



The role of TRPC6 in α 1-AR activation-induced calcium signal changes in human podocytes

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Background: An accumulating amount of evidence has suggested that there is a contributive role of sympathetic nervous hyperactivity in the pathogenesis of chronic kidney disease (CKD). α 1-AR promotes an increase in calcium levels in podocytes and adjusts podocyte contraction. Changes in TRPC6 expression and function can directly affect the podocyte cytoskeleton, which is a key component in podocyte injury. This study proposed to clarify the correlation between α 1-AR activation-induced signal cascade reaction and TRPC6 in human podocytes.

Methods: Human podocytes were incubated with the calcium probe Fluo-3/AM. Next, the effects of the α 1-AR agonists or antagonists and nonselective TRPC6 blockers on intracellular calcium were observed under laser confocal microscopy. FITC-phalloidin was employed to stain podocytes, and the change of F-actin under the α 1-AR activation condition was observed.

Results: The α 1-AR agonist PE (phenylephrine hydrochloride) induced an increase in intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) in human podocytes. Moreover, the downregulation of TRPC6 by siRNA or TRPC6 blocker could attenuate the PE-induced $[\text{Ca}^{2+}]_i$ elevation in a phospholipase C (PLC)-dependent pattern. When podocytes were stimulated to the PE, their F-actin fiber cytoskeletal structure was lost. PE subsequently increased the expression of RhoA, and the TRPC6-dependent Ca^{2+} influx was involved in this process. The abnormal activation of RhoA could result in disturbance of the podocyte skeleton structure, thus leading to podocyte injury.

Conclusions: We concluded that TRPC6 is involved in α 1-AR activation-induced calcium signal changes in podocytes. Meanwhile, the α 1-AR agonists can destroy the cell's cytoskeletal structure, which is mediated by TRPC6 via the RhoA/ROCK pathway.

Keywords: TRPC6; α 1-AR; cell cytoskeletal structure; podocytes

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Introduction

Strong evidence has shown that both the kidney and the sympathetic nervous system are vitally involved in the progression and maintenance of hypertension (1). Several studies have demonstrated that sympathetic nerves are involved in controlling the glomerular filtration rate and

renin secretion (2). Additionally, sympathetic nerves in the kidney are also involved in the pathogenesis of hypertension, diabetes, and ischemia (3). Nevertheless, the exact effects of sympathetic nervous system activation in podocyte function is unknown. The calcium signal, the most important signaling pathway, participates in the regulation of cell functions after

the activation of $\alpha 1$ -adrenoceptor ($\alpha 1$ -AR) (3). Renal $\alpha 1$ -AR activation can increase the calcium levels in podocytes, thereby regulating podocyte function (4). Our team has shown that there is an expression of $\alpha 1$ -AR in mesangial cells, which can regulate cell proliferation (5). These findings may shed new light on the potential novel therapeutic options for delaying progressive kidney disease. However, at present, $\alpha 1$ -AR activation-triggered signaling cascades have rarely been illustrated in podocytes.

Transient receptor potential 6 (TRPC6), a prominent member of TRPC channels, is extensively distributed in the glomeruli and renal tubules and is mainly expressed in podocytes (2). As a calcium ion-permeable non-specific cation channel, TRPC6 is involved in multiple intracellular calcium signal changes. A previous study has shown the involvement of TRPC6 upregulation in Ca^{2+} signaling as well as actin reorganization in podocytes following oxygen-glucose deprivation (6). Moreover, gene mutation or abnormal expression of TRPC6 not only leads to genetic focal segmental glomerular sclerosis but also plays a vital role in numerous acquired proteinuric diseases (7,8). $\alpha 1$ -AR is a G protein-coupled receptor, and thus $\alpha 1$ -AR activation can cause G protein coupling. Their interaction leads to the hydrolysis of phosphatidylinositol-4,5-bisphosphate by phospholipase C (PLC), thereby forming inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). Of them, IP3 can activate the cellular store-operated calcium channel to release the intracellular calcium, while DAG can activate the receptor-operated calcium channel to cause persistent inflow of extracellular calcium.

TRPC6 can be activated by PLC-dependent pathway and belongs to the receptor-operated calcium channel, which has been shown to be an important component of intracellular calcium changes following $\alpha 1$ -AR activation in rabbit venous smooth muscle cells, mouse myocardial cells, nerve cells and vascular smooth muscle cells (9). Therefore, in this study, we aimed to explore the possible role of $\alpha 1$ -AR activation to stimulate intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) increase by TRPC6 in human podocytes and identify the underlying mechanisms.

Filamentous actin (F-actin) plays a vital role in healthy podocyte foot processes (FPs). By contrast, the disruption of dynamin can trigger the absence of the functional actin network and incongruous membrane signaling, leading to an effacement of FPs and subsequent proteinuria (10,11). RhoA, a member of the Rho protein family, can regulate numerous cell processes, including those involved in the actin cytoskeleton (12,13). TRPC6 and RhoA consist of

a molecular complex, and TRPC6-mediated Ca^{2+} influx increases RhoA activity (2). RhoA can affect the morphology and function of podocytes. Lipid radicals regulate podocyte homeostatic alterations via redox-sensitive RhoA signaling by causing a shortage of F-actin fibers (14). To this end, we explored whether $\alpha 1$ -AR activation could induce TRPC6-dependent Ca^{2+} influx and whether Ca^{2+} influx could further mediate RhoA activation and as participate in $\alpha 1$ -AR activation-triggered actin cytoskeleton alterations.

Methods

Cell culture and transfection

The human podocyte cell line was kindly offered by Dr. Moin Saleem of Children's Renal Unit and Academic Renal Unit, University of Bristol, Southmead Hospital, Bristol, UK. The cell line was subsequently maintained in line with previous protocols (15). In brief, cells were maintained at 33 °C in RPMI-1640 medium (HyClone, USA), containing 10% fetal bovine serum (FBS; HyClone) as well as insulin, transferrin, sodium selenite (ITS; Sigma). Human podocytes between passages 4 and 15 were utilized for experimental assays. Cells were transiently transfected with TRPC6 siRNA (10 μM ; Santa Cruz, CA, USA) or scrambled siRNA (10 μM ; Santa Cruz, CA, USA) using X-treme GENE siRNA Transfection Reagent (Roche) in line with the standard protocols. After transfection for 24–48 h, related *in vitro* assays were performed on the transfected cells.

Fluorescence measurement of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$)

$[\text{Ca}^{2+}]_i$ levels were established according to previous protocols (16). Briefly, human podocytes were incubated with 1% normal saline supplemented with Pluronic F-127 (0.03%, Sigma) as well as Fluo-3/AM (3 μM , Molecular Probes) at 37 °C for 45 min, followed by stimulation with diverse inhibitors agonists or antagonists as indicated in the result section, including U73122 (a PLC inhibitor, Sigma), and nonselective TRPC6 blockers including 2-APB (Sigma) and SKF-96365 (Sigma). Afterward, a laser confocal scanning microscope (FV300; Olympus, Japan) was used to determine the fluorescence intensity of Fluo-3 in podocytes. Additionally, the actual fluorescence intensity was divided by the average baseline fluorescence intensity to display the pseudo-ratio value of $[\text{Ca}^{2+}]_i$. Results from 20 to 40 cells were summarized in a single run, and this assay was performed in triplicate.

Western blot (WB)

WB was conducted in line with standard protocols. In brief, the membranes reacted with appropriate primary antibodies, including polyclonal rabbit anti-TRPC6 (1:200, Alomone Labs, Israel), anti-RhoA (1:500, Santa Cruz, CA, USA) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:1,000, ZSGB-Bio). The Odyssey infrared imaging system (LI-COR, Lincoln) was used for visualization and quantification of the bands.

Fluorescence staining of F-actin

For immunofluorescence (IF) staining, podocytes were first cultured on coverslips and further stained according to the manufacturers' instructions. Cells were fixed with 4% paraformaldehyde (PFA) for 15 min, permeabilized with 0.4% Triton X-100, then blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), followed by staining with phalloidin-FITC (5 $\mu\text{g}/\text{mL}$, Sigma). Afterward, a laser scanning confocal microscope (Olympus, Japan) was used to observe and photograph representative images.

RhoA activation assay

The Rho activation assay kit (Cytoskeleton, Inc.) was purchased to observe the RhoA activity according to the manufacturer's instructions. After cell lysis and centrifugation, the supernatant was collected for incubation with Rhotekin-Rho binding domain (RBD) glutathione affinity beads, which specifically bound to GTP-bound RhoA. WB was then employed to measure the protein expression of total RhoA after normalization to GTP-bound RhoA densitometric units. All results were repeated 3 times.

Statistical analysis

All the data are displayed as means \pm standard error (SEM). Two-tailed Student's *t*-test or one-way analysis of variance (ANOVA) was utilized for comparison between groups, along with Dunnett's test. A *P* value <0.05 suggested statistical significance.

Results

$\alpha 1$ -AR agonists stimulate an elevation in $[\text{Ca}^{2+}]_i$ in human podocytes

To explore the possible relationship between the protein

expression of $\alpha 1$ -AR and functional receptors presence, $\alpha 1$ -AR specific agonist phenylephrine hydrochloride (PE, 10 μM) was employed to stimulate Fluo-3/AM-loaded human podocytes. The calcium ion in podocytes was rapidly increased and reached a peak under PE stimulation at 100 s, remained stable continuously, and finally returned to the baseline level (*Figure 1A*). Pretreatment of podocytes with 200 nM prazosin, a selective $\alpha 1$ -AR antagonist, could lead to significant suppression of PE-induced $[\text{Ca}^{2+}]_i$ elevation (*Figure 1B*). To further investigate the potential role of Ca^{2+} influx in PE-induced $[\text{Ca}^{2+}]_i$ elevation, PE was used to stimulate podocytes without extracellular Ca^{2+} . Consequently, the application of PE stimulation in podocytes still led to the transient increase of $[\text{Ca}^{2+}]_i$ in podocytes (*Figure 1C*). However, the peak of PE-induced $[\text{Ca}^{2+}]_i$ elevation was slight without calcium induction, almost without a steady plateau, suggesting that an extracellular Ca^{2+} entry was very likely to participate in $[\text{Ca}^{2+}]_i$ elevation by $\alpha 1$ -AR activation in human podocytes.

The role of the PLC signaling pathway in $\alpha 1$ -AR activation-induced $[\text{Ca}^{2+}]_i$ increase in podocytes

To further determine whether the PLC-dependent pathway participated in $\alpha 1$ -AR activation-induced $[\text{Ca}^{2+}]_i$ elevation, $\alpha 1$ -AR agonists were utilized to stimulate the PLC inhibitor U73122 (10 μM). As shown in *Figure 1D*, the administration of U73122 almost completely abolished the PE-induced $[\text{Ca}^{2+}]_i$ elevation, confirming the PLC-dependent pattern in PE-induced $[\text{Ca}^{2+}]_i$ elevation in podocytes.

Activation of $\alpha 1$ -AR induces TRPC6-dependent Ca^{2+} influx

Nonselective TRPC channel blockers, SKF96365 (30 μM) and 2-APB (100 μM) were employed to pharmacologically abolish TRPC, which further significantly suppressed the PE-induced $[\text{Ca}^{2+}]_i$ increase (*Figure 2A,B,C,D*). The above outcomes collectively indicate that TRPC-dependent Ca^{2+} entry participated in the PE-induced $[\text{Ca}^{2+}]_i$ elevation ($P < 0.001$; $n = 3$). In order to determine whether TRPC6 contributed to the $\alpha 1$ -AR activation-induced $[\text{Ca}^{2+}]_i$ elevation, siRNA targeting TRPC6 was transfected into human podocytes to downregulate TRPC6 expression, followed by analysis to validate the knockdown efficiency of TRPC6-siRNA (data not shown). As displayed in *Figure 2E,F*, PE-induced $[\text{Ca}^{2+}]_i$ elevation was significantly suppressed by transfection with TRPC6 siRNA ($P < 0.001$;

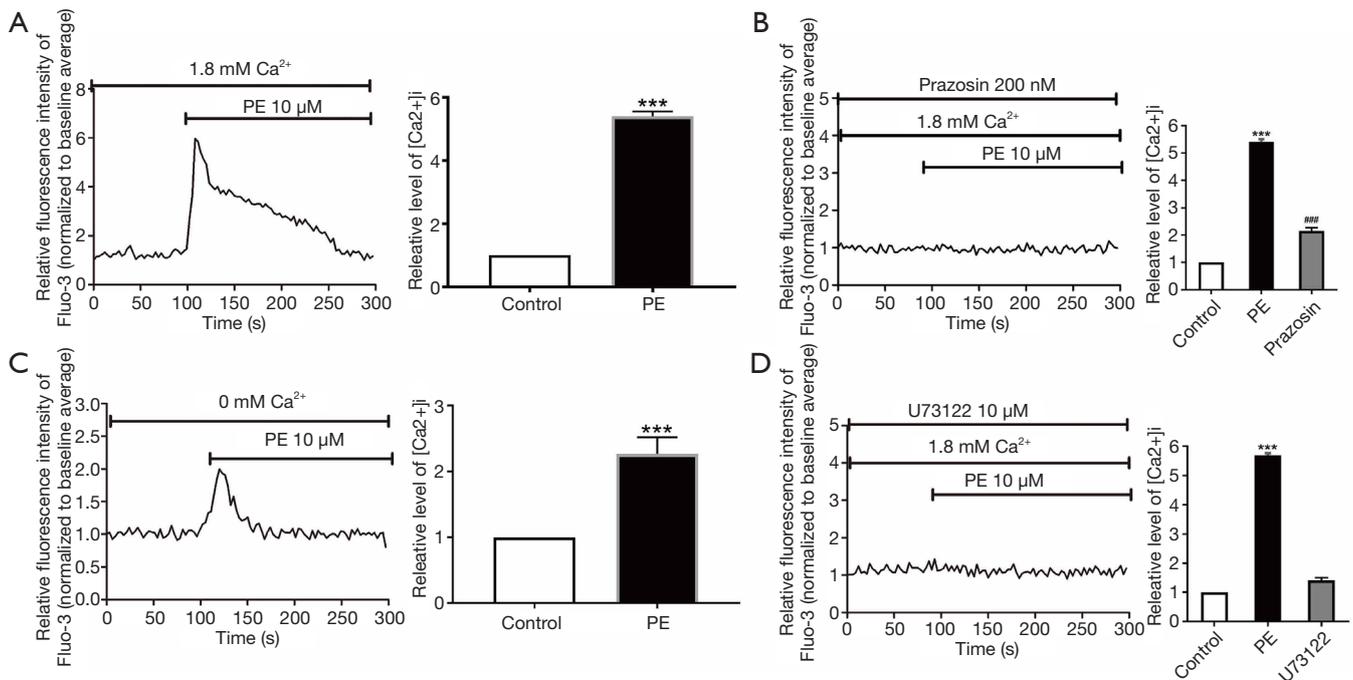


Figure 1 $\alpha 1$ -AR agonist-induced the increased $[Ca^{2+}]_i$ in human podocytes. Confocal microscopy using Fura-3 fluorescent dye was used to observe changes in the Ca^{2+} concentration. (A) A representative trace showing that $\alpha 1$ -AR agonist PE induced a $[Ca^{2+}]_i$ increase in the presence of 1.8 mM extracellular Ca^{2+} . (B) Prazosin, a selective $\alpha 1$ -AR antagonist, significantly inhibited the PE-induced $[Ca^{2+}]_i$ increase. (C) The PE-induced $[Ca^{2+}]_i$ increase peak was small in the absence of calcium induction, which almost had no steady plateau. (D) The PLC inhibitor U73122 could almost completely suppress the PE-induced $[Ca^{2+}]_i$ increase. The results were from at least 3 independent experiments; 20–30 cells were selected in each trial. ***, $P < 0.001$ vs. Ctrl $n = 3$; ###, $P < 0.001$ vs. PE.

$n = 3$), in comparison with transfection with scrambled small interfering RNA (siRNA). The above outcomes demonstrated that TRPC6 was involved in the $\alpha 1$ -AR activation-induced increase of $[Ca^{2+}]_i$.

$\alpha 1$ -AR activation increases the protein expression of TRPC6 in podocytes

At present, the impacts of $\alpha 1$ -AR activation on inducing the protein expression of TRPC6 in podocytes remains largely undefined. Herein, WB was employed to examine the changes in TRPC6 protein expression in human podocytes following the administration of $\alpha 1$ -AR specific agonist PE. As shown in Figure 3A, the administration of 10 μM PE for 48 h could elevate the protein expression of TRPC6 in podocytes.

$\alpha 1$ -AR activation induces the TRPC6-dependent Ca^{2+} inflow to increase the RhoA activation

Affinity pull-down assay through the GST fusion protein

rhotekin was used to directly examine the RhoA activity, and was able to recognize only the active form of RhoA (GTP-RhoA). As displayed in Figure 3B, the administration of $\alpha 1$ -AR agonist PE for 48 h significantly increased RhoA activity compared with the normal group. This was likely be due to PE-induced TRPC6 activation, as PE-induced elevation of RhoA activity following transfection with TRPC6 siRNA was attenuated compared with the normal group, non-transfection group, and scramble siRNA group ($P < 0.05$; $n = 3$). The above outcomes suggested that TRPC6 plays a role in the $\alpha 1$ -AR activation-induced Ca^{2+} inflow, further contributing to RhoA activation.

TRPC6 destroys the cytoskeleton stress fiber via the RhoA pathway in response to $\alpha 1$ -AR activation

To investigate the functional effects of TRPC6 on $\alpha 1$ -AR activation-induced skeleton stress fiber, FITC-phalloidin was employed to stain podocytes. As shown in Figure 4A, well-developed F-actin fibers were detected in normal

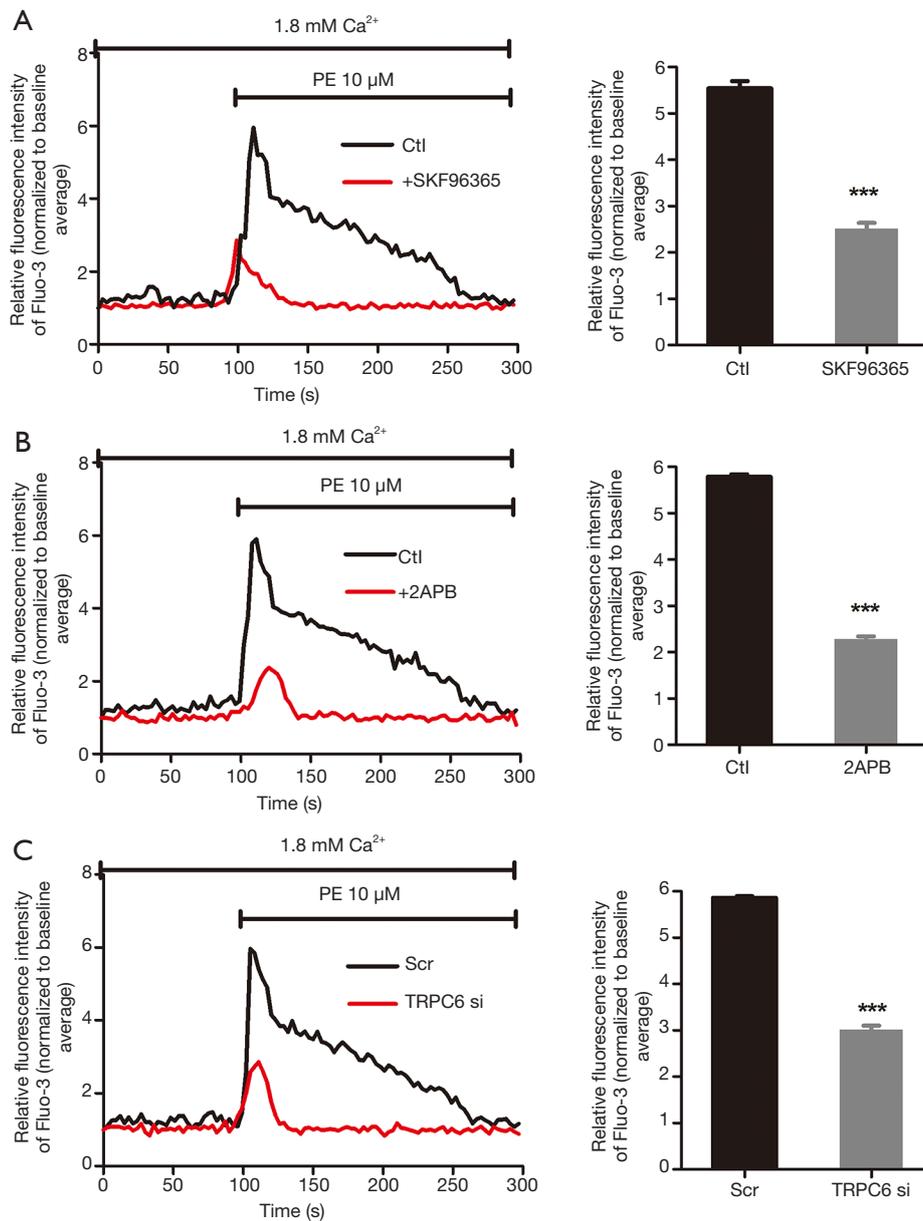


Figure 2 Activation of $\alpha 1$ -AR induces TRPC6-dependent Ca^{2+} influx. Confocal microscopy using Fura-3 fluorescent dye was used to observe changes in the Ca^{2+} concentration. (A and B) SKF96365 (30 μM) significantly inhibited the $[\text{Ca}^{2+}]_i$ expression induced by PE (***, $P < 0.001$ vs. Ctrl $n=3$). (C and D) 2-APB (100 μM) significantly inhibited the expression of $[\text{Ca}^{2+}]_i$ induced by PE (***, $P < 0.001$ vs. Ctrl $n=3$). (E and F) TRPC6 siRNA could remarkably inhibit the expression of $[\text{Ca}^{2+}]_i$ increased by PE ($P < 0.001$; $n=3$). (***, $P < 0.001$ vs. Scr $n=3$). The number of trials =3; 20–30 cells were selected in each trial.

podocytes. F-actin manifested as the yellow-green, long, and fine filament running along the whole-length of podocytes after FITC-phalloidin staining. The treatment with the culture medium containing 10 μM $\alpha 1$ -AR agonist PE caused a rearrangement of F-actin, rupture of stress

fibers, and reduction of the number of orderly arranged fibers, in comparison with those in the normal group. As expected, after transfection with TRPC6-siRNA and the administration of TRPC channel inhibitor SKF-96365, the stress fibers partially ruptured, the number of orderly

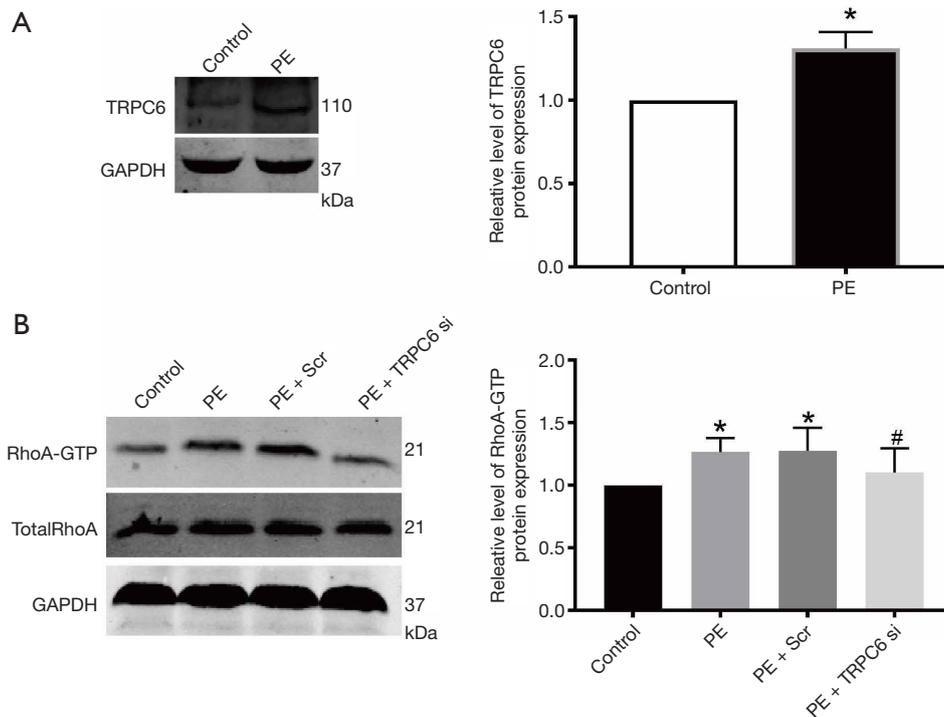


Figure 3 α 1-AR activation induced the TRPC6-dependent Ca^{2+} inflow to increase the RhoA activation. (A) Compared with normal podocytes, the application of 10 μM PE to treat podocytes for 48 h could upregulate the TRPC6 protein expression (*, $P < 0.05$ vs. Ctrl, $n=3$). (B) Compared with the normal group, RhoA activity was markedly enhanced after 48 h of treatment with α 1-AR agonist PE; relative to the normal group, the non-transfection group and scramble siRNA group attenuated the PE-induced increase in RhoA activity (*, $P < 0.05$ vs. Ctrl; #, $P < 0.05$ vs. PE + Scr, $n=3$).

arranged fibers slightly increased, and the PE-induced F-actin rearrangement slightly recovered, compared with those in the PE stimulation group (Figure 4A,B). To determine whether the RhoA pathway was the downstream pathway of the involvement of TRPC6 in the PE-induced podocyte skeleton destruction, the RhoA/ROCK inhibitor Y-27632 was used for intervention. Consequently, the number of ruptured stress fibers decreased, and the number of orderly arranged fibers slightly increased, in comparison with those in the PE stimulation group (Figure 4C). The above results indicate the relationship between TRPC6-mediated RhoA pathway and α 1-AR activation-induced alterations in the cytoskeleton.

Discussion

The kidneys, which are rich in afferent and efferent sympathetic nerves, play crucial physiological roles in the modulation of renal functions. Evidence from several studies supports the speculation autosomal dominant

polycystic kidney disease manifests as sympathetic renal hyperactivity (17). Moreover, recent studies have shown that renal sympathetic inhibition or denervation is likely to partially reduce or even prohibit renal destruction triggered by ischemia/reperfusion injury (18). In addition, the importance of renal nerves has been recently discussed in relation to sympathetic activity in other vascular beds, arterial pressure, and long-term hypertension (19). However, the possible roles of renal sympathetic nerves in podocytes have not been extensively investigated. To this end we here demonstrate that α 1-AR agonists can trigger $[\text{Ca}^{2+}]_i$ elevation in human podocytes.

α 1-AR participates in diverse physiological functions controlled by endogenous catecholamines, noradrenaline, and adrenaline, which is also a useful therapeutic target (20). Functionally, α 1-AR utilizes intracellular Ca^{2+} as a second messenger for the regulation of cellular function in multiple types of cells. For example, smooth muscle contractions to exogenous agonists are particularly mediated by α 1-AR (7). Nakladal *et al.* suggested that (R)-enantiomer of

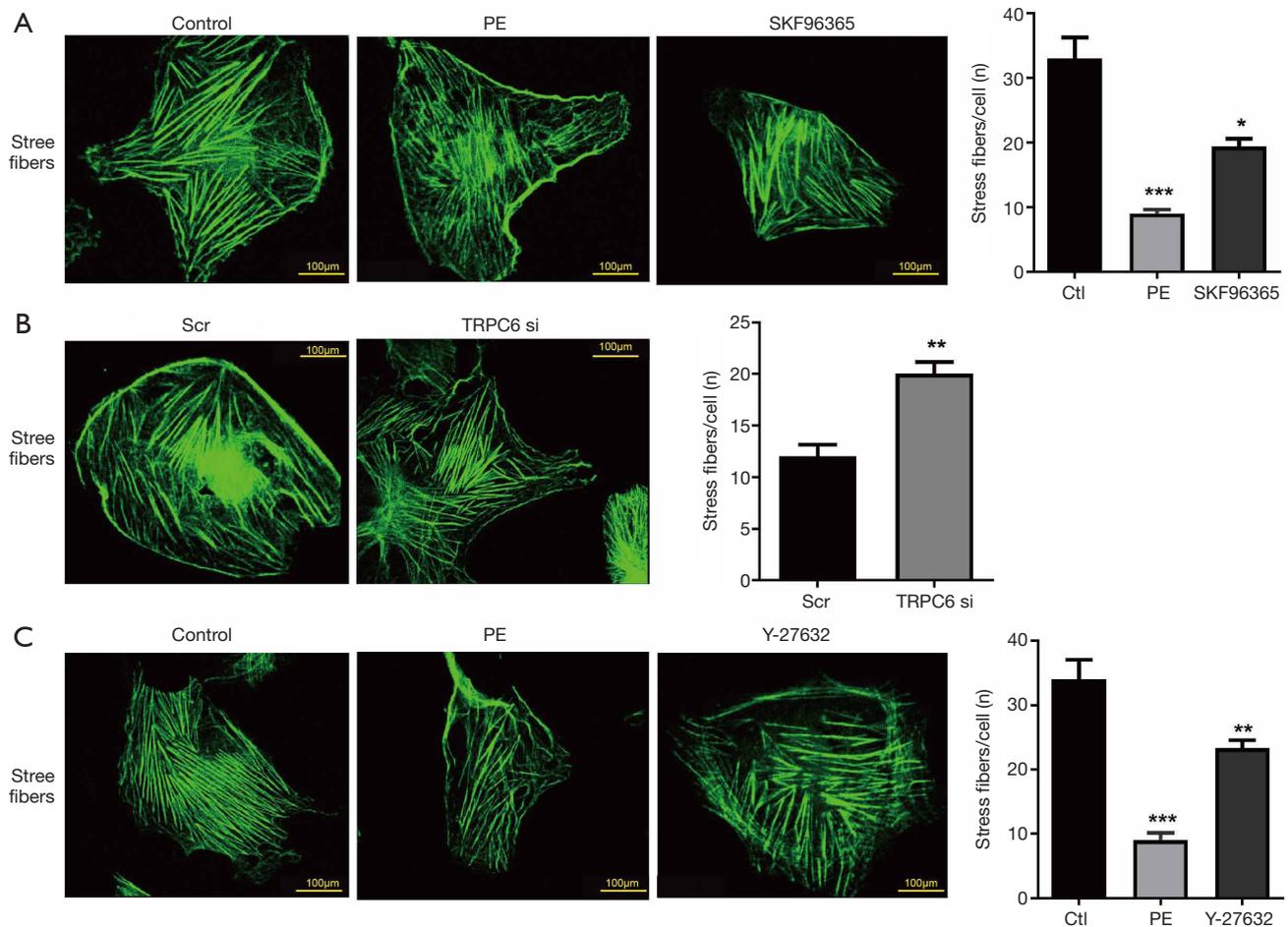


Figure 4 α 1-AR activation induced TRPC6 to destroy the cytoskeleton stress fiber through the RhoA pathway. FITC-labeled phalloidin was employed to evaluate the changes of the podocyte skeleton by stress fiber. (A) SKF-96365 could rupture the stress fibers induced by PE (***, $P < 0.001$ vs. Ctrl; *, $P < 0.05$ vs. PE, $n = 3$). (B) TRPC6-siRNA ruptured the stress fibers compared with the scramble siRNA group (**, $P < 0.01$ vs. Scr, $n = 3$). (C) Y-27632 increased the stress fiber number induced by the PE group (***, $P < 0.001$ vs. Ctrl; **, $P < 0.01$ vs. PE, $n = 3$).

the 6-chromanol derivative SUL-121 improves renal graft perfusion via antagonism of the α 1-AR (4). Cabrita *et al.* showed that Ca^{2+} signaling is important in TMEM16A driving renal cyst growth (21). Our experimental findings reveal that activation of α 1-AR could mediate Ca^{2+} influx through TRPC6 in human podocytes. Consistent with research on other cell types (22), we also found that the PE-induced increase in intracellular calcium was derived from the release of intracellular calcium and inflow of extracellular calcium.

The physiological function in podocytes vitally relies on appropriate intracellular calcium handling, while excessive calcium influx in podocytes might cause FP effacement, apoptosis and subsequent glomeruli injury.

TRPC6 is considered a critical protein that is responsible for calcium flux in podocytes, while hyper-activation or mutation of TRPC6 channel activity can result in glomeruli injury progression (23). Moreover, TRPC6 is related to the pathogenesis of familial forms of focal segmental glomerulosclerosis (FSGS) (7), while α 1-AR belongs to the G protein-coupled receptor, whose activation can produce IP₃ and DAG. TRPC6 is widely acknowledged to function as a DAG-activated channel. Therefore, it is reasonable to speculate that the PLC-dependent generation of DAG could trigger TRPC6 following α 1-AR stimulation. Herein, we have shown that the PLC inhibitor almost entirely suppressed the α 1-AR activation-induced $[Ca^{2+}]_i$ elevation in podocytes, which is consistent with previous

outcomes observed in myocardial cells (24). Moreover, we demonstrate that $\alpha 1$ -AR agonist PE stimulation triggers not only Ca^{2+} release but also TRPC6-dependent Ca^{2+} entry in human podocytes. Considering the glomerular sclerosis induced by changes in the TRPC6, we preliminarily posit that $\alpha 1$ -AR activation-induced changes in TRPC6 activity is vitally involved in podocyte injury and glomerular sclerosis development.

The specialized morphology of podocytes, visceral epithelial cells of the renal glomerulus, form the selective glomerular filtration barrier. The cytoskeleton of podocytes consists of intermediate filaments, microtubules, and F-actin. Loss of over 30% of podocytes is harmful to the glomerulus, subsequently causing glomerulosclerosis and chronic kidney disease (CKD), a common complication of diabetes. Thus, it is of great clinical significance to preserve podocytes (25). Cytoskeletal structure plays an essential role in maintaining appropriate FP and the filtration barrier integrity (26). According to our present findings, the exposure of podocytes to $\alpha 1$ -AR agonist PE could result in a loss of cytoskeletal structure of F-actin fiber. After knockdown of TRPC6 expression by transfecting TRPC6siRNA, the $\alpha 1$ -AR agonist PE could attenuate the podocyte cytoskeletal structure destruction. Additionally, the administration of $\alpha 1$ -AR agonist PE in podocytes could also induce the activation of RhoA, which was notably attenuated by TRPC6 siRNA. The abnormal activation of RhoA could result in disturbance of the cytoskeletal structure and further suppress the formation in podocytes (27). However, previous studies have demonstrated that excessive or scarce RhoA activity is equally harmful to podocytes by increasing or decreasing RhoA activity to subsequently cause proteinuric kidney disease (28,29). In our experiments, hyperactive RhoA resulted in a cortical rearrangement of F-actin fibers. When the RhoA/ROCK inhibitor Y27632 was used, the $\alpha 1$ -AR agonist PE could decrease the destruction of the podocyte skeleton. On the other hand, we can speculate that a hyperactive RhoA is destructive and can cause cytoskeletal rearrangement and F-actin fiber loss. Consistent with this, our present outcomes also show the loss of F-actin fibers and RhoA activation in a similar fashion. If the actin cytoskeleton were repaired, the early injury of podocytes might be irreversible, while persistent and chronic podocyte injury would result in the loss of glomerular function. However, the precise mechanism for TRPC6 over-load $\alpha 1$ -AR activation-induced calcium signal changes in podocytes

is still unclear, and is of great significance to our future research.

Conclusions

The activation of $\alpha 1$ -AR can upregulate TRPC6 expression and increase calcium inflow in podocytes. Meanwhile, TRPC6 can induce RhoA activation, which participates in the $\alpha 1$ -AR agonist PE-induced podocyte injury through the RhoA mechanism. Moreover, these outcomes offer a new pathway that could be the first step for a better understanding of how $\alpha 1$ -AR activation affects podocytes, which is of great clinical significance in CKD.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/apm-19-602>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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