Spironolactone inhibits podocyte motility via decreasing integrin β1 and increasing integrin β3 in podocytes under high-glucose conditions

ZHUO LI[1,2], LI ZHANG[2], WEI SHI[2], YUANHAN CHEN[2], HONG ZHANG[2], SHUANGXIN LIU[2], XINLING LIANG[2], TING LING[2], CHUNPING YU[2], ZHONGSHUN HUANG[1,2], XIAOFAN TAN[1,2], XINCHEN ZHAO[1,2], ZHIMING YE[2], BIN ZHANG[2], WENJIAN WANG[2], RUIZHAO LI[2] and JIANCHAO MA[2]

[1]Graduate School, Southern Medical University, Guangzhou, Guangdong 510515; [2]Department of Nephrology, Guangdong General Hospital, Guangdong Academy of Medical Sciences, Guangzhou, Guangdong 510080, P.R. China

Abstract. Integrin β1 and β3 expression by podocytes is required to maintain glomerular structural integrity. Previous studies have shown that aldosterone (ALD) is involved in glomerular podocyte injury, and mineralocorticoid receptor (MR) blocker spironolactone effectively reduces proteinuria in patients with diabetic nephropathy. The present study was designed to observe the effects of spironolactone on β1 and β3 integrin expression and podocyte motility under in vitro diabetic conditions. Immortalized mouse podocytes were cultured in media containing normal glucose (NG) levels, high glucose (HG) or HG plus spironolacton. The expression of β1 and β3 integrin in podocytes was detected by reverse transcription quantitative polymerase chain reaction, immunofluorescence and western blot analyses. The effects of spironolacton on podocyte motility was further evaluated using a wound healing assay. HG stimulation markedly decreased mRNA and protein expression of integrin β1, and significantly increased mRNA and protein expression of integrin β3 in cultured podocytes. However, simultaneous treatment with spironolacton (10^{-7} mol/l) significantly attenuated HG-mediated increases in integrin β3 and decreases in integrin β1 expression. Furthermore, the migration of podocytes induced by HG was abrogated by concomitant treatment with spironolacton. In conclusion, the present study suggested that HG decreased the expression of integrin β1 in cultured podocytes, accompanied with an increase of integrin β3. Spironolactone inhibited cell motility and stabilized podoctyes treated with HG, probably through partly normalizing the expression of integrin β1 and decreasing the expression of integrin β3.

Introduction

Diabetic nephropathy (DN) is a serious and common complication of diabetes type I and II, leading to end-stage renal disease (ESRD) (1,2). Accumulating evidence suggested that glomerular podocytes have a pivotal role in the pathogenesis of diabetic kidney disease (3,4). Podocytes are terminally differentiated cells residing on the outer surface of the glomerular basement membrane (GBM) and have a key role in maintaining the structure and function of the glomerular filtration barrier (3). Previous studies have demonstrated podocyte depletion and loss in the early stages of DN (5,6). These stages of podocyte depletion are accompanied by corresponding degrees of proteinuria (7,8). Therefore, establishment of novel and innovative therapeutic strategies targeted to block the shedding of podocytes, decrease proteinuria and delay the progression of DN has become increasingly urgent.

Integrins are heterodimeric transmembrane adhesion receptors composed of α- and β-sub-units. Binding of extracellular matrix molecules or other ligands to the extracellular domain of integrins delivers a variety of signals into the cell. Integrins have key roles in a number of important biological processes, including migration, survival, proliferation, gene expression and receptor tyrosine kinase signaling (9,10). Integrin α3β1 is the principal adhesion complex which is responsible for the attachment of podocytes to the GBM. An in vitro study has demonstrated that high-glucose conditions decreased α3β1 integrin expression in rat and human podocytes (11). Genetic ablation of β1 integrin in mice was shown to cause embryonic lethality shortly after implantation (12). Mice featuring knockout of podocyte-specific *Itgb1*, which encodes β1 integrin, were shown to develop a similar phenotype to podocyte-specific *Itga3*-knockout mice, with massive proteinuria soon after birth, a laminated GBM with extensive splitting, and foot process effacement followed by mortality within 1-5 weeks due to ESRD (13.14). Besides β1 integrins, αvβ3 integrin is highly expressed in glomerular

Correspondence to: Professor Wei Shi, Department of Nephrology, Guangdong General Hospital, Guangdong Academy of Medical Sciences, 106 Zhongshan No. 2 Road, Guangzhou, Guangdong 510080, P.R. China
E-mail: shiweigd139@163.com

podocytes (15,16). In mice and humans, activation of αvβ3 by the (soluble) urokinase receptor (uPAR) was demonstrated to result in foot process effacement, proteinuria and focal segmental glomerulosclerosis (15,17). Inhibition of αvβ3 with an anti-β3 antibody or the small-molecule inhibitor cilengitide alleviated proteinuria induced by urokinase receptor (15,17).

It has been suggested that aldosterone has an important role in the pathogenesis of DN (18). Podocytes are one type of target cells for the deleterious effects of aldosterone (19). Spironolactone (SPL), a non-selective aldosterone receptor blocker, was reported to exert beneficial effects not only by reducing proteinuria but delaying DN progression (20,21). Similarly, a previous study demonstrated that SPL prevents podocytic adhesion in streptozotocin-induced diabetic rats, decreases urine albumin and podocyte levels, and upregulates integrin α3 expression (22). However, whether spironolactone has an effect on β1 and β3 integrin has remained elusive.

Based on the abovementioned findings, the present study was designed to observe whether spironolactone has an impact on integrin β1 and β3 expression, and podocyte motility under *in vitro* diabetic conditions.

Materials and methods

Drugs and reagents. Spironolactone (S3378), D-glucose (G7021) and mannitol (M9647) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Corning, Inc. (Corning, NY, USA). Fetal bovine serum (FBS), recombinant interferon γ and rat tail collagen type I were purchased from Invitrogen Life Technologies, Inc., (Carlsbad, CA, USA), ProSpec Tany Technogene Ltd. (East Brunswick, NJ, USA) and BD Biosciences (Franklin Lakes, NJ, USA), respectively. All primary antibodies, including rabbit polyclonal antibody integrin β1 (cat. no. sc-8978), rabbit polyclonal anti-integrin β3 (cat. no. sc-14009), goat polyclonal anti-synaptopodin (N-14; cat. no. sc-21536) and mouse monoclonal anti-GAPDH (cat. no. sc-365062), were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Fluorescein isothiocyanate-conjugated (FITC)-donkey anti-goat immunoglobulin (Ig)G (H+L; cat. no. sc-2024) and goat anti-rabbit Alexa Fluor 555 (cat. no. sc-362272) were obtained from Santa Cruz Biotechnology, Inc., and Cell Signaling Technology, Inc. (Beverly, MA, USA), respectively. Dimethyl sulfoxide (DMSO) and TRIzol were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Invitrogen Life Technologies, Inc, respectively. PrimeScript 1st Strand cDNA Synthesis kit (cat. no. RR001A) and SYBR Premix Ex Taq Green kit (cat. no. RR420A) were purchased from Takara Biotechnology Inc. (Dalian, China). A radioimmunoprecipitation assay (RIPA) protein extraction kit (cat. no. C1053) and bicinchoninic acid (BCA) Protein Assay kit (cat. no. FD2001) were purchased from Puli Lai company (Beijing, China) and Fabio Science company (Hangzhou, China), respectively.

Cell culture and treatment. The conditionally immortalized mouse podocyte cell line (MPC) was kindly provided by Dr. Jochen Reiser (Rush University Medical Center, Chicago, IL, USA) and were cultured as previously described (23). Differentiated podocytes (passage 13-18) were serum-starved for 24 h and then treated with normal glucose (NG; 5.3 mM), high glucose (HG; 20 mM) or with NG (5.3 mM) plus mannitol (14.7 mM; osmolality control) for 48 h prior to the assays. For intervention experiments, aldosterone receptor blocker SPL (Sigma-Aldrich) at concentrations of 10^{-8} mol/l and 10^{-7} mol/l was respectively added to cells treated with HG at a concentration of 20 mM for 48 h.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). TRIzol reagent was used to extract the total RNA from the cultured podocytes from the experimental groups according to the manufacturer's instructions (Invitrogen Life Technologies, Inc.). The complementary DNA was synthesized using 1,000 ng RNA in 20 µl using the PrimerScript™ RT Regent kit (cat. no. RR001A; Takara Biotechnology Inc.) according to the manufacturer's instructions. RT-PCR was performed with a Bio-Rad CFX96 Touch q-PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using Power SYBR Green PCR Master Mix with the cDNA and primer pairs (Invitrogen Life Technologies). The sequences of primer pairs used for real-time PCR were as follows: Integrin β1 forward, 5'-GGCTGAAGATTACCCTAT-3' and reverse, 5'-CATTCA TCAAATCCGTTC-3'; integrin β3 forward, 5'-GCCTTCGTG GACAAGCCTGTA-3' and reverse, 5'-GGACAATGCCTG CCAGTCTTC-3'; GAPDH forward, 5'-TGTGTCCGTCGT GGATCTGA-3' and reverse, 5'-TTGCTGTTGAAGTCGCAG GAG-3'. The PCR conditions were as follows: An initial step of 2 min at 95°C, followed by 40 cycles of 5 sec at 95°C, 25 sec at 60°C, and 40 sec at 72°C. Every reaction was amplified in triplicate and the fold change in the expression of each gene was calculated using ΔΔCt method with GAPDH mRNA as an internal control.

Immunofluorescence staining. After being subjected to various treatments, podocytes were fixed with 4% paraformaldehyde at -20°C for 20 min and then incubated with 5% BSA for 20 min at RT to block non-specific binding. Samples were incubated with the primary antibodies overnight at 4°C, followed by 1 h of incubation with secondary antibodies goat anti-rabbit Alexa Fluor 555 (1:1,000) or FITC-donkey anti-goat IgG (H+L) (1:250) at room temperature, followed by counterstaining with DAPI (Roche Diagnostics, Basel, Switzerland) for 5 min to visualize the nuclei. After being washed, the slides were mounted with anti-fade mounting medium (Beyotime Institute of Biotechnology, Haimen, China). Photomicrographs were captured using confocal microscopy (Leica SP5-FCS; Leica Microsystems, Oberkochen, Germany). The primary antibodies used in the present study were as follows: Rabbit polyclonal anti-integrin β1 (1:100), rabbit polyclonal anti-integrin β3 (1:100) and goat polyclonal anti-synaptopodin (N-14) (1:100). All images were analyzed by two investigators blinded to the identity of the samples.

Western blot analysis. Differentiated podocytes, subjected to various experimental conditions, were washed twice with cold phosphate-buffered saline (PBS). The washed podocytes were drained and scraped with RIPA lysis buffer using a cold plastic cell scraper. Protein concentration was quantified using the BCA Protein Assay Reagent kit. An aliquot of cell lysates containing 30 µg protein was separated by 8% SDS-PAGE
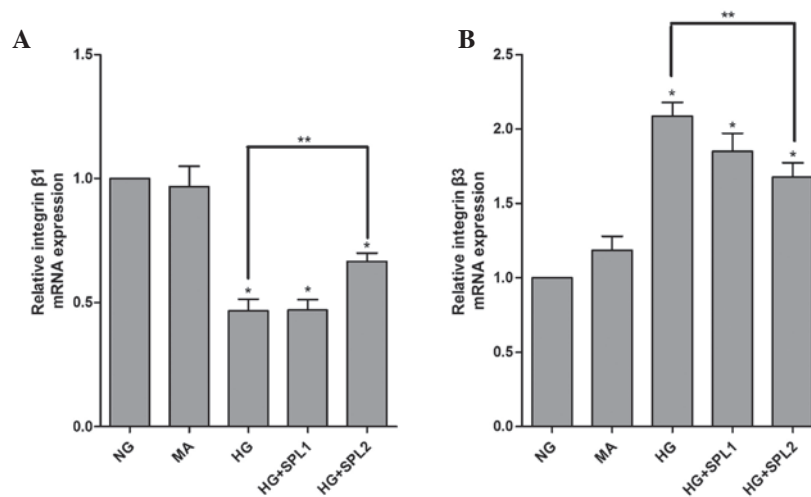
Figure 1. mRNA levels of integrin β1 and β3 in cultured podocytes under various experimental conditions. (A) mRNA levels of integrin β1 and (B) integrin β3 were examined using reverse transcription quantitative polymerase chain reaction analysis. GAPDH mRNA was used as an internal control. Quantitative values were obtained using the $2^{-\Delta\Delta CT}$ method. Values are expressed as the mean ± standard deviation of assays performed in triplicate. *P<0.05 vs. NG group. **P<0.05, HG + SPL2 vs. HG. Groups: NG, normal glucose (5.3 Mm); HG, high glucose (20 mM); MA, normal glucose (5.3 mM) + mannitol (14.7 mM), as an osmolality control; HG + SPL1, high glucose (20 mM) + spironolactone (10^{-8} mol/l); HG + SPL2, high glucose (20 mM) + spironolactone (10^{-7} mol/l).

and transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). Membranes were blocked with 5% fat-free milk for 1 h at room temperature and then incubated overnight at 4°C with the following primary antibodies: Rabbit polyclonal anti-integrin β1 (1:1,000), rabbit polyclonal anti-integrin β3 (1:1,000) and mouse monoclonal anti-GAPDH (1:2,000). After washing, HRP-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Jackson Immuno Research, West Grove, PA, USA, 1:5,000) was added and incubated 1 h at room temperature. The immunoblots were washed three times with Tris-buffered staline containing Tween 20 and immersed in ECL Plus Western Blotting Detection Reagents (Beyotime Institute of Biotechnology). Results were analyzed using Image J v1.47 software (National Institutes of Health, Bethesda, MD, USA) and normalized to the protein expression of GAPDH.

Wound healing assay. Cultured differentiated podocytes (1x10^5/ml) were seeded overnight on vitronectin-coated coverslips in six-well plates. Each coverslip was then scratched with a sterile 200-μl pipette tip, washed with PBS and placed into fresh medium. After 24 h, cells were fixed with cold methanol, permeabilized with 0.5% Triton X-100 in PBS and cell nuclei were stained with DAPI (Roche Diagnostics). Images were captured by phase-contrast microscopy under a x10 objective on a Leica SP5-FCS microscope (Leica Microsystems) at 0 and 24 h after scratching, and the numbers of cells that had migrated into the same-sized square fields were counted. Results are presented presented as the mean ± standard deviation (SD) of six independent experiments.

Statistical analysis. Values are expressed as the mean ± SD. Statistical analysis was performed by SPSS 17.0 (SPSS Inc, Chicago, IL,USA) using analysis of variance followed by Bonferoni's multiple comparisons test. P<0.05 was considered to indicate a significant difference between values.

Results

HG decreases integrin β1 and increases integrin β3 expression in cultured podocytes. To determine the effects of HG on integrin β1 and integrin β3, podocytes were cultured in DMEM containing 5.3 mM glucose (NG group), 5.3 mM glucose plus 14.7 mM mannitol (M group, as an osmolality control) or 20 mM glucose (HG group) for 48 h. The mRNA and protein expression of integrin β1 and integrin β3 were determined using RT-qPCR and western blot analysis. As shown in Fig. 1A, the mRNA expression of integrin β1 was significantly decreased in the HG group compared to that in the NG group (P<0.001). By contrast, the mRNA levels of integrin β3 were markedly increased in podocytes incubated with HG for 48 h (Fig. 1B). Immunofluorescent staining and western blot analysis also showed a decreased protein expression of integrin β1 and an increased protein expression of integrin β3 in the HG group (Figs. 2 and 3). As expected, mannitol had no effect on the mRNA expression of integrin β1 and integrin β3, suggesting that the decreased integrin β1 and β3 mRNA expression under HG did not result from high osmolality.

SPL normalizes integrin β1 and integrin β3 expression in podocytes under HG. To evaluate the effects of SPL on the expression of integrin β1 and integrin β3 in HG-cultured podocytes, cells incubated under various conditions were analyzed using RT-qPCR, immunoblotting and immunofluorescence. As shown in Fig. 1A, the HG-mediated decrease in integrin β1 mRNA expression in podocytes was significantly attenuated by SPL (10^{-7} mol/l) (P<0.01). Similarly, immunofluorescence and western blot analysis demonstrated that decreases in integrin β1 protein expression in HG-cultured podocytes were restored by SPL (10^{-7} mol/l) (Fig. 2A, and 3A and B). Furthermore, the significant HG-induced increase in the expression of integrin β3 was reduced by treatment with SPL (10^{-7} mol/l) at the mRNA level (Fig. 1B) and at the protein level (Figs. 2B, and 3A and C). However, a low dose of SPL
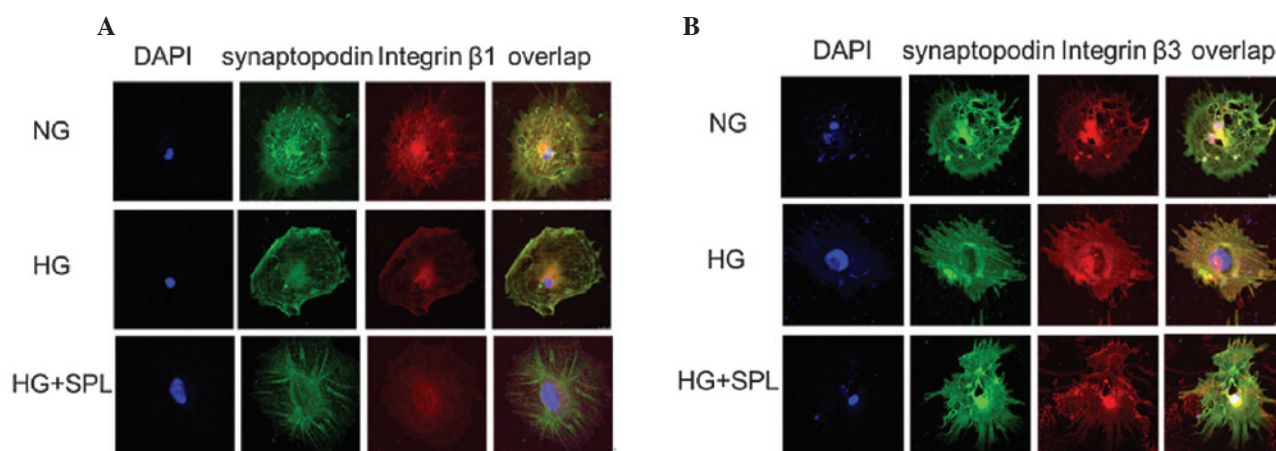
Figure 2. Effects of spironolactone on integrin β1 and β3 expression were analyzed by immunofluorescent staining in podocytes under HG conditions. (A) Double immunofluorescent staining of integrin β1 (red), synaptopodin (green), DAPI-stained nuclei (blue) and merged images in cultured podocytes treated with NG, HG and HG plus SPL for 48 h, respectively. (B) Double immunofluorescent staining of integrin β3 (red), synaptopodin (green), DAPI-stained nuclei (blue) and merged images in cultured podocytes treated with NG, HG and HG plus SPL for 48 h, respectively. NG, normal glucose (5.3 Mm); HG, high glucose (20 mM); HG + SPL, high glucose (20 mM) + spironolactone (10^{-7} mol/l).
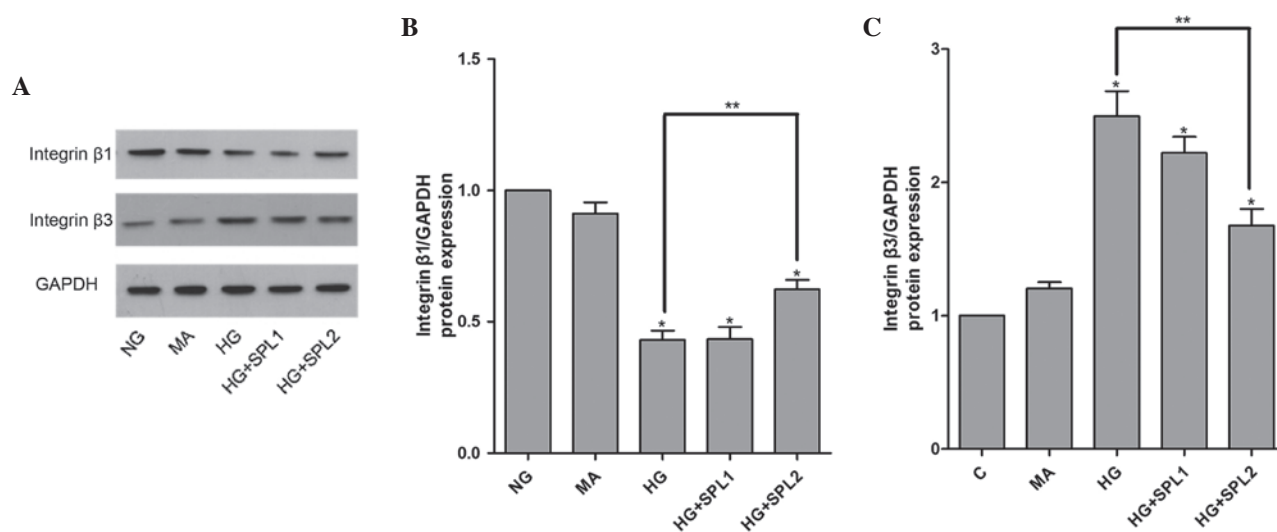

Figure 3. Effects of spironolactone on integrin β1 and β3 expression in podocytes under HG conditions were analyzed by immunoblotting. (A) Protein levels of integrin β1 and β3 expression in podocytes under various conditions were analyzed using immunoblotting. Densitometric analysis of three repetitions of (B) integrin β1 and (C) integrin β3 protein. All Values are expressed as the mean ± standard deviation. *$P<0.05$ vs. NG; **$P<0.05$ vs. HG. Groups: C, control; NG, normal glucose (5.3 Mm); HG, high glucose (20 mM); MA, normal glucose (5.3 mM) + mannitol (14.7 mM), as an osmolality control; HG + SPL1, high glucose (20 mM) + spironolactone (10^{-8} mol/l); HG + SPL2, high glucose (20 mM) + spironolactone (10^{-7} mol/l).
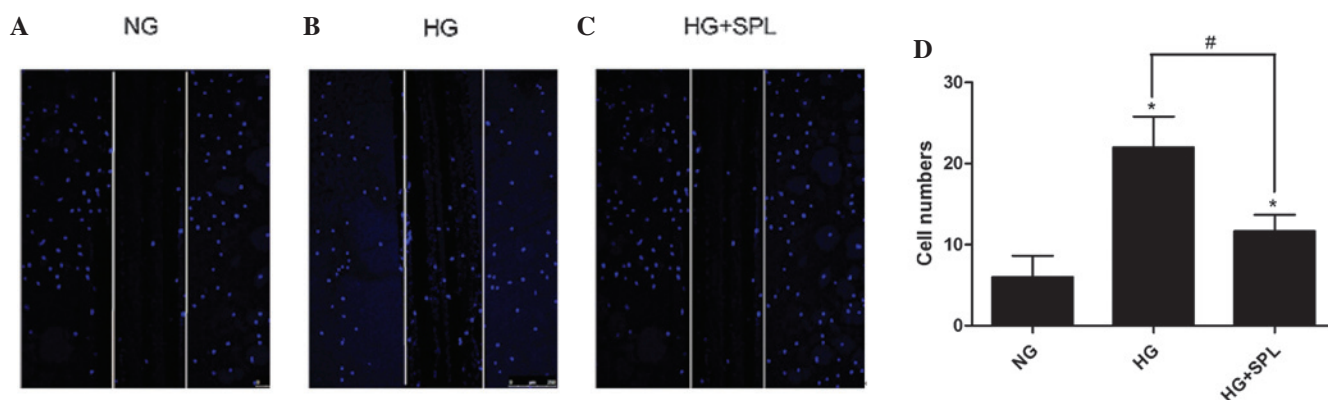

Figure 4. Spironolactone inhibits podocyte motility induced by HG. (A) NG group. (B) HG treatment significantly promoted podocyte wound closure (22.0±4.0 vs. 6.0±3.0; P<0.001). (C) By contrast, treatment with spironolactone reduced HG-induced podocyte motility (12.0±2.0 vs. 22.0±4.0; P<0.05). (D) Quantified numbers of migrated podocytes. All Values are expressed as the mean ± standard deviation. Magnification, x100. *$P<0.05$ vs. NG; #$P<0.05$ vs. HG. NG, normal glucose (5.3 Mm) group; HG, high glucose (20 mM); HG + SPL, high glucose (20 mM) + spironolactone (10^{-7} mol/l).

(10^{-8} mol/l) did not affect integrin β1 and integrin β3 expression in podocytes cultured under HG (P>0.05) (Figs. 1 and 3).

SPL inhibits podocyte motility induced by HG. Podocyte motility is regarded as a surrogate indicator for proteinuria and effacement of podocyte foot processes *in vivo* (15,17,24). Therefore, the present study next explored whether SPL has a role in inhibiting cell motility of podocytes *in vitro*. The effects of SPL on the spatial motility of podocytes was analyzed using a scrape-wound assay (Fig. 4). As compared with the control (6.0±3.0), HG treatment significantly promoted podocyte wound closure (22.0±4.0; P<0.05) (Fig. 4A, B and D). By contrast, treatment with SPL reduced HG-induced podocyte motility (12.0±2.0; P<0.05) (Fig. 4C and D). These results demonstrated that SPL inhibits podocyte motility induced by HG.

Discussion

The incidence of diabetes has been increasing on a yearly basis; in addition, DN has gradually become a severe complication in patients with diabetes (1,2). Experimental and clinical studies have shown that a decrease in the number of podocytes due to shedding or apoptosis leads to proteinuria in DN (5,6,25). Apart from the administration of angiotensin-converting enzyme inhibitors, angiotensin receptor blockers and other drugs, no other treatments for effectively lowering urinary protein and delaying disease progression are currently available. As a novel approach for the development of therapies for the treatment of DN, the prevention or inhibition of podocyte shedding or apoptosis is an obvious and promising therapeutic target.

Under normal circumstances, podocytes are anchored to the GBM through the α3β1-integrin complex that is present in the sole of the foot processes. However, under pathological conditions, resembled by a rat model of diabetes or podocytes cultured under HG, podocyte shedding or podocye motility were increased, accompanied by changes in the levels of integrin, which was observed at early stages of DN (5,6,26). Therefore, blocking this process by increasing the levels of integrin β1 is expected to enable podocytes to closely attach to the GBM. In addition, a recent study by our group showed that uPAR expression was increased in HG-cultured podocytes and DN patients (27). Previous studies have demonstrated that uPAR and integrin β3 co-localize in podocytes and form a lipid-dependent complex with integrin β3, thereby causing a structural configurational change of integrin β3, resulting in its activation with increased affinity for ligand binding (5,6). *In vivo* gene delivery of a structurally fixed and constitutively active integrin β3 was shown to be sufficient to induce proteinuria in mice; conversely, the inhibition of uPAR expression and integrin β3 activation improved the structure of the podocyte foot process and had an anti-proteinuric effect (24). Therefore, the present study addressed the question whether decreased expression of integrin β3 by drug intervention under diabetic conditions can inhibit podocyte motility.

The results of the present study demonstrated that HG decreased integrin β1 expression and increased integrin β3 expression, accompanied by increased podocyte motility. In HG-cultured podocytes treated with various concentrations of

SPL, a non-selective aldosterone receptor antagonist, changes in integrin β1 and integrin β3 expressions were partially normalized by high concentrations of SPL (10^{-7} mol/l). HG-induced decreases in integrin β1 expression increases in integrin β3 expression and increases in podocyte motility were significantly attenuated by SPL. However, a low concentration of SPL (10^{-8} mol/l) had no marked effects on HG-induced changes in integrin expression and podocyte motility. Whether the attenuating effects of SPL on the HG-induced effects on podocytes are based on respective interactions with the two integrin β sub-units or on mutual reactions between integrin β1 and integrin β3 themselves remains elusive. A cancer-associated study reported that integrin α3β1 inhibits integrin αvβ3 expression (28). Hence, it was hypothesized in the present study that, under physiological conditions, integrin β1 expression in podocytes may inhibit the expression of integrin β3. However, under pathological conditions, the role of inhibited integrin β3 is expected to be attenuated due to decreased levels of integrin β1. In the present study, decreases in integrin β1 and increases in integrin β3 expression were attenuated by co-incubation with SPL in HG-cultured podocytes.

Recent clinical and experimental studies have demonstrated that aldosterone has pathogenetic roles in podocyte injury and DN (18,19,29). Aldosterone is a potent inducer of proteinuria. Siragy and Xue (30) demonstrated that diabetes increased local aldosterone production in the kidney, which contributed to the development of renal inflammation, matrix formation and albuminuria. Another previous study also showed that the local aldosterone system is activated and is involved in podocyte apoptosis under diabetic conditions (31). These results suggested that blockade of the aldosterone system may represent a novel therapeutic strategy to prevent proteinuria and podocyte injury under hyperglycemic conditions. In fact, experimental as well as clinical studies have demonstrated renoprotective effects of SPL in DN (20,21,32-37). However, the mechanisms by which SPL attenuates proteinuria and podocyte injury in DN remain to be fully elucidated. The results of the present study revealed that under HG conditions, the expression of integrin β1 was increased and integrin β3 expression was decreased by SPL, accompanied with the inhibition of podocyte motility. This effect may be one of the possible mechanisms of the protective effects of SPL on podocyte injury.

In conclusion, the present study showed that HG conditions decreased integrin β1 and increased integrin β3 expression in podocytes, accompanied with enhanced podocyte motility. Treatment with SPL markedly inhibited podocyte motility and partly restored integrin β1 and integrin β3 expression in this cell model. These results indicated that the effects of SPL on podocyte motility may, proceed via restoring integrin β1 and β3 expression, which may be one of the underlying mechanisms of its protective effects against podocyte injury under HG conditions.

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