 μg/ml) for 12 h and with 100 μg/ml of AS-IV for 3, 6, 12 and 24 h, respectively. All the glucose used in the present study was D-glucose. All experimental groups were cultured in quadruplicate.

2.3. Cell adhesion assay

Cell adhesion assay (Calbiochem, USA; Cat. No. CBA025) was designed for the analysis of the relative attachment of adherent cell lines to basement membrane protein complex (BMC) [14]. The kit was supplied with a 96-well strip plate coated with mouse BMC. BSA-coated wells served as a negative control and poly-L-lysine-coated wells served as a positive control. Podocytes were suspended in the desired duplicate wells of the BMC-coated 96-well plate at 3×10^4 cells/well and incubated at 37 °C under different experimental conditions. Following incubation, non-adherent cells were removed by gently washing twice with PBS and the attached cells were labelled the green fluorescent dye by adding 100 μl Calcein-AM working solution to each well. Cells were incubated for 1 h at 37 °C in culture incubator. Finally, relative cell attachment was assessed using a fluorescence plate reader at an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

The cell adhesion assay was also performed by centrifugation method as described previously [15]. Cell adhesion to protein-coated surfaces was measured using a centrifugation assay that applies controlled detachment forces. Tissue culture polystyrene 96-well plates (Corning 3595, Corning, NY, USA) were coated with BMC in deionized H_2O for 1 h at room temperature. Conditions were replicated in four separate columns on the same microplate. All wells were then blocked in 1% heat-denatured BSA for 1 h to prevent non-specific cell adhesion. Podocytes under different experi-

mental conditions were carefully aspirated to remove undetached cells and refilled with fresh PBS–dextrose for an initial count to determine the density of cells before detachment. The cells were observed under a microscope (Olympus IX70, Japan) equipped with a Hoffman Modulation Contrast system and recorded with a DVC-1310C Magnafire digital camera (Optronics). The lid was removed, and the plate was covered with sealing tape (Nalge Nunc) and centrifuged upside down at 1500 × *g* for 10 min on a Beckman Allegra centrifuger (GH 6.8 rotor) to detach the cells. The wells were carefully aspirated with a Finnpipette electronic multichannel pipettor set to the slowest flow rate and refilled with fresh PBS–dextrose for the count under the same conditions to determine the density of remaining adherent cells. Cell adhesion was determined on quadruplicate wells by counting attached cells in three different fields. Two individuals recorded cell attachment for these experiments. We normalized experiments to compare different repetitions of the assay on different days. Because of day-to-day variation in experimental setup, we normalized attachment assays by the average cell attachment/binding for that day. The adhesive capacity was measured as the ratio of adherent cells/cells before detachment.

All the adhesion assays were performed in quadruplicate and the experiment was repeated three times.

2.4. Real-time quantitative RT-PCR

Total RNA was isolated by the Trizol procedure (Invitrogen, Carlsbad, CA, USA). Then, 1 μg of total RNA was reverse transcribed using the SuperScript RT kit from Invitrogen (Carlsbad, CA, USA). The synthesized cDNA was quantified by Rotor-Gene 3000 Sequence Detection System (Corbett Research, Australia) using SYBR Green I as a double-strand DNA-specific dye, according to the manufacturer's protocol. The PCR amplification reaction mixtures (25 μl) contained 40 ng cDNA, 1× SYBR Green I, 25 mM $MgCl_2$ and 0.35 μM forward and reverse primers. Primer concentrations were determined by preliminary experiments that analyzed the optimal concentrations of each primer. The following sequence-specific oligonucleotide primers were used for PCRs: α_3 integrin sense 5′-CCCTCGCTTTGTAGGTTA-3′ and anti-sense 5′-GTCCCTGTCAGCCTCCAC T-3′; β_1 integrin sense 5′-GTTCCATGCGTAGCGACAA-3′ and anti-sense 5′-TTCTCC-CTGCTTTCCACTTTAG-3′; integrin-linked kinase (ILK) sense 5′-CATCAATGCAGTGAATGAGC-3′ and anti-sense 5′-GACATT-CCTCATTGAAGTCC-3′; and β-actin sense 5′-CCTCTATGC-CAACACAGTGC-3′ and anti-sense 5′-GTACTCCTGCTTGC-TGATCC-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. The reaction conditions were designed as follows: initial denaturation at 95 °C for 5 min followed by 40 cycles with 10 s at 95 °C for denaturing, 15 s at 58 °C for annealing and 20 s at 72 °C for extension. After real-time PCR, the temperature was increased from 72 to 99 °C at a rate of 12 °C/min to construct a melting curve. A control without cDNA was run in parallel with each assay. Results were collected and analyzed with Rotor-Gene 6.0 software (Corbett Research, Australia). Each reaction was amplified in triplicate and ratio results were calculated based on the $\Delta\Delta C_t$ method [16]. Relative mRNA levels were normalized to those of β-actin. Signals from NG podocytes were assigned a relative value of 1.0. The experiments were repeated independently three times.

2.5. Immunoblotting

Cells under different experimental conditions were lysed in a cell lysis buffer (50 mM Tris–HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1% Triton X-100; 1 mM PMSF; 1 μg/ml leupeptin; 1 mM Na_3VO_4; 1 mM NaF) with a sonicator. Crude lysates were centrifuged at 10,000 rpm for 5 min at 4 °C. Total protein concentration from the resultant supernatant was determined by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). Protein samples (40 μg) were electrophoresed on a graded (4–20%) sodium dodecyl sulfate (SDS)/polyacrylamide gel under reducing conditions, transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA, USA), blocked in 2% non-fat dried milk in Tris-buffered saline Tween (TBS and 0.1% Tween 20) and incubated overnight (4 °C) with the following primary antibodies: anti-α_3 integrin, anti-β_1 integrin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-ILK antibody (Upstate Biotechnology Inc., Lake Placid, NY, USA). Negative controls were performed without primary antibody. After washing, secondary antibody was added and incubated 1 h at room temperature and protein bands were visualized by ECL Plus (Amersham, Arlington Heights, IL, USA). The membranes were then stripped and re-probed with mouse anti-β-actin antibody (Sigma, St. Louis, MO, USA). Densitometric quantitation was performed using a Bio-Rad VersaDoc imaging system model 5000 with Bio-Rad Quantity One software. Protein expression was quantified as the ratio of specific band to β-actin. Three independent experiments were performed.

2.6. ILK activity assay

Cells incubated for 12 h under different experimental conditions were lysed in a cell lysis buffer mentioned above. The lysates were precleaned with protein A-agarose (Amersham Pharmacia Biotech, Buckinghamshire, UK) and incubated with affinity purified rabbit anti-ILK (Upstate Biotechnology Inc., Lake Placid, NY, USA) overnight at 4 °C. Immune complexes were then purified once again by using protein-A agarose, and a kinase assay was performed by incubating the complexes with Akt (Upstate Biotechnology Inc., Lake Placid, NY, USA) and ATP (Sigma, St. Louis, MO, USA). ILK activity was measured by phosphorylation of Akt with anti-phospho-PKB/Akt (Cell Signaling Technology, Beverly, MA, USA).

2.7. Statistical analysis

Statistics were conducted by SPSS 11.5 software. Results are expressed as mean ± standard error of the mean (S.E.M.). The significance of differences among experimental groups was determined by ANOVA analysis. When a significant difference was detected, the data were further analyzed by Dunnett's multiple range test. A value of $P < 0.05$ was considered statistically significant.

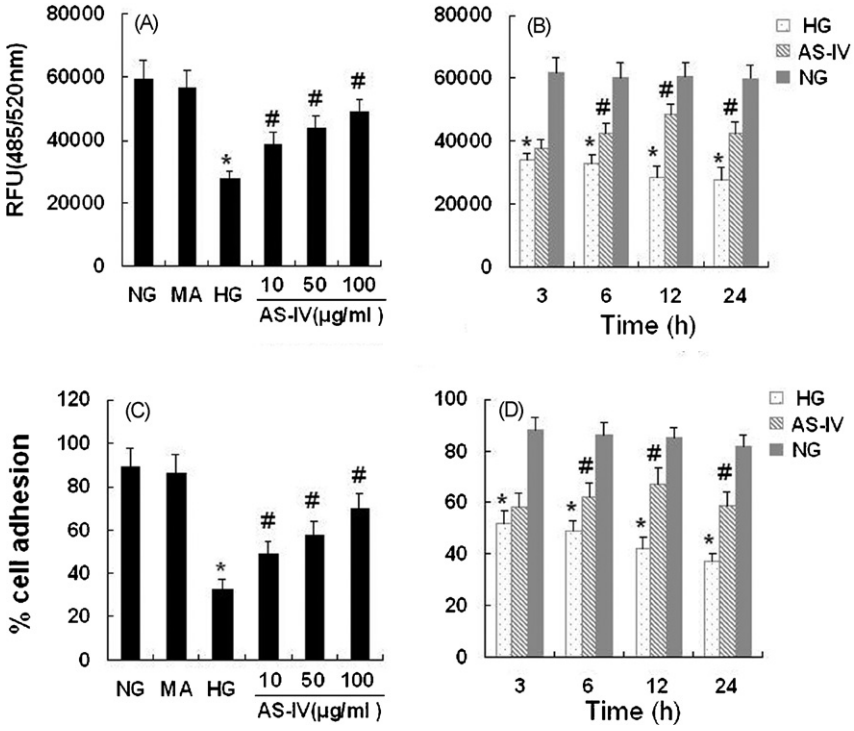

Fig. 2 – Effects of AS-IV on attachment of adherent podocytes to BMC assessed by fluorescence cell adhesion assay (A and B) and centrifugation cell adhesion assay (C and D). Podocytes were exposed to NG, MA, HG, HG with 10, 50, 100 μg/ml of AS-IV for 12 h and HG with 100 μg/ml of AS-IV for 3, 6, 12 and 24 h, respectively. The adhesion of podocytes to BMC was assessed by relative fluorescence unit (RFU) and by the ratio of adherent cells/cells before detachment, respectively. AS-IV caused a dose-dependent increase (A and C) and a time-dependent increase (B and D) in adherent podocytes to BMC. Results are expressed as the mean ± S.E.M. of three independent experiments. $^{*}P < 0.05$ vs. NG; $^{\#}P < 0.05$ vs. HG.

3. Results

3.1. *Effects of AS-IV on HG-stimulated adhesion of podocytes to BMC*

The relative attachment of adherent podocytes to BMC was examined by two different cell adhesion assays. As shown in fluorescence-labelled cell adhesion assay, exposure to HG for 12 h resulted in a significant decrease in the adhesion of podocytes to BMC compared with incubation in NG (27,783 ± 2351 vs. 59,548 ± 5480 RFU, $P < 0.05$). MA had no effect on cell adhesion. However, AS-IV strongly prevented the HG-induced inhibition of cell adhesion to BMC in a concentration-dependent manner, with the maximal effect achieved at 100 μg/ml of AS-IV (Fig. 2A). The effect of AS-IV was also time-dependent. The podocyte adhesion, which reached a maximal level of 1.7-fold of HG control at 12 h, was increased 6 h after 100 μg/ml of AS-IV and remained high throughout the 24 h treatment (Fig. 2B). The effect of AS-IV on podocyte adhesion was further confirmed by centrifugation cell adhesion assay. The adhesion of the podocytes to BMC under HG condition was only 37% compared with that of NG control. MA did not affect podocyte adhesion to BMC apart from the effect of osmolarity. However, AS-IV increased the adhesion of podocytes to BMC by 50, 75 and 110% at a concentration of 10, 50 and 100 μg/ml, respectively, when compared with HG (Fig. 2C). Treatment of 100 μg/ml of AS-IV also induced a time-dependent increase in adherent podocytes to BMC, which increased by 25% at 6 h and reached a peak of 1.6-fold of HG control at 12 h (Fig. 2D). Taken together, treatment with AS-IV resulted in significantly increased podocyte adhesion and this effect was evident in as little as 6 h and at a dose as low as 10 μg/ml.

3.2. *Effects of AS-IV on HG-stimulated mRNA expression of α_3 and β_1 integrin subunits in podocytes*

Because the $\alpha_3\beta_1$ integrin mediates the adhesion of podocytes to GBM [14], we investigated the effects of AS-IV on $\alpha_3\beta_1$ integrin expression. Compared with incubation in NG, exposure to HG for 12 h significantly decreased the mRNA levels of both β_1 and α_3 integrin subunits by approximately 70% ($P < 0.05$). Exposure to MA for 12 h did not change the mRNA expression of α_3 and β_1 integrin subunits in podocytes. However, the HG-induced decrease in the mRNA levels of both the β_1 and α_3 integrins was partially prevented by AS-IV in a dose- and time-dependent manner (Fig. 3). Podocytes treated with 10 μg/ml of AS-IV showed a 100% increase in β_1 integrin mRNA and a maximal increase of 2.1-fold of HG control was seen with a concentration of 100 μg/ml of AS-IV (Fig. 3A). AS-IV also induced a time-dependent increase in the gene expression of β_1 integrin which reached a peak of 2.7-fold of HG control at 12 h (Fig. 3C). Likewise, the mRNA level of α_3 integrin in podocytes under HG stimulation was upregulated by AS-IV

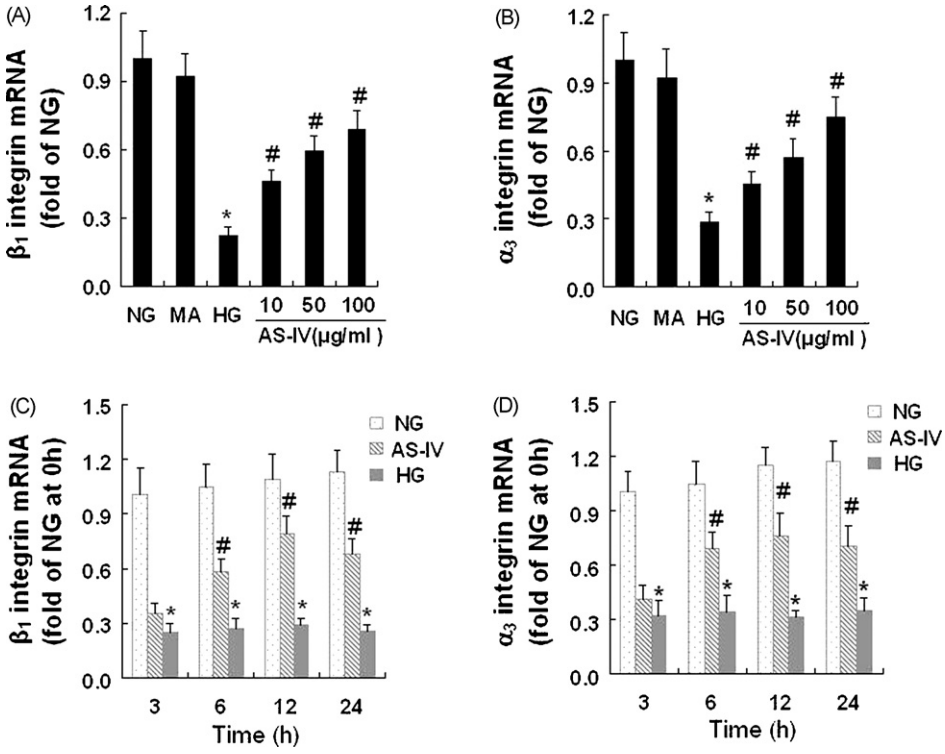

Fig. 3 – Effects of AS-IV on $\alpha_3\beta_1$ integrin mRNA expression in cultured mouse podocytes. Podocytes were exposed to NG, MA, HG, HG with 10, 50, 100 μg/ml of AS-IV for 12 h and HG with 100 μg/ml of AS-IV for 3, 6, 12 and 24 h, respectively. The $\alpha_3\beta_1$ integrin mRNA expression was examined by real-time RT-PCR. AS-IV induced a dose-dependent increase (A and B) and a time-dependent increase (C and D) in $\alpha_3\beta_1$ integrin mRNA expression. Results are expressed as the ratio to NG and are the mean ± S.E.M. of three independent experiments. $^{*}P < 0.05$ vs. NG; $^{\#}P < 0.05$ vs. HG.

both in a dose-dependent manner (Fig. 3B) and in a time-dependent manner (Fig. 3D).

3.3. *Effects of AS-IV on HG-stimulated protein production of α_3 and β_1 integrin subunits in podocytes*

Consistent with these effects on mRNA expression, HG for 12 h reduced the amount of β_1 integrin protein by 80% ($P < 0.05$) and that of α_3 integrin by 48% ($P < 0.05$) compared with NG. MA had no effect on protein production of α_3 and β_1 integrins while AS-IV significantly increased the protein production of β_1 integrin in podocytes under HG condition ($P < 0.05$). This stimulation was both time- and dose-dependent, become evident in as little as 3 h (Fig. 4B) and at a dose as low as 10 μg/ml (Fig. 4A). Podocytes treated with 10 μg/ml of AS-IV showed a 1.8-fold increase in β_1 integrin protein and the maximal level of 2.8-fold of HG control was obtained at 100 μg/ml of AS-IV (Fig. 4A). Moreover, treatment with 100 μg/ml of AS-IV increased the quantity of β_1 integrin protein by 80% at 3 h and the maximal level of 4-fold of HG control was observed at 24 h (Fig. 4B). Similarly, exposing the podocytes to AS-IV for 12 h raised the protein production of α_3 integrin in a dose-dependent manner. This stimulation became evident at a dose as low as 10 μg/ml and reached a peak of 1.6-fold of HG at 100 μg/ml (Fig. 4A). The upregulatory effects of AS-IV on protein production of α_3 integrin were also time-dependent. Treatment with 100 μg/ml of AS-IV elevated HG-induced α_3 integrin protein production by 35% at 3 h and the maximal level of 2.4-fold of HG control was observed at 24 h (Fig. 4B). These findings indicated that AS-IV attenuated the HG-induced reduction in $\alpha_3\beta_1$ integrin protein expression in podocytes.

3.4. *Effects of AS-IV on HG-stimulated ILK activity and mRNA expression in podocytes*

Since ILK has been shown to mediate cell adhesion, an in vitro ILK kinase assay was performed. After 12 h of incubation, ILK activity was elevated by HG. However, AS-IV treatment abrogated the HG-induced ILK activation in a dose-dependent manner. As little as 10 μg/ml AS-IV inhibited the activity of ILK, and maximal inhibition was achieved by 100 μg/ml AS-IV. In contrast, MA had no effect on ILK activity (Fig. 5A).

Compared with NG, HG for 12 h induced a 3.8-fold increase in mRNA levels of ILK in podocytes while MA had no effect. However, AS-IV partially inhibited the HG-induced over-expression of ILK mRNA in a dose- and time-dependent manner. There was a 32, 46 and 63% reduction in ILK mRNA in podocytes treated with 10, 50 and 100 μg/ml of AS-IV compared with podocytes treated with HG (Fig. 5B). AS-IV also decreased ILK mRNA level in HG-stimulated podocytes by 25, 40 and 60% at 6, 12 and 24 h, respectively (Fig. 5C).

3.5. *Effects of AS-IV on HG-stimulated ILK protein expression in podocytes*

Similarly, HG for 12 h caused a twofold increment in the amount of ILK protein compared with NG ($P < 0.05$) while MA

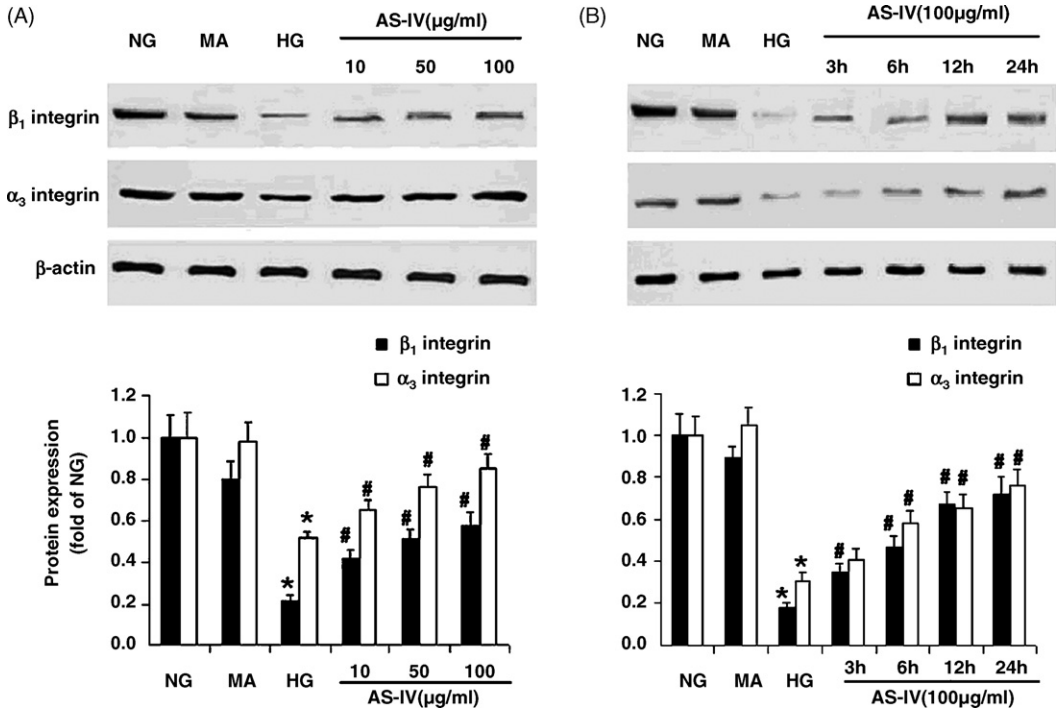

Fig. 4 – Effects of AS-IV on $\alpha_3\beta_1$ integrin protein expression in cultured mouse podocytes. Podocytes were exposed to NG, MA, HG, HG with 10, 50, 100 μg/ml of AS-IV for 12 h and HG with 100 μg/ml of AS-IV for 3, 6, 12 and 24 h, respectively. The $\alpha_3\beta_1$ integrin protein expression was examined by Western blotting. AS-IV upregulated the β_1 integrin and α_3 integrin protein expression in a dose-dependent manner (A) and in a time-dependent manner (B). Results are expressed as the ratio to NG and are the mean ± S.E.M. of three independent experiments. $^{*}P < 0.05$ vs. NG; $^{\#}P < 0.05$ vs. HG.

did not change the ILK protein expression. However, AS-IV significantly decreased the ILK protein production in HG-stimulated podocytes ($P < 0.05$). This inhibitory effects were both time- and dose-dependent, become evident in as little as 12 h and at a dose as low as 50 μg/ml. Podocytes treated with AS-IV showed 35 and 50% decrease in ILK protein production at 50 and 100 μg/ml, respectively (Fig. 6A). Moreover, AS-IV lowed the ILK protein production by 35 and 60% at 12 and 24 h, respectively (Fig. 6B). These findings indicated that AS-IV effectively inhibited the HG-induced overexpression in ILK protein expression in podocytes.

4. Discussion

We firstly reported that AS-IV protected HG-induced podocyte adhesion in vitro. This effect was shown using two different types of adhesion assays. AS-IV induced a significant dose- and time-dependent increase in cell–matrix adhesion of podocytes under HG stimulation. These findings showed AS-IV as a novel drug for protecting podocyte adhesion to GBM, which provided a potential new therapy in the treatment of DN and other renal diseases affecting podocytes.

We performed fluorescence-labelled and centrifugation cell adhesion assays to exactly measure the cell–matrix adhesion. The BMC, a solubilized basement membrane preparation extracted from Engelbreth–Holm–Swarm (EHS) mouse sarcoma [17], was used in cell adhesion assays. The major component of BMC is laminin, collagen IV, heparan sulfate proteoglycans and entactin. The use of BMC allowed us to more accurately model key GBM components (fibronectin, laminins and collagen IV isoforms), thus provided us a physiologically relevant environment for studies of cell adhesion between podocyte and GBM. In the present study, HG strongly inhibited adhesion of podocytes to BMC, which consisted with previous studies [8,9]. This inhibitory action was not due to an osmotic effect of HG because an equivalent concentration of D-mannitol did not produce the above result. However, AS-IV effectively prevented the HG-induced inhibition of podocytes adhesion to BMC in a dose- and time-dependent manner and this effect was evident in as little as 6 h and at a dose as low as 10 μg/ml. The maximal increase of podocyte adhesion by AS-IV was obtained at 12 h after treatment and at a dose of 100 μg/ml.

Morphologically, podocyte detachment from the underlying GBM may be related to the alteration of adhesion molecules [18]. The $\alpha_3\beta_1$ integrin, which is the only β_1 integrin in podocytes, mediates cell adhesion and regulates glomerular permeability by maintaining the shape and adhesion of the podocytes [14,19]. Loss of cell anchorage to the GBM may result from downregulation of the $\alpha_3\beta_1$ integrin receptor [20]. Decreased expression of $\alpha_3\beta_1$ integrin expression on podocytes may serve to foot process detachment, podocyte loss and proteinuria in humans with primary FSGS and chronic PAN-treated rats [21]. Reduced expression of $\alpha_3\beta_1$ integrin on podocytes has also been reported in patients with diabetes mellitus and streptozotocin-induced diabetic rats [22,23]. In vitro experiments have shown decreased expression of the

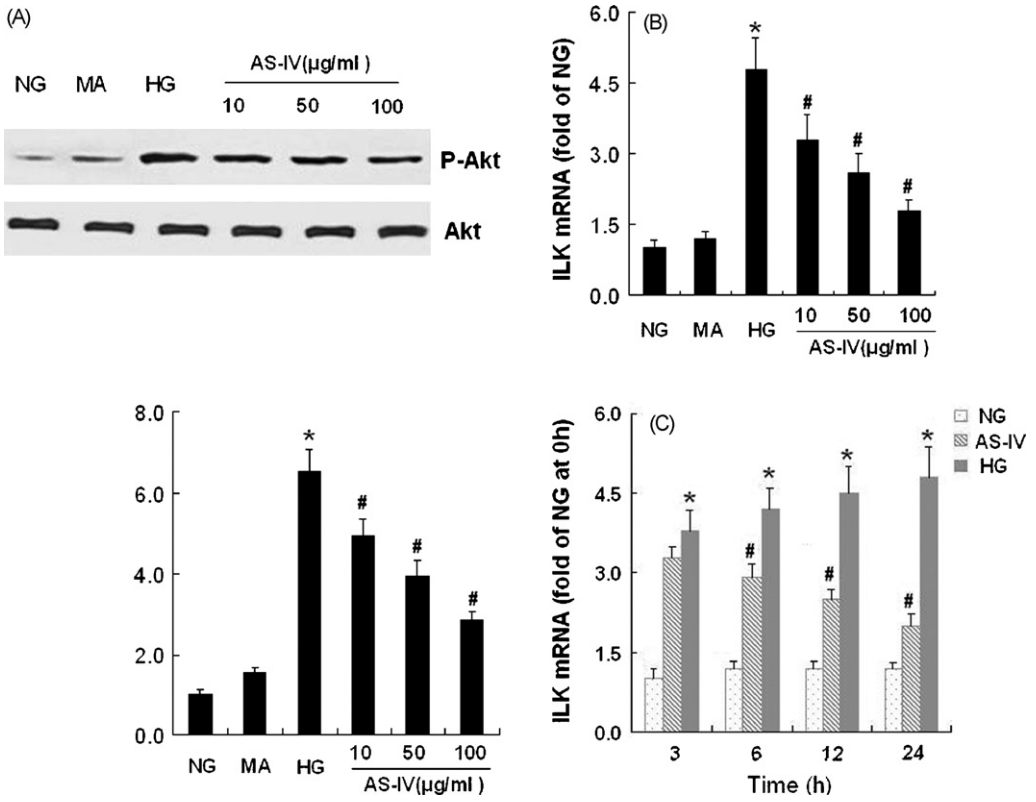

Fig. 5 – Effects of AS-IV on ILK activity and mRNA expression in cultured mouse podocytes. Podocytes were exposed to NG, MA, HG, HG with 10, 50, 100 μg/ml of AS-IV for 12 h and HG with 100 μg/ml of AS-IV for 3, 6, 12 and 24 h, respectively. ILK activity was determined by the phosphorylation of Akt at Ser-473 in protein immunoprecipitated with ILK antibody. ILK mRNA expression was examined by real-time RT-PCR. (A) Dose-dependent inhibition of ILK activity by AS-IV. (B) Dose-dependent downregulation of ILK mRNA by AS-IV. (C) Time-dependent downregulation of ILK mRNA by AS-IV. Results are expressed as the ratio to NG and are the mean ± S.E.M. of three independent experiments. *P < 0.05 vs. NG; #P < 0.05 vs. HG.

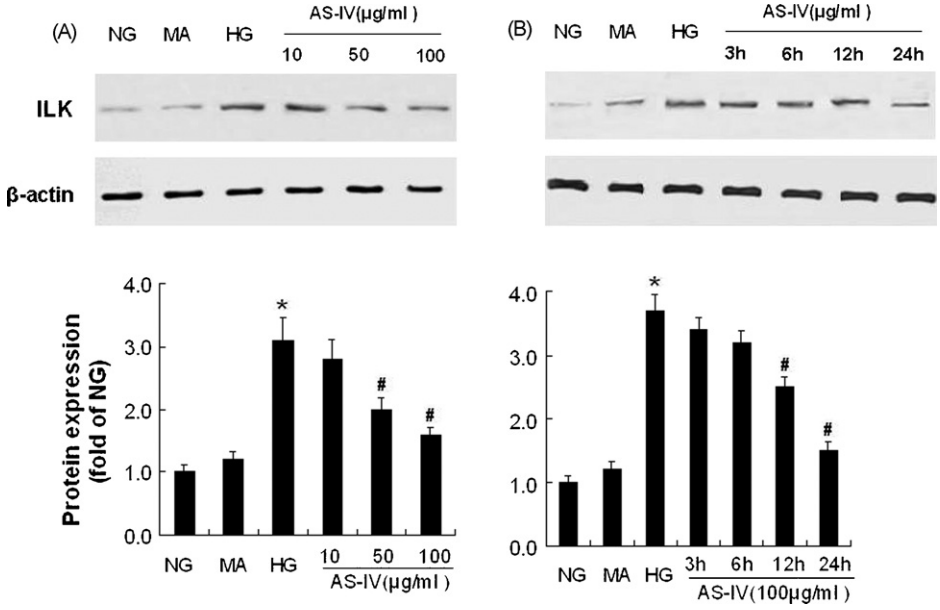

Fig. 6 – Effects of AS-IV on ILK protein levels in cultured mouse podocytes. Podocytes were exposed to NG, MA, HG, HG with 10, 50, 100 μg/ml of AS-IV for 12 h and HG with 100 μg/ml of AS-IV for 3, 6, 12 and 24 h, respectively. The ILK protein expression was examined by Western blotting. (A) AS-IV downregulated the ILK protein expression in a dose-dependent manner. (B) AS-IV also downregulated the ILK protein expression in a time-dependent manner. Results are expressed as the ratio to NG and are the mean ± S.E.M. of three independent experiments. *P < 0.05 vs. NG; #P < 0.05 vs. HG.

$\alpha_3\beta_1$ integrin in rat and human podocytes cultured in HG media [9,22]. In our experiments, HG also induced a significant reduction in $\alpha_3\beta_1$ mRNA and protein expression. In contrast, one study demonstrated that HG increased the production of β_1 integrin in cultured podocytes [24]. These different results may be attributed to the use of different species of cultured podocytes and different culture conditions.

ILK is a recently identified integrin cytoplasmic-binding protein that has been implicated in the regulation of cell adhesion [25]. ILK is regarded as a candidate downstream effector in proteinuria and ILK overexpression lead to anchorage-independent growth and reduced matrix adhesion [26]. ILK expression has been reported to be dramatically increased in diabetic glomeruli [27] and in the mouse podocytes cultured in HG media [24]. Conversely, the small molecular ILK inhibitor MC-5 was able to rescue podocyte matrix adhesion close to control levels in cultured human podocytes [28]. In this study, HG also increased ILK activity and expression in cultured mouse podocytes. These findings support that ILK activation and overexpression leads to impaired cell–matrix adhesion in podocytes under HG stimulation. Several studies have suggested a close relationship between the dysregulation of the integrin β_1–ILK cascade and DN in human and experimental diabetic animal models [23,27,29]. Thus, podocytes under HG stimulation show decreased $\alpha_3\beta_1$ integrin expression, as well as increased ILK activity and expression, leading to impaired cell–matrix adhesion. The integrin–ILK cascade might even represent a new therapeutic target in the treatment of DN.

We demonstrated that AS-IV induced a significant increase in $\alpha_3\beta_1$ integrin expression and decrease in ILK expression, which are shown on protein and mRNA level. The increase in $\alpha_3\beta_1$ integrin mRNA due to AS-IV treatment correlated with an increase in the protein production of $\alpha_3\beta_1$ integrin, measured by Western blotting of cell lysate. These results support the possibility that AS-IV, directly or indirectly, increases $\alpha_3\beta_1$ integrin synthesis in podocytes, thus maintaining the adhesion of podocytes to GBM. The expression of mRNA and protein for ILK, which is an important β_1-integrin-mediated intracellular signaling molecule [30], was rapidly downregulated after AS-IV treatment for 6 and 12 h, peaking at 24 h. AS-IV also significantly inhibited overexpression of ILK mRNA and protein in a dose-dependent manner, which reached a peak at 100 μg/ml. Moreover, AS-IV markedly inhibited the activation of ILK, with the maximal inhibition at 100 μg/ml. Taken together, the regulatory effects of AS-IV on integrin–ILK system are likely to be accountable for its action of protecting podocyte adhesion.

Oxidative stress plays a pivotal role in the development of DN. HG increases reactive oxygen species (ROS) generation in podocytes [31]. Susztak et al. [32] also demonstrate a role for a hyperglycemia-induced ROS in podocyte depletion in vitro and in vivo. Downregulation of $\alpha_3\beta_1$ integrin and alterations in cell–matrix adhesion in podocytes might depend on extracellular ROS [33]. Treatment of animals with phosphatidylcholine-bound superoxide dismutase reduced proteinuria and preserved the α_3 integrin expression in podocytes [34]. Oxidative stress also induced ILK activation in a dose-dependent manner, followed by increased proliferation and reduced matrix adhesion of podocytes [26]. Conversely, a small molecular ILK inhibitor was also able to block transcriptional activation of MMP-9 after oxidative stress in podocytes [35]. Interestingly, both in vivo and in vitro studies have provided evidences that AS-IV has an antioxidant effect [36]. AS-IV has been shown to be an aldose reductase inhibitor and a free radical scavenger for preventing the development of DN [12]. Thus, the protective effect of AS-IV on podocyte adhesion and its regulatory effects on integrin–ILK system may be associated with its antioxidant action.

In conclusion, AS-IV improves cell–matrix adhesion of podocytes under HG stimulation and this effect may be involved in $\alpha_3\beta_1$ integrin upregulation and ILK inhibition.

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