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The role of TRPC6 in oxidative stress-induced podocyte ischemic injury

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ABSTRACT

Increasing evidence suggests that ischemia and hypoxia serve important functions in the development of renal diseases. However, the underlying mechanism of ischemic injury has not been fully understood. In this study, we found that renal ischemia-reperfusion injury induced podocyte effacement and the upregulation of TRPC6 mRNA and protein expression. In in vitro experiments, oxygen glucose deprivation (OGD) treatment enhanced the expression of TRPC6 and TRPC6-dependent Ca^{2+} influx. TRPC6 knockdown by siRNA interference attenuated the OGD-induced $[Ca^{2+}]_i$ and actin assembly. OGD treatment also increased ROS production. Furthermore, inhibition of ROS activity by N-acetyl-L-cysteine (NAC) eliminated the OGD-induced increase in TRPC6 expression and Ca^{2+} influx. H_2O_2 treatment, which results in oxidative stress, also increased TRPC6 expression and Ca^{2+} influx. We conclude that TRPC6 upregulation is involved in Ca^{2+} signaling and actin reorganization in podocytes after OGD. These findings provide new insight into the mechanisms underlying the cellular response of podocytes to ischemic injury.

1. Introduction

Kidneys are susceptible to ischemia reperfusion injury, which may result in high morbidity and mortality. However, most studies mainly focus on renal tubular cells, and less attention has been directed toward glomerular damage. The processes of glomerular visceral epithelial cells (called podocytes) form a slit diaphragm and serve crucial functions in the maintenance of the glomerular filtration barrier [1]. Several lines of evidence indicate that ischemia and hypoxia can affect podocytes' structure and function [2]. A recent study found glomerular capillary permeability changes following renal ischemic injury in rats [3]. Another study showed that renal ischemia promotes the loss of interactions between slit diaphragm proteins [4]. Although increasing evidence shows that ischemic injury in podocytes contributes to the progression of renal disease, the underlying molecular mechanism remains unclear.

TRPC is a member of the transient receptor potential superfamily, which are non-selective cation channels that play an important role in the regulation of various cell functions [5]. TRPCs are Ca^{2+}-permeable cation channels that are primarily distributed in the brain, lungs, and kidneys. These channels can be divided into three subfamilies based on their amino acid sequence similarity, including C1, C4\C5, and C3\C6\C7 [6]. TRPC1, TRPC3, and TRPC6 have been detected in podocytes. It has been reported that TRPC6 gene mutations were associated with focal segmental glomerulosclerosis [7]. TRPC6 expression was upregulated in several secondary renal diseases, and the overexpression of TRPC6 in mice caused transient proteinuria [8]. There is evidence that TRPC6 is primarily involved in the formation of receptor-operated calcium entry (ROCE) through phospholipase C-coupled receptors [9]. TRPC6 also serves as a downstream effector of reactive oxygen species (ROS) [10]. In addition, the actin cytoskeleton is important in stabilizing cell morphology and maintaining the internal structure of a cell, and available evidence suggests that TRPC6 can regulate actin cytoskeleton function [11]. Recently, TRPC6-deficient mice exhibited protective effects in the lung ischemia-reperfusion model [12]. However, the cellular responses of TRPC6-mediated calcium influx in kidney have not been fully understood.

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We hypothesize that TRPC6 channels are involved in the podocytes' response to ischemic injury. In this study, we sought to investigate alterations in TRPC6 expression following renal ischemia-reperfusion injury in vivo, and examine the possible underlying mechanisms of oxygen-glucose deprivation-induced modulation of TRPC6 channels in vitro.

2. Materials and methods

2.1. Animal studies

Male Kunming mice weighing 18–22 g were obtained from the Experimental Animal Center of Harbin Medical University. The use of animals was approved by the Institutional Animal Care and Use Committee of Harbin Medical University. Renal ischemia reperfusion injury was produced as previously reported [13]. Briefly, after mice were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneal injection), the bilateral renal pedicles were occluded with non-traumatic vascular clips for 30 min. During the operation, the mice were kept well hydrated, and their body temperature was maintained at 37 °C. Reperfusion was then allowed to occur for 3 h. The sham group was induced by separating, but not clamping, the renal pedicles. After the stated perfusion times, blood samples were collected, and both kidneys were removed for mRNA, protein and histological analyses. Glomeruli were isolated by graded sieving as previously described [14].

2.2. Measurement of biochemical parameters

Serum creatinine concentration was measured using an automatic biochemical analyzer (7070A, Tokyo, Japan).

2.3. Morphological detection

Scanning and transmission electron microscopy examinations were performed according to standard methods. At least 10 glomeruli from each kidney were counted, and representative images were selected.

2.4. Cell culture, transfection and oxygen-glucose deprivation

Conditionally immortalized human podocytes were cultured using a previously described method [15]. The siRNA oligonucleotide targeting TRPC6 was purchased from Santa Cruz Biotechnology (sc-42672, USA). Podocytes were transfected with siRNA using the X-Tremegene siRNA transfection reagent (Roche, Germany) according to the manufacturer's instructions. For OGD, the culture medium was replaced with a glucose-free Earle's balanced salt solution. The cells were then incubated in an anoxic chamber (2% O_2/93% N_2/5% CO_2) at 37 °C for 1.5, 3 or 4 h. The reoxygenation group was established by subjecting the podocytes to OGD for 4 h followed by 6 or 24 h of culture under normoxic conditions. The control group was cultured in normal medium and maintained in a 95% air/5% CO_2 incubator.

2.5. Quantitative real-time PCR

Real-time PCR was performed in accordance with standard methods. Briefly, total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), and 0.5 mg RNA sample was transcribed with reverse transcriptase to make the template cDNA. The PCR primer sequences used were (5′-3′): TRPC1 sense, CGCCGAACGAGGTGAT and antisense GCACGCCAGCAAGAAA; TRPC3 sense CGGCAACATCCCAGTG and antisense CGTAGAAGTCGTCGTCCTG; and TRPC6 sense GCCAATGAGCATCTGGAAAT and antisense TGGAGTCACATCATGGGAGA. PCR assays were performed using the ABI PRISM 7500 (ABI Applied Biosystems, USA). Data were normalized to GAPDH in individual samples to correct for sample variability.

2.6. Western blot analysis

Western blot analysis was performed as described previously [15]. Polyclonal rabbit anti-TRPC6 (Alomone Labs, Israel) or mouse anti-actin (Santa Cruz, USA) antibodies were used as the primary antibodies. The secondary antibodies used in the analysis were fluorescence-conjugated goat anti-rabbit IgG or goat anti-mouse IgG antibodies (Invitrogen, USA). The immunoreactive proteins were detected using the Odyssey infrared imaging system (Li-COR, Lincoln, USA).

2.7. Immunofluorescence staining

To detect TRPC6 and synaptopodin in kidney samples, the renal tissue was cut into 4-μm frozen sections, fixed with cold acetone for 10 min, permeabilized with 0.3% Triton X-100, blocked with goat serum containing 5% BSA, and incubated with rabbit anti-TRPC6 antibody (diluted 1:100, Alomone Labs, Israel) at 4 °C overnight. The sections were then incubated with a mouse anti-synaptopodin antibody (diluted 1:10, PROGEN Biotechnik) for 1 h at room temperature. After rinsing thrice with PBS, the sections were incubated in a mixture of Alexa 488- or Alexa 594-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies (diluted 1:200) for 1 h at room temperature. The images were visualized under a fluorescence microscope (Olympus, Tokyo, Japan).

2.8. Ca^{2+} imaging

The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was monitored using the Ca^{2+}-sensitive fluorescent dye Fluo-3/AM as previously described [15]. Briefly, podocytes were grown to confluence on glass slides prior to the experiments. The cells were then treated with a physiological saline solution containing the intracellular Ca^{2+} indicator Fluo-3/AM (3 μM/L, Molecular Probes, USA) and 0.03% Pluronic F-127 (Sigma, USA) for 45 min at 37 °C. The changes in fluorescence of the Fluo-3- loaded cells were recorded using a laser scanning confocal microscope (Olympus, Japan). The $[Ca^{2+}]_i$ levels were calculated using a pseudo-ratio value of the actual fluorescence intensity divided by the average baseline fluorescence intensity.

2.9. Intracellular ROS detection

ROS production in podocytes was determined using the fluorescent probe 2′,7′-dichlorofluorescin diacetate (DCF-DA; Sigma). The podocytes were grown in 24-well plates for 24 h prior to the experiment. The cells were washed with PBS and then loaded with 10 μmol/L of DCF-DA at 37 °C for 45 min. The cells were subsequently washed with PBS three times. DCF fluorescence images were detected using a confocal microscope (FV300; Olympus, Japan).

2.10. Direct fluorescence staining of F-actin

Podocytes were seeded on glass coverslips for 24 h before use. After treatment, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton X-100. F-actin was detected using FITC-conjugated phalloidin (5 μg/mL in PBS; Sigma–Aldrich, St. Louis, MO). The F-actin fluorescence was visualized using a fluorescence microscope (Olympus, Tokyo, Japan).

2.11. Statistical analysis

Data are expressed as the means ± SE. Comparisons between groups were analyzed using Student's t-test or one-way ANOVA followed by Bonferroni's multiple-range test. GraphPad Prism 5 software was used for statistical analysis, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Ischemic injury to mouse kidney induces podocyte effacement and the upregulation of TRPC6 mRNA and protein expression

To study the effects of ischemia on podocyte injury, we clamped the renal pedicles of Kunming mice to establish a model of renal ischemia. Kidney sections were examined by scanning and transmission electron microscopy. After 30 min of ischemia, the electron micrographs showed capillary network collapse and structural damage to the integrity of the podocytes, with narrowed slit diaphragms. By 3 h of reperfusion, the podocyte cell body was flattened, with shorter foot processes and focal areas even showed effacement, as shown in Fig. 1B. We also detected serum creatinine at 3 h of reperfusion; as shown in Fig. 1A, serum creatinine levels were significantly increased. Our reverse transcription–polymerase chain reaction results showed that TRPC6 mRNA was significantly increased after 30 min of ischemia and 3 h of reperfusion by 156% and 93%, respectively (Fig. 2A). Consistent with the mRNA results, western blot analysis showed that 30 min of ischemia in the kidney induced an increase in TRPC6 protein expression, which was slightly reduced after 3 h of reperfusion (Fig. 2B). This was further confirmed by immunofluorescence results; the expression of the TRPC6 protein in glomerular podocytes was demonstrated by colocalization with the podocyte marker synaptopodin (red). TRPC6 protein expression (green) was increased in the ischemia and reperfusion group compared with the sham group (Fig. 2C).

3.2. OGD selectively increases the levels of TRPC6 mRNA and protein expression

Oxygen glucose deprivation (OGD) is a well-established model to mimic ischemic injury. We observed podocytes that had been

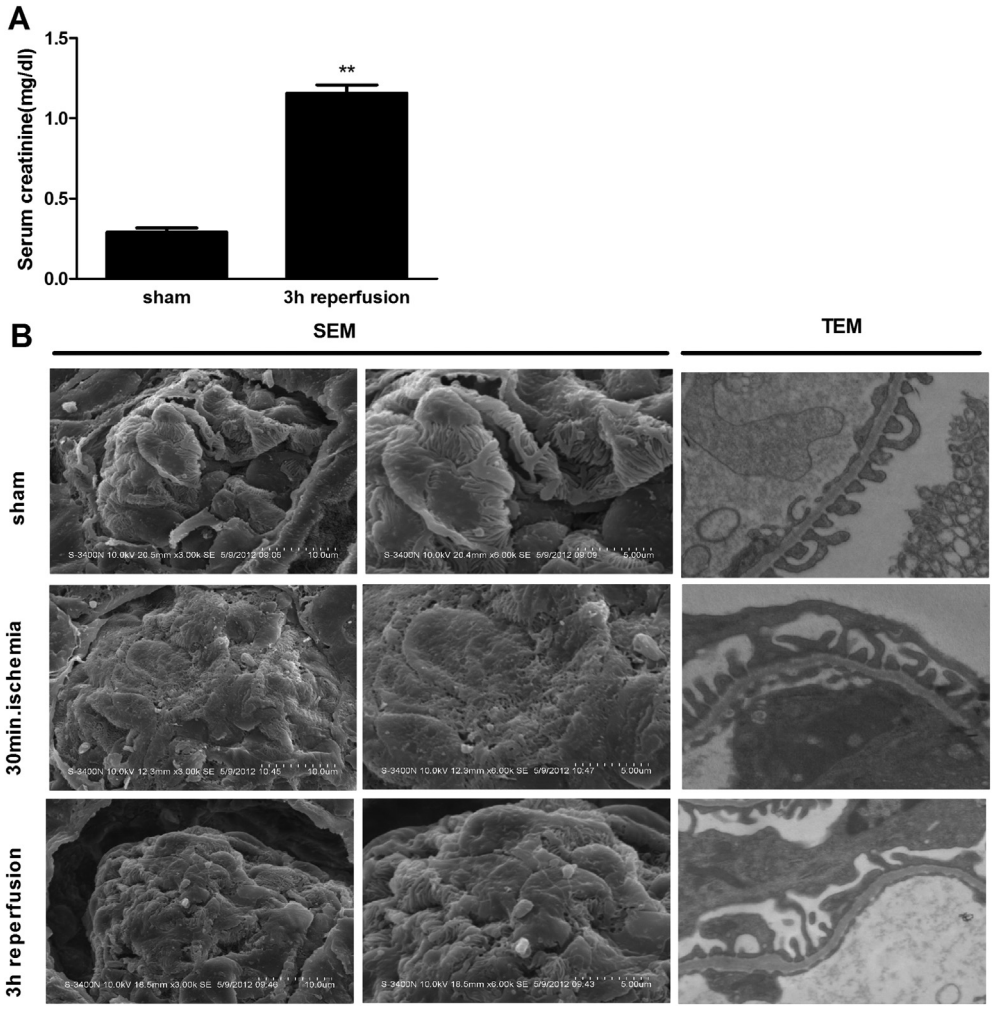

Fig. 1. Ischemic injury to mouse kidney induced damage to the podocytes' structural integrity. Renal ischemia reperfusion injury was induced by clamping the bilateral renal pedicles of Kunming mice, which were randomly divided into sham-treated, 30 min ischemia and 3 h reperfusion groups. (A) Serum creatinine (mg/dl) was significantly increased in the 3 h reperfusion mice. (B) Scanning and transmission electron micrographs of the kidneys from the three different groups. After 30 min of ischemia, capillary network collapse and damaged structural integrity of podocytes and the slit diaphragm were observed. After 3 h of reperfusion, the podocyte cell body was flattened, the foot processes were shortened, and some focal areas were effaced. Data are reported as the means ± SE ($n = 8$). Asterisks indicate the statistical significance ($^{**}p < 0.01$) with respect to the sham-treated group. SEM, scanning electron micrograph; TEM, transmission electron micrograph.

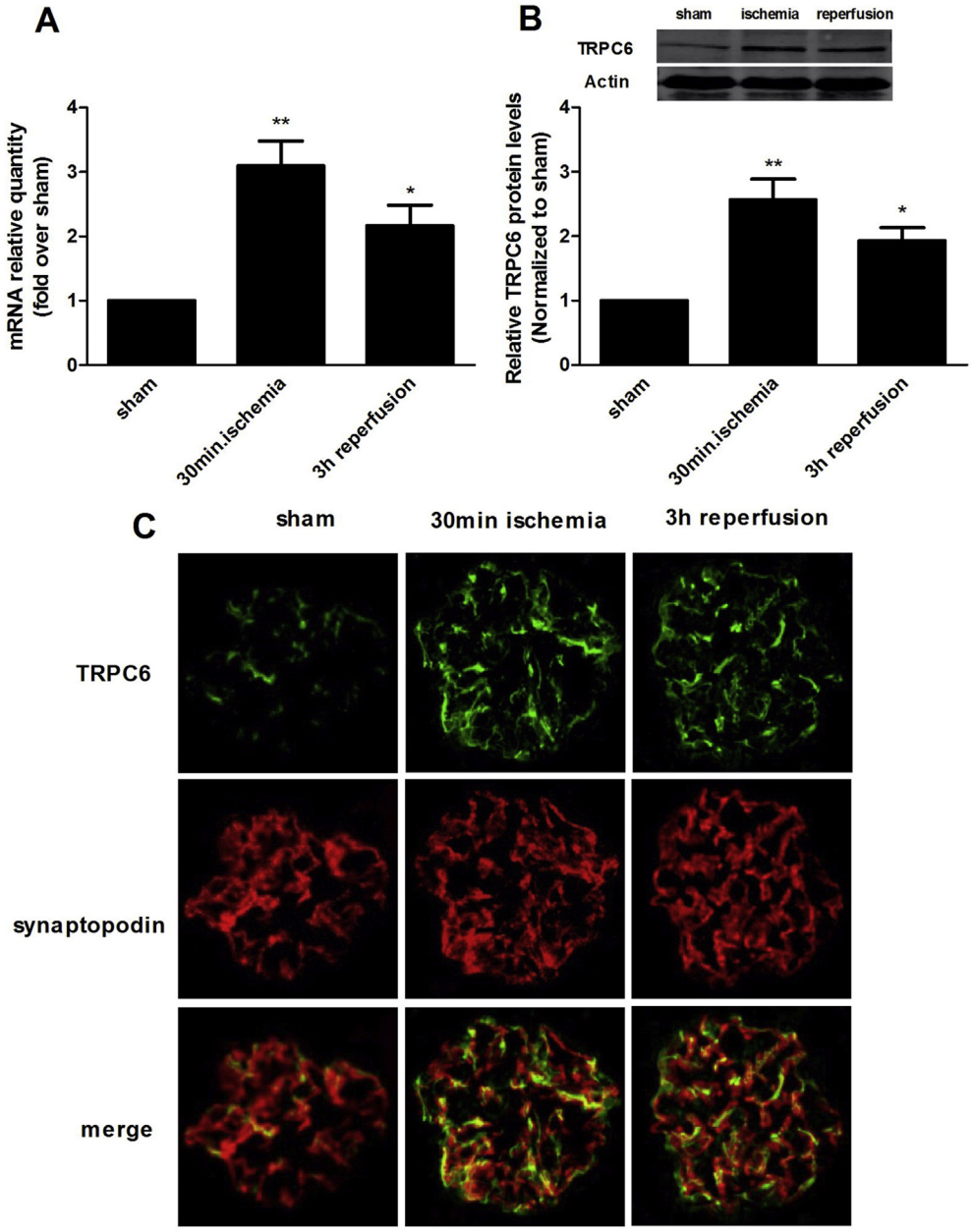

Fig. 2. Ischemic injury to mouse kidney induced the upregulation of TRPC6 expression in glomerular podocytes. Renal ischemia reperfusion injury was induced by clamping the bilateral renal pedicles of Kunming mice, which were randomly divided into sham-treated, 30 min ischemia and 3 h reperfusion groups. (A) TRPC6 mRNA levels in isolated glomeruli were determined using real-time PCR analysis. Thirty minutes of ischemia induced a significant increase of TRPC6 mRNA expression, whereas 3 h of reperfusion restored the initial change to some extent. (B) Representative western blots (upper panel) and summarized data for TRPC6 protein levels in glomeruli from the different groups of mice. (C) Frozen mouse kidney sections were examined by indirect immunofluorescence with anti-TRPC6 and anti-synaptopodin primary antibodies. Synaptopodin was used as podocyte marker. Data are reported as the means ± SE ($n = 8$). Asterisks indicate the statistical significance ($^{*}p < 0.05$, $^{**}p < 0.01$) with respect to the sham-treated group.

exposed to OGD for 3 h significantly increased TRPC6 mRNA and protein expression by 138% and 85%, respectively, whereas TRPC1 and TRPC3 were unaffected (Fig. 3A and D). Subsequently, we examined the time course of OGD and reoxygenation on TRPC6 mRNA and protein expression. The effects of OGD on TRPC6 expression are time-dependent (Fig. 3B and E). Furthermore, the administration of reoxygenation following OGD for 4 h induced higher levels of TRPC6 mRNA, with a peak at 6 h and a reversal to some extent at 24 h (Fig. 3C). Western blot analysis showed that reoxygenation increased TRPC6 protein expression with a maximum at 6 h, consistent with the mRNA results (Fig. 3F).

3.3. OGD enhances the TRPC6-dependent Ca^{2+} influx

TRPC6 channels can be activated by the diacylglycerol analog 1-oleoyl-2-acetyl-sn-glycerol (OAG), thus resulting in an elevation of intracellular calcium via ROCE. The cells were initially incubated in a medium lacking extracellular Ca^{2+}. Internal Ca^{2+} stores were depleted by incubating the podocytes with 1 μM thapsigargin (TG) for 5 min. Subsequently, the addition of 1.8 mM Ca^{2+} into the bath solution stimulated a remarkable increase in $[Ca^{2+}]_i$ (Fig. 4A). The TG-induced Ca^{2+} leak flux after store depletion occurs via store-operated channels (SOCE), as previously described. Following TG-induced SOCE, treatment with

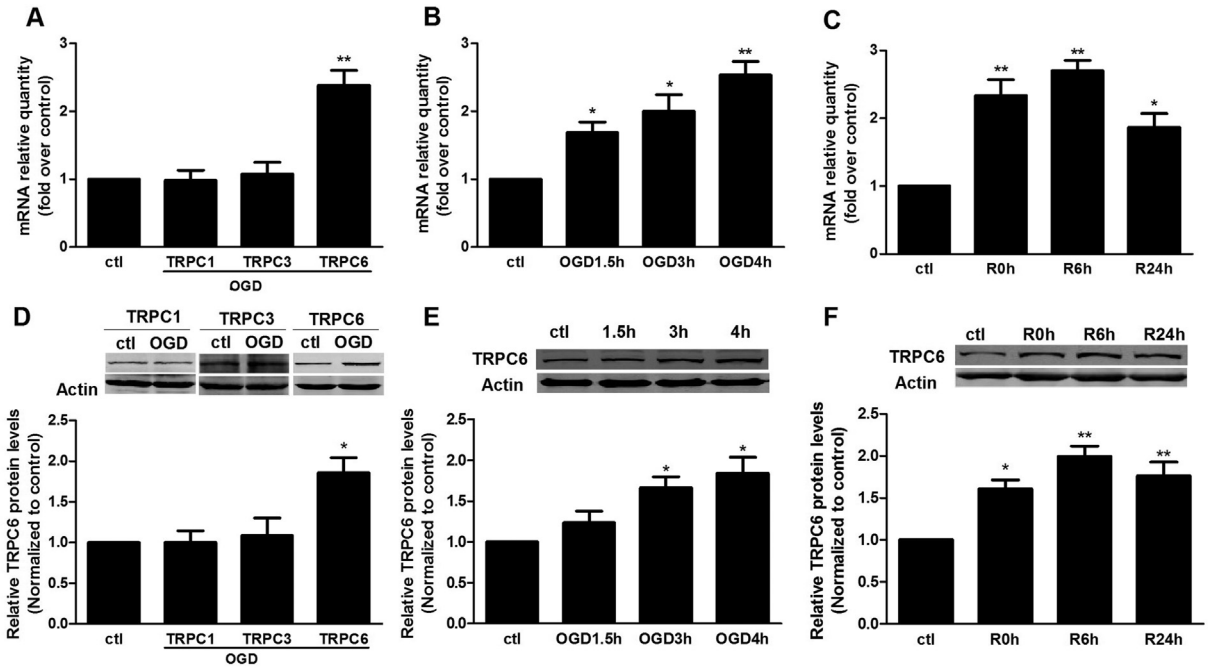

Fig. 3. Influence of OGD treatment on TRPC6 mRNA levels and protein expression. Podocytes were treated with OGD for 1.5, 3, or 4 h, and then subjected to reoxygenation for 0, 6, or 24 h after OGD for 4 h. OGD for 3 h selectively increased the mRNA (A) and protein expression (D) of TRPC6, but not TRPC1 and TRPC3. TRPC6 mRNA levels were significantly upregulated during the time course of OGD (B) and reoxygenation treatment (C), with the peak observed at 6 h and reversing to some extent at 24 h. OGD for 1.5 h did not affect TRPC6 protein expression, whereas OGD for 3 h–4 h (E) and reoxygenation treatment (F) significantly upregulated TRPC6 protein expression, with the maximum at 6 h of reoxygenation. Data are reported as the means ± SE ($n = 5$ for each condition). Asterisks indicate the statistical significance ($^*p < 0.05$, $^{**}p < 0.01$) with respect to the control sample. OGD, oxygen glucose deprivation; R, reoxygenation treatment.

100 μM OAG stimulated an apparent elevation of $[Ca^{2+}]_i$, which can be suppressed by the application of 100 μM 2-APB, a nonselective TRPC channel blocker (Fig. 4A). Therefore, OAG-induced Ca^{2+} influx occurred via the plasmalemmal channels or, at least, in addition to SOCE. Podocytes lack voltage-gated calcium channels and exhibited no spontaneous $[Ca^{2+}]_i$ variations during these experiments. Furthermore, no changes were observed when extracellular Ca^{2+} was re-added in the absence of agonists (data not shown). As shown in Fig. 4C, transfection with TRPC6 siRNA significantly reduced the OAG-induced Ca^{2+} influx by 41%, in contrast to transfection with the scrambled siRNA, where TG-induced SOCE was unaffected. In addition, treatment with the scrambled siRNA had no effect on the TG-induced SOCE or OAG-induced ROCE compared with the non-transfected cells (data not shown). Real-time PCR and Western blot analyses were utilized to assess the specificity and efficiency of the TRPC6 siRNA as shown in Fig. 4B. Data from the two analyses suggested that OAG-induced Ca^{2+} influx is TRPC6-dependent. Moreover, the exposure of podocytes to OGD4h-R6h significantly increased the OAG-induced ROCE by 190% ($p < 0.01$; $n = 3$; Fig. 4D), but had no effect on the TG-induced SOCE. These results indicate that OGD enhanced TRPC6-dependent Ca^{2+} influx.

3.4. OGD-induced ROS generation mediates TRPC6 expression and Ca^{2+} influx

There is agreement regarding the function of ischemic injury-induced ROS generation. In this study, we examined whether ROS is implicated in OGD-induced TRPC6 upregulation. ROS production was measured using the dye DCFH-DA. As shown in Supplementary Fig. 1A, the exposure of podocytes to OGD4h-R0h markedly increased ROS generation. Furthermore, R6h following OGD4h enhanced this change. In addition, pretreatment with 10 mmol/L of N-Acetyl-L-cysteine (NAC), a ROS inhibitor, prevented ROS generation and inhibited the OGD-induced TRPC6 protein expression (Supplementary Fig. 1A and 1B). Consistent with these findings, OGD-stimulated TRPC6-dependent Ca^{2+} influx was also eliminated by the application of NAC (Supplementary Fig. 1D). To demonstrate further the function of ROS associated with TRPC6 activation, we detected TRPC6 protein expression and Ca^{2+} influx after podocytes were exposed to 500 μM H_2O_2. As shown in Supplementary Fig. 1C and 1E, H_2O_2 induced a remarkable increase in TRPC6 protein expression and Ca^{2+} influx. These results strongly suggest that OGD-associated ROS generation increases TRPC6 expression and Ca^{2+} influx.

3.5. TRPC6 mediates OGD-induced actin assembly and reorganization

Actin superstructures were analyzed using phalloidin staining of F-actin. As shown in Supplementary Fig. 2A, the actin filaments were organized in cell spanning bundles called stress fibers under normoxic conditions, whereas OGD4h-R0h induced the disruption of F-actin. Furthermore, after R6h, the F-actin arrangement became prominent. The cells did not recover their baseline actin structure after 24 h of reoxygenation (data not shown). The OGD-induced actin assembly and reorganization were significantly inhibited by TRPC6 knockdown with siRNA. As shown in Supplementary Fig. 2B, OGD treatment induced a decrease in the number of stress fibers in podocytes, whereas TRPC6 knockdown prevented this decrease. Moreover, incubation with 2-APB also attenuated the influence of OGD on F-actin (Supplementary Fig. 2A and 2B). These results demonstrated the role of TRPC6 in actin assembly and reorganization in response to OGD treatment.

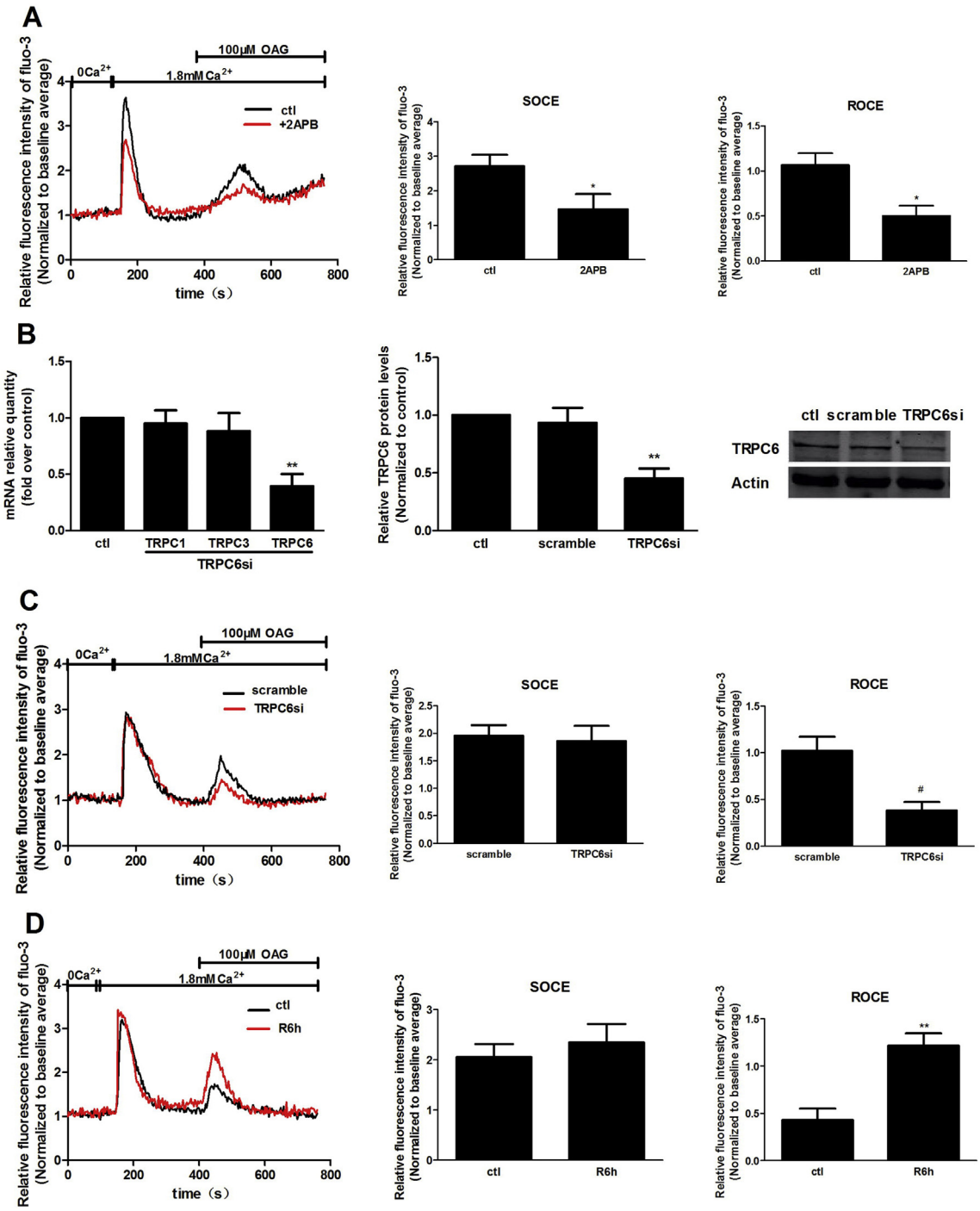

Fig. 4. Effects of TRPC6 knockdown and OGD treatment on TG-induced SOCE and OAG-induced ROCE. Changes in $[Ca^{2+}]_i$ were monitored using Fluo-3 fluorescence methods. (A) Representative traces (left) and summary data (right) show that pretreatment with 100 μM of 2-APB inhibited TG-induced SOCE and OAG-induced ROCE, respectively ($n = 3$). (B) Real-time PCR experiments suggest that TRPC6 siRNA significantly reduced the mRNA levels of TRPC6 but not TRPC1 and TRPC3 ($n = 3$). Western blot analyses indicate that transfection with TRPC6 siRNA significantly reduced TRPC6 protein expression compared with the scrambled siRNA ($n = 3$). (C) Representative traces (left) and summary data (right) show that TRPC6 siRNA transfection significantly inhibited OAG-induced ROCE compared with the scrambled siRNA, but did not affect TG-induced SOCE ($n = 4$). (D) Representative traces (left) and summary data (right) reveal that reoxygenation for 6 h after OGD for 4 h enhanced OAG-induced ROCE, whereas TG-induced SOCE was unaffected ($n = 4$). Data are reported as the means ± SE. Asterisks indicate the statistical significance ($^{*}p < 0.05$ vs. control; $^{**}p < 0.01$ vs. control; $^{\#}p < 0.01$ vs. scramble). OGD, oxygen glucose deprivation; TG, thapsigargin; OAG, 1-oleoyl-2-acetyl-sn-glycerol; SOCE, store-operated calcium entry; ROCE, receptor-operated calcium entry; 2-APB, 2-aminoethoxydiphenyl borate; R, reoxygenation.

4. Discussion

A growing body of evidence has indicated that ischemic injury in podocytes contributes to the progression of renal disease [16,17]. In this study, we found that TRPC6 expression was increased after renal ischemia reperfusion injury. In cultured human podocytes, OGD and reoxygenation treatment, a well-established model that mimics ischemic injury [18], enhanced

TRPC6 mRNA and protein expression. Functional evidence revealed that the podocytes exposed to OGD displayed an increase in TRPC6-dependent Ca^{2+} entry. In addition, TRPC6 knockdown suppressed the OGD-induced $[Ca^{2+}]_i$ increase. These results suggest that TRPC6-mediated Ca^{2+} entry is responsible for the elevated $[Ca^{2+}]_i$ induced by ischemic injury in podocytes. These findings provide a new perspective on the potential mechanisms of podocyte ischemic injury.

Hypoxia and ischemia are known to induce ROS production, resulting in apoptosis and even cell death. Furthermore, previous studies have reported that TRPC6 can act as an important downstream effector of ROS in a variety of cell types, such as vascular myocytes [10], central neurons [19], smooth muscle cells [20], and kidney podocytes [21]. Our data reveal that OGD-induced ROS increase TRPC6 expression and activity, which is consistent with previous studies. Notably, BK_{Ca} channels colocalize with TRPC6 in podocytes, and TRPC6 knockdown reduced the steady-state surface expression of endogenous BK_{Ca} channels [22]. Additionally, we recently reported that BK_{Ca} channels are involved in the podocytes' response to hypoxic injury via the upregulation of the β4-subunit [23]. Taken together, we speculate that the interaction between the BK_{Ca} channels and TRPC6 channels may provide positive feedback for Ca^{2+} influx in podocytes.

Given the unique importance of Ca^{2+} signaling in various cell types, the rise in intracellular Ca^{2+} via the TRPC6 channel is unsurprisingly causal to, or at least involved in, the regulation of podocyte function in health and disease. In central neurons, hypoxic stress evokes TRPC activation and triggers the alterations of the cytoskeleton structure, subsequently resulting in blood–brain barrier disruption [24]. Consistent with these results, this study showed that TRPC6 knockdown attenuated the OGD-induced reorganization of the actin cytoskeleton. A number of studies indicated that the TRPC6 channel interacts with actin through RhoA, a member of the Rho GTPase family [25]. Furthermore, RhoA is also activated during periods of hypoxia [26]. Therefore, we hypothesize that OGD-induced TRPC6 activation may promote actin reorganization through RhoA signaling pathways. Alternatively, other signaling pathways may also contribute to TRPC6-associated actin reorganization. For example, the changes in Ca^{2+} levels in the slit diaphragm (SD) also requires the presence of calcium-binding proteins, such as calmodulin, another crucial downstream effector of TRPC6 [27]. Notably, as an SD protein, TRPC6 may also interact with other proteins in the SD, such as nephrin and podocin, to form signaling scaffolds that can facilitate TRPC6 signaling [11]. In addition to acting as Ca^{2+} entry pathways in the plasma membrane, TRPC6 may also affect cell behavior through ion-transport-independent mechanisms [28]. Future studies are required to elucidate the crosstalk among the various signaling pathways that converge on the TRPC6 channels in podocytes.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.054.

Transparency document

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