

Original Article

Triptolide potentiates the cytoskeleton-stabilizing activity of cyclosporine A in glomerular podocytes via a GSK3 β dependent mechanism

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Abstract: Tripterygium wilfordii Hook F. (TwHF) is a traditional Chinese herb and has a broad spectrum of biological functions including immunosuppression and anti-inflammatory effects. When used in combination with other standard of care medications, such as glucocorticoids and calcineurin inhibitors like cyclosporine A, for treating glomerular diseases, TwHF demonstrates a remarkable dose-sparing effect, the molecular mechanism for which remains largely unknown. In an *in vitro* model of podocytopathy elicited by a diabetic milieu, triptolide, the major active component of TwHF, at low doses, potentiated the beneficial effect of cyclosporine A, and protected podocytes against diabetic milieu-elicited injury, mitigated cytoskeleton derangement, and preserved podocyte filtration barrier function, entailing a synergistic cytoskeleton-preserving and podocyte protective effect of triptolide and cyclosporine A. Mechanistically, inhibitory phosphorylation of GSK3 β , a key molecule recently implicated as a convergence point of podocytopathic pathways, is likely required for the synergistic effect of triptolide and cyclosporine A on podocyte protection, because the synergistic effect was largely blunted in cells expressing the constitutively active GSK3 β . Ergo, a synergistic podocyte cytoskeleton-stabilizing mechanism seems to underlie the cyclosporine A-sparing effect of triptolide in glomerulopathies. Combined triptolide and cyclosporine A therapy at reduced doses may be an invaluable regimen for treating diabetic nephropathy.

Keywords: Triptolide, podocyte, kidney, glomerulus, cytoskeleton

Introduction

Tripterygium wilfordii Hook F. or thunder god vine is a traditional Chinese herb that has a broad spectrum of biological functions such as immunosuppression, anti-inflammatory, anti-fertility and neuroprotective effects [1, 2]. For thousands of years, this herbal medicine has been used successfully for treating kidney diseases, rheumatoid arthritis, psoriasis, systemic lupus erythematosus, inflammatory bowel disease, and as a male contraceptive [3-6]. For glomerular diseases like minimal change disease (MCD) and focal segmental glomerulosclerosis (FSGS), Tripterygium wilfordii Hook F. is highly efficacious as monotherapy. When used in combination therapy with other standard of care medications, such as glucocorti-

coid, mycophenolate mofetil and calcineurin inhibitors like cyclosporine A, for treating glomerular diseases, Tripterygium wilfordii Hook F. demonstrates a remarkable dose-sparing effect that is able to potentiate the therapeutic activity at reduced doses while minimize the adverse effect [5, 7-9]. This makes Tripterygium wilfordii Hook F. a great choice of therapy for long-term maintenance treatment of refractory glomerulopathies. However, despite clinical observations in support of the dose-sparing effect of Tripterygium wilfordii Hook F. in glomerular disease, the underlying molecular mechanism remains largely obscure.

Central to the pathogenesis of nephrotic glomerulopathies like MCD and FSGS is the injury of glomerular podocytes, which enwrap glomer-

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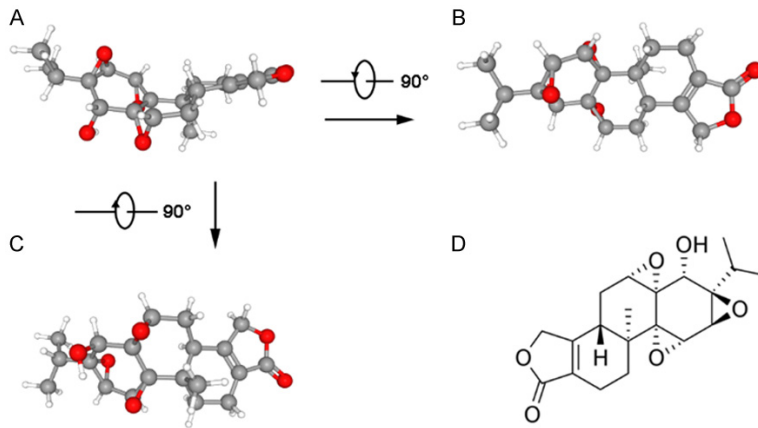


Figure 1. The chemical structure of triptolide, a biologically active oxygenated diterpenoid epoxide and the major pharmacologically active component of *Tripterygium wilfordii* Hook F. A-C. Front, top and bottom views of the 3-D conformer of triptolide; D. The 2-D view of the chemical structure of triptolide.

ular capillary tufts via the unique interdigitating foot processes and play a key role in governing glomerular permselectivity, fulfilling blood ultrafiltration, i.e. the extraction of urine and the retention of protein. In case of podocyte injury featured by podocyte cytoskeleton derangement and foot process effacement, massive plasma protein leaks from circulation into urine, resulting in heavy proteinuria and the ensuing nephrotic syndrome [10]. Calcineurin inhibitors like cyclosporine A have been known to confer a direct protective effect on podocyte cytoskeleton and thereby improve podocyte injury via a mechanism independent of its systemic immune suppression [11].

Triptolide is a biologically-active oxygenated-diterpene derived from *Tripterygium wilfordii* Hook F. (Figure 1) and is the major pharmacologically active metabolite that mediates the therapeutic effects, including the beneficial effects on kidney cells *in vitro* and *in vivo* [12-15]. Though cyclosporine A has been commonly used in combination with *Tripterygium wilfordii* Hook F. to treat glomerular diseases, whether and how triptolide interacts with cyclosporine A in kidney parenchymal cells has been barely studied. This study examined the impact of triptolide on the effect of cyclosporine A in glomerular podocytes in an *in vitro* model of podocytopathy elicited by a diabetic milieu. We found that triptolide is able to potentiate the cytoskeleton preserving effect of cyclosporine A and reinforce the podocyte protective activity. The synergistic effect of trip-

tolide and cyclosporine A on protecting against podocyte injury is likely dependent on enhancing the inhibitory phosphorylation of glycogen synthase kinase (GSK)3 β , a key molecule recently implicated as a convergence point of multiple podocytopathic signaling pathways.

Materials and methods

Reagents

Triptolide, cyclosporine A, mannitol and D-glucose were purchased from Sigma-Aldrich (St. Louis, MO, USA). TGF β 1 was acquired from R&D Systems (Minneapolis, MN, USA). Lipofectamine 2000 was purchased from Life Technologies (Carlsbad, CA, USA). The antibodies against p-GSK3 β and GSK3 β were purchased from Cell Signaling Technology, and those against synaptopodin, haemagglutinin (HA) and GAPDH were acquired from Santa Cruz Biotechnology. The counterstaining reagent 4',6-diamidino-2-phenylindole (DAPI) was purchased from Vector Laboratories (Burlingame, CA, USA). Phalloidin was purchased from Invitrogen (Skokie, IL, USA). G-Actin/F-Actin *In Vivo* Assay Biochem kit was acquired from Cytoskeleton, Inc. (Denver, CO, USA).

Cell culture and transient transfection

Conditionally immortalized mouse podocytes in culture (courtesy of Dr. Stuart Shankland, University of Washington, Seattle, WA) were cultured in RPMI 1640 medium (Invitrogen, Skokie, IL, USA) supplemented with 10% FBS in a humidified incubator with 5% CO $_2$. The cells were cultured at 33°C with 50 units/ml recombinant mouse interferon- γ (Millipore, Billerica, MA, USA) on collagen-coated plastic Petri dishes and were transferred to a 37°C incubator without interferon- γ to induce differentiation for 14 days. Podocytes were pretreated with cyclosporine A (0.2 μ g/ml), triptolide (0.5, 1 or 3 ng/ml) or vehicle for 30 min and then exposed to the diabetic milieu consisting of glucose (25 mM) and TGF β 1 (2 ng/ml), or a high osmolality control medium containing mannitol (20 mM) for 36 h.

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The eukaryotic expression vectors encoding the Empty Vector (EV) or the haemagglutinin (HA)-tagged constitutively active (S9A) mutant (S9A-GSK3 β -HA/pcDNA3) were employed as previously described [16] and transfected to podocytes by using Lipofectamine 2000. After transfection, the cells were cultured under non-permissive conditions in normal growth medium for 36 h before transfection efficiency was assessed by immunoblot analysis for target molecules. Cells were then exposed to the diabetic milieu or control medium, and other treatments for 36 h. Cells were subsequently collected and prepared for Western blot analysis or other assays.

Western immunoblot analysis

Cultured cells were lysed in radioimmunoprecipitation assay buffer containing protease inhibitors. Protein samples were processed for immunoblot analysis as previously described [23]. In brief, samples of equal amounts of proteins (40 μ g) were fractionated by 10% or 7.5% SDS-PAGE and transferred to 0.45 μ m PVDF membranes (Thermo Fisher Scientific, Waltham, MA, USA). The membranes were then blocked and incubated overnight with primary antibodies against the following molecules: synaptopodin, p-GSK3 β , GSK3 β , HA, actin and GAPDH. Finally, the membranes were detected by HRP-labelled secondary antibody and visualized with an enhanced ECL substrate kit (Thermo Fisher Scientific, Waltham, MA, USA) on G:Box imaging system (Syngene, MD, USA).

Phalloidin staining of F-actin

Podocytes in culture were fixed in 4% paraformaldehyde in PBS and permeabilized. Filamentous actin (F-actin) was stained by incubation with rhodamine-conjugated phalloidin (Invitrogen, IL, USA). Then, cells were counterstained with DAPI and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Images were documented using the Leica TCS SP5 multiphoton laser scanning confocal microscope and analyzed in a blinded fashion.

Measurement of the F-actin/G-actin ratios

The amounts of intracellular G-actin and F-actin were measured using the G-Actin/F-Actin *in vivo* assay kit (Cytoskeleton Inc., Denver, CO,

USA). In brief, podocytes were lysed and F-actin filaments stabilized according to the manufacturer's instructions by using the F-actin stabilization buffer. Subsequently, after ultracentrifugation, cell lysates containing equal amounts of proteins, as determined by using a bicinchoninic acid protein assay, were separated into soluble and insoluble fractions. Both fractions were analyzed in parallel by immunoblot analysis with an anti-actin antibody. G- and F-actin bands on western blots were scanned by densitometry. The F-actin to G-actin ratio was calculated by dividing the F-actin density regulated by the G-actin binding, and was expressed as a percentage of that in the control group.

Wound healing assay

Confluent monolayers of differentiated podocytes were scraped with a 10 μ l pipette. Phase-contrast micrographs were obtained at 0 h and 24 h after scratching using an inverted microscope (EVOS XL, Thermo Fisher Scientific, Waltham, MA, USA). The migration and wounding areas were calculated and analyzed using Image J version 1.52a (NIH, Bethesda, MD) image processing program.

Paracellular permeability assay

An albumin influx assay was used to evaluate the filtration barrier function of podocyte monolayers. Primarily cultured podocytes were seeded into the collagen-coated Corning Transwell inserts (3 μ m pore, Corning, NY, USA) in the top chamber. After different treatments for 24 h, podocytes were washed with PBS. The top chambers were then refilled with 0.15 ml RPMI 1640 and the bottom chambers were refilled with 1 ml RPMI 1640 supplemented with 40 mg/ml of bovine serum albumin and then incubated at 37°C. At 3 h, 20 μ l medium in the top chambers were collected and prepared for albumin concentration assay by using a bicinchoninic acid protein assay kit (GenDEPOT, Barker, TX, USA).

Detection of reactive oxygen species (ROS) generation by fluorescence

Intracellular levels of ROS were monitored by measuring the changes in fluorescence resulting from the oxidation of 5',6'-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate-ace-

tyl ester (CM-H₂DCFDA, Molecular Probes, Eugene, OR, USA). CM-H₂DCFDA diffuses into cells, and intracellular fluorescent dichlorodihydrofluorescein (DCF) derivatives were generated by cleavage of the ester groups by cellular esterases and oxidation by ROS. Briefly, cells were loaded with 20 μ M CM-H₂DCFDA and fluorescence was determined at baseline or at indicated times following different treatments as previously described [17]. The relative DCF fluorescence value was expressed as the percent change in that of cells treated with the diabetic milieu alone.

Statistics

For immunoblot analysis, bands were scanned and the integrated pixel density was determined using a densitometer and the ImageJ analysis program (NIH, Bethesda, MD). All data are expressed as mean \pm SD. Unless otherwise indicated, all experimental observations were repeated three times. Statistical analysis of the data from multiple groups was performed by repeated measures ANOVA followed by Tukey tests. $P < 0.05$ was considered statistically significant.

Results

Triptolide potentiates the protective effect of cyclosporine A on podocytes, resulting in an improved integrity of actin cytoskeleton

The conditionally immortalized murine podocytes in culture were well-differentiated under nonpermissive conditions, characterized by abundant expression of synaptopodin, a podocyte homeostatic marker, as shown by western immunoblot analysis (**Figure 2A-D**), as well as typical arborized morphologic features, accompanied by intense ventral stress fibers marked by phalloidin-labeled filamentous actin (F-actin) (**Figure 2E**). Glomerular podocytes are known to be a target for diabetes-related injuries, and as a matter of fact, diabetic podocytopathy has been regarded as the origin of diabetic kidney disease [18, 19]. In our hands, exposure to a diabetic milieu consisting of high ambient glucose (25 mM) and transforming growth factor (TGF) β 1 (2 ng/ml) caused prominent podocyte injury, as evidenced by drastic loss of synaptopodin expression (**Figure 2A-D**). This was concomitant with reduced arborization, podocyte shrinkage, aster like cell shape and an impaired

actin cytoskeleton integrity that manifested as increased expression of cortical filaments and diminished ventral stress fibers, indicative of a more migratory phenotype (**Figure 2E**). In contrast, as an osmolality control, mannitol treatment resulted in a phenotype no different from the resting podocytes. In agreement with previous reports [20, 21], triptolide protected podocytes against the diabetic milieu-elicited loss of synaptopodin in a dose-dependent fashion (**Figure 2A**). Despite the trivial beneficial effect exerted by triptolide (0.5 ng/ml) or cyclosporine A (0.2 μ g/ml) alone treatment at low doses, combined treatment with triptolide and cyclosporine A, in stark contrast, largely abrogated the injurious effect of the diabetic milieu on synaptopodin expression (**Figure 2C, 2D**), podocyte shape, and the integrity of actin cytoskeleton (**Figure 2E**), suggesting a synergistic protective effect.

Actin cytoskeleton exists in podocytes in a state of dynamic equilibrium between assembly and disassembly, corresponding to two forms of actin proteins, i.e. filamentous(F)-actin polymer microfilaments and globular-actin monomers [22]. To validate the morphologic findings of cytoskeleton, the *in vivo* F-actin/G-actin assay was conducted. Shown in **Figure 2F** and **2G**, F-actin was found to be predominant in differentiated podocytes under resting conditions. Exposure to the diabetic milieu reversed the F-actin/G-actin ratio and made G-actin predominant. At low doses, triptolide or cyclosporine A alone treatment had a marginal impact on diabetic milieu-elicited injury. In contrast, in combination with triptolide, cyclosporine A strikingly mitigated the diabetic milieu effect and largely restored the F-actin/G-actin ratio in podocytes, implying that triptolide potentiates the cytoskeleton stabilizing activity of cyclosporine A.

Triptolide synergizes with cyclosporine A to restore the homeostatic motility of podocytes and preserve podocyte filtration barrier function

Actin cytoskeleton integrity is critical for maintaining the homeostasis of the podocyte foot processes, a key element of the glomerular filtration barrier [23]. *In vivo*, actin cytoskeleton derangement entails foot process effacement, podocyte hypermotility and impaired glomeru-

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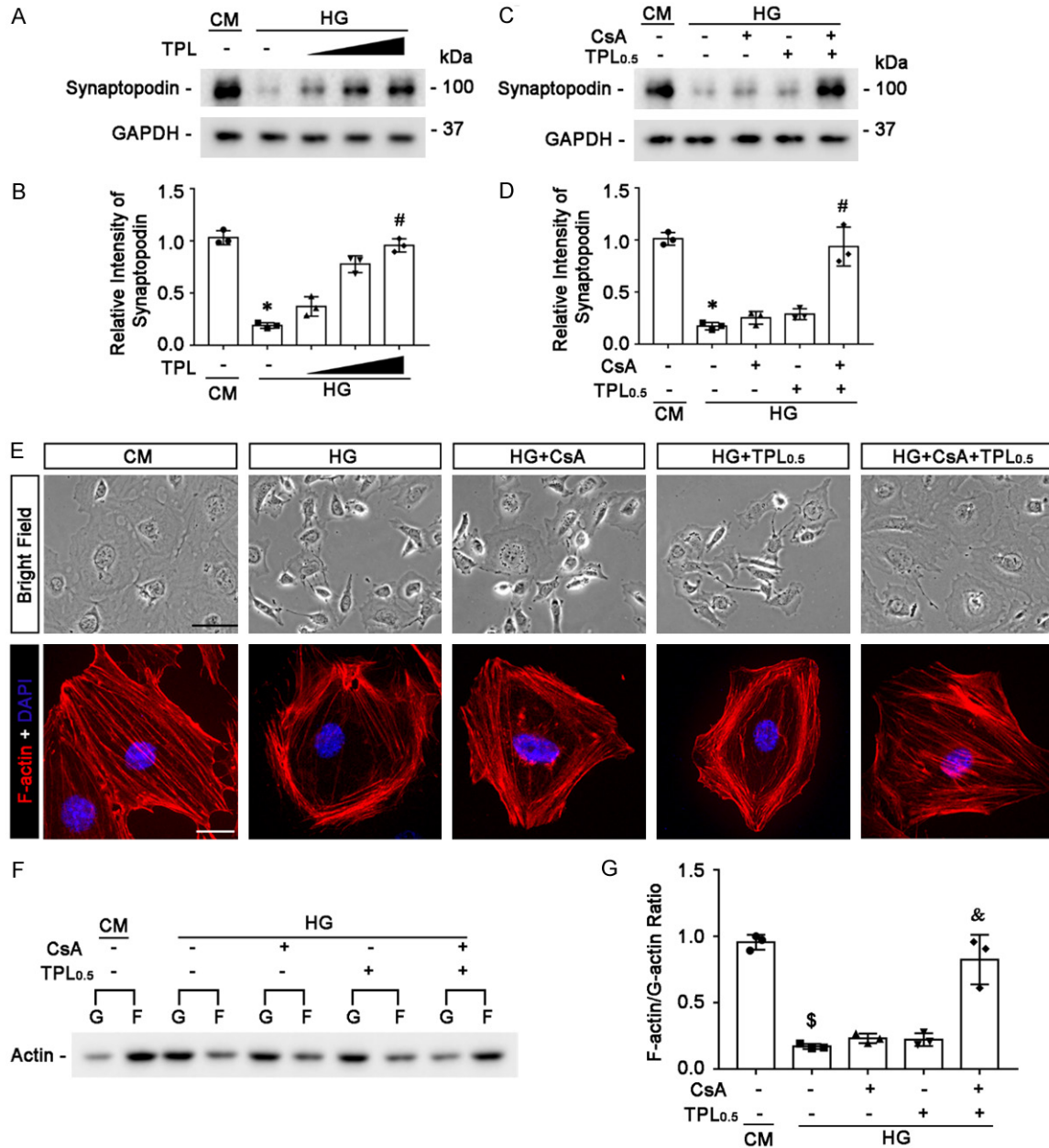


Figure 2. The podocyte protective and cytoskeleton-stabilizing effect of cyclosporine A is amplified by triptolide. (A) Differentiated podocytes were treated with triptolide (TPL) (0.5, 1 and 3 ng/ml) or vehicle for 30 minutes and then exposed to a diabetic milieu (HG) consisting of high glucose (25 mM) and TGFβ1 (2 ng/ml), or to a high osmolality control medium (CM) containing 20 mM mannitol for 36 h. Cell lysates were subjected to immunoblot analysis for synaptopodin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (cropped blots are shown as indicated by the lines and uncropped blots are included in the [Supplementary Figure 1](#)). (C) Differentiated podocytes were treated with low-dose cyclosporine A (CsA, 0.2 μg/ml) in the presence or absence of low-dose triptolide (TPL_{0.5}, 0.5 ng/ml) for 30 minutes and then exposed to the diabetic milieu (HG) or control medium (CM) for 36 h. Cell lysates were subjected to immunoblot analysis for synaptopodin and GAPDH (cropped blots are shown as indicated by the lines and uncropped blots are included in the [Supplementary Figure 1](#)). (B and D) Immunoblots were subjected to densitometric analysis and arbitrary units were expressed respectively as immunoblot densitometric ratios of synaptopodin to GAPDH as folds of the control group. **P* < 0.01 versus the CM group. #*P* < 0.01 versus HG alone treatment (n = 3). (E) Representative phase contrast micrographs show podocyte shape changes. Podocytes were large, flat and arborized cells with evidently rich stress fibers in cytoplasm and well-developed processes under basal conditions. Following exposure to the diabetic milieu, podocytes demonstrated prominent cytopathic changes, marked by podocyte shrinkage, reduced arborization, and asterlike cell shape. Combined cyclosporine A and triptolide treatment strikingly attenuated changes in podocyte morphology. Bar = 100 μm. Alternatively, podocytes were processed for fluorescent labeling of cytoskeletal filamentous actin (F-actin) with rhodamine-conjugated phalloidin and counter-

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stained with 4',6-diamidino-2-phenylindole (DAPI). Representative fluorescent microscopic images show changes in F-actin cytoskeleton. Bar = 25 μm . (F and G) Podocytes were lysed and subjected to the F-Actin/G-Actin in vivo assay. Representative immunoblots were shown (F), cropped blots are shown as indicated by the lines and uncropped blots are included in the [Supplementary Figure 1](#), and subjected to densitometric analysis (G). The F-actin to G-actin ratio was calculated as immunoblot densitometric ratios of F-actin to G-actin as folds of the CM group. $^{\$}P < 0.01$ versus the CM group. $^{\&}P < 0.01$ versus HG alone treatment ($n = 3$).

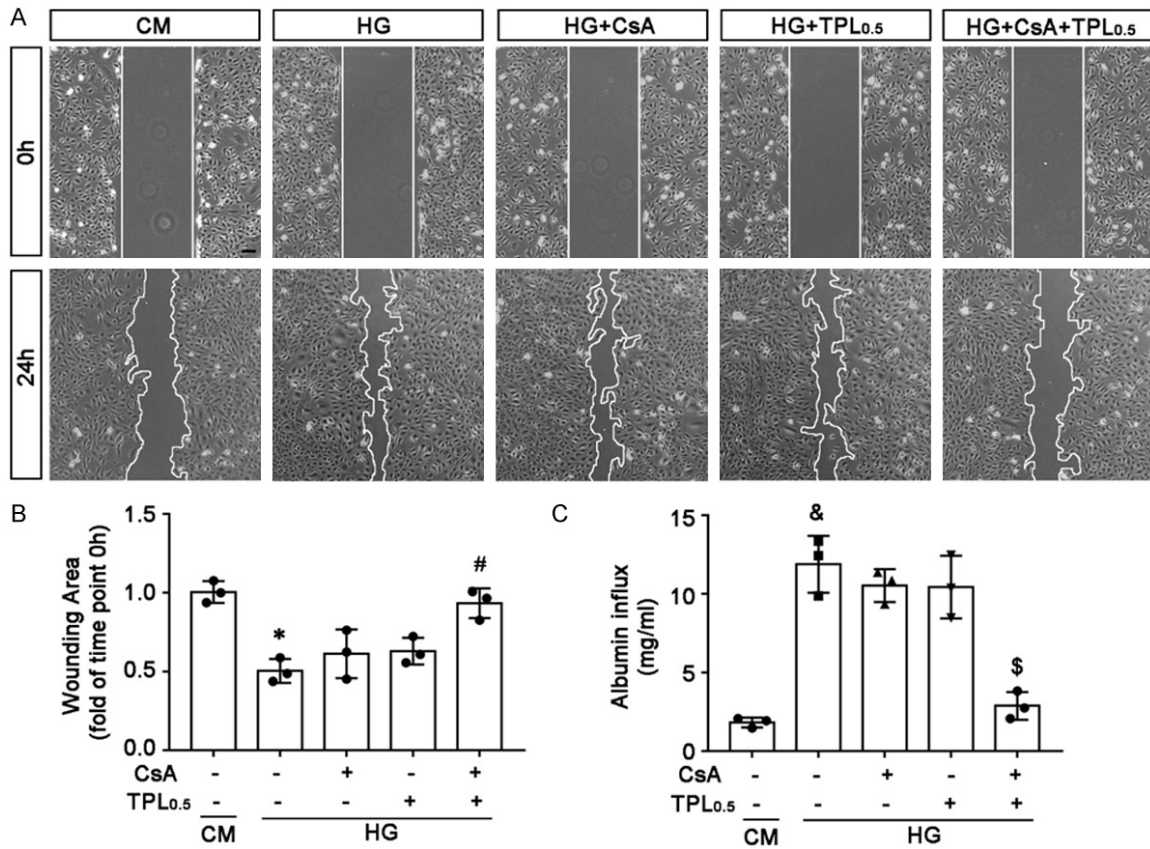


Figure 3. Triptolide potentiates the effect of cyclosporine A to counteract the diabetic milieu-elicited podocyte hypermotility and impairment of podocyte filtration barrier function. **A.** Confluent monolayers of differentiated podocytes were scraped with a 10 μl pipette and treated with low-dose cyclosporine A (CsA, 0.2 $\mu\text{g}/\text{ml}$) in the presence or absence of low-dose triptolide (TPL_{0.5}, 0.5 ng/ml) for 30 minutes before stimulation with the diabetic milieu (HG) or control medium (CM) for 24 h. Representative phase contrast micrographs were obtained at 0h and 24h after scratching (Bar = 100 μm). **B.** Computerized morphometric analysis of the cell migration and wounding areas following the indicated treatments. $^*P < 0.01$ versus the CM group ($n = 3$); $^{\#}P < 0.01$ versus HG alone treatment ($n = 3$). **C.** Podocyte monolayers on collagen-coated Transwell filters were treated with cyclosporine A (0.2 $\mu\text{g}/\text{ml}$) in the presence or absence of triptolide (TPL_{0.5}, 0.5 ng/ml) for 30 minutes before stimulation with the diabetic milieu (HG) or control medium (CM) for 24 h. Albumin influx across podocyte monolayers was then determined by the paracellular permeability assay for 3 h. $^{\&}P < 0.01$ versus the CM group ($n = 3$); $^{\$}P < 0.01$ versus HG alone treatment ($n = 3$).

lar filtration barrier [24]. To discern if the synergistic effect of triptolide and cyclosporine A has any functional consequences, podocytes were subjected to a traditional cellular wound-healing assay for assessing cellular migration and motility in the presence of various treatments. Shown in **Figure 3A**, podocytes had a basal level of motility and migratory capacity that lessened the distances between the leading edges of the migrating podocyte sheets. Exposure to the diabetic milieu substantially

accelerated the closure of the gap between the invading fronts of the cells. Triptolide or cyclosporine A alone treatment at low doses minimally affected the effect of diabetic milieu. However, combined triptolide and cyclosporine A treatment markedly attenuated the effect of the diabetic milieu and mitigated podocyte hypermotility. The morphologic findings were corroborated by computerized morphometric analysis of the cell migration and wounding areas (**Figure 3B**). To further evaluate the

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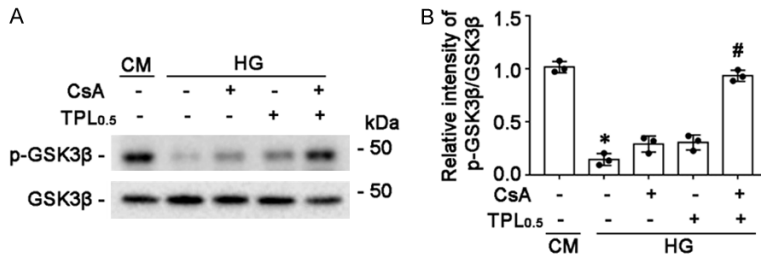


Figure 4. Cyclosporine A and triptolide synergize on reinstating the inhibitory phosphorylation of GSK3 β in podocytes exposed to the diabetic milieu. A. Differentiated podocytes were treated with low-dose cyclosporine A (CsA, 0.2 μ g/ml) in the presence or absence of low-dose triptolide (TPL_{0.5}, 0.5 ng/ml) for 30 minutes before stimulation with a diabetic milieu (HG) consisting of glucose (25 mM) and TGF β 1 (2 ng/ml), or a high osmolality control medium (CM) containing 20 mM mannitol for 36 h. Cell lysates were subjected to immunoblot analysis for p-GSK3 β and GSK3 β (cropped blots are shown as indicated by the lines and uncropped blots are included in the [Supplementary Figure 2](#)). B. Immunoblots were subjected to densitometric analysis and arbitrary units were expressed as immunoblot densitometric ratios of p-GSK3 β to GSK3 β as folds of the control group. * $P < 0.01$ versus the CM group (n = 3); # $P < 0.01$ versus HG alone treatment (n = 3).

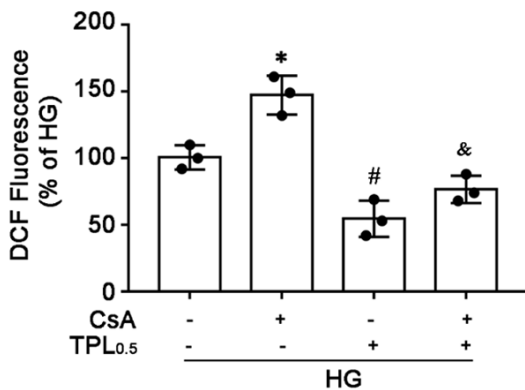


Figure 5. Triptolide mitigates the cyclosporine A exacerbated oxidative stress in podocytes exposed to the diabetic milieu. Differentiated podocytes were treated with cyclosporine A, triptolide and exposed to a diabetic milieu (HG), as elaborated in [Figure 4](#), in the presence of 5',6'-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate-acetyl ester (CM-H₂DCFDA, 20 μ M) for 36 h, followed by dichlorodihydrofluorescein (DCF) fluorometric analysis. * $P < 0.01$ versus HG alone treatment (n = 3); # $P < 0.01$ versus the CsA-treated group (n = 3).

changes in the barrier function of podocyte monolayers, podocytes were subjected to the paracellular permeability assay, which measures the albumin flux rate across the monolayers of podocytes cultured on collagen-coated Transwell filters. Shown in [Figure 3C](#), exposure to the diabetic milieu resulted in drastic albumin influx across the podocyte monolayers. This effect was minimally affected by triptolide or cyclosporine A alone treatment at low doses,

but was considerably overridden by combined triptolide and cyclosporine A treatment.

Triptolide potentiates the cyclosporine A induced inhibitory phosphorylation of GSK3 β , concomitant with an amplified antioxidant effect

Recent evidence suggests that GSK3 β plays a key role in podocyte injury and cytoskeleton derangement [25]. Many injurious stimuli, including high ambient glucose, are known to cause podocytopathy, at least in part via over-activating GSK3 β [26, 27]. Calcineurin has been known to dephosphorylate and thus

over-activate GSK3 β [28], while cyclosporine A is a standard inhibitor of calcineurin. Indeed, in cultured podocytes, cyclosporine A alone treatment, even at a low dose (0.2 μ g/ml), apparently counteract the effect of the diabetic milieu and noticeably restored the inhibitory phosphorylation of GSK3 β , as measured by immunoblot analysis ([Figure 4A](#)) followed by densitometric analysis ([Figure 4B](#)), denoting a promoted inhibition of GSK3 β . This effect of cyclosporine A was drastically augmented by co-treatment with low-dose triptolide ([Figure 4A and 4B](#)), which alone also demonstrated a noticeable but trivial effect on the inhibitory phosphorylation of GSK3 β , thus suggesting a synergistic effect of triptolide and cyclosporine A on the inhibitory phosphorylation of GSK3 β .

GSK3 β is a prototypical redox-sensitive kinase and its inhibitory phosphorylation is highly affected by redox status [29]. In addition, cyclosporine A has been shown to promote oxidative stress in a number of cells [30, 31], whereas triptolide has been reported to exert an antioxidant activity in podocytes [32, 33]. Thus, it is plausible to hypothesize that the triptolide sensitized inhibitory phosphorylation of GSK3 β in cyclosporine A-treated podocytes may be associated with redox changes. To test this hypothesis, the levels of ROS in podocytes after different treatments were assessed by measuring oxidation and conversion of CM-H₂DCFDA into fluorescent DCF derivatives, a process that is highly dependent on the redox status. Shown in

Figure 5, exposure to the pro-oxidant diabetic milieu resulted in a basal level of ROS production in podocytes. Cyclosporine A augmented, while triptolide lessened the ROS generation. The pro-oxidant effect of cyclosporine A was completely mitigated by concomitant triptolide treatment, in parallel with the potentiated inhibitory phosphorylation of GSK3 β (**Figure 4A** and **4B**), inferring that the combined antioxidant effect may underlie the synergistic effect of triptolide and cyclosporine A on the inhibitory phosphorylation of GSK3 β .

GSK3 β inhibition is required for the synergistic effect of triptolide on cyclosporine A-mediated podocyte protection

To determine if the synergistic phosphorylation of GSK3 β triggered by triptolide and cyclosporine A has functional corollaries, podocytes were subjected to transient transfection with an empty vector (EV) or a vector encoding the dominant active GSK3 β (S9A), in which the serine 9 residue is substituted by alanine thus resulting in a constitutively active and uninhibitable mutant. Podocytes were satisfactorily transfected with the vectors, as evidenced by immunoblot analysis for hemagglutinin (HA) (**Figure 6A**), which is tagged to the mutant GSK3 β . S9A-expressing cells were subsequently exposed to the diabetic milieu or mannitol in the presence or absence of triptolide and cyclosporine A treatments. Shown in **Figure 6B**, neither triptolide or cyclosporine A alone treatment nor the combined triptolide and cyclosporine A treatment restored the synaptopodin expression in cells exposed to the diabetic milieu. In parallel, the diabetic milieu-disrupted actin cytoskeleton in S9A-expressing cells was negligibly improved by the combined treatment with triptolide and cyclosporine A, as shown by phalloidin-labeled F-actin (**Figure 6D**) and quantified by F-actin/G-actin ratios (**Figure 6E**, **6F**). Collectively, these data suggest that the above observed synergistic effect of triptolide on cyclosporine A-induced podocyte protection and cytoskeletal preservation is abolished in cells expressing the dominant active GSK3 β .

Discussion

Clinical evidence suggests that *Tripterygium wilfordii* Hook F. has a potent dose-sparing effect on kidney protective medications and is able to reduce the dose of cyclosporine A while achieve a better therapeutic efficacy and mini-

mize the adverse effects [5, 7-9]. The molecular mechanism accounting for this dose-sparing effect remains largely unknown. The present study demonstrated that the diterpenoid epoxide triptolide, a major active component of *Tripterygium wilfordii* Hook F. is able to sensitize the cytoskeleton-preserving effect of cyclosporine A in glomerular podocytes. Even at very low doses, triptolide synergizes with cyclosporine A to mitigate cytoskeleton derangement, protect podocytes against the diabetic milieu-elicited injury and preserve the podocyte filtration barrier function. In current clinical practice, the use of triptolide and cyclosporine A is considerably limited by their serious toxic and side effects, including opportunistic infection due to immune over-suppression, cyclosporine A nephrotoxicity, and triptolide toxicity, such as hepatotoxicity, reproductive toxicity, etc. [1, 31, 34, 35]. Our finding suggests that combined use of triptolide and cyclosporine A at much-reduced doses may achieve an equal, if not better, therapeutic effect in patients with glomerular disease than the use of triptolide or cyclosporine A alone. Thus, our study is of great clinical relevance and translational potential because it provides a mechanistic basis for developing a new regimen that maximizes the kidney protective efficacy and minimizes the adverse effects.

Our data indicated that the synergistic protective effect of triptolide and cyclosporine A on podocyte is likely mediated, at least in part, *via* suppressing GSK3 β . GSK3 β is a highly-conserved, and constitutively active serine/threonine protein kinase that was originally coined about 40 years ago as a key cellular signaling transducer involved in glycogenesis and mediating inhibitory phosphorylation of glycogen synthase [36]. Interest in GSK3 β expanded greatly with the realization that it is also a pivotal regulator of many cellular processes well beyond glycogen metabolism, such as tumorigenesis, cell-cycle progression, cytoskeletal organization, development control, inflammation and immunity, mitochondria permeability transition, adaptive response to oxidative stress, and GSK3 β has since been implicated in a multitude of pathophysiologic processes, including tissue injury, repair and regeneration [36]. In mammals, GSK3 exists in two distinct, but closely related isoforms, namely GSK3 α and GSK3 β . They display 84% overall identity and 98% identity within their catalytic domain

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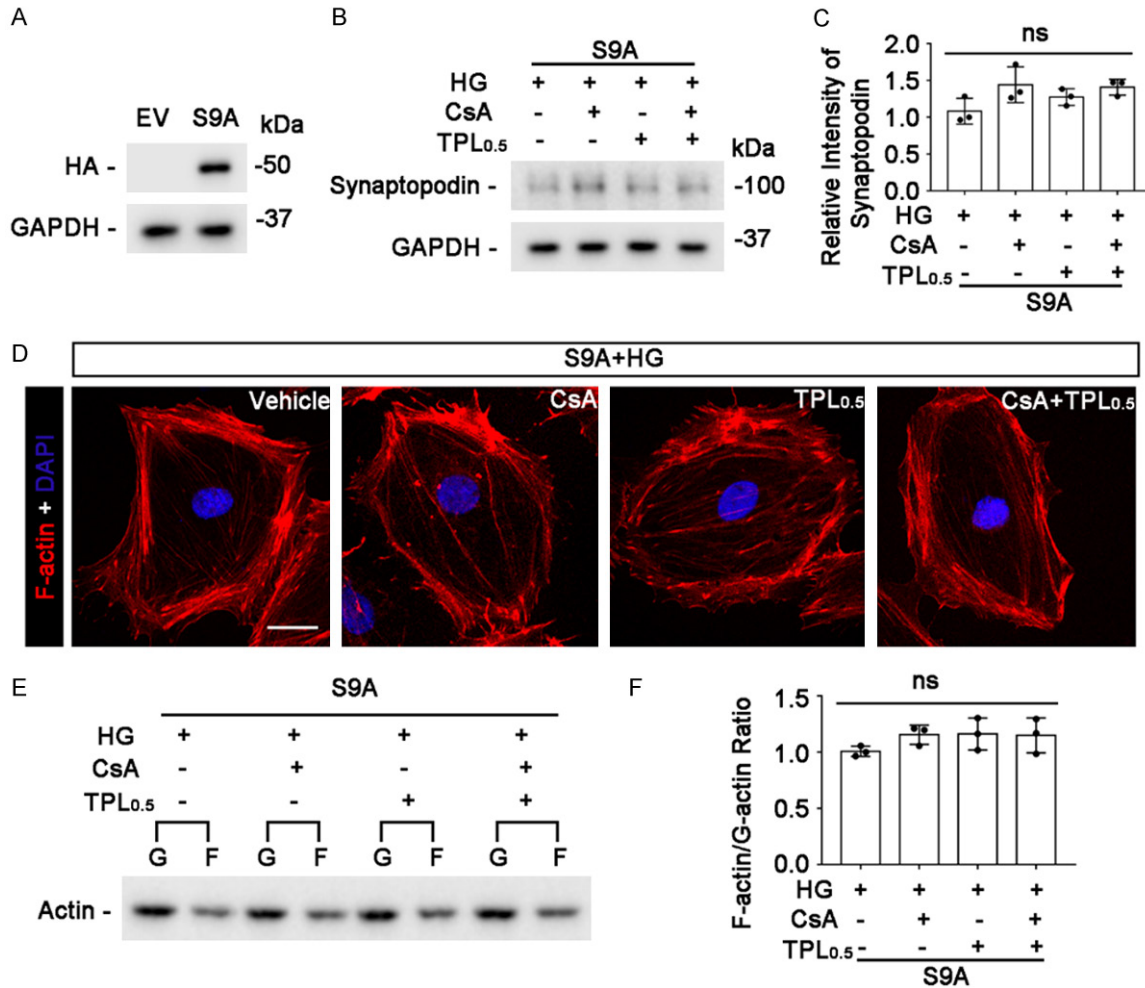


Figure 6. Inhibitory phosphorylation of GSK3 β is essential for the synergistic effect of triptolide on cyclosporine A-promoted podocyte protection and cytoskeletal stabilization. (A) Podocytes were subjected to transient transfection with vectors encoding an empty vector (EV) or a vector encoding the hemagglutinin (HA)-conjugated dominant active GSK3 β (S9A). Cells were cultured under nonpermissive conditions in normal growth medium for 48 h before transfection efficiency was assessed by immunoblot analysis for HA and GAPDH (cropped blots are shown as indicated by the lines and uncropped blots are included in the [Supplementary Figure 3](#)). (B) After transfection, cells were treated with cyclosporine A (CsA, 0.2 μ g/ml) in the presence or absence of triptolide (TPL_{0.5}, 0.5 ng/ml) for 30 minutes and then stimulated with the diabetic milieu (HG) for 36 h. Cell lysates were subjected to immunoblot analysis for synaptopodin and GAPDH (cropped blots are shown as indicated by the lines and uncropped blots are included in the [Supplementary Figure 3](#)). (C) Immunoblots were subjected to densitometric analysis and arbitrary units were expressed as immunoblot densitometric ratios of synaptopodin to GAPDH as folds of the HG alone treatment group (ns, not significant). (D) After the above treatments, podocytes were processed for fluorescent labeling of F-actin with rhodamine-conjugated phalloidin and counterstained with DAPI. Representative fluorescent microscopic images show changes in F-actin cytoskeleton. Bar = 25 μ m. (E and F) Podocytes were lysed and subjected to the F-Actin/G-Actin *in vivo* assay. Representative immunoblots were shown (E, cropped blots are shown as indicated by the lines and uncropped blots are included in the [Supplementary Figure 3](#)) and subjected to densitometric analysis (F). The F-actin to G-actin ratio was calculated as immunoblot densitometric ratios of F-actin to G-actin as folds of the HG alone treatment group ($n = 3$; ns, not significant).

[37]. However, they are not interchangeable functionally, as demonstrated by the embryonic-lethal phenotype observed when the gene that encodes GSK-3 β is knocked out [38]. In different organs and tissues, the two isoforms are differentially expressed and the kidney

glomeruli predominantly express the β isoform, which is mainly located to podocytes [39]. A growing body of evidence recently reveals that GSK3 β is a convergence point of multiple podocytopathic signaling pathways and mediates podocyte autonomous injury by integrating mul-

multiple pathogenic processes, including those disrupting the integrity of actin and microtubule cytoskeleton [40]. In consistency, genetic or pharmacological targeting of GSK3 β has been shown to substantially preserve podocyte cytoskeleton *in vitro* and *in vivo* and ameliorate podocyte injury and glomerular damage in experimental glomerular diseases [16].

The activity of GSK3 β could be modulated by a number of signaling cascades. Calcineurin has been found to be a key modifier. Calcineurin physically interacts with GSK3 β and is able to form a stable complex with GSK3 β and dephosphorylate GSK3 β at serine 9, resulting in an increase in the activity of GSK3 β , and an increase in the phosphorylation of the substrates of GSK3 β like tau [28]. High ambient glucose is a potent activator of calcineurin [41, 42] and may thereby cause GSK3 β hyperactivity. Conversely, as the standard inhibitor of calcineurin, cyclosporine A is able to elevate the inhibitory phosphorylation of GSK3 β and suppress GSK3 β activity in kidney cells [43]. Triptolide has also been described to regulate GSK3 β activity [44], although the exact mechanism remains to be defined. But as a redox sensitive kinase, the inhibitory phosphorylation and activity of GSK3 β is highly dependent on the intracellular redox status, and ROS are able to activate GSK3 β [45]. Triptolide possesses a potent anti-oxidant activity and suppresses the generation of ROS in multiple cell types [32, 33], including glomerular podocytes [46, 47]. Therefore, it is conceivable that triptolide may be able to suppress GSK3 β indirectly *via* its anti-oxidant effect. Taken together, there is good reason to believe that the podocyte protective effects of both cyclosporine A and triptolide are conveyed partially *via* intercepting the GSK3 β -mediated podocytopathic signaling. Indeed, in our study, the trivial beneficial effect of the very low dose of cyclosporine A or triptolide on diabetic milieu-elicited cellular injury and cytoskeleton derangement was completely blunted in podocytes expressing the dominant-active mutant of GSK3 β .

Then, how do triptolide and cyclosporine A synergize to inhibit the podocytopathic GSK3 β signaling pathway? Although the exact mechanism is unknown, the following possibilities are consistent with our data and previous findings. It is likely that the GSK3 β -inhibitory activity of triptolide may be only applicable to cells under

oxidative stress or exposed to oxidative injuries like the diabetic milieu [48], which was used in this study. Indeed, Matsui et al found that triptolide induced inhibitory phosphorylation of GSK3 β only in cells injured by cisplatin, a prototypical pro-oxidant stimulus, but not in cells under the resting condition [44]. Cyclosporine A is widely known as a pro-oxidant and is able to promote the production of reactive oxygen and nitrogen species and cause oxidative stress [30, 31], despite a general mitochondrial protective property [49] and a podocyte protective effect that is largely attributable to the cytoskeleton-stabilizing activity [11]. To this end, cyclosporine A has been reported to cause oxidative damage in glomerular mesangial cells [50], renal tubular cells [51] and vascular endothelial cells [52] *via* increased production of reactive oxygen and nitrogen species and free radicals. This pro-oxidant effect has also been implicated in the pathogenesis of cyclosporine A nephrotoxicity and other serious side effects [53]. Provided that triptolide has a potent antioxidant activity, it is tempting to speculate that the combined use of triptolide is capable of mitigating the oxidative stress elicited by cyclosporine A in podocytes and thereby amplifying the suppressive effect on GSK3 β to protect against podocyte cytoskeleton disruption and injury. Indeed, the DCF assay in our study revealed that triptolide and cyclosporine A did exhibit a combined antioxidant activity, which may contribute to the synergistic effect on the inhibitory phosphorylation of the redox-sensitive GSK3 β .

In conclusion, triptolide synergizes with cyclosporine A at very low doses to preserve cytoskeletal integrity and protect against diabetic milieu-induced injury in glomerular podocytes *via* an amplified inhibitory effect on the podocytopathic GSK3 β signaling. Our findings reveal the molecular mechanism underlying the cyclosporine A-sparing effect of triptolide in treating glomerular disease and suggest that combined use of triptolide and cyclosporine A at reduced doses may be an invaluable regimen to treat diabetic glomerulopathy with improved efficacy and minimized adverse effect.

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Disclosure of conflict of interest

None.

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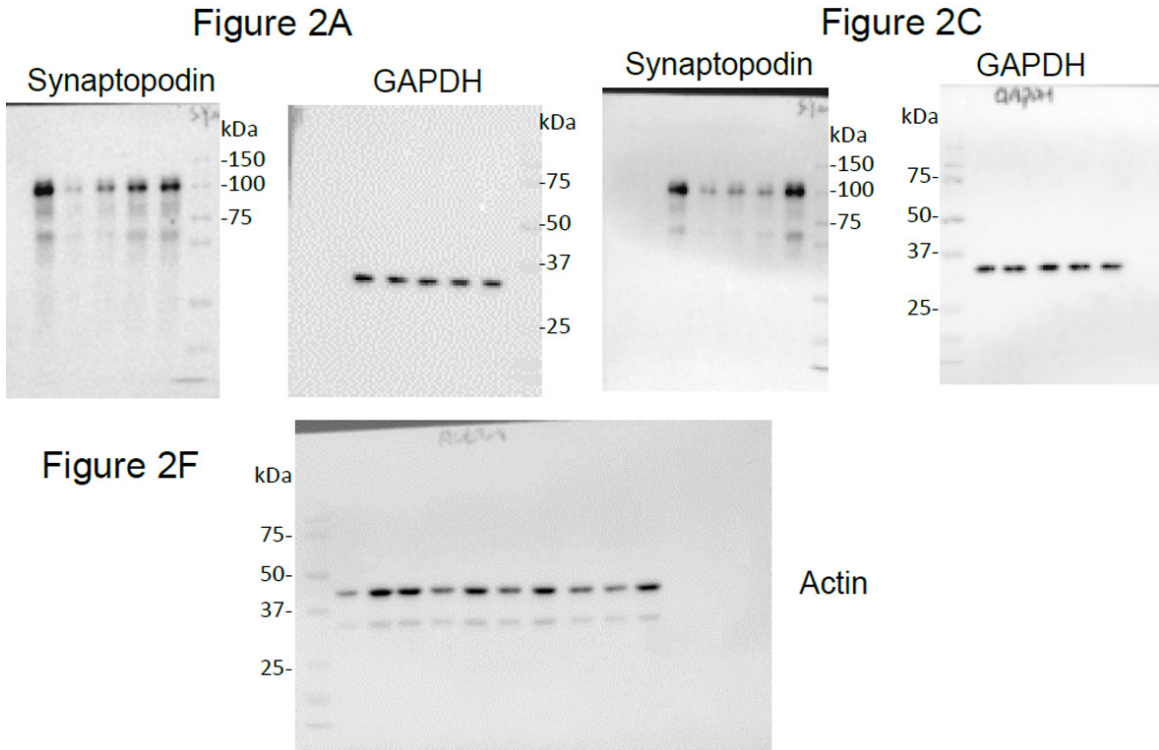
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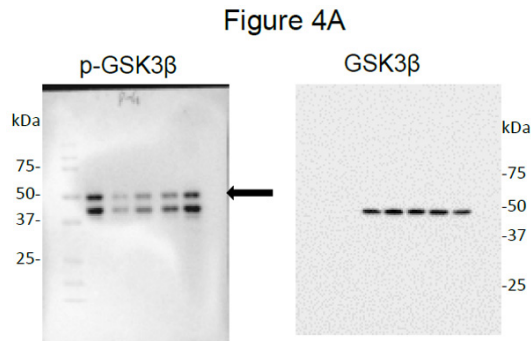
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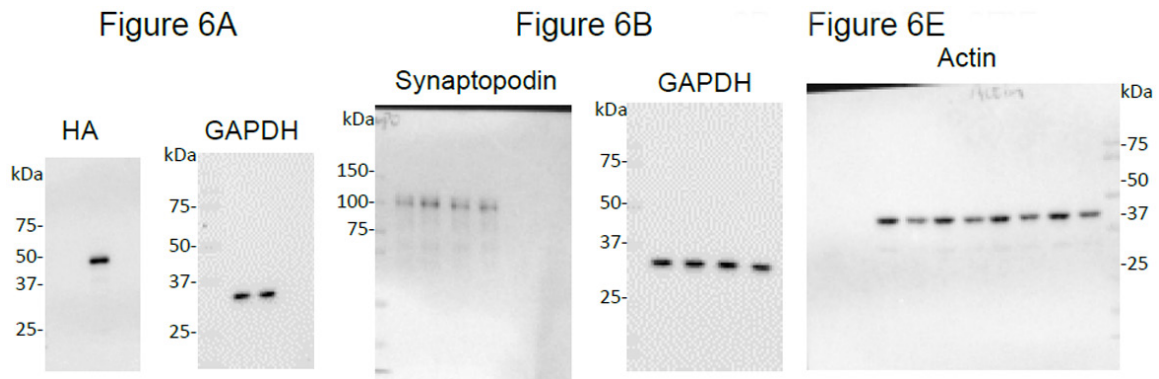
Triptolide synergizes with CsA on podoprotection



Supplementary Figure 1. Original images of WB in Figure 2.



Supplementary Figure 2. Original images of WB in Figure 4.



Supplementary Figure 3. Original images of WB in Figure 6.