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Research article

Ginseng total saponin modulates the changes of α-actinin-4 in podocytes induced by diabetic conditions

Tae-Sun Ha*, Ji-Young Choi, Hye-Young Park, Ja-Ae Nam, Su-Bin Seong

Department of Pediatrics, College of Medicine, Chungbuk National University, Cheongju, Korea

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ABSTRACT

Background: The actin cytoskeleton in podocytes is essential for the maintenance of its normal structure and function. Its disruption is a feature of podocyte foot-process effacement and is associated with proteinuria. α-Actinin-4 in podocytes serves as a linker protein binding the actin filaments of the cytoskeleton.

Methods: To investigate the effect of ginseng total saponin (GTS) on the pathological changes of podocyte α-actinin-4 induced by diabetic conditions, we cultured mouse podocytes under normal glucose (5mM) or high glucose (HG, 30mM) conditions, with or without the addition of advanced glycosylation end products (AGE), and treated with GTS.

Results: In confocal imaging, α-actinin-4 colocalized with the ends of F-actin fibers in cytoplasm, but diabetic conditions disrupted F-actin fibers and concentrated α-actinin-4 molecules at the peripheral cytoplasm. GTS upregulated α-actinin protein in a time- and dose-dependent manner, and suppressed the receptor for AGE levels in western blotting. Diabetic conditions, including HG, AGE, and both together, decreased cellular α-actinin-4 protein levels at 24 h and 48 h. Such quantitative and qualitative changes of α-actinin-4 protein induced by diabetic conditions were mitigated by GTS.

Conclusion: These findings imply that both HG and AGE have an influence on the distribution and amount of α-actinin-4 in podocytes that can be recovered by GTS.

1. Introduction

Diabetic nephropathy is a serious chronic complication of diabetes mellitus that affects around 30% of diabetic patients and is the most common cause of end-stage renal disease in the Western world [1]. Early glomerular pathological changes of diabetic nephropathy include glomerular hypertrophy, an increase in mesangial matrix, thickening of the glomerular basement membrane (GBM), and morphological changes of podocytes such as effacement, denudation, and loss, which manifest as hyperfiltration and microalbuminuria [1,2]. Diabetic renal changes are characterized by a progressive loss of renal function, oxidative stress, chronic inflammation, vascular remodeling, glomerulosclerosis, tubulointerstitial fibrosis, and overt proteinuria [1,2]. These pathological and hemodynamic changes are caused by biochemical alterations, including hyperglycemia, secondary glycated proteins, or irreversible advanced glycosylation end products (AGEs), leading to an increase in glomerular permeability as a result of the impaired glomerular filtration structure [3–5].

The glomerular capillary is a knot of specialized capillaries with a fairly high pressure flow (60 mmHg), and are an efficient and selective barrier allowing the filtration of large amounts of plasma and small solutes into the urinary space. The glomerular-slit diaphragm, a slit between the interdigitating foot processes of podocytes, serves as a size-selective barrier and is linked to the actin-based cytoskeleton by adaptor proteins [6–10]. The podocyte foot processes contain a dense network of actin filaments, which are linked by linker proteins, such as α-actinin-4, synaptopodin, and cortactin [6–11]. The α-actinin molecule is an elongated, symmetrical, and antiparallel dimeric rod with actin-binding sites at either end that enables cross-linkage of F-actin filaments into contractile bundles [10,12] and forms an anchoring complex for the

* Corresponding author. Department of Pediatrics, College of Medicine, Chungbuk National University, Cheongju 361-763, Korea.
E-mail address: tsha@chungbuk.ac.kr (T.-S. Ha).

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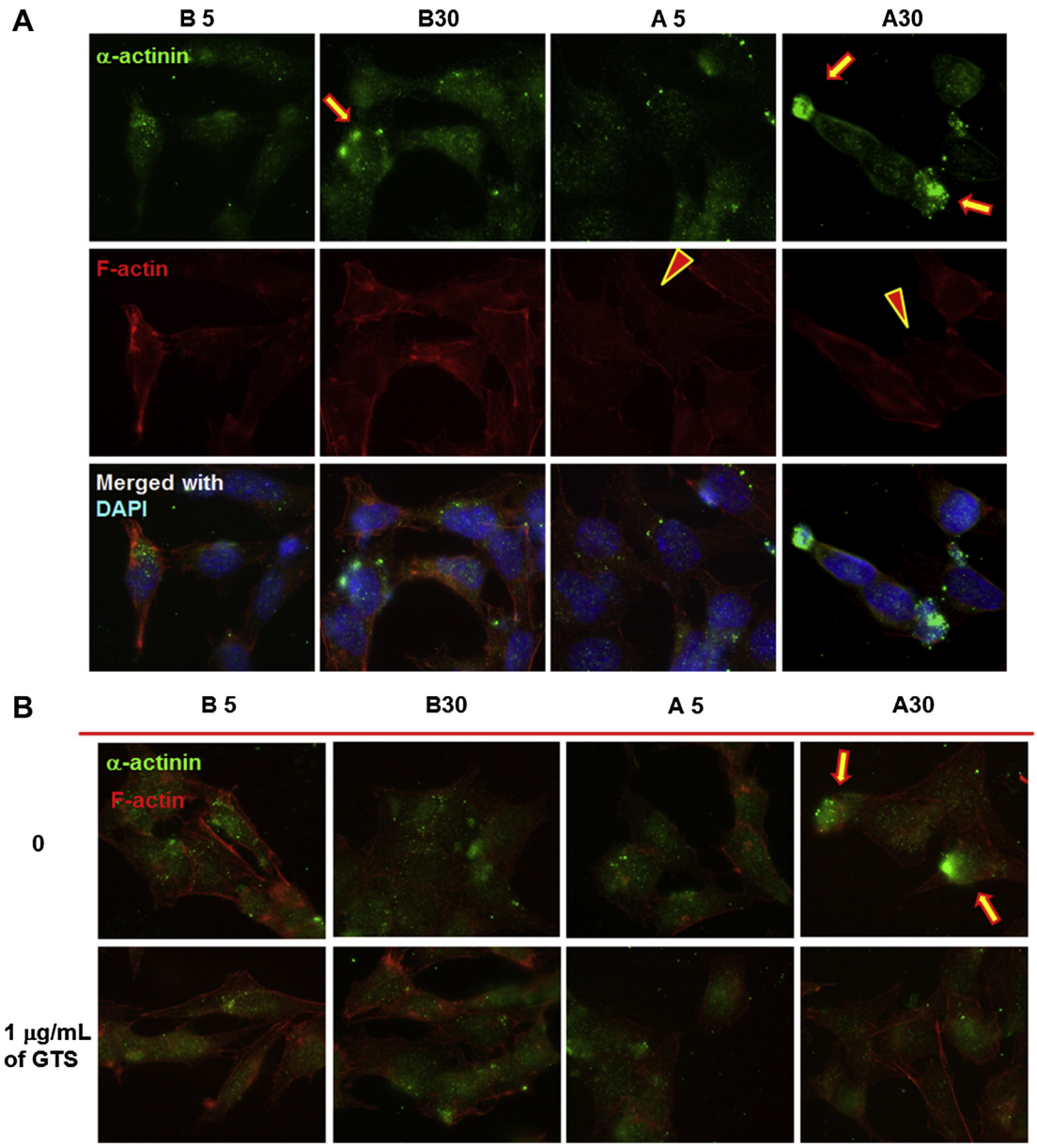

Fig. 1. Localization of α-actinin-4 and F-actin in podocytes. (A) Fluorescence spots of α-actinin-4 are located in the peripheral cytoplasm and processes of podocytes and are co-localized at the terminal ends of actin filaments. Diabetic conditions tend to concentrate α-actinin-4 staining at the peripheral cytoplasm (arrows) and disrupt F-actin fibers (arrow heads). (B) Ginseng total saponin (GTS, 1 μg/mL) improves the distributional changes of α-actinin-4 and F-actin fibers induced by diabetic conditions. (Scale: ×400.) DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride.

actin cytoskeleton at focal contacts on the plasma membrane. Of the four highly homologous α-actinin molecules, nonmuscle type α-actinin-4 is highly expressed at the foot processes of the podocytes and is required for normal podocyte adhesion [11,13].

Ginseng has been reported to be effective in the prevention and treatment of diabetic nephropathy in type 1 and type 2 diabetic animal models. In type 1 insulin-dependent diabetic nephropathy induced by streptozotocin animal models, Sun ginseng [14], heat-processed American ginseng [15], 20(*S*)-ginsenoside Rg3 [16], and Korean Red Ginseng (KRG) [17] ameliorated elevated serum glucose and renal damage. In particular, KRG ameliorated abnormalities associated with diabetic nephropathy through suppression of the inflammatory pathways activated by tumor necrosis factor-α and AGE [17]. In type 2 insulin-resistant diabetic nephropathy animal models, 20(*S*)-ginsenoside Rg3 decreased the elevated blood glucose and proteinuria, and augmented creatinine clearance in type 2 diabetic Otsuka Long-Evans Tokushima Fatty rats [18].

Although the renoprotective effect of ginseng components in diabetic models has been reported, there are a few reports that have attempted to elucidate the changes of the podocyte cytoskeleton in diabetes. Recently, we reported that *in vitro* diabetic conditions induced the distributional change and suppressed the production of adaptor proteins, such as ZO-1 [19], p130Cas [20], and β-catenin [21], thus causing the phenotypical changes and hyperpermeability of podocytes, which could be rescued by ginseng total saponin (GTS) [19–21].

In this study, we investigated the effect of GTS on the pathological changes of podocyte cytoskeletal α-actinin-4, an important cytoskeletal linker protein, induced by diabetic conditions.

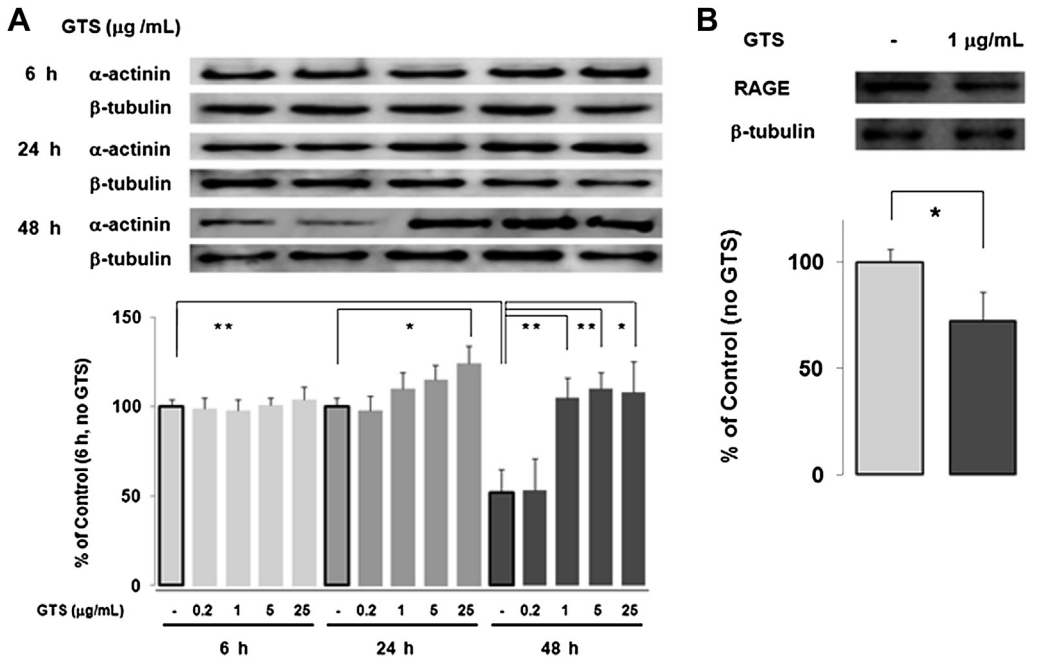

Fig. 2. Effects of ginseng total saponin (GTS) on the α-actinin-4 protein and receptor for advanced glycosylation end products (RAGE) assayed by western blotting. (A) GTS upregulates α-actinin in a dose-dependent manner. (B) GTS downregulates RAGE levels in podocytes. Data on the densitometric analysis of α-actinin-4 or RAGE/β-tubulin ratio are expressed as mean ± standard deviation ($n = 3$). Control (100%), the value without GTS condition. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus control.

2. Materials and methods

2.1. Cell culture of mouse podocytes

Conditionally immortalized mouse podocytes were kindly provided by Dr Peter Mundel (University of Harvard, Boston, MA, USA) and were cultured and differentiated as described previously [22]. Briefly, cells were cultivated at 33°C (permissive conditions) in a culture medium supplemented with 10 U/mL mouse recombinant γ-interferon (Roche, Mannheim, Germany) to induce the expression of temperature-sensitive large T antigens for proliferation. To induce differentiation, podocytes were maintained at 37°C without γ-interferon (non-permissive conditions) for at least 2 wk.

2.2. Culture additives

Mouse podocytes were serum-deprived to reduce the background of serum 24 h before each experiment. The podocytes were then exposed to glucose and/or AGEs. Cells were incubated in culture medium containing either 5mM glucose (normal glucose) or 30mM glucose (high glucose, HG) without insulin. AGEs were produced by a technique previously described by Ha et al [23]. To imitate a long-term diabetic condition, AGEs were added (5 μg/mL) and controls were established using unmodified bovine serum albumin (5 μg/mL). To exclude the effect of additionally produced glycated proteins during culturing, incubation did not last longer than 48 h. For identification purposes, AGEs and bovine serum albumin are denoted as A and B, and 5mM and 30mM glucose are denoted as 5 and 30, respectively. Briefly, B5 is normal, B30 is a short-term diabetic condition, A5 is a long-term normoglycemic or aged condition, and A30 is a long-term diabetic condition.

For ginseng treatment, podocytes were incubated with GTS at various concentrations (0.2, 1, 5, 25 μg/mL) for 6 h, 24 h, and 48 h. GTS was kindly provided by the Korea Ginseng Corporation (Daejeon, Korea).

2.3. Confocal image analysis

Podocytes that were grown on type I collagen-coated glass cover slips incubated for 24 h were fixed in 4% paraformaldehyde, permeabilized in a phosphate buffer solution, blocked with 10% normal goat serum, and labeled with polyclonal goat antimouse α-actinin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Primary antibody-bound specimens were incubated with 1:500 (v/v) Alexa 488 (Molecular Probes, Invitrogen, Eugene, OR, USA) conjugated secondary antibodies at room temperature for 1 h. F-actin was visualized with TRITC-phalloidin (Sigma Chemical, St. Louis, MO, USA) and nuclei were stained with 2mM 4′,6-diamidino-2-phenylindole dihydrochloride (Sigma Chemical). Coverslips were mounted in an aqueous mounting medium and viewed with a fluorescence microscope (BX51, OLYMPUS, Tokyo, Japan).

2.4. Western blotting

The confluently-grown cell layers incubated with additives for α-actinin and various durations for AGE were extracted, and then the protein concentrations were determined as previously described [23]. For the western blotting of α-actinin and the receptor for AGE (RAGE), 30 μg of boiled extracts were applied to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (Bio-Rad Laboratories, Hercules, CA, USA) and transferred to polyvinylidene fluoride membranes. Then, the membranes were air-dried and blocked in 3% fat-free milk before incubation with antiα-actinin antibody or antiRAGE antibody (Santa Cruz Biotechnology). After incubation with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology), bands were detected using the ECL chemiluminescence system (Amersham Biotech Ltd., Bucks, UK). Data on the densitometric analysis of respective proteins/β-tubulin ratio are expressed as mean ± standard deviation.

2.5. Statistical analysis

The results are presented as mean values ± standard deviation, as required under different conditions. The statistical significance was assessed using a nonparametric Kruskal-Wallis analysis of varience or Student *t* test using the SPSS 9.0.0 (SPSS, Chicago, IL, USA) software program. A p-value < 0.05 was considered significant.

3. Results

3.1. Confocal image changes of α-actinin

The α-actinin staining, located in the peripheral cytoplasm and processes of podocytes, was co-localized at the terminal ends of actin filaments. Diabetic conditions, especially in more pathological A30 at 24 h, concentrated α-actinin-4 staining of the peripheral cytoplasm and disrupted F-actin fibers (Fig. 1A). Such distributional change of α-actinin-4 and F-actin fibers was reversed by 1 μg/mL of GTS (Fig. 1B).

3.2. Effects of GTS on podocyte α-actinin and RAGE

In western blotting, GTS significantly ($p < 0.05$) upregulated the α-actinin-4 protein of the podocytes at longer durations (24 h and 48 h) in a dose-dependent manner compared with the control (B5) (Fig. 2A). GTS also downregulated RAGE levels in podocytes by 28.1% ($p < 0.05$) compared with B5 (Fig. 2B). GTS therefore might have a positive influence on the α-actinin protein of podocytes partly by inhibiting RAGE expression.

3.3. Western blotting of α-actinin in cultured podocyte

The bands for α-actinin protein at 100 kDa were compared to those of β-tubulin. Density values for the α-actinin protein of representative immunoblots from each group revealed that HG (B30) suppressed the amount of α-actinin protein by 26.8% at 24 h and 24.1% at 48 h. These reductions were significant when compared with the control (B5). AGE (A5) alone or HG and AGE (A30) conditions also significantly suppressed the amount of α-actinin protein at 24 h and 48 h ($p < 0.05$) compared with B5 at 6 h after correcting for the β-tubulin levels (Figs. 3A and 3B). GTS (1 μg/mL) rescued the quantitative changes in the amount of α-actinin protein induced by diabetic conditions at 48 h ($p < 0.05$). Results on B5 and A30 podocytes were compared according to the exposure times given in Fig. 3C. These observations suggest that both HG and AGE induced cytoplasmic relocalization and concentration and suppressed the production of α-actinin-4 in an *in vitro* diabetic milieu, which could be mitigated by GTS (Fig. 4).

4. Discussion

The podocyte consists of a cell body, major processes, secondary processes, and finely interdigitating foot processes [10,11]. The podocyte cell body and major and secondary foot processes contain vimentin-rich intermediate filaments, and the larger microtubules form organized structures along the major and secondary processes [10,11,24]. The podocyte foot processes contain long, dense actin fiber bundles that run cortically and contiguously to link adjacent processes and are connected with an array of linker proteins to both the slit diaphragm and the GBM anchor proteins [8–11]. These interactions are an essential prerequisite to maintain the highly ordered foot process architecture, and hence the filtration barrier. The foot process effacement, a morphological change in proteinuric conditions, including advanced diabetic nephropathy, leads to

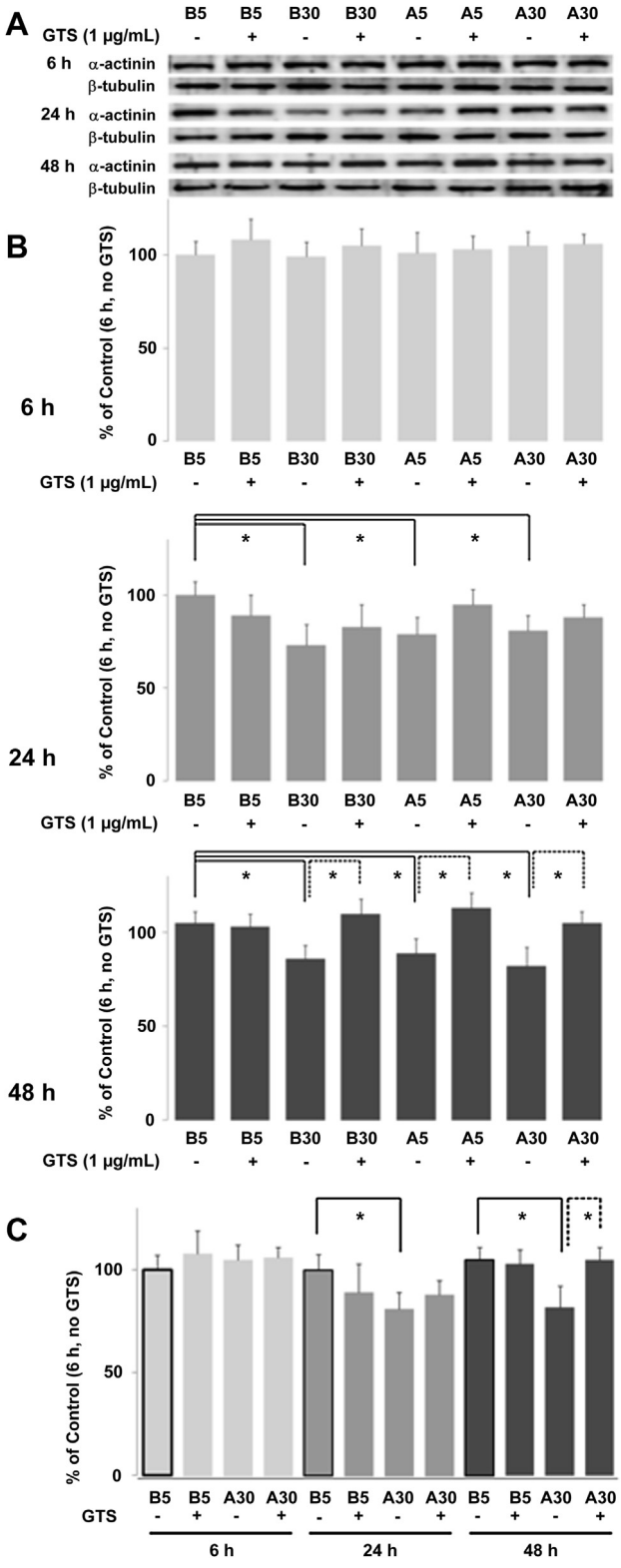

Fig. 3. Effects of diabetic conditions and ginseng total saponin (GTS) on the α-actinin-4 protein assayed by western blotting. (A) The bands for α-actinin protein at 100 kDa were compared with those of β-tubulin. (B) After 24 hours of incubation time, high glucose (B30), advanced glycosylation end products (AGE) (A5), or high glucose plus AGE (A30) conditions significantly suppress the amount of α-actinin protein. (C) Results on B5 and A30 were compared according to the exposure times. Data on the densitometric analysis of the α-actinin/β-tubulin ratio are expressed as mean ± standard deviation ($n = 3$). Control (100%) the value of the B5. *$p < 0.05$ versus. control.

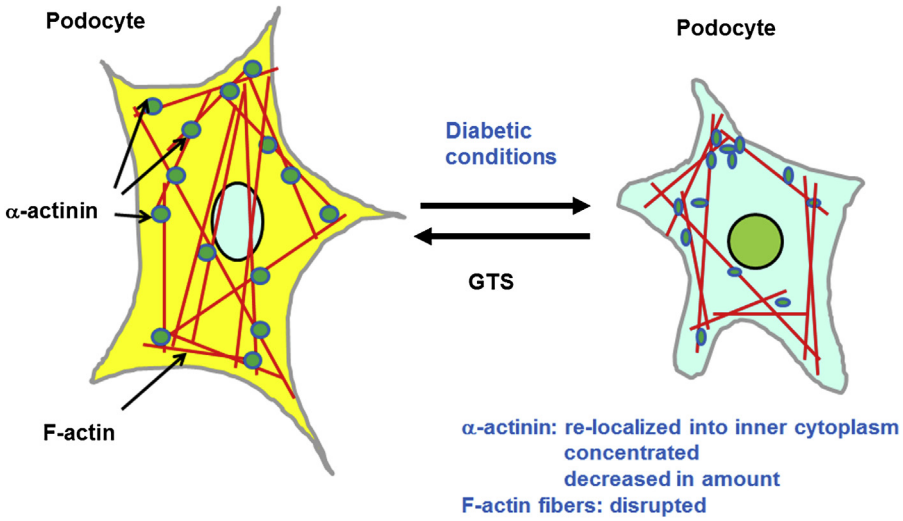

Fig. 4. Schematic view of cytoskeletal changes in the podocyte. Both high glucose and advanced glycosylation end products (AGE) induce the inner cytoplasmic relocalization and suppress the production of α-actinin-4 with the disruption of actin fibers, which were mitigated by ginseng total saponin (GTS).

alterations in the cell–cell contacts at the slit diaphragm and mobilization of the cell-matrix contacts [8,25].

The actin filaments of the podocyte foot processes are linked by linker proteins, such as α-actinin-4, synaptopodin, and cortactin [6–11]. The α-actinin molecule is an elongated, symmetrical, and anti-parallel dimeric rod with actin-binding sites at focal contacts on the plasma membrane that enable cross-linkage of F-actin filaments into contractile bundles [10,12,26]. The α-actinin molecule is highly expressed in podocytes and is required for normal podocyte adhesion.

A form of human familial autosomal-dominant focal and segmental glomerulosclerosis is known to be associated with function mutations of the *ACTN4* gene. The mutant actinins showed increased F-actin affinity [13], and α-actinin-4 was noted as a key molecule in maintaining podocyte cytoskeletal integrity in physiological and pathological conditions. Knockout [27] and transgenic studies [28] have also emphasized the critical role of α-actinin-4 in maintaining podocyte integrity in animals. These genetic results demonstrate that podocyte damage and proteinuria can result from cytoskeletal alterations. The fact that loss-of-function mutations can lead to proteinuria and focal and segmental glomerulosclerosis supports further investigation of the subtle inherited and acquired changes in α-actinin-4 that may be involved in the development of human and animal kidney diseases. We reported that *in vitro* both HG and AGE can induce cytoplasmic relocalization and condensation and also suppress the production of α-actinin-4 at the transcriptional level. These changes may explain the cytoskeletal changes of the podocyte in diabetic conditions [12]. The results reported by Ha [12] relating to podocyte α-actinin-4 are similar to this study and are mitigated by GTS.

Ginseng has been reported to be effective in the prevention and treatment of diabetic nephropathy of type 1 diabetic animal models. Sun ginseng [14], heat-processed American ginseng [15], 20(*S*)-ginsenoside Rg3 [16], and KRG [17] ameliorated elevated serum glucose and renal damage in streptozotocin-induced type 1 insulin-dependent diabetic nephropathy animal models. In particular, KRG decreased serum glucose and significantly reduced the AGE formation and secretion, the levels of N-(carboxymethyl) lysine, and the expression of RAGE in the diabetic kidney. KRG also prevented the streptozotocin-induced destruction of glomerular structure and significantly suppressed HG-induced fibronectin production [17]. In this study, we found that GTS downregulated the RAGE levels in podocytes, which could explain the protective role of ginseng substances on diabetic glomerular pathology.

Although the renoprotective effect of ginseng components in diabetic models has been reported, there were a few reports which elucidated the changes of glomerular filtration structures. Focusing on glomerular filtration structures, ginsenoside Rgl improved the diabetic pathological changes of glomerular filtration, such as GBM thickness and podocytopenia with the reduction of urine protein and serum creatine [29]. Ginsenoside Rgl also improved the overexpressed levels of serum monocyte chemotactic protein-1 and tumor necrosis factor-α, which correlated with the improved clinical and pathological indices. Each gram of GTS, assayed by HPLC and provided by Korea Ginseng Corporation, contains Rg1 (94.6 mg), Re (87.0 mg), Rf (28.5 mg), Rh1 (5.6 mg), Rg2 (23.7 mg), Rb1 (161.6 mg), Rc (81.1 mg), Rb2 (76.9 mg), Rd (39.7 mg), Rg3 (22.5 mg), and Rh2 (24.8 mg). The effects of each GTS component on the glomerular structure in the pathological condition need to be examined.

Recently, we reported that *in vitro* diabetic conditions induced the distributional change and suppressed the production of ZO-1 [19], p130Cas [20], and β-catenin [21], which adapted the slit diaphragm and the GBM to the cytoskeleton. We also found that such distributional and quantitative changes in ZO-1 were associated with podocyte hyperpermeability at early incubation times (2–8 h) [19], however, there were no significant changes in α-actinin-4 at 6 h incubation in this study. The apparent decreases in α-actinin protein were observed at 24 h and 48 h. We therefore suggest that *in vitro* diabetic conditions induced the distributional and quantitative changes of adaptor proteins at an early stage, causing podocyte hyperpermeability, and thereafter distributional and quantitative changes in the cytoskeletal proteins.

In this study, we found that in an *in vitro* diabetic milieu, both HG and AGE induced the cytoplasmic translocation and suppressed the production of α-actinin-4, which could be mitigated by GTS (Fig. 4); therefore GTS would be helpful in the prevention of cytoskeletal changes of podocytes in diabetic nephropathy.

Conflict of interest

We declare that no financial conflict of interest exists in relation to the publication of this work.

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